DISS. ETH Nr. 21129

New insights into host specialization and mycoparasitic interaction between powdery mildew fungi and *Ampelomyces quisqualis*

A dissertation submitted to

ETH ZURICH

For the degree of

Doctor of Sciences

Presented by

DARIO ANGELI

Doctor of Agronomic Science and Technology, UniPD Padua

Born February 08th, 1976

Citizen of Mezzolombardo (IT)

Accepted on the recommendation of

Prof. Dr. Cesare Gessler, examiner

Prof. Dr. Bruce A. McDonald, co-examiner

Dr. Monika Maurhofer and Dr. Ilaria Pertot, co-examiners

2013

Table of contents

Table of contents

Abstract

Pycnidial fungi belonging to the genus *Ampelomyces quisqualis* are the most widespread natural antagonists of *Erysiphales*. *A. quisqualis* is a specific mycoparasite of many species of *Erysiphales* and the most studied biocontrol agent of powdery mildews. Genetically different *A. quisqualis* strains are available from culture collections and one strain has been already commercialized under the trade name of AQ10. Report data on the aggressiveness and morphology and cultural patterns of *A. quisqualis* strains found in the literature are controversial and incomplete. Screening is a crucial step in the selection of strains capable of providing highly effective biocontrol. There is a need for further investigations aiming at the identification of phenotypic markers that can be used to differentiate genetically distinct groups within *A. quisqualis* in order to find more effective strains within *A. quisqualis* species, which differ considerably with respect to their biocontrol effectiveness.

The first objective of the present work was to verify the presence of natural strains of *A. quisqualis* in a wide viticulture area (Trentino Alto Adige region). We aimed to isolate and select new strains better adapted to the local environmental conditions than commercial strain AQ10 and highly aggressive against *Erysiphaceae* for a potential development as biocontrol agents. During a three-year survey, a limited amount of natural parasitism of *E. necator* by *Ampelomyces* spp. (0.17−3.51 %) was observed. Pycnidia and conidia of *Ampelomyces* spp. parasitizing *E. necator* chasmothecia were found both in conventional, organically grown on and untreated vineyards. Some of the isolated *A. quisqualis* strains have conidia that are shaped differently than those of the commercial *A. quisqualis* strain (AQ10) and are phylogenetically different from AQ10.

Second objective of the thesis was to characterize several *A. quisqualis* strains from different hosts and geographic regions and possessing different ITS rDNA sequences and investigate whether the host or site of origin of the strains or their cultural, morphological and/or growth characteristics are related to their phylogenetic group which would indicate an adaptation to the host or geographic area. Strains were molecularly characterized by sequencing the ITS rDNA and sequence polymorphisms were used to classify and group the strains. The results revealed some significant variation among the selected strains, which provides evidence for the existence

of different physiological forms within the *A. quisqualis* species. Phylogenetic analysis revealed that these *A. quisqualis* strains can be classified into five different genetic groups, which generally correlate with the fungal host of origin and morphological and growth characteristics.

Finally, the pathogenicity, virulence and host range of a group of *A. quisqualis* strains was assessed on different powdery mildew agents. Strains were screened both for their ability to colonize different powdery mildews (mycoparasitic activity) and for *in vitro* production of cell wall degrading enzymes (CWDEs). This study showed a positive correlation between mycoparasitic activity and production of chitobiases and proteases. *A. quisqualis* strains with similar levels of mycoparasitic activity originate from the same host species and share an identical ITS rDNA sequence.

In the 5th chapter it was investigated whether it is possible to enhance the efficiency of this fungus in the biological control of powdery mildews by increasing the conidial germination rate of the fungus. The obtained results revealed that some natural extracts can stimulate the germination of *A. quisqualis* conidia and enhance its biocontrol ability of the powdery mildew. This part of the thesis demonstrates that the conidial germination efficacy of *A. quisqualis* strains is positively related to virulence against powdery mildew and can, therefore, be considered as relevant factor in the selection of biocontrol agents.

The results obtained in this thesis provide a deeper understanding of the process of mycoparasitism and a sound basis for developing new screening strategies for detecting highly effective *A. quisqualis* strains for the biocontrol of powdery mildews. Moreover, we demonstrated the existence of *A. quisqualis* strains well adapted to local environmental conditions. Their discovery may be the starting point for their development as biocontrol agents to control powdery mildew under the environmental conditions found in Northern countries.

Riassunto

I funghi che producono picnidi appartenenti al genere *Ampelomyces quisqualis* sono i più diffusi antagonisti naturali degli *Erysiphales*, gli agenti causali degli oidi. *A. quisqualis* è un micoparassita specifico di molte specie di *Erysiphales* e risulta essere l'agente di biocontrollo degli oidi più noto. Alcuni isolati di *A. quisqualis* diversi geneticamente sono disponibili in collezioni di colture ed uno di essi è già stato commercializzado con il nome di AQ10. I dati presenti in letteratura risultati di test colturali, morfologici e di prove di aggressività con diversi isolati di *A. quisqualis* sono piuttosto contrastanti ed incompleti. La selezione di isolati altamente efficaci rappresenta un passo cruciale per il biocontrollo degli oidi. C'è la necessità di ulteriori investigazioni che mirino all'identificazione di caratteri fenoticipi che possano essere impiegati per la differenziazione di diversi gruppi genetici di *A. quisqualis* con lo scopo di individuare isolati più efficaci all'interno della stessa specie che differiscono per la loro capacità di biocontrollo.

Il primo obiettivo del presente lavoro era quello di verificare la presenza di isolati naturali di *A. quisqualis* all'interno di un ampia area viticola (regione Trentino Alto Adige). Lo scopo era di isolare e selezionare nuovi isolati meglio adattati alle caratteristiche climatiche locali rispetto all'isolato commerciale AQ10 ed altamente aggressivi nei confronti dei funghi appartenenti alla famiglia delle *Erysiphaceae*, da poter sviluppare quali nuovi agenti di controllo biologico. Durante i tre anni di monitoraggio è stata osservata una ridotta parassitizzazione naturale di *E. necator* con *Ampelomcyes* spp. (0.17−3.51 %). Chasmoteci di *E. necator* parassitizzati da picnidi e conidi di *Ampelomyces* spp. sono stati individuati sia in vigneti biologici che incolti ma anche in vigneti sottoposti ai trattamenti chimici convenzionali. Alcuni degli isolati di *A. quisqualis* identificati producevano conidi di forma diversa rispetto a quelli dell'isolato commerciale (AQ10) ed erano filogeneticamente diversi da AQ10.

Il secondo obiettivo della tesi era quello di caratterizzare isolati di *A. quisqualis* provenienti da ospiti e regioni geografiche diverse e con una diversa sequenza ITS rDNA ed indagare se l'ospite o regione di origine degli isolati o le loro caratteristiche colturali, morfologiche e/o di crescita sono correlate al loro gruppo filogenetico indicando in tal caso un adattamento all'ospite oppure all'area geografica. La caratterizzazione molecolare degli isolati è stata fatta seguendo la

metodologia descritta da Szentivanyi attraverso la determinazione della lunghezza dei polimorfismi della regione amplificata rDNA-ITS. I polimorfismi di sequenza sono stati utilizzati per classificare e raggruppare gli isolati. I risultati hanno rivelato molte variazioni significative tra gli isolati selezionati, evidenziando in tal modo l'esistenza di diverse forme fisiologiche all'interno della specie *A. quisqualis.* Le analisi filogenetiche hanno rivelato che questi isolati di *A. quisqualis* possono essere classificati in cinque differenti gruppi genetici, i quali in linea generale correlano con l'ospite fungino di origine e le caratteristiche morfologiche e di crescita.

Infine, la patogenicità, virulenza e spettro d'ospite di un gruppo di isolati di *A. quisqualis* è stato determinato su diversi agenti di oidio. Gli isolati sono stati selezionati sia per la capacità di colonizzazione di diversi oidi (attività micoparassitica) sia per la produzione in vitro di enzimi degradanti pareti cellulari (CWDEs). Questo studio ha mostrato una correlazione positiva tra l'attività micoparassitica e la produzione di chitobiasi e proteasi. Isolati di *A. quisqualis* con livelli simili di attività micoparassitica erano originati dalle stesse specie ospiti e mostravano una sequenza ITS rDNA identica tra loro.

Un paragrafo addizionale è stato aggiunto per verificare la possibilità di migliorare l'efficacia di questo fungo nel controllo biologico degli oidi attraverso un aumento del tasso di germinazione dei conidi del fungo. I risultati attuali hanno rivelato l'esistenza di alcuni estratti naturali in grado di stimolare la germinazione dei conidi di *A. quisqualis* e migliorare la sua capacità di biocontrollo degli oidi. Questo studio ha dimostrato che la capacità di geminazione dei conidi di *A. quisqualis* è legata a differenze di virulenza nei confronti dell'oidio e può perciò essere considerata come un fattore importante nella selezione di nuovi efficaci agenti di biocontrollo.

Il lavoro presentato in questa tesi fornisce una più profonda comprensione del processo di micoparassitismo e offre una base solida per lo sviluppo di nuove strategie di selezione per l' individuazione di isolati di *A. quisqualis* altamente efficaci nel biocontrollo degli oidi. Inoltre, noi abbiamo accertato l'esistenza di isolati di *A. quisqualis* bene adattati alle condizioni ambientali locali e la loro scoperta potrebbe rappresentare il punto di partenza per il loro sviluppo quali agenti di controllo biologico dell'oidio nei Paesi del Nord.

Chapter 1

General Introduction

1.1 Natural antagonists of powdery mildew fungi

Powdery mildews (Ascomycotina, Erysiphales) are some of the world's most frequently encountered plant pathogenic fungi and they are among the most significant plant diseases, despite extensive research on their pathogenesis, epidemiology and control. They are often conspicuous owing to the profuse production of conidia that give them their common name. Among the economically important plants, important crops like grapevine, apple, strawberry, but also cereals and several vegetables and ornamentals, grown in the field or greenhouses, are the major targets of powdery mildew fungi. They infect leaves, stems, flowers, and fruits of nearly 10,000 species of angiosperms (Braun, 1987). Uncontrolled epidemics of *Erysiphales* may result in yield losses, as well as a reduction in the quality of the production. During the winter, the fungus survives either as mycelia in the dormant buds of grapevine or as chasmothecia, which are the fruiting bodies arising from the sexual stage. In the spring, primary infections originating from ascospores commonly appear randomly as scattered whitish and powdery spots on leaves (1-3 mm in diameter) that mainly appear close to the trunk. The two main methods for disease control currently available in crop production are repeated applications of fungicides and the use of cultivars resistant or tolerant to powdery mildews. However, both methods have their own limitations (Hewitt, 1998). Public attitude and environmental concerns towards the use of pesticides as well as the development of powdery mildew strains resistant to different fungicides have reduced the appeal of chemicals (Whipps & Lumsden, 2001; Bélanger & Benyagoub, 1997; Ishii et al., 2001). Moreover, in some countries a number of fungicides effective against powdery mildews are no longer registered for greenhouse production, due to restrictions in pesticide usage (Jarvis & Slingsby, 1977; Menzies & Bélanger, 1996). Cultivars resistant or tolerant to powdery mildew infections have been developed in a number of crops, but their use is limited, especially in fruit and vegetable crops (Bélanger & Benyagoub, 1997). All these constraints have led to the search of alternative methods to control powdery mildews. Non-fungicide products, such as soluble silicon, oils, salts and plant extracts, inducing resistance in plants infected with powdery mildews or acting as prophylactic and/or curative factors are in focus, especially in greenhouse production (Bélanger & Benyagoub, 1997; Menzies & Bélanger, 1996; Pasini et al., 1997; McGrath & Shishkoff, 1999). On the other hand, the exploitation of antagonistic trophic interactions between plant inhabiting microorganisms offers an opportunity for their use in biological control of plant diseases, yet few examples have reached the market. Microbial

biocontrol agents (BCAs) of plant pathogen, if carefully selected, may offer a valid alternative to chemical fungicides in crop protection against weed, insects, and disease in both agriculture and forestry (Cook & Baker, 1983; Andrews, 1992). This method is based on the knowledge of natural interaction between pests or pathogen and their natural enemies. Some of the beneficial organisms used in the biocontrol are mass produced and available for large-scale distribution in crops with the aim to reduce damage population in organic farming. Microbial biocontrol agents have several mechanisms to antagonize their hosts. Four different mechanisms for controlling pests or diseases by BCAs are known: i) inhibition of the pathogen by the production of metabolites produced by another organism (antibiosis); ii) consuption of the same resource present in limited quantity, insufficient for the survival of both organisms (competition); iii) direct attack on the pathogen and using it as nutrition source (mycoparasitism); iv) production of several metabolites reducing damages due to the plant respons to the pathogen (induction of resistence). Attempts have been made to use mycolytic bacteria, mycophagous arthropods and other possible non-fungal biological control agents against powdery mildews, but these studies have provided no promise of practical control to date (Kiss, 2003). The most promising biological control trials have involved a number of fungi antagonistic to powdery mildews.

1.1.1 Antagonistic fungi in nature

The names of all the fungal species reported as natural antagonists of powdery mildews and/or used in biocontrol experiments against them are presented in Table 1. Some of these fungi are well-known natural antagonists of powdery mildews. For example, *Ampelomyces* spp. or *Tilletiopsis* spp. were repeatedly isolated from plants infected with powdery mildew worldwide (Kiss, 1998; Falk et al., 1995; Knudsen & Skou, 1993; Klecan et al., 1990; Urquhart et al., 1994). Other fungi, such as *Cephalosporium* spp., *Cladosporium* spp. or *Trichothecium* spp. are cited as being frequently associated with powdery mildew colonies (Braun, 1987). Furthermore, *Pseudozyma flocculosa* is known worldwide and this species is considered one of the most efficient biocontrol agent of powdery mildews (Paulitz & Bélanger, 2001). A number of species included in Table 1, such as *Aphanocladium album* (Hijwegen & Buchenauer, 1984) were also reported to inhibit naturally the sporulation and growth of powdery mildews, but available data on their natural occurrence and/or biocontrol activity are very limited. Some of these data need

further confirmation. Other fungi, such as *Trichoderma* spp. (Elad, 2000; Elad et al., 1998), *Penicillium chrysogenum* or *Fusarium oxysporum* (Hijwegen, 1988) were used in some biocontrol trials against powdery mildews, but they were never found to be naturally associated with powdery mildew colonies.

Due to the biotrophic nature of powdery mildews, fungal antagonists can act against them through antibiosis and mycoparasitism only. Competition for nutrients and/or space, the third major mechanism of microbial antagonism, is not feasible against powdery mildews. The niche they occupy in the phyllosphere could be colonized only by other plant pathogens and certainly not by biocontrol agents. Mycoparasitism of powdery mildews by *Ampelomyces* spp. is one of the best known mechanisms of fungal antagonism. These intracellular mycoparasites suppress the sporulation of the attacked powdery mildew mycelia and kill all the parasitized cells (Kiss, 1998; Falk et al., 1995; Hashioka & Nakai, 1980). When the sporulation rate of the pathogen is high, the mycoparasites usually cannot stop the spread of powdery mildew colonies. In contrast, other phyllosphere fungi acting through antibiosis, such as *Pseudozyma* spp. or *Tilletiopsis* spp., can kill powdery mildew colonies rapidly and completely, causing plasmolysis of their cells (Hajlaoui et al., 1994; Choudbury et al., 1994). Some authors have proposed to enlarge the possible modes of action of antagonists by considering induced resistance as a part of the antagonistic effect (Bélanger & Labbé, 2002). The induction of plant defence might be a plausible mechanism in cases when neither mycoparasitism nor antibiosis can explain the efficacy of a biocontrol fungus (Elad, 2000; Elad et al., 1998). Most probably, in many biocontrol agents the antagonistic effect is based on more than one mode of action (Cook & Baker, 1983; Bélanger & Labbé, 2002). *Verticillium lecanii*, for example, was reported as a mycoparasite of powdery mildews that penetrate their cells either directly or by means of appressoria (Heintz & Blaich, 1990). However, according to recent studies, antibiosis also plays an important role in this interaction (Bélanger & Labbé, 2002). However, in most antagonists, the modes of action against powdery mildews are largely unknown.

Table 1. A list of fungi tested as potential biocontrol agents against powdery mildews.

Antagonist	Powdery mildew	Plant host	Mode of action
Acremonium alternatum	*Sphaerotheca fuliginea*	*Cucumis sativus*	Mycoparasitism ? [a]
A.strictum	*S. fuliginea*	*C. sativus*	?
A.lanosoniveum	*S. macularis*	Strawberry	?
Ampelomyces quisqualis	Many species	Many species	Mycoparasitism
Aphanocladium album	*Erysiphe cichoracearum*	*C. sativus*	?
	S. fuliginea	*C. sativus*	?
	S. pannosa	*Rosa sp.*	?
Aspergillum fumigatus	*E. cichoracearum*	*C. sativus*	?
Cladosporium spongiosum	*Phyllactinia dalbergiae*	*Dalbergia sissoo*	?
	Ph. corylea	*Morus alba*	?
Cladosporium sp.	*S. fuliginea*	*Cucurbita pepo*	?
	Leveillula taurica	*Capsicum annuum*	?
Cephalosporium sp.	*S. fuliginea*	*Citrullus lanatus*	?
Calcarisporium arbuscola	*S. fuliginea*	*C. sativus*	Antibiosis ?
Cladobotryum varium	*S. fuliginea*	*C. sativus*	?
Chaetomium spp.	*Podosphaera leucotricha*	*Malus domestica*	?
Drechslera spicifera	*E. cichoracearum*	*C. maxima*	?
Fusarium oxysporum	*E. cichoracearum*	*C. maxima*	?
Paecilomyces farinosus	*S. fuliginea*	*C. sativus*	Antibiosis ?
	E. marti	*Lupinus polyphyllus*	Antibiosis ?
	L. taurica	*C. annum*	Antibiosis ?
Penicillium chrysogenum	*S. fuliginea*	*C. sativus*	?
P. fellutanum	*E. cichoracearum*	*C. maxima*	?
Peziza ostracoderma	*S. fuliginea*	*C. sativus*	?
Pseudozyma spp.	*S. fuliginea*	*C. sativus*	Antibiosis ?
	E. polygoni	*Trifolium pratense*	Antibiosis ?
	S. pannosa	*Rosa sp.*	Antibiosis ?
	Blumeria graminis	*Triticum aestivum*	Antibiosis ?
Scopulariopsis brevicaulis	*S. fuliginea*	*C. sativus*	?

Table 1. (continued)

Antagonist	Powdery mildew	Plant host	Mode of action
Sesquicillium candelabrum	*S. fuliginea*	*C. sativus*	?
Sepedonium chrysospermum	*S. fuliginea*	*C. sativus*	?
Tilletiopsis albescens	*S. fuliginea*	*C. sativus*	Antibiosis
	B. graminis	*Hordeum vulgare*	Antibiosis
T. minor	*S. fuliginea*	*C. sativus*	Antibiosis
	E. marti	*L. polyphyllus*	Antibiosis
	B. graminis	*H. vulgare*	Antibiosis
T. pallescens	*B. graminis*	*Hordeum vulgare*	Antibiosis
	S. pannosa	*Rosa sp.*	Antibiosis
	S. fuliginea	*C. sativus*	Antibiosis
T. washingtonensis	*S. fuliginea*	*C. sativus*	Antibiosis
Tilletiopsis sp.	*S. fuliginea*	*C. sativus*	Antibiosis
Trichoderma harzianum	*S. fusca*	*C. sativus*	Induced resistence ?
T. viride	*S. fuliginea*	*C. sativus*	?
Verticillium lecanii	*S. fuliginea*	*C. sativus*	Mycoparasitism ?
	E. necator	*Vitis sp.*	Mycoparasitism ?
V. fungicola	*S. fuliginea*	*C. sativus*	?

[a] The mechanism of action is unknown

1.1.2 Potential relevance for biocontrol

Crops are constantly under the attack of pest and pathogens. In the past harvest losses due to pest and diseases often caused food shortage and severe famines (i.e. *Phytophthora infestans* on potatoes in Ireland). It is therefore understandable how the development of chemical pesticides were applauded in the past century. Soon after the initial enthusiasm, synthetic compounds showed their serious drawbacks: toxicity and negative effect on human health, environmental pollution, but also outbreaks of uncontrolled not-target pests due to the disruption of ecological balance and loss of efficacy related to resistant strains in the pathogen population. In EU, consumers and policy makers concerns on pesticides encouraged, through specific and stringent regulations, the removal of highly toxic pesticides from the market. Incorporation of transgenes to obtain genetically modified plants resistant to pests and pathogens can help in future to solve point wise particular problems. Therefore, chemical industry is nowadays looking for new generation pesticides, which must be safe for consumers and have a low or null impact on the environment. The exploitation of antagonistic trophic interactions between plant inhabiting microorganisms offers an opportunity for their use in biological control of plant diseases, yet few examples have reached the market. Microbial biocontrol agents (BCAs) of plant pathogen, if carefully selected, may offer a valid alternative to chemical fungicides, being them safe, biodegradable and renewable. Many potential BCAs have been tested against several plant pathogens and various mechanism of action have been described (competition for space and nutrients, antibiosis, induced resistance, hyperparasitism), but they are far from being fully understood. The most promising biological control trials have involved a number of fungi antagonistic to powdery mildews and have resulted in the development of two biofungicide products, AQ10 Biofungicide and Sporodex, which have been registered and commercialized in some countries. AQ10 contains the conidia of a strain of a pycnidial fungus *Ampelomyces quisqualis* (Hofstein et al., 1996) while Sporodex is based on the conidia of a basidiomycetous yeast, *Pseudozyma flocculosa* (Paulitz & Bélanger, 2001). Other biocontrol fungi have also been studied extensively for the same purpose.

Integrated management programs were also developed combining the use of some biocontrol agents, such as *A. quisqualis* in grapevine and cucumber (Hofstein & Fridlender, 1994; Sundheim, 1982) and *P. flocculosa* in rose (Bélanger & Benyagoub, 1997) together with a

reduced amount of fungicide. The results obtained with the two, already registered biocontrol agents, as well as with other extensively studied fungal antagonists of powdery mildews such as other *Ampelomyces* and *Pseudozyma* species, *Verticillium lecanii*, *Tilletiopsis* spp. and *Acremonium alternatum* have been thoroughly reviewed (Paulitz & Bélanger, 2001; Bélanger & Benyagoub, 1997; Elad et al., 1996; Menzies & Bélanger, 1996; Bélanger & Labbé, 2002). However, the list of antagonistic fungi tested against powdery mildews is much longer. A number of fungal antagonists were used in only one or two trials, in which they were reported to be potentially useful against powdery mildews, but they were not included in any further studies. This may be due to undisclosed limitations or to other factors. Most probably, the lack of data on the biocontrol potential of a number of antagonists reflects only the degree of their evaluation.

1.2 Mycoparasitism by *Ampelomyces quisqualis*

Pycnidial fungi belonging to the genus *Ampelomyces* Ces. are the oldest known and the commonest natural antagonists of powdery mildews that have been intensively studied in crop protection practice. It is specific to the fungi belonging to the Erysiphales (Powdery mildews). The interactions between host plants, powdery mildew fungi and *Ampelomyces* mycoparasites are one of the most evident cases of tritrophic relationships in nature, because this relationship is common world-wide and takes place exclusively on aerial plant surfaces, thus facilitating its direct observation (Kiss, 1998). However, it has received little attention in fungal and plant ecology, although it could be used as a model to study the significance of mycoparasitism in the natural dynamics of plant parasitic fungi. *A. quisqualis* is naturally present worldwide with seemingly a wide host range (16 spp. of Erysiphales on 27 different host plants). *A. quisqualis* has been considered to be a single species for a long time, but its taxonomy has become controversial and probably merits extensive revision (Sutton, 1980; Kranz, 1981). Variation in the morphology of conidia, cultural characteristics, and rDNA ITS sequence of different *A. quisqualis* strains has been described, suggesting that the binomial '*A. quisqualis* ' has been applied to a range of strains representing a species complex. In the absence of a formal taxonomic reassessment, the use of the binomial '*A. quisqualis*' for all the pycnidial hyperparasites of powdery mildew fungi is technically not correct and a taxonomic revision is clearly needed. Until recently, *A. quisqualis* was often confused with several other species: *A.*

quercinus, *A. humuli*, *A. heracli* and *Phoma glomerata*. During recent decades, the species *A. quisqualis* has undergone several taxonomic reorganizations resulting in the assignment of the fast-growing strains (*in vitro* radial growth of 3–4 mm day^{-1}) to *P. glomerata* and other *Ampelomyces* spp., the slow-growing strains (0.5–1.0 mm day^{-1}) have been assigned to *A. quisqualis* sensu stricto (Kiss, 1997; Kiss & Nakasone, 1998). Molecular analyses based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (rDNA) have revealed a high level of genetic diversity among *A. quisqualis* sensu stricto strains (Angeli et al., 2009; Kiss, 1997; Kiss and Nakasone, 1998; Kiss et al., 2011; Liang et al., 2007; Nischwitz et al., 2005; Sullivan and White, 2000; Szentivanyi et al., 2005). Recently, phylogenetic studies have indicated that ITS groups could be related to the host fungus, suggesting, in most cases, a degree of mycohost specialization, although no evidence for a strict association has been found (Park et al., 2010; Pintye et al., 2012). Biocontrol potential of different *A. quisqualis* strains has been assessed on more than 15 powdery mildew fungi (Kiss et al. 2004). There is no evidence for host specificity, so it is current practice to consider *A. quisqualis* a pycnidial intracellular mycoparasite of all powdery mildews world-wide (Kiss et al. 2004).

1.2.1 An overview of the biology and natural occurrence

A. quisqualis is a naturally occuring mycoparasite specific against different powdery mildew agents. It can grow saprophytically, but has little chance to longer survive in natural environment without the host. Conidia of *A. quisqualis* are produced in pycnidia (fruiting bodies) developed intracellularly within powdery mildew hyphae, conidiophores (specialized spore-producing hyphae), and chasmothecia (the closed fruiting bodies of powdery mildews). In ca. 10-20 h under conditions of high humidity (Jarvis & Slingsby, 1977) conidia germinate and the hyphae of the mycoparasites can then penetrate the host. The early stage of mycoparasitism is apparently biotrophic, the later coincide with the death of the invaded cytoplasm (Hashioka & Nakai 1980, Sundheim & Krekling, 1982). Parasitized powdery mildew colonies can continue their radial growth, but their sporulation stops soon after *A. quisqualis* penetrated their mycelia. The conidia concentration on the leaves is relevant: germination rapidly decreases above a concentration of 10^6 cfu ml^{-1} due to the production of a self inhibitor (Gu & Ko 1997). There is no indication that in vitro toxic metabolites or allergenes are produced, however there is no report on absence of

toxicity and allergenicity of metabolites produced during the parasitic phase. Also the specificity to the powdery mildew is questioned as under high inoculum dosages it may be possible for this BCA to attack non-target fungal species and until its host range is identified, it is difficult to determine the risk to beneficial fungi and other soil organisms (Brimner & Boland 2003). Moreover, toxin production has not been detected (Beuther et al. 1981) in contrast to other pycnidial mycoparasites. The presence of host fungi is recognized by *A. quisqualis*; a water-soluble substance from powdery mildew conidia stimulates the germination of its conidia in vitro (Gu & Ko 1997), and growth directed to the host hyphae has also been observed. As with phytopathogenic fungi, penetration of the host cell wall involves both enzymatic and mechanical processes with appressorium-like structures (Sundheim & Krekling 1982). Extracellular lytic enzymes have been identified in liquid cultures of *A. quisqualis*, which may play a role in the degradation of the powdery mildew hyphal walls during penetration.

A. quisqualis has been found on more than 64 species of powdery mildew on 256 species of plants (Kiss, 1997; Kiss, 2003; Kiss et al, 2004). A 4-year study of the natural incidence of *A. quisqualis* in the field in a total of 27 species of powdery mildew fungi infecting 41 host plant genera showed that, in 16 out of the 27 powdery mildew species studied, pycnidia of *A. quisqualis* were present. The intensity of the mycoparasitism, defined as a percentage of the powdery mildew mycelia parasitized by *A. quisqualis* ranged from 0 to 65 % (Kiss, 1998). This wide host range, combined with tolerance to a number of fungicides used against powdery mildews, makes *A. quisqualis* the ideal candidate for use as a biological control agent (Falk et al., 1995; Sundheim & Tronsmo, 1988; Sztejnberg et al., 1989).

1.2.2 Biocontrol potential and its exploitation in sustainable agriculture

Biological control, a phenomenon based on the antagonism between micro-organisms, is considered as an alternative way to prevent or suppress powdery mildews in some crops. Microbial biocontrol agents have several mechanisms to antagonize their hosts and mycoparasitation is an effective tool to control plant pathogens. It is demonstrated by the fact that an hyperparasite *A. quisqualis* of plant pathogen fungi was the first microbial fungicide to be commercially developed and it is currently the most widely used, not only in organic agriculture, but also on conventional integrated pest management (IPM). Infact, a product based on *A.*

quisqualis conidia (AQ10 Biofungicide) is registered and commercialized. *A. quisqualis* is one of the most successful commercialized biocontrol agents, effective against powdery mildews on several crops (Whipps & Lumsden, 2001). Yarwood (1932) was the first author to identify the potential role of *A. quisqualis* as a biocontrol agent, although the first important efficacy trial was reported by Jarvis and Slingsby (1977) who used a conidial suspension of the mycoparasite to control cucumber powdery mildew in greenhouse. AQ10 is widely exploited to control powdery mildew of various crops but report data on the effectiveness in the powdery mildew control by AQ10 application are contradictory (Sztejnberg, 1993). In some experiments, good control of powdery mildews of various crops was achieved but other trials showed that the biocontrol was ineffective, although parasitism of powdery mildew colonies on the treated crops did occur (Angeli et al., 2009; Gilardi et al., 2012). There are a number of biotic and abiotic factors that do not seem favourable for the widespread and activity of *A. quisqualis* against powdery mildew fungi and temperature and relative humidity represents two important limiting factor in its use in biocontrol. However a number of examples of acceptable disease control have been reported for greenhouse and field-grown vegetable crops. Repeated applications are generally necessary, and high humidity and rainfall aid in spread to developing mycoparasite. The use of mycoparasites, entrains the tolerance of a certain level of disease as they can only attack established infections. Evolutionary considerations indicate also that mechanisms allowing the host to survive must be active such as faster growth and spread than that the hyperparasite or a mechanism that induces a reduction of aggressiveness on a weakened host. As many other BCAs its activity is often inconsistent, due to the lack of information of its interactions with the host and environment. A good knowledge of the mechanisms involved in mycoparasitism would help to design formulation additives or to select strains with enhanced abilities.

1.3 Aim of the thesis

This work fulfils the task 1 and task 2 of the activity of AMPELO, a project founded by the Autonomous Province of Trento and started in 2008. The wide objective of the project was to provide the basis for an advanced approach for developing low impact fungicides, being them microbial, enzymatic or chemical, through understanding the molecular mechanism of host

recognition, parasitisation and virulence regulation of a mycoparasite (*A. quisqualis*) of commercially worldwide important diseases (powdery mildews).

Grapevine is one of the most important crops in Trentino-Alto Adige (Northern Italy), with a cultivated area of approximately 10,000 ha. *Erysiphales* are among the most damaging plant diseases in Trentino Alto adige region. Powdery mildew, caused by the obligate biotrophic fungus *Erysiphe necator* (Schw.) Burr., is the most important diseases because of the presence of pathogen inoculum and favorable environmental conditions for its development. Uncontrolled epidemics of *E. necator* may result in yield losses, as well as a reduction in the quality of the produced wine (Gadoury et al., 2001; Ough & Berg, 1979). Control diseases relies mainly on the use of chemical fungicides alternative methods for controlling powdery mildews have been studied, including the use of microbial biocontrol agents (Paulitz and Bélanger, 2001; Elad et al., 1995). *A. quisqualis* is a widespread hyperparasite of powdery mildews (Falk et al. 1995; Kiss 2003; Kiss 1998) and it is the most studied biocontrol agent of powdery mildews (Kiss, 1997). This thesis has two principal objectives. The first is the investigation on the natural occurence of *A. quisqualis* on various *Erysiphaceae* species cultivated in environments with a moderate climate using the Alpine Valleys of Northern Italy (Trentino region) as case study. The second is the biological and mycoparasitic characterization of the genetically different *A. quisqualis* strains in order to develop a quick and simple method for selecting new strains better adapted to the local environmental conditions than commercial strain AQ10 and highly aggressive against *Erysiphaceae* for a potential development as biocontrol agents.

To achieve this, the work was structured in the following order:

In chapter 2 we evaluated the occurrence of *Erysiphe necator* chasmothecia in Trentino-Alto Adige region and monitored their development in the vineyards during a three-year survey. Moreover, the presence of *A. quisqualis* on grapevine powdery mildew in a wide viticulture area in Northern Italy (Trentino-Alto Adige region) was assessed. Wild strains morphologically distinct from the commercial *A. quisqualis* (AQ10) isolated from parasitized chasmothecia, commercial AQ10 strain and other strains obtained from genetic resource collection or other sources (CBS) are included in the following investigations.

In chapter 3 the population of *A. quisqualis* present in the vineyards of Trentino region together with AQ10 and other strains were morphologically and physiologically characterized. Furthermore, all strains were molecularly characterized following the methodology described by Szentivanyi (2005) determining the length polymorphisms of the amplified rDNA-ITS region and the amplicons fully sequenced. Sequence polymorphisms were used to classify and group the strains and the phylogenetic relationship among them was evaluated.

Chapter 4 regarded evaluation of some traits associated with strain aggressiveness and testing the hypothesis that the ability of *A. quisqualis* strains in colonizing powdery mildew pathogens and the *"in vitro"* production of cell wall degrading enzymes (CWDEs) secreted by the fungi are important factor in selecting *A. quisqualis* strains for biocontrol. Mycoparasitic and enzymatic assays were developed for the rapid and specific detection of new, highly effective strains for biocontrol.

In the last chapter (5) a general protocol was derived for optimizing the production of pure, high concentration *A. quisqualis* spore suspensions. We attemped to improve the efficiency of the hyperparasite in the biological control of powdery mildews by adding a certain number of additives to the inoculum in order to overcome humidity requirements of the fungus.

1.4 Literature

Andrews, J.H. (1992). Biological control in the phyllosphere. Annu Rev Phytopathol 30:603-635.

Angeli, D., Pellegrini, E., Micheli, S., Ress, D., Maurhofer, M., Pertot, I. and Gessler, C. (2009). Molecular characterization of *Ampelomyces* spp. strains from different hosts and geographic origins and evaluation of their potential to control powdery mildew of cucumber. IOBC/WPRS Bulletin, 43, 40-44.

Bélanger, R.R. and Benyagoub, M. (1997). Challenges and prospects for integrated control of powdery mildews in the greenhouse.Canad J Plant Pathol 19:310-314.

Bélanger, R.R. and Labbé, C. (2002). Control of powdery mildews without chemicals: prophylactic and biological alternatives for horticultural crops, in The powdery mildews: a comprehensive treatise, ed by Bélanger, R.R., Bushnell, W.R., Dik, A.J. and Carver, T.L.W., American Phytopathological Society, St Paul, USA, pp 256-267.

Beuther, E., Philipp, W.D. and Grossmann, F. (1981). Untersuchungen zum Hyperparasitismus von *Ampelomyces quisqualis* auf Gurkenmehltau (Sphaerotheca fuliginea). Phytopathologische Zeitschrift 101, 265-270.

Braun, U. (1987). A monograph of the Erysiphales (powdery mildews). B. Nova Hedwigia 89: 1-700.

Brimner, T. and Boland, G. (2003). A review of the non-target effects of fungi used to biologically control plant diseases. *Agriculture, Ecosystems & Environment*, 100(1), 3-16.

Choudbury, S.R., Traquair, J.A. and Jarvis, W.R. (1994). 4-Methyl-7,11-heptadecadenal and 4-methyl-7,11-heptadecadienoic acid: new antibiotics from Sporothrix flocculosa and Sporothrix rugulosa. J Nat Prod 57:700-704.

Cook, R.J. and Baker, K.F. (1983). The nature and practice of biological control of plant pathogens, American Phytopathological Society, St Paul, MN.

Elad, Y., Malathrakis, N.E. and Dik, A.J. (1996). Biological control of *Botrytis*-incited diseases and powdery mildews in greenhouse crops. Crop Protect 15:229-240.

Elad, Y., Kirshner, B. and Sztejnberg, A. (1998). Management of powdery mildew and gray mold of cucumber by *Trichoderma harzianum* mT39 and *Ampelomyces quisqualis* AQ10. BioControl 43:241-251.

Elad, Y. (2000). Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Protect 19:709-714.

Falk, S.P., Gadoury, D.M., Cortesi, P., Pearson, R.C. and Seem R.C. (1995). Parasitism of *Uncinula necator* cleistothecia by the mycoparasite *Ampelomyces quisqualis*. Phytopathology 85:794-800.

Gadoury, D.M., Seem, R.C., Pearson, R.C., Wilcox, W.F. and Dunst, R.M. (2001). Effects of powdery mildew on vine growth, yield, and quality of Concord grapes. Plant Dis. 85:137-140.

Gilardi, G., Baudino, M., Garibaldi, A., Gullino, M.L. (2012). Efficacy of biocontrol agents and natural compounds against powdery mildew of zucchini. Phytoparasitica 40, 147-155.

Gu, Y.H. and Ko, W.H. (1997). Water agarose medium for studying factors affecting germination of conidia of *Ampelomyces quisqualis*. Mycological Research 101, 422-424.

Hajlaoui, M.R., Traquair, J.A., Jarvis, W.R. and Bélanger R.R. (1994) Antifungal activity of extracellular metabolites produced by Sporothrix flocculosa. Biocontrol Sci Technol 4:229-237.

Hashioka, Y. and Nakai, Y. 1980. Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. Trans. Mycol. Soc. Jpn 21:329-338.

Heintz, C. and Blaich, R. (1990). *Verticillium lecanii* als Hyperparasit des Rebmehltaus (*Uncinula necator*). Vitis 29:229-232.

Hewitt, H.G. (1998). Fungicides in crop protection, CABI Publishing;Wallingford, UK.

Hijwegen, T. and Buchenauer, H. (1984). Isolation and identification of hyperparasitic fungi associated with Erysiphaceae. Neth J Pl Path 90:79-84.

Hijwegen, T. (1988). Effect of seventeen fungicolous fungi on sporulation of cucumber powdery mildew. Neth J Pl Path 94:185-190.

Hofstein, R. and Fridlender, B. (1994). Development of production, formulation and delivery systems, in Proc Brighton Crop Protect Conf Pests Dis, BCPC, Farnham, Surrey, UK, pp 1273-1280.

Hofstein, R., Daoust, R.A. and Aeschlimann, J.P. (1996). Constraints to the development of biofungicides: the example of 'AQ-10', a new product for controlling powdery mildews. Entomophaga 41:455-460.

Kiss, L. (1997). Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. Mycol. Res. 101:1073-1080.

Kiss, L. (1998). Natural occurrence of *Ampelomyces* intracellular mycoparasites in mycelia of powdery mildew fungi. New Phytol. 140:709-714.

Kiss, L. and Nakasone, K. K. (1998). Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi. Curr. Gen. 33:362-367.

Kiss, L. (2003). A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Management Science, 59, 475-483.

Kiss, L., Russell, J.C., Szentivanyi, O., Xu, X., Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonist of powdery mildew fungi. Biocontrol Science and Technology 14, 635-651.

Kiss, L., Pintye A., Kovacs G.M., Jankovics T., Fontaine M.C., Harvey N., Xu X., Nicot P.C., Bardin M., Shykoff J.A. and Giraud T. (2011). Temporal isolation explains host-related genetic differentiation in a group of widespread mycoparasitic fungi. Molecular Ecology, 20, 1492-1507.

Klecan, A.L., Hippe, S. and Somerville, S.C. (1990). Reduced growth of *Erysiphe graminis* f sp hordei induced by *Tilletiopsis pallescens*. Phytopathology 80:325-331.

Knudsen, I.M.B. and Skou, J.P. (1993). The effectivity of *Tilletiopsis albescens* in biocontrol of powdery mildew. Ann Appl Biol 123:173-185.

Kranz, J. (1981). Hyperparasitism of biotrophic fungi, in Microbial Ecology of the Phylloplane (Blakeman, J. P., Ed.). Academic Press, London, UK, pp. 327-352.

Ishii, H., Fraaije, B.A., Sugiyama, T., Noguchi, K., Nishimura, K., Takeda, T., Amano, T. and Hollomon, D.W. (2001). Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. Phytopathology 91:1166-1171.

Jarvis, W.R. and Slingsby, K. (1977). The control of powdery mildew of greenhouse cucumber by water sprays and *Ampelomyces quisqualis*. Plant Dis Rep 61:728-730.

Liang C., Yang J., Kovacs G.M., Szentivanyi O., Li B., Xu X.M. & Kiss L. (2007). Genetic diversity of *Ampelomyces* mycoparasite isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences. Fungal Diversity, 24, 225-240.

McGrath, M.T. and Shishkoff, N. (1999). Evaluation of biocompatible products for managing cucurbit powdery mildew. Crop Protect m18:471-478.

Menzies, J.G. and Bélanger, R.R. (1996). Recent advances in cultural management of diseases of greenhouse crops. Canad J Plant Pathol 18:186-193.

Nischwitz, C., Newcombe, G., Anderson, C.L. (2005). Host specialization of the mycoparasite *Eudarluca caricis* and its evolutionary relationship to *Ampelomyces*. Mycological Research 109, 421-428.

Ough, C.S. and Berg, H.W. (1979). Powdery mildew sensory effect on wine. Am. J. Enol. Vit. 30:321.

Park M.J., Choi Y.J., Hong S.B. & Shin H.D. (2010). Genetic variability and mycohost association of *Ampelomyces quisqualis* isolates inferred from phylogenetic analyses of ITS rDNA and actin gene sequences. Fungal Biology, 114, 235-247.

Pasini, C., D'Aquila, F., Curir, P. and Gullino, M.L. (1997). Effectiveness of antifungal compounds against rose powdery mildew (*Sphaerotheca pannosa* var rosae) in glasshouses. Crop Protect 16:251-256.

Paulitz, T.C. and Bélanger, R.R. (2001). Biological control in greenhouse systems. Annu Rev Phytopathol 39:103-133.

Pintye, A., Bereczky, Z., Kovács, G.M., Nagy, L.G., Xu, X., Legler, S.E., Váczy, Z., Váczy, K.Z., Caffi, T., Rossi, V., Kiss, L. (2012). No indication of strict host associations in a widespread mycoparasite: grapevine powdery mildew (*Erysiphe necator*) is attacked by phylogenetically distant *Ampelomyces quisqualis* strains in the field. Phytopathology 102:707-716.

Sullivan, R.F. and White, Jr. J.F. (2000). *Phoma glomerata* as a mycoparasite of powdery mildew. Applied and Environmental Microbiology, 66, 425-427.

Sundheim, L. (1982). Control of cucumber powdery mildew by the hyperparasite *Ampelomyces quisqualis* and fungicides. Plant Pathol 31:209-214.

Sundheim, L., Krekling, T. (1982). Host-parasite relationships of the hyperparasite *Ampelomyces quisqualis* and its powdery mildew host *Sphaerotheca fuliginea.* I. Scanning electron microscopy. Phytopathology 104, 202-210.

Sundheim, L. and Tronsmo, A. (1988). Hyperparasites in biological control. Pages 53-69 in: Biocontrol of Plant Diseases. Vol. I. CRC Press, Boca Raton, FL.

Sutton, B.C. (1980). The Coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata. Commonwealth Mycological Institute, Kew, UK.

Szentiványi, O., Kiss, L., Russell, J.C., Kovács, G.M., Varga, K., Jankovics, T., Lesemann, S., Xu, X.M. and Jeffries, P. (2005). *Ampelomyces mycoparasites* from apple powdery mildew identified as a distinct group based on single-stranded conformation polymorphism analysis of the rDNA ITS region. Mycological Research, 109, 429-438.

Sztejnberg, A., Galper, S., Mazar, S., and Lisker, N. (1989). *Ampelomyces quisqualis* for biological and integrated control of powdery mildews in Israel. J. Phytopath. 124:285-295.

Sztejnberg, A. (1993). *Ampelomyces quisqualis* AQ10, CNCM I-807, for biological control of powdery mildew. US patent no. 5190754.

Urquhart, E.J., Menzies, J.G. and Punja, Z.K. (1994). Growth and biological control activity of *Tilletiopsis* species against powdery mildew (*Sphaerotheca fuliginea*) on greenhouse cucumber. Phytopathology 84:341-351.

Whipps, J.M. and Lumsden, R.D. (2001). Commercial use of fungi as plant disease biological control agents: status and prospects, in Fungi as Biocontrol Agents: Progress, Problems and Potential (Butt, T.M., Jackson, C. And Magan, N., Eds.). CABI Publishing, Wallingford, UK, pp. 9-22.

Yarwood, C.E. (1932). *Ampelomyces quisqualis* on clover mildew. Phytopathology 22, 31 (Abstr.).

Chapter 2

Occurrence of *Erysiphe necator* chasmothecia and their natural parasitization by *Ampelomyces quisqualis* in Northern Italy (Trentino Alto Adige region)

Published as

Angeli D., Pellegrini E., Pertot I. 2009. Occurrence of *Erysiphe necator* Chasmothecia and Their Natural Parasitization by *Ampelomyces quisqualis*. Phytopathology, 99: 704-710.

Abstract

In Northern Italy, *Erysiphe necator* overwinters almost exclusively as chasmothecia. From 2004 to 2008, we investigated the occurrence of natural parasitism of grapevine powdery mildew chasmothecia by *Ampelomyces quisqualis* in the Trentino-Alto Adige region, in northern Italy. The survey was conducted in 18 vineyards in autumns 2004 and 2005 and in 45 vineyards in autumns 2006 and 2007. The incidence of powdery mildew signs (white powdery mycelia and conidia), the number of chasmothecia and their development pattern, and the incidence of parasitism by *A. quisqualis* were assessed. The production of *E. necator* chasmothecia on leaves is related to the incidence and severity of the disease on leaves at the end of the season and is not correlated with the elevation of the vineyard, which is inversely related to the temperature. A limited amount of natural parasitism of *E. necator* by *Ampelomyces* spp. (0.17−3.51 %) was observed in all of the years of the survey. Pycnidia and conidia of *Ampelomyces* spp. parasitizing *E. necator* chasmothecia were found both in conventional, organically grown on and untreated vineyards. Some of the isolated *Ampelomyces* strains have conidia that are shaped differently than those of the commercial *A. quisqualis* strain (AQ10) and are phylogenetically different from AQ10.

2.1 Introduction

Grapevine powdery mildew, caused by the obligate biotrophic fungus *Erysiphe necator* (Schw.) Burr., is one of the most important grapevine diseases in Italy, because of the presence of pathogen inoculum and favorable environmental conditions for its development. Uncontrolled epidemics of *E. necator* may result in yield losses, as well as a reduction in the quality of the produced wine (Gadoury et al., 2001; Ough & Berg, 1979). During the winter, the fungus survives either as mycelium in the dormant buds of grapevine or as chasmothecia, which are the fruiting bodies arising from the sexual stage (Bulit & Lafon, 1978; Pearson & Goheen, 1988). Mycelium preserved inside the bud is thought to give rise to so called flag shoots in spring. The infected flag shoots are stunted, deformed and covered with white powdery mycelium and conidia (Rumbolz & Gubler, 2005). In contrast, chasmothecia represent the main source of primary inoculum in regions with cold winters, such as are found in northern Italy. In the spring,

ascosporic infections originating from chasmothecia commonly appear randomly in the vineyard as scattered whitish and powdery spots on leaves (1 to 3 mm in diameter) that mainly appear on leaves close to the trunk (Grove, 2004; Pearson & Gadoury, 1987; Sall & Wrysinski, 1982; Ypema & Gubler, 2000). Grapevine is one of the most important crops in Trentino-Alto Adige (northern Italy), with a cultivated area of approximately 10,000 ha. Chasmothecia are thought to be the main overwintering form of *E. necator* in this region (Angeli et al., 2006).

Ampelomyces quisqualis Ces. is a naturally occurring mycoparasite of several powdery mildew species (*Erysiphales*), including *E. necator* (Falk et al., 1995a; Kiss, 1998; Sundheim & Krekling 1982). *A. quisqualis* has been considered to be a single species for a long time, but its taxonomy has become controversial and probably merits extensive revision (Kiss & Nakasone, 1998). The morphology of the conidia and pycnidia of different *A. quisqualis* isolates and the growing patterns of colonies of these isolates on laboratory media are highly variable (Kiss et al., 2004). The pycnidia of *Ampelomyces* spp. also vary in shape depending upon the fungal structure in which they were formed. They are pear-shaped, spindle-shaped or nearly spherical when they are formed inside *E. necator* conidiophores, hyphae or chasmothecia, respectively. Pycnidia contain cylindrical to spindle-shaped conidia, which are occasionally curved and two-spotted (Falk et al., 1995a). Recent molecular studies have hypothesized the existence of more than one species in the genus *Ampelomyces*. Analyses of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (nrDNA) of several putative *A. quisqualis* strains have uncovered a high level of genetic diversity (Kiss, 1997; Kiss & Nakasone, 1998; Sullivan & White, 2000; Szentiványi et al., 2005), which suggests that the binomial *A. quisqualis* should be regarded as a species complex (Kiss & Nakasone, 1998).

A. quisqualis has been found on more than 64 species of powdery mildew on 256 species of plants (Kiss, 1997; Kiss, 2003; Kiss et al., 2004). This wide host range, combined with tolerance to a number of fungicides used against powdery mildews, makes *A. quisqualis* the ideal candidate for use as a biological control agent (Falk et al., 1995b; Sundheim & Tronsmo, 1988; Sztejnberg et al., 1989). An *A. quisqualis* strain isolated in Israel has been formulated, registered, and commercialized in several countries under the trade name “AQ10” (Sztejnberg, 1993).

A. quisqualis can grow saprophytically for short periods of time, but has little chance of surviving for longer periods in natural environments without parasitizing a powdery mildew

host. It requires water to germinate and to infect powdery mildew colonies. Infections can occur in less than 24 h at 25 °C (Kiss et al., 2004; Sundheim & Krekling, 1982). *A. quisqualis* invades and grows within powdery mildew hosts (Hashioka & Nakai, 1980; Sundheim & Krekling, 1982). Parasitized powdery mildew colonies are dull, flattened and off-white to gray in color. Pycnidia are formed within hyphae, conidiophores, conidia and the immature chasmothecia of powdery mildews. Once the mycoparasite has begun to produce pycnidia, the hyphae and conidiophores swell to several times their normal diameter and the amber color of the pycnidial walls of *A. quisqualis* may be visible through the cell walls of the host (Falk et al., 1995a). Several studies have shown that *A. quisqualis* cannot parasitize mature *E. necator* chasmothecia (Falk et al., 1995a; Kiss et al., 2004). Parasitized chasmothecia are typically dull, fawn-colored, and flaccid and range from 64 to 130 μm in diameter (5). *A. quisqualis* parasitism reduces powdery mildew sporulation, as well as the production of chasmothecia and may eventually kill the entire mildew colony (Falk et al., 1995a; Falk et al., 1995b; Hashioka & Nakai, 1980; Kiss et al., 2004; Sundheim & Krekling, 1982).

While a few studies have reported the presence of *A. quisqualis* on several powdery mildews on several plants (Kiss, 1998; Kiss et al., 2004), no study has reported on the extent of the natural occurrence of *A. quisqualis* on grapevine powdery mildew. The aims of this study were i) to assess and quantify the presence of *Ampelomyces* spp. on grapevine powdery mildew in a wide viticulture area in northern Italy (Trentino-Alto Adige region); and ii) to characterize the isolates present in the area. We also evaluated the occurrence of chasmothecia in Trentino-Alto Adige region and monitored their development in the vineyard.

2.2 Material and Methods

2.2.1 Study sites, assessment, and sampling

The study was carried out from 2004 to 2007 in the Trentino-Alto Adige region of northern Italy. Sampling was carried out between 15 and 31 October in 18 vineyards from 2004 to 2007. Additionally, 27 vineyards were sampled in 2006 and 2007, respectively. The vineyards were randomly selected. The sampled vineyards included conventionally and organically managed vines; abandoned (untreated) vineyards were also surveyed. The chemical fungicides used in the

conventionally managed vineyards included mancozeb, folpet, dimethomorph, zoxamide, iprovalicarb, quinoxyfen, spiroxamine, copper, sulphur, acylalanines, strobilurins and triazoles. In the organic vineyards, only sulphur and copper were used. *A. quisqualis* (AQ10; Ecogen, Langhorne, PA, USA) was not used in any of the monitored vineyards during the survey period. The size of each sampled vineyard ranged between 800 and 1200 m^2. In each vineyard and year, four replicates of 25 leaves each were randomly collected at the fifth leaf from the shoot. For each replicate, the percentage of infected area on the upper surface of the leaf (whitish, powdery spots) and the number of infected leaves were visually assessed. Disease severity (percentage of infected leaf area) and incidence (percentage of infected leaves) were calculated. A disk (2 cm in diameter) was cut from the central part of each sampled leaf. The *E. necator* chasmothecia on the upper side of each leaf disk were counted under a stereomicroscope (Nikon SMZ 800, Tokyo, Japan). To identify the optimal times for assessing parasitism by and development of *Ampelomyces* spp., the ripening of chasmothecia was surveyed weekly between August and November in an untreated experimental vineyard (cv. Schiava) in S. Michele all'Adige on a sample of 100 randomly collected leaves. The number of chasmothecia was assessed as described above. Chasmothecia were classified into three categories according to their color, which reflects their development stage: yellow (young), brown (semi-mature), and black (mature). On each leaf (100 leaves per vineyard, collected when black chasmothecia were 50 ± 10%), *Ampelomyces* spp. mycoparasitism of *E. necator* was assessed under a light microscope (Hund Wetzlar H 600LL, Wetzlar, Germany), in terms of the presence of parasitized dull, flaccid and fawn-colored chasmothecia, brownish intracellular pycnidia in *E. necator* hyphae, and/or cylindrical, spindle-shaped, and two-spotted conidia. In detail, *E. necator* mycelium and chasmothecia were transferred into Eppendorf tubes by brushing the upper surface of sampled leaves (four tubes for each replicate of 25 leaves) and stored at 4 °C. One hundred chasmothecia from each tube were mounted in lactophenol and observed under a light microscope to asses the percentage of parasitized cleistothecia, as well as the presence of *Ampelomyces* spp. pycnidia and conidia. Since *Ampelomyces* spp. infect and produce pycnidia only inside young and semi-mature chasmothecia, as reported in several studies of parasitism of *E. necator* (Falk et al., 1995a; Kiss et al., 2004), when black chasmothecia were 50 ± 10 % of the total, we selected yellow and brown chasmothecia and excluded mature dark brown chasmothecia. Chasmothecia were gently crushed by pressing the cover slip over the glass slide on which the sample was

spread, to allow the release of *Ampelomyces* spp. conidia and pycnidia. The possible presence of *Ampelomyces* spp. pycnidia in the sample was recorded. The incidence of *Ampelomyces* spp. in each vineyard was calculated as the percentage of *E. necator* chasmothecia parasitized by *Ampelomyces* spp. During 2005 to 2008, between April and May of each year, the vineyards were monitored weekly to check for the presence of flag shoots.

2.2.2 Isolation, identification, morphological, and molecular characterization of isolates of *Ampelomyces* spp.

Ampelomyces spp. samples on parasitized chasmothecia and mycelia were initially identified by comparing the morphological characteristics of the observed conidia and pycnidia with those described in the literature (Falk et al., 1995a; Kiss, 1998; Kiss et al., 2004). Some of the *Ampelomyces* spp. samples were isolated by transferring the conidia onto potato dextrose agar (PDA, Oxoid, Hampshire, UK) amended with 2 % chloramphenicol (Sigma, St. Louis, MO, USA). Morphological identification and measurements of pycnidia and conidia (length, width, and shape) were carried out on twenty replicates (pycnidia) for each isolate. Five isolates (ITA 1, ITA 2, ITA 3, ITA 4, and ITA 5) were selected for the genetic analysis, each representing the different morphological shapes (fusiform or ellipsoid) found in homogeneous areas of Trentino region (Trento North, Trento South, and Rovereto). The mycelium of each of five 20-day-old *Ampelomyces* spp. isolates grown on PDA was collected from Petri dishes and freeze-dried. DNA was extracted from 2 μg of homogenized lyophilized mycelia, using the Nucleo Spin Plant Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. The ITS region of the nuclear ribosomal DNA was amplified using the specific fungal primers ITS1 and ITS4 (White et al., 1990). PCR was performed in the Gene Amp PCR System 9700 (Perkin Elmer, Waltham, MA, USA) and the following cycling parameters were used: initial denaturing step at 95 °C for 3 min, 35 cycles of denaturing step at 95 °C for 30 s, primer annealing at 60 °C for 30 s, extension step at 72 °C for 30 s and a final extension at 72 °C for 7 min. PCR products were detected by electrophoresis on 1 % agarose gel in TBE buffer supplemented with ethidium bromide (0.5 μl/ml). PCR products were purified using the ExoSAP-IT enzymes (USB Corporation, Staufen, Germany) and sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Both DNA strands were

sequenced with the primers used for PCR amplification; electrophoresis was carried out using an ABI 3130xl Genetic Analyzer (Perkin Elmer). The consensus sequence was assembled using Pregap4 and Gap4 (Staden et al., 2000). Searches were performed in the NCBI/GenBank database to find the closest relatives of the sequenced isolates and the similitude percentages through the BLAST method (Altschul et al., 1990). The ClustalW2 program, available for free on the internet (Larkin et al., 2007), was used to construct multiple sequence alignments using the DNA identity matrix and to visualize the evolutionary relationship between the input sequences. The alignments were checked and edited using Bioedit 7.0.5.2. (Hall, 1999) to generate an alignment of the same length for inferring phylogenies. Maximum-likelihood and neighbor-joining analyses based on ITS region sequences were conducted using respectively the programs BioEdit 7.0.5.2. and Mega4 (Tamura et al., 2007) with the Jukes-Cantor substitution model and with rate uniformity among sites. All positions containing gaps and missing data were eliminated. The branches of the inferred tree were tested by bootstrap analysis (Felsenstein, 1985) with 1000 replicates. The trees were visualized using TreeView 1.6.6 (Page, 1996).

2.2.3 Meteorological data and statistical analysis

Meteorological data (rain, hourly temperature and relative humidity) were collected by the local agro-meteorological service (http://meteo.iasma.it/meteo/). Sum of hours with optimal temperatures (from 20 to 27 °C) for powdery mildew infection and disease development and total rain (Pearson & Goheen, 1988) were calculated from May 1 to October 31. Statistical analyses were performed using Statistica software 6.0 (Statsoft, Tulsa, OK, USA). Disease incidence and severity data were Arcsin-transformed to normalize the data. One-way analysis of variance (ANOVA) was used to compare differences in the incidence and severity in the different years. Means were separated using Tukey's test ($\alpha = 0.05$). The Chi-square test followed by Ryan's multiple comparison test ($P \leq 0.05$) (Ryan, 1960) was used to compare differences in the presence of *Ampelomyces* in the different years.

2.3 Results

2.3.1 Natural occurrence of *E. necator*

The assessment of powdery mildew infections in the sampled vineyards at the end of the growing seasons detected increases in disease severity and incidence between 2004 and 2007 (Fig. 1). The monitoring showed both high incidences and high severity of *E. necator* infections in 2006 and 2007 (34 and 45 % of average severity and 45 and 67 % of average incidence in all vineyards, respectively) probably because of the favorable climatic conditions for powdery mildew (Pearson & Goheen, 1988) present after veraison through the end of the growing season (Fig. 2). In 2006 and 2007, it was cooler and rainier during the last part of the season, as compared to the earlier seasons (data not shown). Similar to the findings concerning disease severity and disease incidence on leaves, the populations of *E. necator* chasmothecia were lower in 2004 and 2005 than they were in 2006 and 2007 (Fig. 3). There was a very high correlation between the severity of powdery mildew infections on leaves in the vineyard (average of the four replicates) and the number of *E. necator* chasmothecia per unit of leaf area (average of the four replicates; Fig. 4). A similar high correlation was observed between powdery mildew incidence and the number of chasmothecia per unit of leaf surface area. In 2004, 2005, 2006, and 2007, R^2 values were 0.938 ($y = 4.3898x - 63.046$), 0.952 ($y = 4.6758x - 65.763$), 0.984 ($y = 5.4574x - 88.023$), and 0.972 ($y = 6.2413x - 156.83$), respectively. There was no correlation between the number of chasmothecia per unit of leaf area and the elevation of the different vineyards, which is inversely related to temperature. No flag shoots were seen in the vineyards in the years of the survey and infections began (seemingly) randomly in the monitored vineyards.

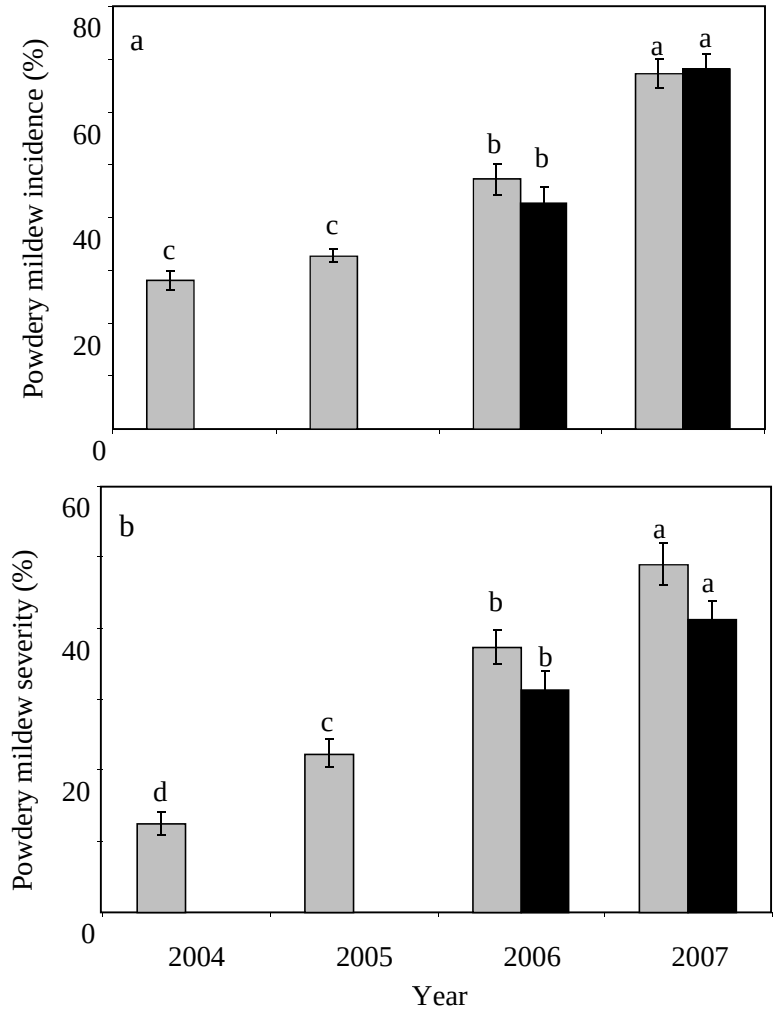


Figure 1. Average powdery mildew incidence (a, percentage of leaves that were infected) and severity (b, percentage of leaf area that was infected) in vineyards in the Trentino-Alto Adige region that were sampled at the end of each growing season (15-31 October). Four replicates (25 leaves per replicate) were assessed in each vineyard. Gray bars indicate the average of the 18 vineyards monitored over the 4 years; black bars indicate the average values in the 27 vineyards that were only surveyed in 2006 and 2007. Error bars represent one standard error of the mean. Columns with the same letter (a-d) are not significantly different from one another ($P \leq 0.05$) according to Tukey's test.

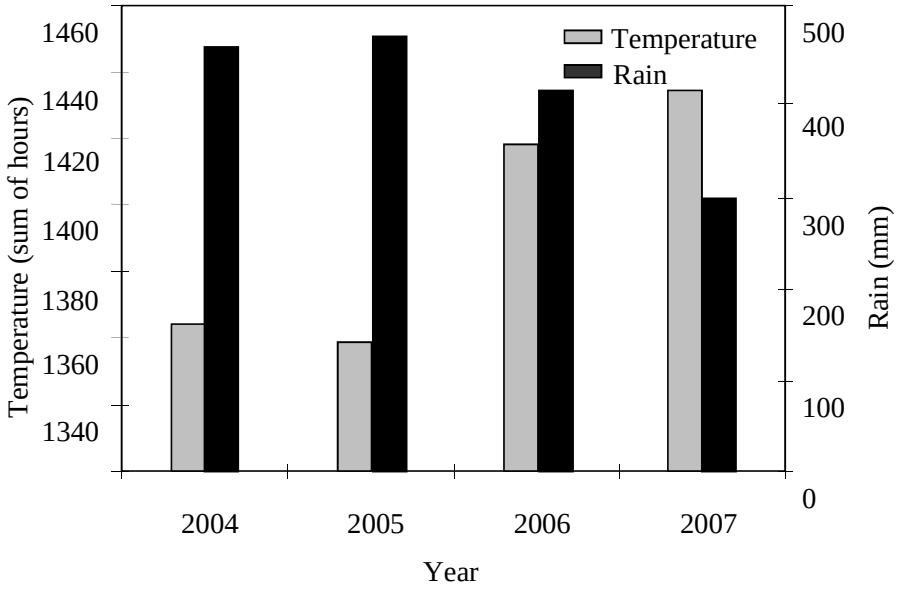


Figure 2. Periods with suitable temperature for powdery mildew (sum of hours with temperature between 20 °C and 27 °C) and total rain from May 1 to October 31 of each year in S. Michele all'Adige (Trentino-Alto Adige region).

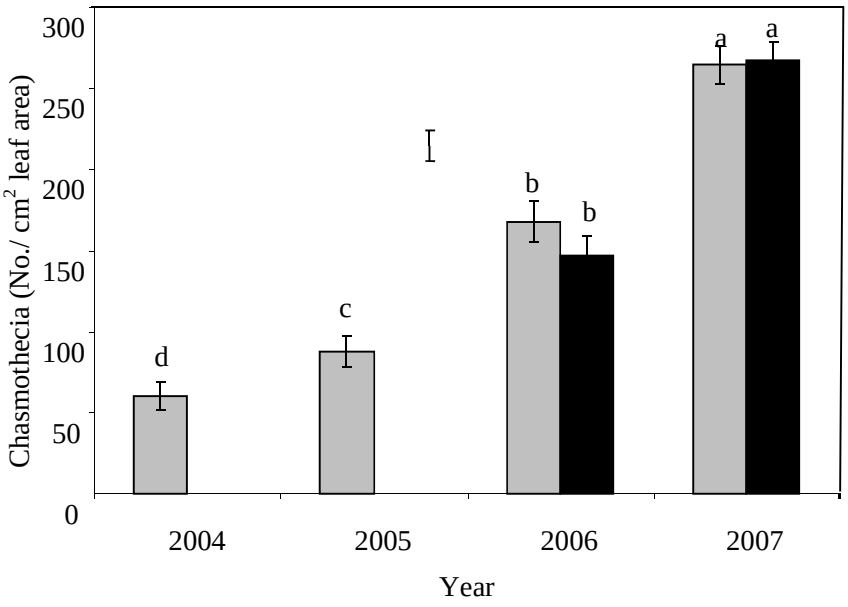


Figure 3. Number of chasmothecia per unit of leaf area (cm^2) in the sampled vineyards in the Trentino-Alto Adige region at the end of each growing season (15-31 October). Four replicates (25 leaves per replicate) were collected and chasmothecia were counted on leaf disk samples cut from the middle of each

leaf. Gray bars indicate the average value for the 18 vineyards monitored for the four years; black bars indicate the average values for the 27 vineyards that were only surveyed in 2006 and 2007. Error bars represent the standard errors of the means. Columns with the same letter (a-d) are not significantly different ($P \leq 0.05$) according to Tukey's test.

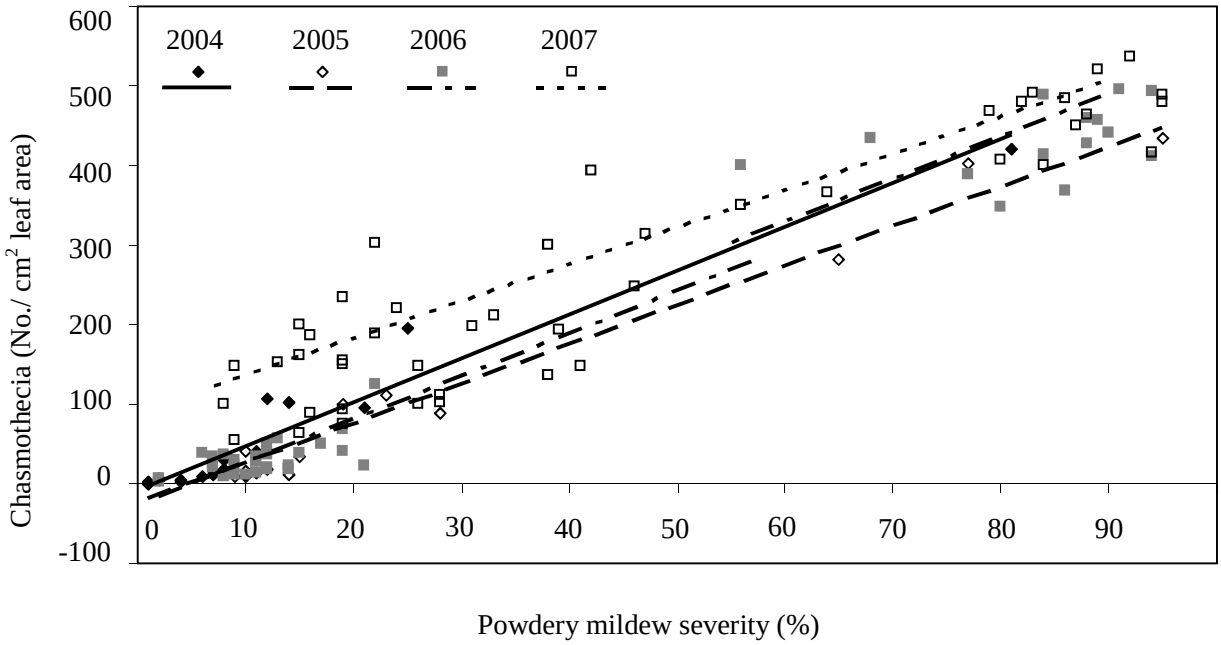


Figure 4. Correlation between powdery mildew severity and the number of chasmothecia per unit of leaf area in the monitored vineyards in the Trentino-Alto Adige region at the end of the growing season (2004-2007). Four replicates (25 leaves per replicate) were surveyed for disease incidence and one disk was excised from each leaf for the chasmothecia counting.

2.3.2 Development of powdery mildew chasmothecia

Almost identical results were obtained during each of the four years of the survey. Therefore, only data collected in 2005 are presented here. The first signs of the initial development of chasmothecia were observed on *E. necator*-infected leaves in the experimental vineyard of S. Michele all'Adige in the beginning of August. The number of translucent white masses, which represent the primary stage of chasmothecia formation, quickly increased during August whilst

the amount of dark mature chasmothecia began to increase rapidly after the middle of October (Fig. 5).

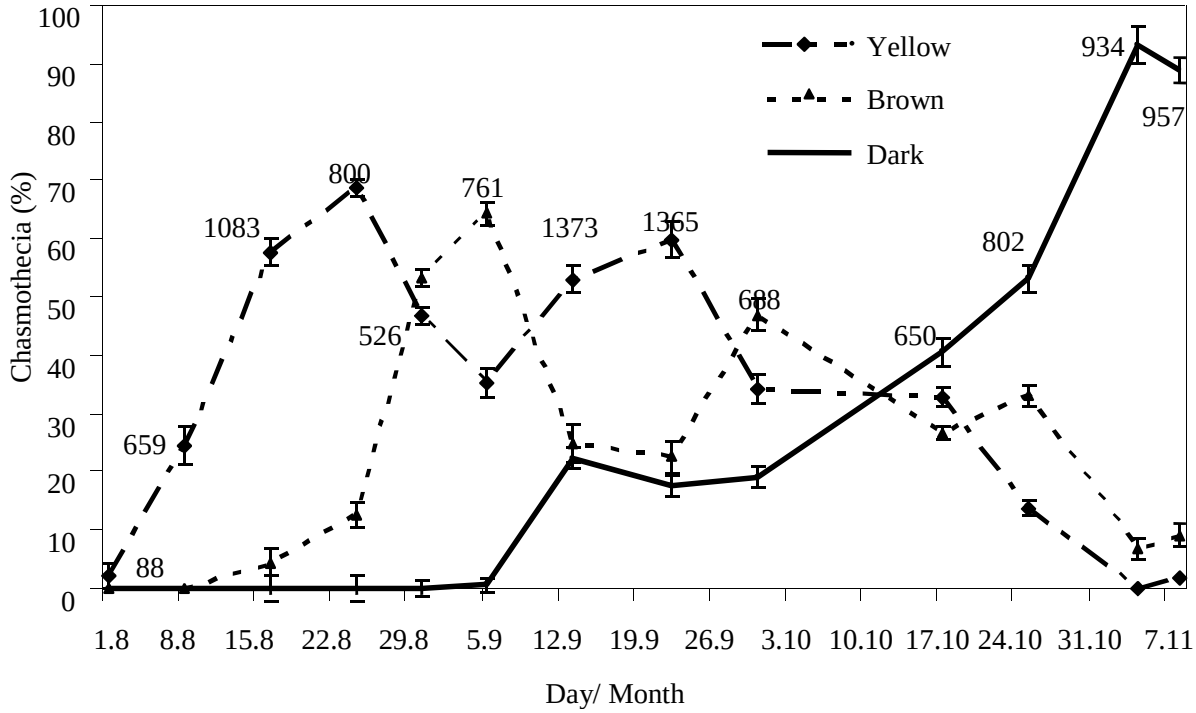


Figure 5. Development of chasmothecia on leaves in an untreated vineyard in S. Michele all'Adige in 2005. Each week, the number of chasmothecia on a sample of 100 infected leaves (one leaf disk, 1 cm in diameter, cut from the central part of each leaf) was counted. The percentages of young (yellow colored), semi-mature (brown colored), and mature (dark-brown colored) chasmothecia per square centimeter of upper leaf surface were determined. Numbers represent the total number of chasmothecia counted on each date.

2.3.3 Occurrence of *Ampelomyces* spp. in *E. necator*

E. necator chasmothecia were naturally parasitized by *Ampelomyces* and fruiting bodies of the mycoparasite (pycnidia and conidia) were found throughout Trentino-Alto Adige region on leaves of different vine cultivars. Some of these cultivars are highly susceptible to powdery mildew (e.g., Schiava, Muller Thurgau, Lagrein, Teroldego, Marzemino, and Chardonnay), some are moderately susceptible (e.g., Nosiola, Traminer, Pinot Gris, and Pinot Noir) and some are not susceptible at all (e.g., Riesling, Merlot, Cabernet Sauvignon, and Moscato Giallo). The results

of this four-year monitoring program underline the generally low natural presence of *Ampelomyces* spp. in the vineyards of the Trentino-Alto Adige region (Table 1).

In the first two years no differences in the percentage of parasitism in the vineyard were seen, but the total number of chasmothecia parasitized by *Ampelomyces* significantly increased over the period of the study (χ^2 = 102.98; df = 3; $P \leq 0.001$). The average rate of parasitism among chasmothecia in all of the monitored vineyards ranged from 0.17 % (2004) to 3.51 % (2007). Once *Ampelomyces* spp. were first found in a vineyard, it was also found in that vineyard in following years. *Ampelomyces* spp. was found on leaves, mainly as conidia in parasitized *E. necator* chasmothecia and, in four vineyards, as pycnidia in the powdery mildew mycelia. Because of the limited number of *E. necator* samples parasitized by *Ampelomyces* spp. and the limited numbers of available untreated/abandoned and organic vineyards in the area, no relationship between treated (conventional and biological treatments) or untreated/abandoned vineyards and *A. quisqualis* parasitism of powdery mildew could be identified. A weak relationship was observed between the number of chasmothecia per unit of leaf surface area and the percentage of chasmothecia parasitized by *Ampelomyces* spp. during the period 2004-2007 (R^2 = 0.366, y = 7E – $0.5x^2$ – 0.0165 + 0.5218).

Table 1. Incidence of *Erysiphe necator* and presence of *Ampelomyces* spp. on grapevine in Trentino-Alto Adige region (2004-2007)

Year	Monitored vineyards		Incidence of powdery mildew[a]	Chasmothecia per unit leaf area[a]	Vineyard with presence of *Ampelomyces* spp.[b]	Chasmothecia parasitized by *Ampelomyces*[c]	Average of chasmothecia parasitized by *Ampelomyces*[d]
	Type	No.	(% ± SE)	(No./cm^2 ± SE)	(No.)	(% ± SE)	(%)
2004	conventional	16	22.8 ± 3.65	35 ± 12.65	0	0	0.17 a
	organic	1	40.0 ± 3.92	102 ± 10.26	0	0	
	untreated	0	-	-	-	-	
	abandoned	1	100 ± 0	420 ± 22.73	1	3.0	
2005	conventional	16	25.0 ± 2.93	46 ± 11.19	1	2.0	0.44 a
	organic	1	90.0 ± 3.01	402 ± 18.65	1	3.0	
	untreated	0	-	-	-	-	
	abandoned	1	100 ± 0	433 ± 24.57	1	3.0	
2006	conventional	35	30.1 ± 3.42	77 ± 12.69	6[e]	9.2 ± 4.37	1.97 b
	organic	2	90.0 ± 4.21	401 ± 20.87	1	5.0	
	untreated	7	95.0 ± 3.29	427 ± 17.56	3	5.7 ± 2.33	
	abandoned	1	100 ± 0	457 ± 21.89	1	4.0	
2007	conventional	35	58.8 ± 2.65	207 ± 15.88	10[e]	10.5 ± 3.58	3.51 c
	organic	2	100 ± 0	502 ± 26.56	1	7.5	
	untreated	7	96.2 ± 2.54	441 ± 20.21	4	6.3 ± 2.55	
	abandoned	1	100 ± 0	521 ± 17.96	1	5.0	

[a] Values are means of four replicates (25 leaves each) per vineyard. Leaves were randomly collected at the end of the growing season (after harvest, in October-November) and examined under the light microscope. Standard errors of the means are reported (SE).

[b] Vineyards where *Ampelomyces* spp. conidia were found in at least one chasmothecia.

[c] Average of parasitized chasmothecia in the vineyards where parasitism was present. The percentage of parasitized chasmothecia was calculated by checking 100 chasmothecia per site.

[d] Average of parasitized chasmothecia in all monitored vineyards. Values followed by the same letter (a-c) are not significantly different ($P \leq 0.05$) according to the χ^2-test followed by Ryan's multiple comparison test.

[e] *Ampelomyces* spp. pycnidia were also found.

2.3.4 Identification and morphological characterization of *Ampelomyces* spp.

Pycnidia of the *Ampelomyces* spp. isolates varied in shape (ovoid, ellipsoid, or globose). They were found only in powdery mildew mycelia. No pycnidia were observed in immature or semi-mature chasmothecia. Moreover, the ranges of pycnidia sizes were quite variable.

Conidia of *Ampelomyces* spp. were found in chasmothecia and in mycelia. Conidia size was variable within isolates. The conidia ranged from 11.5 to 14.5 μm in length (major axis) and from 2.5 to 3.5 μm in width (minor axis). Differences in the shape (fusiform or ellipsoidal) of the conidia were observed among isolates collected in different vineyards (Table 2). Molecular analysis, based on ITS sequencing, showed that all of the collected isolates belong to the *Ampelomyces* genus. The most similar NCBI library strains are listed in Table 3, together with their accession numbers and similarity percentage figures. The ITS sequences of the five selected *Ampelomyces* strains were analyzed together with the sequence obtained from AQ10 and with other *Ampelomyces* sequences from GenBank, from different collections and from different host species, in order to evaluate their phylogenetic diversity. A 511 nucleotides long alignment was used to infer phylogenies. Both the maximum-likelihood (not shown) and the neighbor-joining trees (Fig. 6) showed the same clustering of the strains. The five wild strains grouped into two different clades with an optimal bootstrap support (100 %): the isolates named ITA 1 and ITA 2 grouped together with AQ10 and no differences were observed between these three sequences. The fusiform-shaped conidia of ITA1 are similar to the conidia of the commercial *A. quisqualis* (AQ10). The other three wild isolates, named ITA 3, ITA 4, and ITA 5 grouped together with the sequence from the ATCC collection. There were more than 80 differences between the sequences of these isolates and AQ10's sequence, while these isolates differed from one another by only one base.

Table 2. *Ampelomyces* spp. strains found on grapevine leaves in the Trentino-Alto Adige region in the different homogeneous areas (2004-2007)

Location	Area	Year	Type of vineyard	Conidia shape[a]	Isolate name
Calliano	Trento South	2004	Abandoned	Fusiform	ITA 2
Sorni	Trento North	2005	Organic	Ellipsoidal	SOR
S. Michele all'Adige 1	Trento North	2005	Conventional	Fusiform	SM1
S. Michele all'Adige 2	Trento North	2006	Conventional	Ellipsoidal	ITA 4
Rovere della Luna	Trento North	2006	Conventional	Ellipsoidal	ROV
Lavis	Trento North	2006	Conventional	Fusiform	LAV
Nave S. Rocco	Trento North	2006	Conventional	Ellipsoidal	NAV
Mezzocorona	Trento North	2006	Untreated	Fusiform	MEZ
Pergolese 1	Trento South	2006	Conventional	Ellipsoidal	PER 1
Pergolese 2	Trento South	2006	Untreated	Fusiform	PER 2
Maso Rover	Trento North	2006	Untreated	Fusiform	ITA 3
Pressano	Rovereto	2007	Conventional	Ellipsoidal	PRE
Nomi	Rovereto	2007	Conventional	Ellipsoidal	NOM
Besenello 1	Trento South	2007	Conventional	Ellipsoidal	BES 1
Besenello 2	Trento South	2007	Untreated	Ellipsoidal	ITA 1
Nogaredo	Rovereto	2007	Conventional	Ellipsoidal	ITA 5

[a] Twenty conidia of *Ampelomyces* spp. per each isolate were examined under the light microscope.

Table 3. Comparison of ITS sequences of five wild isolates found in the Trentino-Alto Adige region and other isolates from GenBank with the most similar NCBI library strains

Fungal isolate	Sequence length (bp)	Closest NCBI library strain	Similarity (%)
ITA 1	589	*Ampelomyces* sp. AQ10 (AF035783) [a]	100
		Ampelomyces sp.HMLAC214 (DQ490765)	100
ITA 2	649	*Ampelomyces* sp. AQ10 (AF035783)	100
ITA 3	585	*A. quisqualis* (AF126817)	97
ITA 4	582	*Ampelomyces quisqualis* (AF126817)	97
ITA 5	575	*Ampelomyces quisqualis* (AF126817)	97
AQ10	520	*Ampelomyces* sp.HMLAC214 (DQ490765)	99
CBS 128.79	587	*Ampelomyces* sp.CBS130.79 (U82449)	99
CABI 272851	588	*Ampelomyces* sp.HMLAC214 (DQ490765)	99
ATCC 250	605	*A. quisqualis* (AF126817)	97

[a] Accession number.

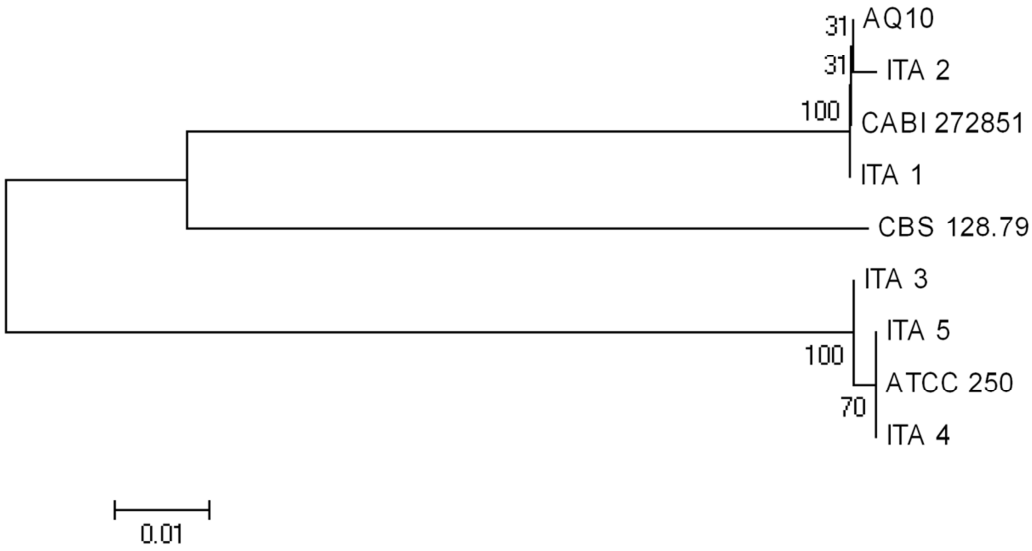


Figure 6. The Neighbour Joining tree of nine *Ampelomyces* spp. sequences as inferred with Mega4 software package (34) using the Jukes-Cantor substitution model and rate uniformity among sites. All gaps and missing data of the 511 characters long alignment were eliminated. On the branches are indicated the bootstrap values. The scale bar represents the number of inferred substitutions per 100 sites (nucleotide residues).

2.4 Discussion

Powdery mildew caused by *E. necator* is an important disease of grapevine in the Trentino-Alto Adige region, second only to downy mildew (*Plasmopara viticola*). If it is not controlled (untreated or abandoned vineyards), the disease can become extremely widespread and devastating (Table 1). Chasmothecia were formed in all the monitored vineyards in each year of the study, confirming that the initiation and development of chasmothecia in Italian vineyards can occur in any year in which conditions are favorable for disease development (Cortesi et al., 1995). The high incidence of chasmothecia in the vineyards of the Trentino-Alto Adige region, which was associated with the absence of flag shoots in the monitored vineyards, could imply that chasmothecia are the primary inoculum and confirm previous results, which had indicated that chasmothecia represent the main source of inoculum in the spring (Cortesi et al., 1995).

The monitoring of powdery mildew infections carried out during the course of this four-year study led to the detection of a highly significant relationship between the level of powdery mildew infection (incidence and severity) and the quantity of overwintering chasmothecia formed on leaves. The amount of chasmothecia was generally low in conventional vineyards and therefore their presence could be probably reduced by applications of an effective method of disease control during the growing season. Even if some reports have stated that most of the chasmothecia die during the winter without having any opportunity to release ascospores (Cortesi et al., 1995), a reduction in the number of overwintering chasmothecia by hyperparasites may nevertheless be useful for reducing the amount of primary inoculum present in the region.

In the Trentino-Alto Adige region, chasmothecia were formed on the surface of infected leaves beginning in the late summer. The many studies on *E. necator* chasmothecia have reported unclear and sometimes contradictory effects of environmental factors on the development of chasmothecia (Gadoury & Pearson, 1988). Reports based on field studies have suggested that the formation of ascocarps is triggered by severe powdery mildew infection, drought, cold, heat or environmental conditions that are generally unfavorable for the parasite. Host nutrition and host resistance have both been reported to affect the formation of chasmothecia (Gadoury & Pearson, 1988). Our results suggest that the concentration of chasmothecia per unit of leaf area was mainly related to the level of disease in the vineyard, which is usually the result of complex interactions among the amount of initial inoculum, the efficacy of disease control measures,

cultivar susceptibility, the physiology of the crop, and the weather conditions during the growing season. The elevation of the vineyard, which is inversely related to temperature, was not related to the concentration of chasmothecia.

A. quisqualis is one of the most successful commercialized biocontrol agents. It acts directly by invading and destroying host cytoplasm (Hashioka & Nakai, 1980). The present study is the first extensive investigation of quantitative aspects of *A. quisqualis* on grapevine powdery mildew in Italy. *Ampelomyces* spp. were observed parasitizing colonies and chasmothecia of *E. necator* on grapevine leaves in the main viticultural areas of Trentino-Alto Adige region suggesting that, although at low levels, *Ampelomyces* spp. was naturally present in the region. This finding also adds support to the claim that chasmothecia parasitism is probably widespread wherever *A. quisqualis* is present (Falk et al., 1995a). In Trentino, the level of natural chasmothecia parasitism by *A. quisqualis* on leaves was shown to be lower than that observed in New York State (Falk et al., 1995a), but similar to what was reported for chasmothecia on vine bark in New York State. The conidia size of the Italian isolates was quite variable and generally longer than reported in literature (Falk et al., 1995a).

Even if *A. quisqualis* (AQ10) was not directly used in the monitored vineyards during the studied period, it is and was used in the surrounding area. The similarity between the ITS sequences of two of the isolates and that of AQ10 may suggest that the commercial strain could have become established in the area. The fact that *Ampelomyces* was found continuously in certain vineyards may indicate that, in some areas, it may find suitable climatic conditions for parasitism. Among the isolated *Ampelomyces* spp., three strains that are probably new were identified (ITA 3/ITA 4, ITA 2, and ITA 5). The ITS sequences of these strains were different from that of the commercial *Ampelomyces* strain (AQ10). In any case, further exhaustive studies are necessary for any inferences concerning *Ampelomyces* spp. population genetics.

The widespread presence of the disease at the end of the growing season did not correspond to high levels of parasitism in the same season. At the levels of infection observed in the Trentino-Alto Adige region, the potential for the mycoparasite to naturally reduce the levels of overwintering inoculum available for the next growing season and/or to impede disease development were relatively low. A few hypotheses can be raised to explain the minimal presence of *A. quisqualis* on *E. necator* in the region. The prevailing environmental conditions

may not meet the requirements of the mycoparasite (Kiss et al., 2004; Sundheim & Krekling, 1982). It is also possible that the low level of powdery mildew disease before the beginning of the survey (from 1999 to 2003) (Mescalchin et al., 2005) may also have reduced the natural population of the mycoparasite in the area.

Numerous *Ampelomyces* mycoparasites are found around the world (Liang et al., 2007). Some of the *A. quisqualis* strains isolated in the Trentino-Alto Adige region were different from AQ10. Therefore, they may represent new isolates that are better adapted to local environmental conditions and their discovery may be the starting point of their development for use as biocontrol agents to control powdery mildew under the environmental conditions found in northern countries. Further analyses are necessary to better characterize the aggressiveness of these isolates against *E. necator* and their environmental requirements for development and parasitism.

2.5 Acknowledgements

We thank L. Maines, D. Ress, E. Di Marino and E. Mescalchin for the technical support and assistance. This research was supported by SafeCrop Centre, funded by Autonomous province of Trento.

2.6 Literature

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic Local Alignment Search Tool. J. Mol. Biol. 215: 403-410.

Angeli, D., Di Marino, E. and Mescalchin, E. (2006). Colonization of grapevine powdery mildew cleistothecia by the mycoparasite *Ampelomyces quisqualis* in Trentino-Alto Adige, Italy. IOBC/WPRS bulletin 29(11):89-92.

Bulit, J. U. and Lafon, R., 1978. Powdery mildew of the vine. Pages 525-648 in: The Powdery Mildews. Academic Press, New York.

Cortesi, P., Gadoury, D. M., Seem, R. C. and Pearson, R. C. (1995). Distribution and retention of cleistothecia of *Uncinula necator* on the bark of grapevines. Plant Dis. 79:15-19.

Falk, S. P., Gadoury, D. M., Cortesi, P., Pearson, R. C. and Seem R. C. (1995a). Parasitism of Uncinula necator cleistothecia by the mycoparasite *Ampelomyces quisqualis*. Phytopathology 85:794-800.

Falk, S. P., Gadoury, D. M., Pearson, R. C. and Seem, R. C. (1995b). Partial control of grape powdery mildew by the mycoparasite *Ampelomyces quisqualis*. Plant Dis. 79:483-490.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Gadoury, D. M. and Pearson, R. C. (1988). Initiation, development, dispersal, and survival of cleistothecia of *Uncinula necator* in New York vineyards. Phytopathology 78:1413-1421.

Gadoury, D. M., Seem, R. C., Pearson, R. C., Wilcox, W. F. and Dunst, R. M. (2001). Effects of powdery mildew on vine growth, yield, and quality of Concord grapes. Plant Dis. 85:137-140.

Grove, G. G. (2004). Perennation of *Uncinula necator* in vineyards of Eastern Washington. Plant Dis. 88:242-247.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Hashioka, Y. and Nakai, Y. (1980). Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. Trans. Mycol. Soc. Jpn 21:329-338.

Kiss, L. (1997). Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. Mycol. Res. 101:1073-1080.

Kiss, L. (1998). Natural occurrence of *Ampelomyces* intracellular mycoparasites in mycelia of powdery mildew fungi. New Phytol. 140:709-714.

Kiss, L. (2003). A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Manag. Sci. 59:475-483.

Kiss, L. and Nakasone, K. K. (1998). Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi. Curr. Gen. 33:362-367.

Kiss, L., Russell, J. C., Szentivanyi, O., Xu, X. and Jeffries, P. 2004. Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonist of powdery mildew fungi. Biocontrol Sci. Technol. 14:635-651.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007). ClustalW2 and ClustalX version 2. Bioinformatics 23:2947-2948.

Liang, C., Yang, J., Kovacs, G. M., Szentivanyi, O., Li, B., Xu, X. M. and Kiss, L. (2007). Genetic diversity of *Ampelomyces* mycoparasite isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences. Fung. Diversity 24:225-240.

Mescalchin, E., Di Marino E. and Angeli, D. (2005). Monitoraggio della presenza di *Ampelomyces* nei vigneti del Trentino. Terra Trentina 5:32-37.

Ough, C. S. and Berg, H. W. (1979). Powdery mildew sensory effect on wine. Am. J. Enol. Vit. 30:321.

Page, R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357-358.

Pearson, R. C. and Gadoury, D. M. (1987). Cleistothecia, the source of primary inoculum for grape powdery mildew in New York. Phytopathology 77:1509-1514.

Pearson, R. C. and Goheen, A. C. (1988). Compendium of Grape Diseases. American Phytopathological Society, St. Paul, MN.

Rumbolz, J. and Gubler, W. D. (2005). Susceptibility of grapevine buds to infection by powdery mildew *Erysiphe necator*. Plant Path. 54:535-548.

Ryan, T. A. (1960). Significance tests for multiple comparison of proportions, variances, and other statistics. Psychol. Bull. 57:318-328.

Sall, M. A. and Wrysinski, J. (1982). Perennation of powdery mildew in buds of grapevine. Plant Dis. 66:678-9.

Staden, R., Beal, K. F. and Bonfield, J. K. (2000). The Staden package, 1998. Bioinform. Meth. Prot. 132:115-130.

Sullivan, R. F. and White, J. F. Jr. (2000). *Phoma glomerata* as a mycoparasite of powdery mildew. Appl. Environ. Microb. 66:425-427.

Sundheim, L. and Krekling, T. (1982). Host-parasite relationships of the hyperparasite *Ampelomyces quisqualis* and its powdery mildew host *Sphaerotheca fuliginea*. I. Scanning electron microscopy. Phytopath. Z. 104:202-210.

Sundheim, L. and Tronsmo, A. (1988). Hyperparasites in biological control. Pages 53-69 in: Biocontrol of Plant Diseases. Vol. I. CRC Press, Boca Raton, FL.

Szentiványi, O., Kiss, L., Russell, J. C., Kovács, G. M., Varga, K., Jankovics, T., Lesemann, S., Xu, X. M. and Jeffries, P. (2005). *Ampelomyces* mycoparasites from apple powdery mildew identified as a distinct group based on single-stranded conformation polymorphism analysis of the rDNA ITS region. Mycol. Res.109:429-438.

Sztejnberg, A. (1993). *Ampelomyces quisqualis* AQ10, CNCM I-807, for biological control of powdery mildew. US patent no. 5190754.

Sztejnberg, A., Galper, S., Mazar, S. and Lisker, N. (1989). *Ampelomyces quisqualis* for biological and integrated control of powdery mildews in Israel. J. Phytopath. 124:285-295.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

White, T., J., Bruns, T., Lee, S. 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. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds), Academic Press, San Diego, California.

Ypema, H. L. and Gubler, W. D. (2000). The distribution of early season grapevine shoots infected by *Uncinula necator* from year to year: A case study in two California vineyards. Am. J. Enol. Vit. 51:1-6.

Chapter 3

Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis*

Published as

Angeli D., Maurhofer M., Gessler C., Pertot I. 2012. Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis*. Phytoparasitica, 40 (1): 37-51.

Abstract

Powdery mildew fungi are parasitized by strains of the genetically distinct *Ampelomyces quisqualis*. To investigate whether differences in the phylogeny and other cultural, morphological and physiological characteristics of these different strains are related to differences in their geographic origins or the host species from which they were isolated, several strains of different species of *Erysiphaceae* collected in different countries and possessing different ITS rDNA sequences were selected and characterized. The results revealed some significant variation among the selected strains, which provides evidence for the existence of different physiological forms within the *A. quisqualis* species. Two groups that display differential growth on artificial media were identified. These groups also differ in the morphology of their mycelium, but not in the morphology of their pycnidia and conidia. Temperature greatly affected the *in vitro* growth of the *A. quisqualis* strains and growth rate was closely correlated to colony color. Differences in the conidial germination of distinct strains were observed during the recognition phase of the parasitic relationship. The germination of each of the investigated strains was greatly stimulated by all of the examined powdery mildew species and not only by the conidia of their original hosts. An Italian strain isolated from grapevine in the Trentino Alto-Adige region was identified as the strain that germinates the most quickly in the presence of powdery mildew conidia. Phylogenetic analysis revealed that these *A. quisqualis* strains can be classified into five different genetic groups, which generally correlate with the fungal host of origin and morphological and growth characteristics.

3.1 Introduction

Ampelomyces quisqualis is a widespread hyperparasite of powdery mildews (Falk et al., 1995a; Kiss, 2003; Kiss, 1998). The natural occurrence of *A. quisqualis* on various *Erysiphaceae* species has been reported in different geographic regions (Angeli et al., 2009a; Kiss, 1997; Kiss et al., 2004; Rankovic, 1997). Most research on *A. quisqualis* has focused on its potential use as a biocontrol agent against powdery mildews of various crops (Bélanger & Labbé 2002; Paulitz & Bélanger, 2001; Sundheim & Tronsmo, 1988; Sztejnberg et al., 1989). Its biology and biocontrol potential were reviewed by Kiss et al. (2004).

This mycoparasite invades and destroys host cytoplasm, killing the parasitized powdery mildew cells (Falk et al., 1995ab; Hashioka & Nakai, 1980; Kiss et al., 2004). Intracellular pycnidia of *A. quisqualis* are commonly found in hyphae, conidiophores and immature ascomata of powdery mildews (Kiss et al., 2004). These pycnidia vary in shape depending upon the fungal structure in which they are formed (Sundheim & Krekling, 1982). Pycnidia contain cylindrical to spindle-shaped conidia, which are occasionally curved and two-spotted (Falk et al., 1995a). Recently, the microcyclic conidiogenesis of powdery mildews has been investigated (Kiss et al., 2009). When *mildew* colonies are treated with a suspension of *A. quisqualis* conidia, pycnidia are formed in microcyclic conidiophores, thereby accelerating the asexual reproduction of *A. quisqualis*.

The concentration of *A. quisqualis* conidia is an important factor affecting their germination. Germination has been shown to dramatically decrease when conidia are at a concentration of more than 10^6 conidia ml^{-1}, due to the production of self-inhibitory substances (Gu & Ko, 1997). *A. quisqualis* conidia do not germinate well in sterile, distilled water. However, their germination is significantly enhanced in the presence of conidia of powdery mildew fungi (Sundheim 1982). The presence of host fungi is recognized by *A. quisqualis* and a water-soluble substance from conidia of powdery mildew fungi has been shown to stimulate the germination of *A. quisqualis* conidia *in vitro* (Gu & Ko, 1997). After penetration, the hyphae of the mycoparasite continue to grow and produce their intracellular pycnidia after 5 to 8 days in the mycelia of their fungal host (Hashioka & Nakai, 1980; Sundheim & Krekling, 1982). High relative humidity and temperatures between 20° and 25 °C enhance the growth and sporulation of *A. quisqualis*, but this mycoparasite survives and is active against powdery mildew even at temperatures below 12°C (Jarvis & Slingsby, 1977; Philipp & Cruger, 1979).

A. quisqualis is known to be a slow-growing fungus with an *in vitro* radial growth rate of 0.5–1.0 mm d^{-1} on Czapek-Dox agar supplemented with 2 % malt extract (MCzA) at 23 °C (Kiss, 1997; Kiss & Nakasone, 1998). Until recently, *A. quisqualis* was often confused with several other species: *A. quercinus*, *A. humuli*, *A. heracli* and *Phoma glomerata*. Unlike *A. quisqualis*, these other powdery mildew mycoparasites do not produce intracellular pycnidia and they also grow more quickly, with *in vitro* radial growth rates of 3–4 mm d^{-1} (Kiss et al., 2004; Sullivan & White, 2000). In the past, scientists identified all of these fast-growing strains as *A. quisqualis (*e.g., Mhaskar, 1974a; Kiss, 1997). Later, a molecular phylogenetic study of rDNA ITS sequences (Kiss & Nakasone, 1998) revealed that the slow-growing and fast-growing strains

belong to two distinct groups. The slow-growing strains are genetically more diverse; whereas the fast-growing ones show a close phylogenetic relationship with *Epicoccum nigrum*, which is known to have a pycnidial, *Phoma*-like state (Kiss et al., 2004). It seems likely that the fast-growing strains are, in fact, *Phoma* species; whereas true *A. quisqualis* strains always grow slowly in culture and produce intracellular pycnidia in powdery mildew mycelium.

Several *A. quisqualis* strains are available from culture collections and one strain has been formulated, registered and commercialized under the trade name of AQ10 (Kiss, 1997; Sztejnberg, 1993). Many molecular analyses based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (nrDNA) have revealed considerable genetic diversity among *A. quisqualis* strains (Angeli et al., 2009b; Liang et al., 2007; Park at al., 2010; Szentiványi et al., 2005). Recently, ITS sequences and microsatellite markers have been used to show that a set of *A. quisqualis* populations found in apple powdery mildew (*Podosphaera leucotricha*) are quite genetically distinct from populations collected from several other powdery mildew species infecting other plant species (Kiss et al., 2011). Although the genetic diversity within the *A. quisqualis* species has already been characterized, we still do not know whether the differences in the phenotypic characteristics of different strains of *A. quisqualis* are related to their phylogeny.

The morphological and cultural characteristics of this hyperparasite on different fungal hosts and plants have been reported to be extremely variable (Belsare et al. 1980; Shin and Kyeung 1994; Speer 1978). Some older studies focused on the shape and size of the pycnidia and conidia on culture media (e.g., Mhaskar, 1974a, Mhaskar & Rao, 1974b) and fungal hosts (e.g., Kiss, 1997; Rankovic, 1997). However, all of these studies were carried out when fast-growing strains (*Phoma glomerata* and *Ampelomyces* spp.) were still classified as *A. quisqualis*. Therefore, a thorough investigation of *A. quisqualis sensu strictu* is lacking. More physiological and phenotypic information is required to complete the picture of the genetic differences that have been identified in previous studies (Liang et al., 2007; Park et al., 2010; Kiss et al., 2011).

In the present work, we selected several *A. quisqualis* strains from different hosts and geographic regions, all characterized by the formation of intracellular pycnidia and slow radial growth at room temperature *in vitro* (defined as Type II; Kiss, 1997), features corresponding to the characterization of *A. quisqualis sensu strictu*. We investigated the cultural, morphological and

growth characteristics and phylogenetic relations (ITS) of these strains. The objectives of this study were to determine whether the host or site of origin of the strains or their cultural, morphological and/or growth characteristics are related to their phylogenetic group which would indicate an adaptation to the host or geographic area. Furthermore, some physiological tests were performed as we looked for evidence of any host specificity or temperature adaptation of strains affecting the recognition phase of the host-parasite interaction.

3.2 Material and Methods

A total of 28 *A. quisqualis* strains isolated from different crops, sites and powdery mildew species were included in this study (Table 1). We selected strains isolated from grapevine (*Vitis vinifera*), cucumber (*Cucumis sativus*) and apple (*Malus domestica*) in different geographic regions, as well as strains isolated from wild and/or ornamental species. Only slow-growing strains [e.g., Type II (0.5–1.0 mm d^{-1}) (Kiss, 1997)] producing intracellular pycnidia within powdery mildew mycelia were selected. The strains were obtained from culture collections (ATCC, American Type Culture Collection, Rockville, MD, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; CABI, Commonwealth Agricultural Bureaux International, Egham, UK; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh, Braunschweig, Germany) or provided by individual scientists (L. Kiss, PPI, Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary; D. Angeli, FEM, Fondazione Edmund Mach, S. Michele all'Adige, Italy).

Table 1. Designation, plant host, area and year of isolation, source and GenBank accession numbers of *A. quisqualis* strains included in this study. All strains are Type II (growth rate: 0.5–1.0 mm d^{-1}) and were acquired from culture collections (ATCC, CBS, CABI, DSMZ) or provided by individual scientists (L. Kiss, PPI; D. Angeli, FEM)

Designation	Host fungus	Host plant	Geographical origin and year of isolation	Source [a]	GenBank accession no. [b]
ATCC200245	*Erysiphe necator*	*Vitis vinifera*	New York, USA, 1989	ATCC	AF126817
ATCC200246	*E. necator*	*V. vinifera*	New York, USA, 1991	ATCC	HQ108030
ATCC200247	*E. necator*	*V. vinifera*	New York, USA, 1991	ATCC	HQ108031
ATCC200248	*E. necator*	*V. vinifera*	New York, USA, 1991	ATCC	HQ108032
ATCC200249	*E. necator*	*V. vinifera*	New York, USA, 1991	ATCC	HQ108033
ATCC200250	*E. necator*	*V. vinifera*	New York, USA, 1991	ATCC	HQ108034
ITA1	*E. necator*	*V. vinifera*	Italy, 2007	FEM	HQ108047
ITA2	*E. necator*	*V. vinifera*	Italy, 2007	FEM	HQ108048
ITA3	*E. necator*	*V. vinifera*	Italy, 2007	FEM	HQ108049
ITA4	*E. necator*	*V. vinifera*	Italy, 2007	FEM	HQ108050
ITA5	*E. necator*	*V. vinifera*	Italy, 2007	FEM	HQ108051
CBS128.79	*Erysiphe cichoracearum*	*Cucumis sativus*	Canada, 1975	CBS	HQ108037
CBS129.79	*E. cichoracearum*	*C. sativus*	Canada, 1975	CBS	HQ108038
CBS130.79	*E. cichoracearum*	*C. sativus*	Canada, 1975	CBS	HQ108039
CBS131.79	*E. cichoracearum*	*C. sativus*	Canada, 1975	CBS	HQ108040
DSM2222	*E. cichoracearum*	*C. sativus*	Germany, unknown [d]	DSMZ	U82450
MYA-3389	*Podosphaera leucotricha*	*Malus domestica*	Hungary, 1995	PPI	AY663815

Table 1. (continued)

Designation	Host fungus	Host plant	Geographical origin and year of isolation	Source [a]	GenBank accession no. [b]
MYA-3391	*P. leucotricha*	*M. domestica*	Hungary, 1995	PPI	HQ108043
MYA-3394	*P. leucotricha*	*M. domestica*	United Kingdom, 2002	PPI	HQ108044
MYA-3395	*P. leucotricha*	*M. domestica*	Germany, 2002	PPI	AY663817
MYA-3398	*P. leucotricha*	*M. domestica*	United Kingdom, 2002	PPI	HQ108045
AQ10	Powdery mildew [c]	*Chata edulis*	Israel, unknown [d]	Ecogen Italia	AF035783
DSM2225	*Erisiphe heracli*	*Daucus* sp.	Germany, unknown [d]	DSMZ	HQ108042
DSM4624	*Sphaerotheca fuliginea*	*Leontodon* sp.	Germany, unknown [d]	DSMZ	HQ108041
CABI272851	Powdery mildew [c]	*Schinus molle*	Ecuador, 1983	CABI	HQ108036
CABI380234	Powdery mildew [c]	*Alliaria petiolata*	Switzerland, 1999	CABI	HQ108035
ATCC201056	*Oidium hortenisae*	*Lycium halimifolium*	Hungary, 1990	ATCC	AF035781
MYA-3401	*Oidium hortenisae*	*Hydrangea macrophylla*	United Kingdom, 1999	PPI	HQ108046

[a] ATCC, American Type Culture Collection, Rockville, MD, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; CABI, Commonwealth Agricultural Bureaux International, Egham, United Kingdom; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh, Braunschweig, Germany); FEM, Fondazione Edmund Mach, S. Michele all'Adige, Italy; PPI, Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary.

[b] GenBank accession numbers of the rDNA ITS sequences.

[c] Unknown species.

[d] Data not available.

Table 2. GenBank accession numbers of the rDNA ITS sequences, plant hosts, area and year of isolation and source of published *A. quisqualis* sequences included in the ITS sequence analysis

GenBank accession no. [a]	Host fungus	Host plant	Geographical origin and year of isolation	Source
GQ324149 [b]	*Erysiphe necator*	*Vitis vinifera*	Seoul, South Korea, 2007	Park et al. (2010)
GQ324144 [b]	*E. necator*	*Vitis flexuosa*	Jeju, South Korea, 2006	Park et al. (2010)
DQ490750	*Podosphaera xanthii*	*Cucurbita pepo*	China, 2002	Liang et al. (2007)
DQ490752	*P. xanthii*	*Cucumis moschata*	China, 2002	Liang et al. (2007)
DQ490755	*P. xanthii*	*Cucumis sativus*	China, 2002	Liang et al. (2007)
DQ490759	*P. xanthii*	*Cucurbita maxima*	China, 2002	Liang et al. (2007)
AY663816	*Podosphaera leucotricha*	*Malus domestica*	Hungary, 2000	Szentivanyi et al. (2005)
AY663818	*P. leucotricha*	*M. domestica*	UK, 2002	Szentivanyi et al. (2005)
AY663819	*P. leucotricha*	*M. domestica*	UK, 2002	Szentivanyi et al. (2005)
AY663820	*P. leucotricha*	*M. domestica*	Germany, 2002	Szentivanyi et al. (2005)
AY663821	*P. leucotricha*	*M. domestica*	Hungary, 2002	Szentivanyi et al. (2005)
GQ324095 [b]	*Erysiphe ranunculi*	*Clematis apiifolia*	Seogwipo, South Korea, 2006	Park et al. (2010)
GU329997 [b]	*Golovinomyces cichoracearum*	*Zinnia elegans*	Yangpyeong, South Korea, 2008	Park et al. (2010)
DQ490762	*Arthrocladiella mougeotii*	*Lycium chinense*	China, 2003	Liang et al. (2007)
DQ490767	*Oidium* sp.	*Castanopsis* sp.	China, 2002	Liang et al. (2007)

[a] GenBank accession numbers of the rDNA ITS sequences.

[b] Strains identified in the maximum-likelihood tree with the same isolate numbers used by Park et al. (2010).

For phylogenetic comparison, 15 sequences of ITS rDNA of strains of *A. quisqualis* were retrieved from GenBank (Table 2). Strains were selected according to the fungal host of origin. The selected strains represent the four major clades reported in a recent study by Park et al. (2010).

3.2.1 Cultural, morphological and physiological characteristics

The cultural behavior and morphological characteristics of the pycnidia and conidia of the 28 selected *A. quisqualis* strains were evaluated on six different types of media: potato dextrose agar (Oxoid, Hampshire, UK, 39 g l^{-1} twice-distilled water), sucrose nutrient agar (50 g sucrose, 28 g nutrient agar, twice-distilled water to 1 l), yeast malt dextrose agar (10 g dextrose, 5 g peptone, 3 g yeast extract, 3 g malt extract, 20 g agar, twice-distilled water to 1 l), nutrient agar (Oxoid, 28 g l^{-1} twice-distilled water), Czapek agar (Oxoid, 33.4 g l^{-1} twice-distilled water) and cornmeal agar (Oxoid, 17 g l^{-1} twice-distilled water).

The Petri-dish cultures of fungus were prepared with 100 µl of a suspension of sterile distilled water containing 10^2 conidia ml^{-1}. The color of the mycelia and pycnidia was assessed by visual observation of colonies grown for 14 days at 25 °C in the dark. We measured the length and width of 60 pycnidia and 60 conidia per strain (20 per replicate) on glass slides under a light microscope (Hund Wetzlar H 600LL, Wetzlar, Germany). The radial growth rate of the *A. quisqualis* strains was evaluated at temperatures of 10°, 15°, 20°, 25° and 30 °C on Petri plates containing potato dextrose agar (PDA) that were inoculated as described above. Cultures were incubated in the dark for 30 days and the radial growth of the colonies (20 per replicate) was evaluated by measuring the diameter of each colony twice a week. All of the experiments were conducted with three replicates (plates) per strain.

Furthermore, the recognition of the fungal host by *A. quisqualis* strains at different temperatures was studied. We evaluated the effects of the conidia of four powdery mildew species, *Podosphaera xanthii* (from cucumber), *E. necator* (from grapevine), *Podosphaera aphanis* (from strawberry) and *Podosphaera leucotricha* (from apple), on the germination rates of five selected *A. quisqualis* strains. Each of the tested strains belongs to a different genetic (ITS) group (identified in this study) and was collected from a different host plant in a different geographic

area (AQ10, ITA3, CBS129.79, MYA-3391 and MYA-3401). Each strain was paired with an isolate of the fungal host from which it was originally isolated and three other powdery mildews. Sterile distilled water suspensions of the conidia of each powdery mildew strain (1×10^5 conidia ml^{-1}) together with each *A. quisqualis* strain (1×10^5 conidia ml^{-1}) were prepared, for a total of 20 combinations. Suspensions of only *A. quisqualis* conidia in sterile distilled water were used as controls. Six drops (10 µl each) per combination (three replicates) were put onto a glass slide, which was placed in a Petri dish (RH = 100 %) and stored in a dark incubator kept at 25 °C, which is the temperature that Gu and Ko (1997) used in their study of the factors affecting the germination of *A. quisqualis* conidia. We evaluated the germination rates and germ-tube elongation of 150 conidia per strain (three replicates, 50 conidia per replicate) under a light microscope after 24 h.

We also evaluated the effect of temperature on the germination of *A. quisqualis* conidia in the presence of the fungal host. In this experiment, we tested two powdery mildew species (*P. xanthii* from cucumber and *E. necator* from grapevine) and the two *A. quisqualis* strains that were the most and least responsive to powdery mildew conidia in the previous experiment. We evaluated both the germination rate and the tube elongation of those strains at three temperatures (15°, 20° and 25 °C) using the method described above.

3.2.2 Phylogenetic analysis

The phylogenetic relations of the 28 strains analyzed in the present study and another 15 strains selected for phylogenetic analysis were inferred from maximum-likelihood and maximum-parsimony analyses in which the sequences of their respective ITS rDNA regions were compared (White et al. 1990). The additional 15 sequences of ITS rDNA from *A. quisqualis* strains that were analyzed by Park et al. (2010) were retrieved from GenBank (Table 2). The strains isolated by Park et al. (2010), whose GenBank accession numbers begin with GQ and GU, were identified here with the same isolate numbers used by Park et al. (2010), to make it easier to recognize them across the two studies (SMKC22963 = GQ324149, SMKC22519 = GQ324144, SMKC22511 = GQ324095, SMKC23914 = GU329997).

The 28 selected strains (Table 1) were grown on PDA for 21 days at 25 °C. DNA was extracted from approximately 200 µg of homogenized, lyophilized mycelium, using the Nucleo Spin Plant Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The ITS region of the nuclear ribosomal DNA was amplified using ITS1 and ITS4 fungal-specific primers (Gardes and Bruns 1993). PCR was performed using the Gene Amp PCR System 9700 (Perkin Elmer, Waltham, MA, USA). PCR products were purified using ExoSAP-IT enzymes (USB Corporation, Staufen, Germany) and sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Both DNA strands were sequenced with the primers that had been used for PCR amplification and electrophoresis was carried out using an ABI 3130xl Genetic Analyzer (Perkin Elmer). The consensus sequence was assembled using Pregap4 and Gap4 (Staden et al. 2000; http://staden.sourceforge.net/). Blast searches were performed in the NCBI/GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/) using the program Megablast to find long stretches of alignment between very similar nucleotide sequences. The ClustalW2 program (Larkin et al. 2007; http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to construct multiple sequence alignments and to identify evolutionary relationships among the input sequences. Maximum-likelihood (ML) and maximum-parsimony (MP) analyses based on ITS region sequences were conducted using the Mega4 program (Tamura et al. 2007; http://www.megasoftware.net/mega.html) and a p-distance model (Nei and Kumar 2000) was used to calculate within-group and between-group distances. A phylogenetic tree inferred from the ITS rDNA region sequences was constructed using the TreeView program, version 1.6.6 (Page 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). *Phoma glomerata* (AF126816), *A. quercinus* (AF035778) and *A. humuli* (AF035779) were also included in the model.

3.2.3 Clustering and statistical analysis

Morphological experiments (size of pycnidia and conidia) were performed in three replicates per strain and medium and standard deviations of the averages were calculated. A one-way analysis of variance (ANOVA) was performed on log-transformed and arcsin-transformed data. If

significant differences were detected, treatment means (strains and media) were separated using Tukey's test ($\alpha = 0.05$).

Growth tests involved two independent experiments with three replicates per experiment. A two-way ANOVA was performed on log-transformed and arcsin-transformed data from two experiments and revealed no significant Experiment × Treatment (temperature) interaction. Therefore, cluster analyses were performed using a software package for multivariate data analysis capable of performing K-means cluster analyses (Multi-Experiment Viewer, TMEV). The Euclidean distance between each of the samples within a given cluster and their respective cluster centroid was calculated. The angular coefficients (a) and the correlation coefficients (r) of the linear regression curves were also calculated. Data from the two experiments were pooled and averages of six replicates are presented in Table 4 and Figure 1. Statistical analyses were performed using Statistica software 7.0 (Statsoft, Tulsa, OK, USA). Significant differences between treatments (strains and temperatures) were separated using Scheffè's test ($\alpha = 0.05$).

Germination tests involved two independent experiments with three replicates per experiment and 50 conidia per replicate. Two-way ANOVA was performed on both the log-transformed and arcsin-transformed data from the two experiments and revealed no significant Experiment × Treatment (temperature) interaction. Therefore, data from the two experiments were pooled. Averages of six replicates are presented in Figures 2a–b and 3a–b. Significant differences between treatments (strains and powdery mildews) were separated using Tukey's test ($\alpha = 0.05$).

3.3 Results

3.3.1 Cultural, morphological and physiological characteristics

Cultural and morphological analyses of *A. quisqualis* strains indicated that two different colony types can be distinguished after 14 days on all tested media: *A. quisqualis* strains that form dark-brown colonies and those that form olive-green colonies (mycelium and pycnidia; Table 3). Furthermore, mycelial growth and pycnidial and conidial formation were all greatly increased on three of the tested media (potato dextrose agar, sucrose nutrient agar and yeast malt dextrose agar), in comparison with the other media. The study on morphology of pycnidia and conidia (Table 3) revealed differentiation in color and dimensions of the fruiting bodies of the tested

strains. In all of the examined strains, the color and shape of the pycnidia varied from light to dark (brown or green) and from ovoid to ellipsoid, with increasing age of the culture. The length and width of the pycnidia ranged from 30 to 95 µm and from 25 to 55 µm, respectively. A great deal of variation in width and length was observed among the pycnidia of individual strains, but no significant differences were found between strains (P = 0.112). The conidia of the different strains also varied in size. They ranged from 5.5 to 14.5 µm in length and from 2.3 to 3.5 µm in width. However, there were no significant differences between strains according to Tukey's test (P = 0.073).

Temperature greatly affected the growth rate of *A. quisqualis* growth on PDA. The highest radial growth rates for all strains were measured between 15° and 25 °C. At the extreme temperatures of 10° and 30 °C, sharp reductions were observed in the growth rates (Fig. 1). K-means clustering based on Euclidean distances showed that at temperatures of 15°, 20° and 25 °C, the slow-growing Type II strains (Kiss 1997) could be further clustered into two different growth-rate groups that were significantly different from each other. At all of these temperatures, the two groups included the same isolates, which we refer to as slow-growing (0.70–0.82 mm d^{-1}) and very slow-growing (0.34–0.63 mm d^{-1}) (Table 4). At 10° and 30 °C, K-means clustering analysis revealed two growth-rate classes, which do not include exactly the same strains as the groups observed at the other tested temperatures (Table 4). However, no statistically significant differences were observed between the two classes (P = 0.09). At 10 °C, the strains grew at a rate of 0.18–0.43 mm d^{-1}; whereas at 30 °C, they grew at a rate of 0.07–0.33 mm d^{-1}.

Our results indicate that, for all of the tested strains except for MYA-3401, there is a relationship between the growth rates at 15°, 20° and 25 °C and colony color. Strains that grew fairly slowly at these temperatures formed dark-brown colonies and strains that grew very slowly at these temperatures formed olive-green colonies. However, even with the limited number of strains examined, it is possible to demonstrate a relationship between growth rate and the original fungal host. Strains isolated from apple, grapevine and cucumber plants belonged to the same growth rate group at temperatures between 15° and 25 °C. On the other hand, no relationship between the growth rate and the geographic origin of the strains was observed.

Table 3. Morphological characteristics of the *A. quisqualis* strains included in this study

Designation	Colony color [a]		Length and width (µm ± SD)[b]	
	mycelia	pycnidia	pycnidium	conidium
ATCC200245	Olive green	Light/dark olive green	65±25 × 38±15	8.9±1.9 × 2.7±0.4
ATCC200246	Olive green	Light/dark olive green	58±18 × 31±12	8.4±1.6 × 2.9±0.4
ATCC200247	Olive green	Light/dark olive green	57±15 × 32±14	7.9±1.3 × 2.8±0.4
ATCC200248	Olive green	Light/dark olive green	58±18 × 31±12	8.5±1.9 × 2.8±0.4
ATCC200249	Olive green	Light/dark olive green	64±23 × 40±11	8.4±1.7 × 2.9±0.5
ATCC200250	Olive green	Light/dark olive green	59±11 × 32± 8	8.5±1.8 × 2.8±0.4
ITA1	Dark brown	Light/dark brown	72±23 × 42±13	8.4±1.6 × 2.8±0.4
ITA2	Dark brown	Light/dark brown	67± 9 × 39±12	9.8±1.8 × 2.9±0.5
ITA3	Olive green	Light/dark olive green	54±15 × 34±11	8.0±1.7 × 2.9±0.4
ITA4	Olive green	Light/dark olive green	56±16 × 33±7	7.3±1.5 × 2.9±0.5
ITA5	Olive green	Light/dark olive green	64±13 × 37±9	7.3±1.5 × 2.7±0.4
CBS128.79	Dark brown	Light/dark brown	59±10 × 35±10	7.1±1.3 × 2.6±0.2
CBS129.79	Dark brown	Light/dark brown	66±14 × 41±7	7.3±1.5 × 2.8±0.5
CBS130.79	Dark brown	Light/dark brown	64±9 × 38±9	9.0±1.6 × 2.9±0.5
CBS131.79	Dark brown	Light/dark brown	71±18 × 37±8	9.1±1.9 × 2.9±0.4
DSM2222	Dark brown	Light/dark brown	67±16 × 41±9	7.9±1.2 × 2.7±0.4
MYA-3389	Olive green	Light/dark olive green	72±20× 38±12	7.9±1.0 × 2.6±0.2
MYA-3391	Olive green	Light/dark olive green	61±16 × 34±9	7.8±1.8 × 2.7±0.4
MYA-3394	Olive green	Light/dark olive green	69±15 × 36±11	8.7±1.8 × 2.8±0.4
MYA-3398	Olive green	Light/dark olive green	65±10 × 34±7	9.1±1.8 × 2.8±0.4
MYA-3395	Olive green	Light/dark olive green	66±17 × 37±10	9.6±1.6 × 2.7±0.4
AQ10	Dark brown	Light/dark brown	55±10 × 32±5	7.5±1.6 × 2.6±0.3
DSM2225	Dark brown	Light/dark brown	62±15 × 36±10	9.6±1.4 × 2.9±0.4
DSM4624	Dark brown	Light/dark brown	58±9 × 36±7	7.9±1.6 × 2.8±0.4
CABI272851	Dark brown	Light/dark brown	66±15 × 35±11	8.5±1.6 × 2.7±0.4
CABI380234	Olive green	Light/dark olive green	56±18 × 34±12	9.5±1.8 × 3.0±0.4
ATCC201056	Olive green	Light/dark olive green	58±17 × 37±12	8.1±1.2 × 2.6±0.2
MYA-3401	Dark brown	Light/dark brown	73±19 × 44±14	10.6±2.6 × 2.9±0.5

[a] Colors of mycelia and pycnidia on the six tested agar media. Three agar plates per strain were examined visually following 14 days of incubation at 25 °C in the dark.

[b] Values are means from cultures grown on the six media (360 pycnidia and 360 conidia per strain). No significant differences between media were detected when Tukey's tests were applied ($P \geq 0.05$). Standard deviations of the means are reported (SD).

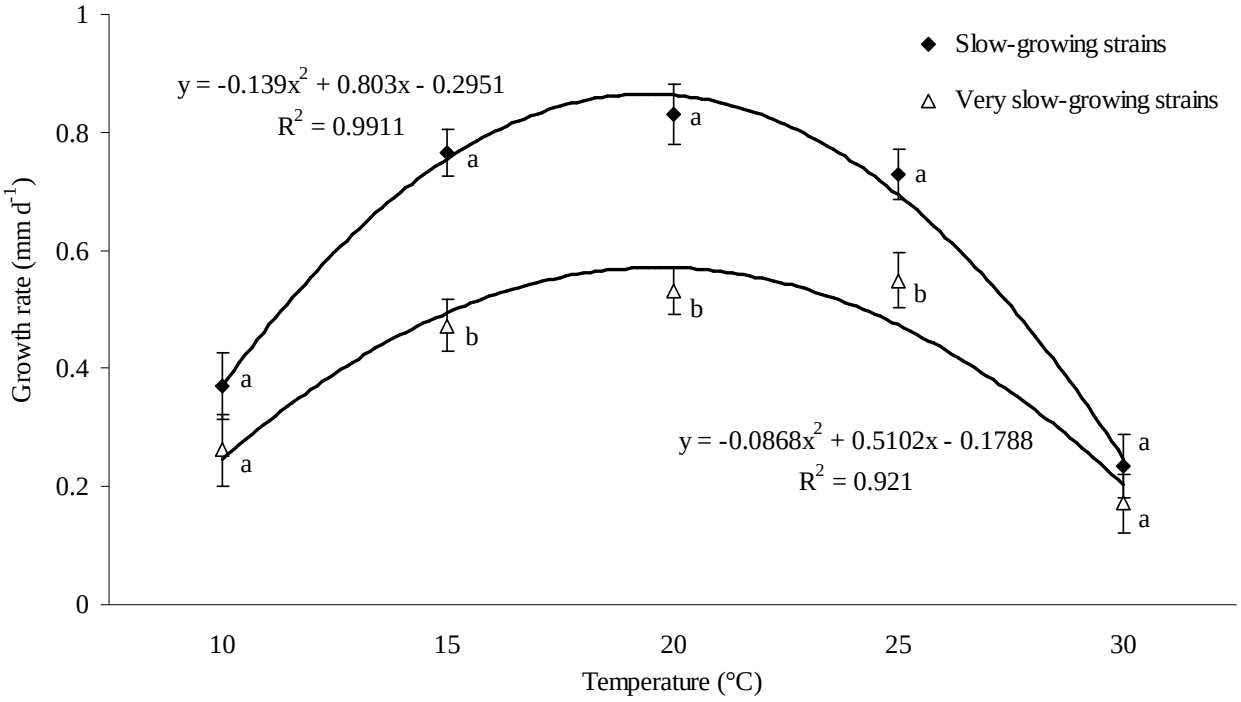


Figure 1. Effect of temperature (10°, 15°, 20°, 25° and 30 °C) on the growth of *A. quisqualis* strains over 30 days on potato dextrose agar. The radial growth rates of 28 strains, which divided into slow-growing and very slow-growing groups at each temperature, are presented (pooled data). K-means clustering using the software TMEV (Multi-Experiment Viewer) was based on the Euclidean distance between each of the strains at each temperature. Significant differences between groups were separated by ANOVA. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment. The standard deviation of the six replicates is presented.

Table 4. Growth rates of the *A. quisqualis* strains at five different temperatures. Data are expressed in terms of the angular coefficients (mm d^{-1}) of the linear regression curves.

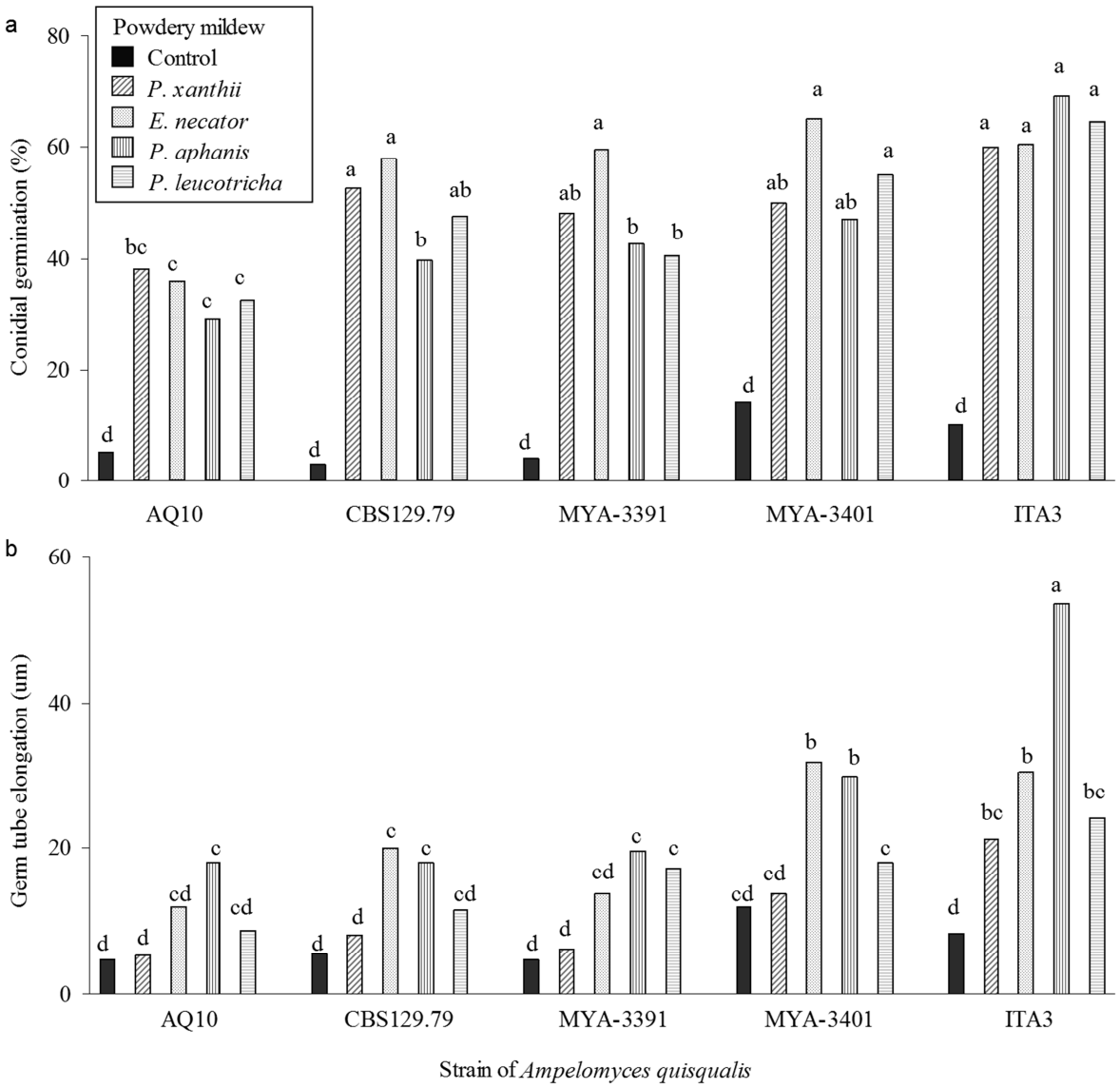
	Temperature (°C)				
ID code	10	15	20	25	30
AQ10	**0.3246** [a]	**0.8187**	**1.0257**	**0.8152**	*0.1384*
ITA1	**0.3699**	**0.7141**	**0.8294**	**0.7525**	*0.0875*
ITA2	**0.3578**	**0.7813**	**0.8526**	**0.6817**	*0.2126*
DSM2222	**0.3686**	**0.6964**	**0.8047**	**0.6698**	*0.1545*
DSM2225	**0.3263**	**0.8272**	**0.8024**	**0.7044**	*0.0799*
DSM4624	**0.4339**	**0.7454**	**0.7851**	**0.6732**	**0.2862**
CBS128.79	**0.4276**	**0.7720**	**0.8145**	**0.7617**	**0.3303**
CBS129.79	**0.4089**	**0.7638**	**0.7545**	**0.7360**	**0.3185**
CBS130.79	**0.3701**	**0.7816**	**0.8032**	**0.6985**	**0.3271**
CBS131.79	**0.3543**	**0.8004**	**0.8473**	**0.7136**	**0.3646**
CABI272851	**0.3224**	**0.7148**	**0.8130**	**0.7700**	**0.2798**
ITA3	**0.3797**	*0.5044*	*0.4922*	*0.5287*	**0.3010**
ITA4	*0.2459*	*0.4308*	*0.4394*	*0.6286*	**0.2697**
ITA5	*0.2637*	*0.4872*	*0.4517*	*0.5222*	**0.2492**
ATCC200246	*0.2343*	*0.5359*	*0.5398*	*0.4925*	**0.3099**
ATCC200245	*0.2567*	*0.3929*	*0.5671*	*0.5349*	*0.1711*
ATCC200247	*0.2809*	*0.4464*	*0.5935*	*0.5977*	*0.0825*
ATCC200248	**0.3292**	*0.4676*	*0.5405*	*0.5447*	*0.2176*
ATCC200249	*0.1957*	*0.4747*	*0.4604*	*0.4932*	*0.1180*
ATCC200250	*0.1805*	*0.3430*	*0.4474*	*0.4816*	*0.0692*
MYA-3389	*0.2448*	*0.4805*	*0.5008*	*0.5270*	*0.1371*
MYA-3391	*0.2856*	*0.5062*	*0.5589*	*0.5738*	*0.1389*
MYA-3394	**0.3191**	*0.4697*	*0.4402*	*0.5269*	*0.1736*
MYA-3395	*0.2061*	*0.5545*	*0.6459*	*0.5956*	*0.1616*
MYA-3398	*0.2763*	*0.5510*	*0.6349*	*0.6290*	*0.1717*
CABI380234	*0.1831*	*0.4107*	*0.5517*	*0.6213*	*0.1074*
ATCC201056	**0.3412**	*0.5259*	*0.5712*	*0.5294*	*0.1092*
MYA-3401	*0.2384*	*0.4486*	*0.5264*	*0.4797*	*0.1249*

[a] Strains belonging to the slow-growing and very slow-growing clusters are listed in bold and italics, respectively. Radial growth was measured twice a week for 30 d. K-means clustering using the software TMEV (Multi-Experiment Viewer) was based on the Euclidean distance measured between each of the strains at each temperature. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment.

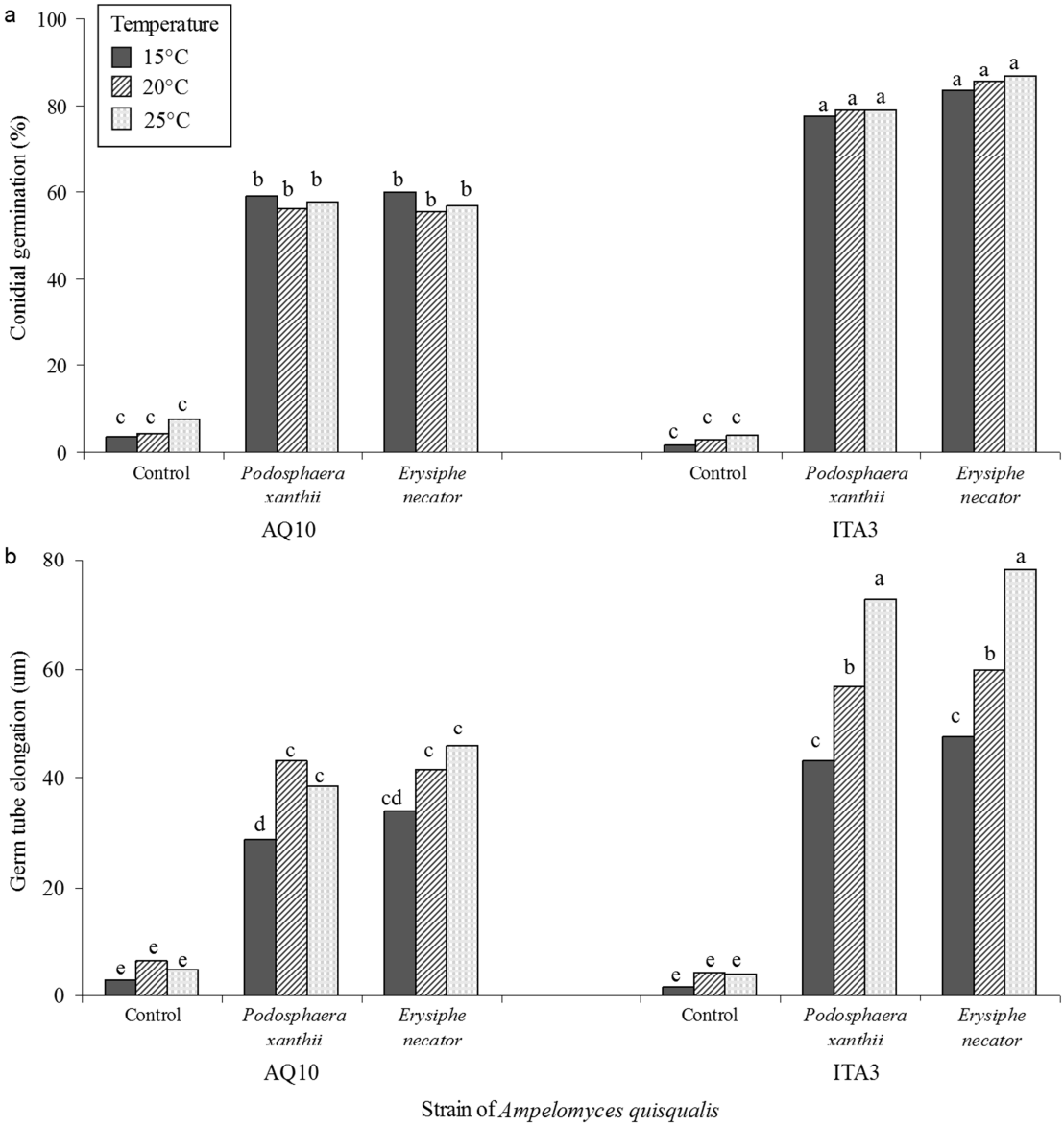
Physiological tests with different powdery mildew species and *A. quisqualis* strains showed that the germination rate and germ-tube length of *A. quisqualis* conidia increased greatly in the presence of powdery mildew conidia. This stimulation of germination is not powdery mildew species-specific. All of the tested powdery mildews increased the germination rate and tube elongation of the *A. quisqualis* strains isolated from different host species (Figs. 2a–b). However, in some cases, significant differences in the induction of germination were detected among the different powdery mildews. For example, the conidial germination of CBS129.79 (originally isolated from cucumber) was stimulated more strongly by grapevine powdery mildew and cucumber powdery mildew than by strawberry powdery mildew. The conidial germination of MYA-3391 (originally isolated from apple) was most strongly induced by grapevine powdery mildew and was less affected by strawberry and apple powdery mildews. We observed big differences among the conidial germination rates of the different strains, independent of the powdery mildew species. The conidial germination of ITA3 and MYA-3401 was strongly induced by conidia of all of the tested powdery mildew species (both in terms of germination rate and tube elongation); whereas AQ10 was generally the least responsive.

In the second experiment, in which we investigated the effect of temperature, we again observed that, in the absence of powdery mildew conidia, the germination rates and germ-tube elongation of strains ITA3 and AQ10 were very poor (Figs. 3a–b). Both parameters were strongly enhanced in the presence of powdery mildew conidia, with no statistical differences between the effects of *E. necator* and those of *P. xanthii*. Just as in the previous experiment, ITA3 was significantly more induced by powdery mildew conidia than AQ10 (Figs. 3a–b). We did not observe any changes in the general behavior of the two *A. quisqualis* strains at the different tested temperatures. In fact, temperature (within the tested range) had no effect on germination rate, but did seem to affect tube length. In the presence of powdery mildew conidia, tube elongation for both strains was always greater at the two higher temperatures (20° and 25 °C) than at 15 °C.

In these two experiments, AQ10 exhibited significantly lower germination rates and less tube elongation in the presence of powdery mildew conidia, as compared to the strain ITA3 (Figs. 3a–b). However, on agar plates incubated at the examined temperatures, AQ10 and ITA3 were classified as slow-growing and very slow-growing, respectively. Thus, the growth rates of strains of *A. quisqualis* on artificial media are not correlated with their germination rates and tube elongation in the presence of a host fungus.



Figures 2a–b. Effects of different powdery mildew species (*Podosphaera xanthii* from cucumber, *Erysiphe necator* from grapevine, *Podosphaera aphanis* from strawberry and *Podosphaera leucotricha* from apple) on the conidial germination (%) (a) and tube elongation (µm) (b) of five selected strains of *Ampelomyces quisqualis*. Measurements were taken in aqueous suspensions containing mixtures of *A. quisqualis* and powdery mildew conidia that had been incubated together for 24 h at 25 °C. Identical column designs indicate exposure to the same powdery mildew species. Columns labeled with the same letter (a-d) are not significantly different ($P \leq 0.05$) according to Tukey's test. Three replicates (50 conidia per replicate) of each strain were evaluated. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment.



Figures 3a–b. Effect of temperature on the conidial germination (%) (a) and tube elongation (µm) (b) of two selected strains of *Ampelomyces quisqualis* in the presence of two different powdery mildew species (*Podosphaera aphanis* from cucumber and *Erysiphe necator* from grapevine). Measurements were taken in aqueous suspensions containing mixtures of *A. quisqualis* and powdery mildew conidia that had been incubated for 24 h at three different temperatures (15°, 20° and 25 °C). Identical column designs indicate incubation at the same temperature. Columns labeled with the same letter (a-e) are not significantly different according to Tukey's test (α = 0.05). Three replicates (50 conidia per replicate) of each strain were evaluated. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment.

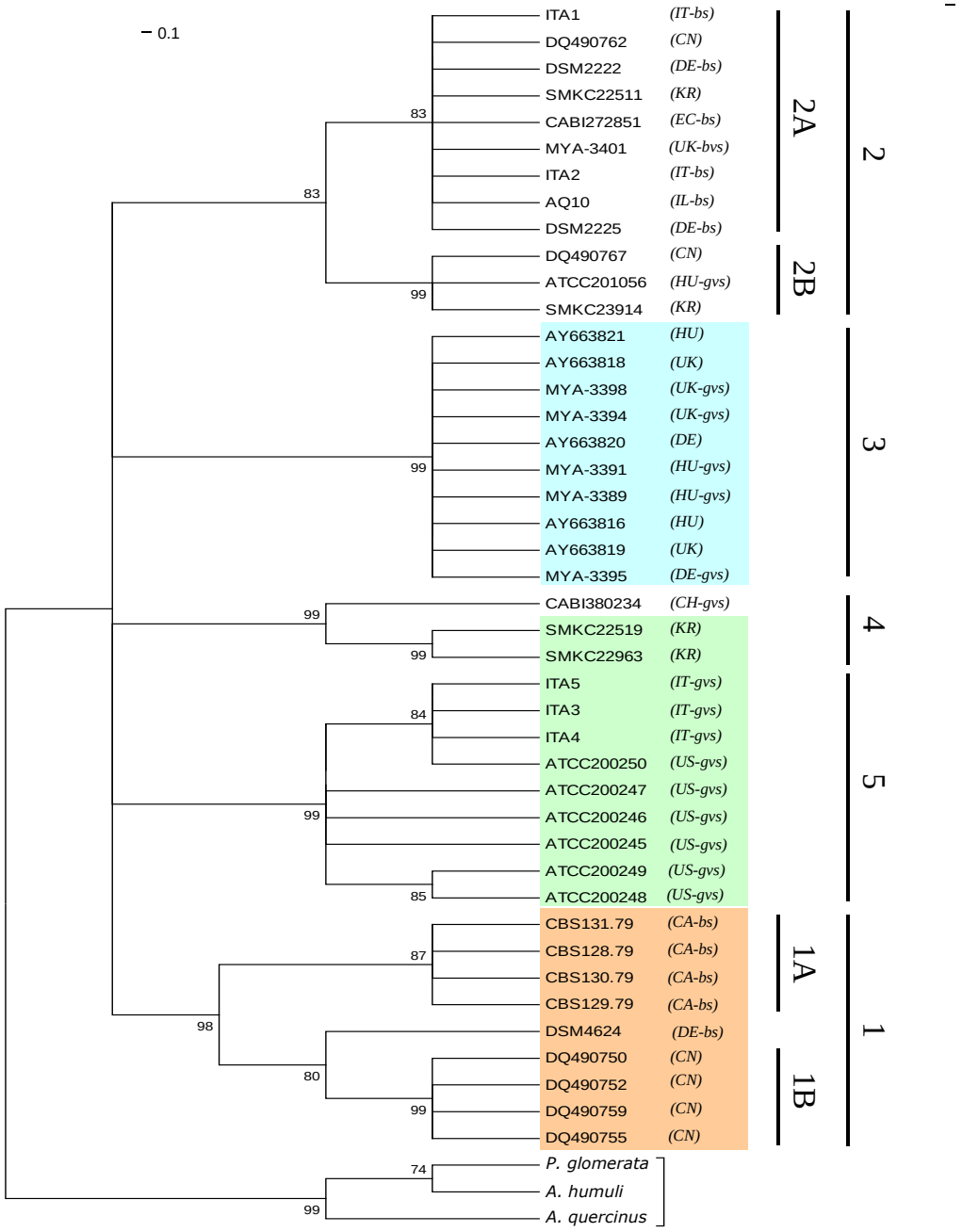
3.3.2 Phylogenetic analysis

In the phylogenetic analysis, the 43 *A. quisqualis* strains clustered into five different groups (1–5), revealing genetic diversity among the A. *quisqualis* strains included in the present work (Fig. 4). *A. quisqualis* strains with similar ITS sequences were often isolated from the same powdery mildew host. In fact, strains isolated from powdery mildew of apple (clade 3), powdery mildew of cucumber (clade 1) and powdery mildew of grapevine (clade 4 and 5) with bootstrap (BS) values between 98 and 99% mainly clustered into four different genetic groups. However, different sequence similarity was observed among strains in clade 1 (92–100 %), clade 3 (99–100 %), clade 4 (95–100 %) and clade 5 (97–100 %). Strains isolated from apple powdery mildew and classified within clade 3 displayed the least ITS diversity, even though they were collected in three different geographical areas.

Comparing clades, clade 1 containing strains isolated from cucumber had the greatest ITS sequence diversity. In fact, these strains can be divided into two different subclades, the first composed of strains collected in Canada (1A) and the second made up of strains collected in China (1B). The strains within each of these subclades share exactly the same ITS sequence (100 %). Comparing strains according to their host, the strains from grapevine clearly displayed the highest level of ITS diversity. These strains fell into two different clades: clade 4, which includes strains isolated in China, and clade 5, which includes strains isolated in the USA and Italy. Taking a closer look at the ITS sequence clustering in clade 5, we see that, with the exception of ATCC200250, the Italian strains seem to differ from the American strains. The only ITS clade which includes strains isolated from different mycohosts and in different geographical regions was clade 2. This clade contains two strains from grapevine powdery mildew (ITA1 and ITA2), one strain from cucumber powdery mildew (DSM2222) and nine strains isolated from several different ornamental plants. The 12 strains in clade 2 (BS value of 83 %) were further divided into two subgroups: subclade 2A (nine sequences) and subclade 2B (three sequences), which were supported by BS values of 83 and 99 %, respectively, and which each had ITS-similarity scores of 98–100 %. Among the strains included in subclade 2A, only the ITS sequence of strains DSM2225 and DQ490767 differed from that of the commercial strain AQ10.

Interestingly, strains isolated from the same fungal host and assigned to the same ITS group (clades 1, 3, 4 and 5) showed similar colony morphology and growth characteristics. Strains

belonging to clades 3, 4 and 5 are green and very slow-growing; whereas strains belonging to clade 1 are brown and grow more quickly. Again, clade 2 is an exception in that it includes strains that differ in the color of their colonies and their *in vitro* growth rates.



Figures 4. The maximum-likelihood tree for the ITS rDNA sequences of 43 *Ampelomyces quisqualis* strains. Sequences were inferred with the Mega4 software package, using the Jukes-Cantor substitution model and rate uniformity among sites. The gaps in the 450-character-long alignment were handled as missing characters. The bootstrap values were obtained from maximum-likelihood and maximum-parsimony analyses. The values shown above the branches are percentages of 1000 replicates; scores below 50 % are not shown. The scale bar represents 0.1 substitutions per nucleotide position. The five groups (1–5) discussed in the text are indicated on the tree. Different colors are used to indicate the different powdery mildew host species: blue = apple, green = grapevine, red = cucumber, no color = other species). In brackets: geographic origin, colony color and growth pattern of each strain. Geographic origin: DE = Germany, UK= United Kingdom, HU = Hungary, CA = Canada, CH = Switzerland, IT = Italy, IL = Israel, EC = Ecuador, CN = China, KR = South Korea, US = United States of America. Colony color and growth pattern: bs = brown, slow-growing; bvs = brown, very slow-growing; gvs = green, very slow-growing).

3.4 Discussion

A. quisqualis strains have mostly been studied for use in the biological control of powdery mildew on different plant species. Data on the morphology and cultural patterns of *A. quisqualis* strains found in the literature are controversial and incomplete. There is a need for further cultural and morphological investigations aiming at the identification of phenotypic markers that can be used to differentiate genetically distinct groups (Park et al. 2010) within the true *A. quisqualis* [e.g., Type II (Kiss 1997)].

With this study, we aimed to understand whether there are relationships between the origins (host, geographic location) of different strains of *A. quisqualis* and their phylogeny and/or other characteristics (cultural, growth, morphology, physiology), in order to establish the existence of any host/geographic adaption of this mycoparasite. Our results provide solid evidence for the existence of different physiological forms among slow-growing *A. quisqualis* strains [e.g., Type II (Kiss 1997)] isolated from different fungal hosts. These physiological forms vary in terms of their cultural, morphological and physiological characteristics. In the present study, the *A. quisqualis* strains cultured on artificial media did not differ in the dimensions or shape of their fruiting bodies (pycnidia and conidia). Thus, these morphological characters are insufficient for accurate identification of different strains in the laboratory. Based on colony characteristics and

growth rates on artificial media, two fairly homogenous groups can be clearly distinguished: dark-brown, slow-growing strains and olive-green, very slow-growing strains. Thus, a clear relationship was detected between the growth rate and colony color of *A. quisqualis* strains. Moreover, we demonstrated the existence of a relationship between the original fungal host and cultural and growth characteristics. In contrast, we did not identify any relationship between the geographic origins of the different strains and their *in vitro* growth rates.

As expected, temperature greatly affects the growth rate of *A. quisqualis*. We observed differences in the behavior of different strains at different temperatures and six strains (DSM4624, CBS128.79, CBS129.79, CBS130.79, CBS131.79, CABI272851) grew faster than the others at all of the tested temperatures.

Powdery mildew strongly affects the germination of *A. quisqualis* conidia during the recognition phase of parasitism, but specialization is indistinct. In fact, the germination rates and tube elongation of all of the examined *A. quisqualis* strains could be stimulated by all of the examined powdery mildew species and not only by the conidia of their original fungal host. However, we did observe statistically significant differences among the tested strains. Strain ITA3 was most strongly stimulated and, interestingly, among all the tested strains, the commercial strain AQ10 was stimulated the least by the presence of conidia of different powdery mildew species.

Furthermore, the present study clearly shows that the growth rate of *A. quisqualis* on artificial media is not related to the conidial germination or the germ-tube elongation of *A. quisqualis* during the recognition phase of parasitism. Therefore, we think that a screening program based on *in vitro* growth rates is not the right way to select strains of this mycoparasitic fungus for use in biocontrol programs.

Finally, phylogenetic analysis of the ITS rDNA sequence revealed a high level of genetic diversity among Type II strains of *A. quisqualis* and suggested that ITS groups could be related to the host fungus. In most cases, a degree of mycohost specialization was present in the *A. quisqualis* strains, as demonstrated by the phylogenetic studies conducted by Park et al. (2010). Our study revealed that strains isolated from the same host species had similar cultural, morphological and growth characteristics and grouped into the same ITS group. Moreover, among the strains isolated from cucumber powdery mildew and grapevine powdery mildew, we observed differentiation among *A. quisqualis* strains that was congruent with differences in

geographic origin. Therefore, it appears that, in some cases, powdery mildew fungi and *A. quisqualis* might have coevolved through the processes of specialization and adaptation without the mycoparasite losing the ability to be stimulated by other hosts.

Our results indicate that the Type II strains of *A. quisqualis* examined in our study are widely scattered among four clades and two subclades identified by Park et al. (2010). Furthermore, this study reveals the presence of an additional clade (5) that includes the Type II strains isolated from grapevine powdery mildew whose morphology and physiology were analyzed in this experiment, as well as all of the American strains of grapevine powdery mildew. Clade 5 appears homogeneous in regard to the mycohost (*E. necator*), as well as colony color and growth pattern. However, the clustering of strains within clade 5 may suggest a certain tendency toward geographic–genetic differentiation, suggesting possible local adaptations of this mycoparasite that should be verified in wider studies. Clade 2A includes the commercial AQ10 strain, as well as five strains (CABI272851, MYA-3401, ITA1, ITA2, SMKC22511) that were isolated after the commercialization of AQ10 and have identical ITS sequences, suggesting that those five strains may have originated from AQ10 treatments applied in the same area. This would indicate that the commercial strain can spread and persist in nature. On the other hand, the strain DQ490762 belonging to Clade 2A which was isolated after the commercialization of AQ10 and having a different nucleotide sequence, probably represents a distinct strain.

Screening is a crucial step in the selection of strains capable of providing highly effective biocontrol. Future studies should attempt to clarify whether the conidial germination rates of *A. quisqualis* strains are related to differences in virulence against powdery mildew, which would be a relevant factor in the selection of biocontrol agents.

3.5 Acknowledgements

The authors thank Dr. Levente Kiss (Plant Protection Institute of the Hungarian Academy of Science, Budapest, Hungary) for valuable comments and suggestions and for providing fungal strains. We are grateful to the following colleagues for their helpful suggestions and assistance with the data analysis: Lorenzo Tosi, Valerio Mazzoni and Alberto Pellegrini. We would also like to thank Susanna Micheli and Denise Ress for their technical support and assistance. This

work was supported by the AMPELO project (morphological and genetic analysis) and the ENVIROCHANGE project (effect of temperature), both funded by the Autonomous Province of Trento.

3.6 Literature

Angeli, D., Pellegrini, E. and Pertot, I. (2009a). Occurrence of *Erysiphe necator* chasmothecia and their natural parasitization by *Ampelomyces quisqualis*. Phytopathology, 99, 704-710.

Angeli, D., Pellegrini, E., Micheli, S., Ress, D., Maurhofer, M., Pertot, I. and Gessler, C. (2009b). Molecular characterization of *Ampelomyces* spp. strains from different hosts and geographic origins and evaluation of their potential to control powdery mildew of cucumber. IOBC/WPRS Bulletin, 43, 40-44.

Bélanger, R.R. and Labbé, C. (2002). Control of powdery mildews without chemicals: prophylactic and biological alternatives for horticultural crops. In: Bélanger RR, Bushnell WR, Dik AJ, Carver TLW (eds), The Powdery Mildews: a Comprehensive Treatise, APS Press, St. Paul, MN, pp. 256-267.

Belsare, S.W., Moniz, L. and Deo, V.B. (1980). The hyperparasite *Ampelomyces quisqualis* Ces. from Maharashtra State, India. Biovigyanam, 6, 173-176.

Falk, S.P., Gadoury, D.M., Cortesi, P., Pearson, R.C. and Seem, R.C. (1995a). Parasitism of *Uncinula necator* cleistothecia by the mycoparasite *Ampelomyces quisqualis*. Phytopathology, 85, 794-800.

Falk, S.P., Gadoury, D.M., Pearson, R.C. and Seem, R.C. (1995b). Partial control of grape powdery mildew by the mycoparasite *Ampelomyces quisqualis*. Plant Disease, 79, 483-490.

Gardes, M. and Bruns, T.D. (1993). ITS primers with enhanced specifity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology, 2, 113-118.

Gu, Y.H. and Ko, W.H. (1997). Water agarose medium for studying factors affecting germination of conidia of *Ampelomyces quisqualis*. Mycological Research, 101, 422-424.

Hashioka, Y. and Nakai, Y. (1980). Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. Transactions of the Mycological Society of Japan, 21, 329-338.

Jarvis, W.R. and Slingsby, K. (1997). The control of powdery mildew of greenhouse cucumber by water spray and *Ampelomyces quisqualis*. Plant Disease Reporter, 61, 728-730.

Kiss, L. (1997). Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. Mycological Research, 101, 1073-1080.

Kiss, L. (1998). Natural occurrence of *Ampelomyces* intracellular mycoparasites in mycelia of powdery mildew fungi. New Phytologist, 140, 709-714.

Kiss, L. (2003). A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Management Science, 59, 475-483.

Kiss, L. and Nakasone, K.K. (1998). Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi. Current Genetics, 33, 362-367.

Kiss, L., Russell, J.C., Szentivanyi, O., Xu, X. and Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonist of powdery mildew fungi. Biocontrol Science and Technology, 14, 635-651.

Kiss, L., Pintye, A., Kovacs, G.M., Jankovics, T., Fontaine, M.C., Harvey, N., Xu, X., Nicot, P.C., Bardin, M., Shykoff, J.A. and Giraud, T. (2011). Temporal isolation explains host-related genetic differentiation in a group of widespread mycoparasitic fungi. Molecular Ecology, 20, 1492-1507.

Kiss, L., Pintye, A., Zséli, G., Jankovics, T., Szentivànyi, O., Hafez, Y.M. and Cook, R.T.A. (2009). Microcyclic conidiogenesis in powdery mildews and its association with intracellular parasitism by *Ampelomyces*. European Journal of Plant Pathology, 126, 445-451.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G. (2007). ClustalW2 and ClustalX version 2. Bioinformatics, 23, 2947-2948.

Liang, C., Yang, J., Kovacs, G.M., Szentivanyi, O., Li, B., Xu, X.M. and Kiss, L. (2007). Genetic diversity of *Ampelomyces* mycoparasite isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences. Fungal Diversity, 24, 225-240.

Mashkar, D.N. (1974a). Mycoparasite – *Ampelomyces* in artificial culture I. Morphology and cultural behaviour. Mycopathologia et Mycologia applicata, 52, 55-64.

Mashkar, D.N. and Rao, V.G. (1974b). The mycoparasite *Ampelomyces quisqualis* Ces. in artificial culture. II. Effect of environmental factors. Phytopathologia Mediterranea, 13, 147-154.

Nei, M. and Kumar, S. (2000). Molecular Evolution and Phylogenetics. New York, Oxford Univ. 333 p.

Page, R.D.M. (1996). Tree View: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences, 12, 357-358.

Paulitz, T.C. and Bèlanger, R.R. (2001). Biological control in greenhouse systems. Annual Review of Phytopathology, 39, 103-133.

Park, M.J., Choi, Y.J., Hong, S.B. and Shin, H.D. (2010). Genetic variability and mycohost association of *Ampelomyces quisqualis* isolates inferred from phylogenetic analyses of ITS rDNA and actin gene sequences. Fungal Biology, 114, 235-247.

Philipp, W.D. and Cruger, G. (1979). Parasitismus von *Ampelomyces quisqualis* auf echten mehltaupilzen an gurken und anderen gemusearten. Zeitchrift fur Pflkanzenkrankheiten und Pflanzenschutz, 86, 129-142.

Rankovic, B. (1997). Hyperparasites of the genus *Ampelomyces* on powdery mildew fungi in Serbia. Mycopathologia, 139, 157-164.

Shin, H.D. and Kyeung, H.Y. (1994). Isolation of hyperparasitic fungi to control powdery mildews and selection of superior isolates for biocontrol of cucurbit powdery mildew. RDA Journal of Agricoltural Science, 36, 141-151.

Speer, E.O. (1978). Beitrag zur morphologie von *Ampelomyces quisqualis* Ces. Sydowia, 31, 242-246.

Staden, R., Beal, K.F. and Bonfield, J.K. (2000). The Staden package, 1998. Methods in Molecular Biology, 132, 115-130.

Sullivan, R.F. and White, Jr. J.F. (2000). *Phoma glomerata* as a mycoparasite of powdery mildew. Applied and Environmental Microbiology, 66, 425-427.

Sundheim, L. (1982). Control of cucumber powdery mildew by the hyperparasite *Ampelomyces quisqualis* and fungicides. Plant Pathology, 31, 209-214.

Sundheim, L. and Krekling, T. (1982). Host-parasite relationships of the hyperparasite *Ampelomyces quisqualis* and its powdery mildew host *Sphaerotheca fuliginea.* I. Scanning electron microscopy. Phytopathology, 104, 202-210.

Sundheim, L. and Tronsmo, A. (1988). Hyperparasites in biological control. Biocontrol of Plant Diseases, Vol. I. CRC Press, Boca Raton, FL, pp. 53–69.

Szentiványi, O., Kiss, L., Russell, J.C., Kovács, G.M., Varga, K., Jankovics, T., Lesemann, S., Xu, X.M. and Jeffries, P. (2005). *Ampelomyces* mycoparasites from apple powdery mildew identified as a distinct group based on single-stranded conformation polymorphism analysis of the rDNA ITS region. Mycological Research, 109, 429-438.

Sztejnberg, A. (1993). *Ampelomyces quisqualis* AQ10, CNCM I-807, for biological control of powdery mildew. US patent no. 5190754.

Sztejnberg, A., Galper, S., Mazar, S. and Lisker, N. (1989). *Ampelomyces quisqualis* for biological and integrated control of powdery mildews in Israel. Journal of Phytopathology, 124, 285-295.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24, 1596-1599.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (eds), PCR Protocols: a Guide to Methods and Applications, Academic Press, San Diego, CA, pp. 315-322.

Chapter 4

Is the mycoparasitic activity of *Ampelomyces quisqualis* strains related to phylogeny and hydrolytic enzyme production?

Published as

Angeli D., Puopolo G., Maurhofer M., Gessler C., Pertot I. 2012. Is the mycoparasitic activity of *Ampelomyces quisqualis* strains related to phylogeny and hydrolytic enzyme production? Biological Control, 63 (3): 348-358.

Abstract

The use of mycoparasites is a highly elegant method of biocontrol, as the agent is specific and can expand its activity on its own, and it presents no risk to the environment. *Ampelomyces quisqualis* best typifies the potential of mycoparasites, as it is highly specific to powdery mildews, does not produce any toxic metabolites and can easily be mass-produced *in vitro*. However, the level of parasitization varies between strains, and the cause of this variation is unknown. In this study, twenty four selected strains isolated from different host mildews and possessing different ITS rDNA sequences were evaluated for their ability to colonize cucumber, grapevine and strawberry powdery mildews and for *in vitro* production of cell wall degrading enzymes (CWDEs). Individual strains differed significantly in enlargement of the colonization area by intrahyphal formation of pycnidia within powdery mildew colonies and in inhibition of host conidiation. Pronounced differences in the *in vitro* activity of chitobiases, proteases and β-1,3-glucanases were also observed between strains. We found a positive correlation between mycoparasitic activity and chitobiases and proteases but not glucanases. Furthermore, principal component analysis showed that *A. quisqualis* strains with similar levels of mycoparasitic activity originated from the same host species and shared an identical ITS rDNA sequence. These results provide a deeper understanding of the process of mycoparasitism and provide a sound basis for developing new screening strategies for detecting highly effective *A. quisqualis* strains in the biocontrol of powdery mildews.

4.1 Introduction

Biotrophic fungi belonging to the *Erysiphaceae* family are the causal agents of powdery mildews that are among the most damaging plant diseases worldwide (Glawe, 2008). Control of powdery mildew diseases relies mainly on the use of chemical fungicides, yet intensive use of these can result in the accumulation of toxic compounds that are potentially hazardous to humans and the environment. In order to reduce the use of chemical pesticides, alternative methods for controlling powdery mildews have been studied, including the use of microbial biocontrol agents (Paulitz & Bélanger, 2001; Elad et al., 1995). *Ampelomyces quisqualis* is a specific pycnidial mycoparasite of several species of *Erysiphales* and has a wide distribution in tropical and

temperate climates (Angeli et al., 2009a; Kiss et al., 2004; Rankovic, 1997). It is the most studied biocontrol agent of powdery mildews (Kiss, 1997) and a formulation based on an *A. quisqualis* strain originally isolated in Israel (CNCM I-807) has been registered and is marketed under the trade name of AQ10 (Sztejnberg, 1993).

During recent decades, the species *A. quisqualis* has undergone several taxonomic reorganizations resulting in the assignment of the fast-growing strains (*in vitro* radial growth of 3–4 mm day^{-1}) to *Phoma glomerata* and other *Ampelomyces* spp., the slow-growing strains (0.5–1.0 mm day^{-1}) have been assigned to *A. quisqualis* sensu stricto (Kiss, 1997; Kiss & Nakasone, 1998). Molecular analyses based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (rDNA) have revealed a high level of genetic diversity among *A. quisqualis* sensu stricto strains (Angeli et al., 2009b; Kiss, 1997; Kiss & Nakasone, 1998; Kiss et al., 2011; Liang et al., 2007; Nischwitz et al., 2005; Sullivan & White, 2000; Szentivanyi et al., 2005). Recently, phylogenetic studies have indicated that ITS groups could be related to the host fungus, suggesting, in most cases, a degree of mycohost specialization, although no evidence for a strict association has been found (Angeli et al., 2012; Park et al., 2010; Pintye et al., 2012). Furthermore, different forms in terms of cultural, morphological and physiological characteristics have been observed within *A. quisqualis* sensu stricto strains isolated from different powdery mildew agents (Angeli et al., 2012).

A. quisqualis invades the powdery mildew host and grows within the mycelium and fruiting bodies. It forms pycnidia within the hyphae, conidiophores and immature chasmothecia of the host, and its development suppresses asexual and sexual sporulation of the powdery mildew (Hashioka & Nakai, 1980; Sundheim & Krekling, 1982). Growth of *A. quisqualis* germ-tubes towards powdery mildew hyphae has also been observed (Sundheim & Krekling, 1982). *A. quisqualis* recognizes the presence of the host and a water-soluble substance from powdery mildew conidia has been shown to stimulate germination of *A. quisqualis* conidia *in vitro* (Gu & Ko, 1997). Toxin production has not been detected in *A. quisqualis* (Kiss et al., 2004), in contrast to another pycnidial mycoparasite, *Coniothyrium minitans* (Machida et al., 2001; McQuilken et al., 2003).

Mycoparasitism is a complex mechanism and hydrolytic enzymes have been reported as key factors responsible for fungal cell wall lysis and cell degradation (Cook & Baker, 1983). Several

fungi have been shown to produce these enzymes and they play an important role in biological control (Adams, 2004; Elad et al., 1982; Elad et al., 1985; McQuilken & Gemmell, 2004; Mucha et al., 2006). Under *in vitro* conditions, exposure of phytopathogenic fungi to lytic enzymes such as chitinases, proteases or glucanases can result in degradation of the structural matrix of fungal cell walls (Lorito et al., 1994a; Lorito et al., 1994b; Oppenheim & Chet, 1992; Pozo et al., 2004; Viterbo et al., 2004 Gruber & Seidl-Seiboth, 2012). The role played by lytic enzymes produced by another mycoparasitic fungus, *Trichoderma harzianum*, in biological control has been explored in depth in many studies which have demonstrated the importance of chitinases, proteases and β-1,3-glucanases in degradation of the fungal cell wall of phytopatogenic fungi (Geremia et al., 1993; Haran et al., 1996; Lorito et al., 1994b; Monteiro et al., 2010; Salmoski et al., 2009).

Penetration of the host cell wall by *A. quisqualis* is likely to involve both enzymatic and mechanical processes. Appressorium-like structures have been reported at the point of penetration (Sundheim & Krekling, 1982), although little is known about lytic extracellular enzymes of *A. quisqualis*. Philipp (1985) identified hydrolytic enzymes in liquid cultures of *A. quisqualis*, which may play a role in degradation of powdery mildew hyphal cell walls during penetration. At that time, however, *P. glomerata* and other *Ampelomyces* spp. (*A. quisqualis* fast-growing strains) had not been separated from *A. quisqualis* sensu stricto (Kiss, 1997), after therefore it is not possible to unequivocally attribute the reported activities to strains of the *A. quisqualis* sensu stricto group. A few years later, Rotem et al. (1999) demonstrated that *A. quisqualis* can excrete an exo-β-1,3-glucanase in culture and during mycoparasitism, and showed that culture filtrates of *A. quisqualis* strain AQ10 could degrade hyphal walls of *Podosphaera xanthii* in the absence of active mycelium.

Yarwood (1932) was the first author to identify the potential role of *A. quisqualis* as a biocontrol agent, although the first important efficacy trial was reported by Jarvis and Slingsby (1977) who used a conidial suspension of the mycoparasite to control cucumber powdery mildew in greenhouse. The commercial strain AQ10 is used to control powdery mildew in various crops but the reported data on its efficacy are contradictory (Sztejnberg, 1993). In some experiments, it effectively controled powdery mildews in various crops; other trials resulted in unsatisfactory levels of biocontrol, although parasitism of powdery mildew colonies on the treated crops did

occur (Sztejnberg, 1993). Repeated applications are generally more effective, while high humidity and rainfall help the mycoparasite to spread and develop. Some preliminary biocontrol experiments suggest there may be different levels of efficacy among different strains of *A. quisqualis* (Angeli et al., 2009b). There is still considerable interest in finding mycoparasitic strains within *A. quisqualis* species that afford more effective plant protection than the existing biofungicide strain.

The present work aims to ascertain whether i) genetically different *A. quisqualis* strains (isolated from different powdery mildew hosts) have different levels of mycoparasitic activity against powdery mildews; ii) the level of mycoparasitic activity is linked to the genotype and/or fungal host of origin; iii) there is a correlation between mycoparasitic activity, genotype and *in vitro* production of CWDEs in *A. quisqualis*. Our approach is based on characterizing the mycoparasitic activity of the *A. quisqualis* strains in terms of their ability to inhibit conidiation in different powdery mildew pathogens and intra-hyphal formation of their intracellular pycnidia within powdery mildews *in vivo*. At the same time, production of cell wall degrading enzymes (CWDEs) was measured under *in vitro* conditions. Analysis of the data indicates which factors are important in effective mycoparasitism of *A. quisqualis* sensu stricto.

4.2 Materials and methods

4.2.1 Fungal strains and pathogens

A total of twenty-four genetically different strains belonging to the *A. quisqualis* sensu stricto group (Kiss, 1997) isolated from different plants, sites and powdery mildew species were included in the present study (Table 1). The strains were obtained from culture collections (ATCC, American Type Culture Collection, Rockville, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; CABI, Commonwealth Agricultural Bureaux International, Egham, United Kingdom; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh, Braunschweig, Germany) or were provided by individual scientists (L. Kiss, PPI, Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary). Five strains were isolated by myself (Angeli et al. 2009b) and belonged to the collection of FEM (Fondazione Edmund Mach, S. Michele all'Adige, Italy). Phenotypic and genetic relationships

among these strains have already been identified (Angeli et al., 2012). During the experiments the strains were grown on Potato Dextrose Agar (PDA, Oxoid, United Kingdom) in the dark at 25 °C (Gu & Ko, 1997; Angeli et al., 2012). For long-term preservation, the strains were kept in glycerol 10 % at −80 °C using microbank vials (Pro-Lab Diagnostic, Richmond Hill, ON, Canada).

Table 1. Designation, hosts, area and year of isolation, source, ITS group and GenBank accession numbers of *Ampelomyces quisqualis* strains included in this study.

ID code [a]	Fungal host	Plant host	ITS group [b]	Geographical origin and year of isolation	Source	GenBank [c]
ATCC 200245	*Erysiphe necator*	*Vitis vinifera*	5	New York (USA), 1989	ATCC	AF126817
ATCC 200246	*E. necator*	*V. vinifera*	5	New York (USA), 1991	ATCC	HQ108030
ATCC 200249	*E. necator*	*V. vinifera*	5	New York (USA), 1991	ATCC	HQ108033
ATCC 200250	*E. necator*	*V. vinifera*	5	New York (USA), 1991	ATCC	HQ108034
ITA 1	*E. necator*	*V. vinifera*	2A	Italy (I), 2007	D. Angeli (FEM)	HQ108047
ITA 2	*E. necator*	*V. vinifera*	2A	Italy (I), 2007	D. Angeli (FEM)	HQ108048
ITA 3	*E. necator*	*V. vinifera*	5	Italy (I), 2007	D. Angeli (FEM)	HQ108049
ITA 4	*E. necator*	*V. vinifera*	5	Italy, 2007	D. Angeli (FEM)	HQ108050
ITA 5	*E. necator*	*V. vinifera*	5	Italy, 2007	D. Angeli (FEM)	HQ108051
CBS 128.79	*Erysiphe cichoracearum*	*Cucumis sativus*	1	Canada (CDN), 1975	CBS	HQ108037
CBS 129.79	*E. cichoracearum*	*C. sativus*	1	Canada (CDN), 1975	CBS	HQ108038
CBS 130.79	*E. cichoracearum*	*C. sativus*	1	Canada (CDN), 1975	CBS	HQ108039
CBS 131.79	*E. cichoracearum*	*C. sativus*	1	Canada (CDN), 1975	CBS	HQ108040
DSM 2222	*E. cichoracearum*	*C. sativus*	2A	Germany (D), ? [e]	DSMZ	U82450
MYA 3389	*Podosphaera leucotricha*	*Malus domestica*	3	Hungary (H), 1995	L. Kiss (PPI)	AY663815
MYA 3391	*P. leucotricha*	*M. domestica*	3	Hungary (H), 1995	L. Kiss (PPI)	HQ108043
MYA 3394	*P. leucotricha*	*M. domestica*	3	United Kingdom (GB), 2002	L. Kiss (PPI)	HQ108044
MYA 3395	*P. leucotricha*	*M. domestica*	3	Germany (D), 2002	L. Kiss (PPI)	AY663817

Table 1. (continued)

ID code [a]	Fungal host	Plant host	ITS group [b]	Geographical origin and year of isolation	Source	GenBank [c]
MYA 3398	*P. leucotricha*	*M. domestica*	3	United Kingdom (GB), 2002	L. Kiss (PPI)	HQ108045
AQ10	Powdery mildew [d]	*Catha edulis*	2A	Israel (IL), ? [e]	Ecogen Italia	AF035783
DSM 2225	*Erysiphe heraclei*	*Daucus* sp.	2A	Germany (D), ? [e]	DSMZ	HQ108042
DSM 4624	*Podosphaera xanthii*	*Leontodon* sp.	2A	Germany (D), ? [e]	DSMZ	HQ108041
CABI 272851	Powdery mildew [d]	*Schinus molle*	2A	Ecuador (EC), 1983	CABI	HQ108036
MYA 3401	*Oidium hortensiae*	*Hydrangea macrophylla*	2A	United Kingdom (GB), 1999	L. Kiss (PPI)	HQ108046

[a] Type II strains deposited in culture collections (ATCC, CBS, CABI, DSMZ) and provided by individual scientists (L. Kiss, D. Angeli).

[b] ITS rDNA grouping according to Angeli et al. 2012.

[c] GenBank accession numbers of the rDNA ITS sequences.

[d] Species is unknown.

[e] Year of isolation is unknown.

The aggressiveness of the *A. quisqualis* strains was tested against the pathogens *Erysiphe necator*, *Podosphaera aphanis* and *P. xanthii*, which were collected in the Trentino-Alto Adige region between 2009 and 2011 from naturally infected grapevine, strawberry and cucumber plants, respectively. Fresh colonies of powdery mildew were maintained under controlled greenhouse conditions at 25 ± 1 °C and 70 ± 10 % relative humidity (RH) at a fixed daily photoperiod of 16 h. Subsequent inoculations of powdery mildew were carried out every 30 days by shaking leaves bearing sporulating mildew over three-week-old plants with at least five true leaves (Angeli et al., 2009b).

The *Trichoderma atroviride* strain SC1 has been used as a positive control in enzymatic experiments as the activity of chitinolytic, proteolytic and glycolytic enzymes in *Trichoderma* spp. has already been extensively characterized (Kubicek et al., 2001; Benitez et al., 2004). *T. atroviride* SC1 was isolated from decaying hazelnut in northern Italy and is currently deposited in the CBS restricted collection (CBS 122089) in accordance with the regulations of the Budapest Treaty.

4.2.2 Mycoparasitic activity: ability of *A. quisqualis* to reduce conidiation of powdery mildews *in vivo*

The ability of the 24 *A. quisqualis* strains to inhibit conidiation of *E. necator*, *P. aphanis* and *P. xanthii* on their respective host plants was assessed under controlled conditions. Two-week-old grapevine, strawberry and cucumber plants were inoculated with their respective powdery mildew pathogens; *P. xanthii* inoculation was carried out by spraying a suspension of distilled water and Tween 80 (0.01 %) containing 1×10^5 conidia/ml, while in the case of *E. necator* and *P. aphanis* dry inoculation was effected by shaking infected leaves bearing sporulating mildew over the plants. The amount of strawberry and grapevine powdery mildew inoculum was determined by counting conidia on glass slides placed between the plants during dry inoculation and was expressed as the number of conidia per cm^2. The choice of different inoculation methods was based on preliminary trials carried out to optimize the methodology (data not shown). Plants were incubated in the greenhouse at 25 ± 1 °C and 70 ± 10 % of RH at a fixed daily photoperiod of 16 h. As soon as powdery mildew conidiation appeared on the upper surface of the leaves (5, 7 and 12 days after infection for cucumber, strawberry and grapevine, respectively) plants were

immediately treated with *A. quisqualis* (see below). Powdery mildew conidiation appeared as a homogenous infection on cucumber and grapevine leaves, while several powdery spots appeared on strawberry leaves.

Conidia suspensions of *A. quisqualis* strains were prepared as follows: *A. quisqualis* strains were grown on PDA in Petri dishes for two weeks at 25 °C in the dark (ten plates per strain); conidia were harvested by adding 20 ml of sterile distilled water and Tween 80 (0.01 %) to each plate and concentration was adjusted to 1×10^6 conidia/ml under a light microscope (Hund Wetzlar H 600LL, Wetzlar, Germany) using a Thoma-Zeiss counting chamber.

Plants bearing sporulating colonies of *P. xanthii* and *E. necator* were homogeneously treated with the suspension containing 1×10^6 conidia/ml of *A. quisqualis* using a hand air-sprayer. Only two basal leaves per plant, previously infected with the relevant powdery mildew, were treated (5 ml of suspension per leaf). For strawberry, a drop of the suspension containing *A. quisqualis* conidia (10 µl each) was applied to powdery mildew spots of approximately 3 mm diameter. Six spots per leaf were treated. Plants with powdery mildew and *A. quisqualis* infections were kept at 25 °C and 95 % RH for 48 h, after which humidity was reduced to 70 ± 10 % RH. Control plants with powdery mildew lesions were sprayed with distilled water and Tween 80 only.

Reduction in powdery mildew fungal conidiation was assessed by counting the number of *E. necator*, *P. aphanis* and *P. xanthii* conidia produced by the pathogens on the leaf surface 10 days after *A. quisqualis* treatment compared with untreated infected plants. The two basal leaves of each grapevine and cucumber plant were collected (12 leaves per strain) and leaf disks of 1.8 cm diameter were cut (6 disks per grapevine leaf, 18 disks per cucumber leaf). The leaf disks were transferred to 50 ml tubes containing 5 ml (grapevine) or 15 ml (cucumber) distilled water with Tween 80 (0.01 %). For strawberry, two leaves per plant were picked and a leaf disk of 3 mm diameter was cut from each lesion and transferred to a 2 ml tube containing 1 ml distilled water with 0.01 % Tween 80. In all three cases, tubes were vortexed for 1 min and for each leaf four droplets of 20 µl were mounted on glass slides. Concentrations of powdery mildew conidia were measured under a light microscope and conidia were counted using a Thoma-Zeiss counting chamber. Results were expressed as the percentage reduction in the number of powdery mildew conidia compared with leaves treated with powdery mildew only. Three independent

experiments each with six replicates (plants with two inoculated leaves) per treatment (*A. quisqualis* strain) were performed.

4.2.3 Mycoparasitic activity: intra-hyphal formation of *A. quisqualis* pycnidia within powdery mildews

Intra-hyphal formation of intracellular pycnidia of the 24 *A. quisqualis* strains in grapevine (*E. necator)*, strawberry *(P. aphanis)* and cucumber (*P. xanthii*) powdery mildew colonies was measured following Kiss's (1998) methodology with some modifications. Potted plants were kept in a greenhouse and their leaves inoculated with their respective powdery mildews. Leaves with powdery mildew infection were detached from the plants and the surfaces checked for the presence of freshly sporulating colonies of *E. necator*, *P. aphanis* and *P. xanthii* under a stereomicroscope. Each sporulating powdery mildew colony (lesion), marked with a permanent label on the lower leaf surface, was inoculated with a 2 µl droplet of an *A. quisqualis* conidial suspension (1×10^5 conidia/ml) spotted in the middle of the lesion. Six leaves bearing five inoculated lesions each were examined for each strain. To assess the inoculum area of each fungal strain, a leaf disk of 5 mm diameter was cut from each lesion from three leaves and put onto a glass slide. The area covered by conidial suspension was measured at 40× magnification with a laser microdissection system (Leica LDM 7600 Light Microscope, Leica, Germany). The average expanse of the 15 inoculation areas measured was then subtracted from the total area covered by pycnidia measured at the end of the experiment (see below). The three other inoculated leaves were kept in Petri dishes (180 mm) and placed on plastic nets floated on water. Petioles were submerged in water in order to guarantee leaf survival and high relative humidity. These plates were placed in a climate chamber with 16 h daily illumination at 25 °C for 14 days. After this period, a leaf disk of 5 mm diameter was cut from each lesion and put onto a glass slide. The total area with *A. quisqualis* pycnidia in the powdery mildew mycelium was measured at 40× magnification under the microscope. The difference between the total area covered by pycnidia and the average surface area below the inoculation drop, determined as described above, was calculated. Intra-hyphal enlargement of *A. quisqualis* strains in the powdery mildew mycelium was expressed as $\mu m^2\ day^{-1}$. Three independent experiments, each with three replicates (leaves with five lesions) per treatment (*A. quisqualis* strain), were carried out.

4.2.4 *In vitro* production of CWDEs by *A. quisqualis* culture filtrates

Glucanase activity was assessed according to the procedure established by Rotem et al. (1999) to characterize exo-β-1,3-glucanase activity in *A. quisqualis* strain AQ10. The amount of µmol of glucose liberated during the reaction was determined with a glucose hexokinase reagent (Sigma), according to the manufacturer's instructions. Glucanase activity was expressed as µmol of glucose h^{-1} mg^{-1} of dried mycelium.

Protease activity was determined using the artificial substrate azocasein according to Girard and Michaud (2002) with some modifications. Briefly, a 6 mm plug of the pycnidia-coated area was excised from 14-day-old *A. quisqualis* PDA cultures. The agar plug was placed in a sterile 1.5 ml tube containing 120 µl of an azocasein solution (1 %, w/v in 50mM Tris-HCl, pH 8.8) and incubated for 7 days at 25 °C. The reaction was then stopped and the undigested substrate was precipitated by the addition of 300 µl of cold trichloroacetic acid solution (10 %, w/v). After centrifugation for 5 min at 13000 rpm, a volume of 100 µl of supernatant was transferred to 96-multiwell plates and 85 µl of NaOH (1N) was added to each well. Absorbance was then read at 440 nm using a spectrophotometer and protease activity was expressed as the ratio of the absorbance value per ml of supernatant.

To induce production of chitinases in *A. quisqualis* strains, 6 mm plugs from pycnidia-coated PDA plates were transferred to sterile tubes containing 200 µl of a carboxymethyl-chitin-remazol brilliant violet (CM-chitin-RBV, Sigma) solution (1 % in 50mM Tris-HCl, pH 7.5) and incubated at 25 °C for 6 days. At the end of the incubation period, the tubes were centrifuged for 5 min at 13000 rpm and supernatants were assessed for their ability to degrade colloidal chitin (endochitinase activity) and for their *N*-acetyl-β-1,4-D-glucosamminidase activity (chitobiase activity). For endochitinase activity, a volume of 100 µl of supernatant was transferred to 96-multiwell plates and absorbance was read at 550 nm using a spectrophotometer according to Quecine et al. (2008). The compound 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc, Sigma), where the 4-methylumbelliferyl group is linked by β-1,4 linkage to the *N*-acetyl-β-D-glucosaminide monosaccharide, was used to assess chitobiase activity (O'Brien & Colwell, 1987). A volume of 50 µl of supernatant was mixed with 200 µl of 4-MU-Glc NAc solution (10 µM in 100 mM sodium phosphate buffer, pH 6.0) and the reaction mixture was incubated at 37 °C for 10 min then stopped by adding 50 µl of Na_2CO_3 (0.2 M). The amount of

free 4-methylumbelliferyl was determined by measuring fluorescence with excitation at 360 nm and emission at 450 nm using a spectrofluorometer. Chitobiase activity was expressed as milliunits (mU) of enzyme ml^{-1}.

In all the enzymatic tests, *T. atroviride* SC1 was included as a positive control under the same conditions. Non-inoculated Potato Broth was used as negative control in the glucanase assays while non-inoculated PDA was used as negative control in the protease and chitinase tests. Three independent experiments each with three replicates per treatment (strain) were performed.

4.2.5 Data evaluation and statistical analysis

The mycoparasitic bioassays were arranged in a randomized complete block design. Data from the mycoparasitic and enzymatic experiments were first tested for homogeneity of variance. A two-way analysis of variance (ANOVA) was then performed on log-transformed data from the three independent experiments and revealed no significant experiment × treatment interactions. Data from the three experiments were therefore pooled. Averages of all replicates are presented in Table 2, Table 3 and Figure 1. Statistical significances among treatments were computed with Statistica software 7.0 (Statsoft, Tulsa, OK, USA) and means were separated using the Tukey's HSD ($P \leq 0.05$).

A Principal Components Analysis (PCA, Wold et al., 1987) was performed using Statistica software 7.0 in order to visualize any potential correlations between the mycoparasitic activity of *A. quisqualis* and the genotype and fungal host of origin of the strain. Four variables were included: ability to reduce powdery mildew conidia, intra-hyphal spread of pycnidia into the host, genotype (ITS group), and the fungal host the strain was originally isolated from. The first two principal components were plotted to visualize the grouping of samples.

Spearman's correlation coefficients (*Rho*) and correlation coefficients (*r*) of the linear regression curves were used to calculate correlations between mycoparasitic and enzymatic activities. The statistical analysis included all tested strains with the exception of ATCC 200245 and ATCC 200249 which were identified as outliers and removed from the data. Data were analyzed for significance using Statistica software 7.0. Means were separated using the Tukey's HSD test after significant F-test ($P \leq 0.01$) with a one-way analysis of variance (ANOVA).

4.3 Results

4.3.1 Mycoparasitic activity: ability of *A. quisqualis* to reduce conidiation of powdery mildews *in vivo*

The trials on the different pathosystems (*E. necator*-grapevine, *P. aphanis*-strawberry and *P. xanthii*-cucumber) showed that all 24 *A. quisqualis* strains tested significantly reduced the number of conidia on treated leaves compared with untreated controls. On strawberry (Fig. 1a), the strains belonging to ITS group 1 isolated from *P. xanthii* (cucumber, Canada, Table 1) and most strains of ITS group 2A, including strains isolated from different fungal hosts, (ITA 1 and ITA 2 from *E. necator*, CABI 272851 from *Schinus molle*, DSM 4624 from *S. fuliginea*, DSM 2225 from *E. heraclei*, MYA 3401 from *Oidium hortensiae*) greatly reduced the number of powdery mildew conidia (46–51 %). AQ10, DSM 2222 (ITS group 2A) and ITS group 3 reduced *P. aphanis* conidia by 28–40 %. Very poor powdery mildew conidia reduction was obtained with the four ATCC strains isolated from *E. necator* and clustering within ITS group 5 (22–27 % reduction). Strain ITA 3 was the only strain of ITS group 5 able to greatly reduce the powdery mildew conidiation rate (53 %). In the experiments with *E. necator*, all the *A. quisqualis* strains tested greatly reduced the number of conidia on treated leaves compared with untreated controls (Fig. 1b), with values ranging from 63 % (ATCC 200245) to 100 % (MYA 3401). Significant differences between strains were, however, detected. With the exception of DSM 2225, all the strains which performed best against strawberry powdery mildew (ITS group 1, some strains from ITS group 2A and ITA 3) were also the most effective against *E. necator*. Strain MYA 3401 even produced complete inhibition of *E. necator* conidiation, while a significantly lower effect was obtained with strains belonging to ITS groups 5 and 3. The greatest variation between *A. quisqualis* treatments was observed (23–93 % control) with cucumber (Fig. 1c). The same strains which were highly effective against *P. aphanis* and *E. necator* (ITS group 1, ITA 1, ITA 2, DSM 4624, CABI 272851 from ITS group 2A and ITA 3 belonging to ITS group 5) greatly reduced the number of powdery mildew conidia (77–93 %). The *A. quisqualis* strains isolated from *P. xanthii* (cucumber, Canada) and belonging to ITS group 1 were the best strains for controlling cucumber powdery mildew. Strains belonging to ITS group 3 isolated from *P. leucotricha* on apple plants and the strains of ITS group 5 (except ITA 3) isolated from *E. necator* were found to have little efficacy .

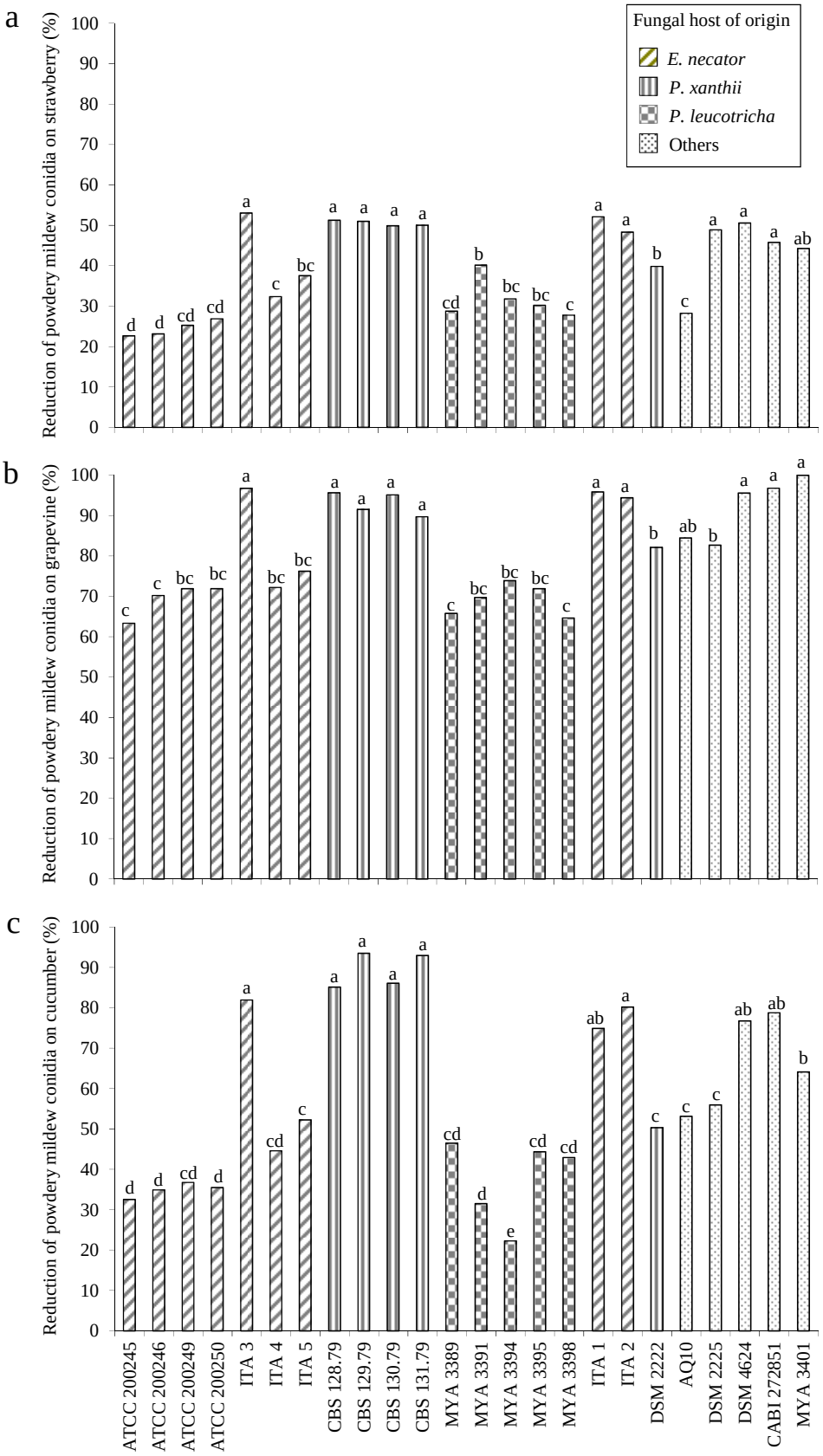

a
b
c
Fungal host of origin
E. necator
P. xanthii
P. leucotricha
Others
Reduction of powdery mildew conidia on strawberry (%)
Reduction of powdery mildew conidia on grapevine (%)
Reduction of powdery mildew conidia on cucumber (%)
ATCC 200245
ATCC 200246
ATCC 200249
ATCC 200250
ITA 3
ITA 4
ITA 5
CBS 128.79
CBS 129.79
CBS 130.79
CBS 131.79
MYA 3389
MYA 3391
MYA 3394
MYA 3395
MYA 3398
ITA 1
ITA 2
DSM 2222
AQ10
DSM 2225
DSM 4624
CABI 272851
MYA 3401

Figure 1. Reduction in the number of powdery mildew conidia of *Podosphaera aphanis* (a), *Erysiphe necator* (b), *P. xanthii* (c), in comparison with untreated controls, on leaves sprayed with *Ampelomyces quisqualis* strains from different host plants, fungal hosts and geographic origins. Columns with the same design represent strains isolated from the same plant and mildew species, with the exception of the dotted columns which represent strains isolated from different hosts (Table 1). The four ITS groups (1, 2A, 3, 5) discussed in the text are indicated in the figure. Columns with the same letters do not significantly differ ($P \leq 0.05$, Tukey's test). Values are means of eighteen replicates derived from three independent experiments with six replicates per experiment.

4.3.2 Mycoparasitic activity: intra-hyphal formation of *A. quisqualis* pycnidia within powdery mildews

The 24 *A. quisqualis* strains were rated for their ability to parasitize powdery mildew mycelia by forming intrahyphal pycnidia. Microscopic examination revealed that intrahyphal pycnidia had already formed outside the inoculation area for all tested strains of *A. quisqualis* 14 days after inoculation. All *A. quisqualis* strains displayed antagonistic activity against *P. xanthii*, *E. necator* and *P. aphanis*, but different levels of fungal growth within the pathogens were observed between strains (Table 2). Among the 24 strains, six (ITA 1, ITA 2 and all strains from cucumber powdery mildew belonging to ITS group 1) induced the largest parasitized areas on the three powdery mildews. Their daily expansion ranged from 182 to 263 $mm^2\ day^{-1}$. Good growth rates were also found with strains ITA 3, CABI 272851 and DSM 4624. CBS strains clustering into ITS group 1 displayed the highest mycoparasitic activity *in vivo* (191–245 $mm^2\ day^{-1}$). Considerably reduced pycnidial development into host cells was observed with strains clustering into ITS group 3 (36–52 $mm^2\ day^{-1}$) and group 5 (except ITA3) (62–93 $mm^2\ day^{-1}$). Analysis of the data according to ITS group showed that strains from ITS groups 1 and 2A spread significantly faster within the powdery mildew host compared with strains belonging to ITS groups 3 and 5 (Table 2).

Table 2. Intra-hyphal spread of *Ampelomyces quisqualis* pycnidia within powdery mildew

Strains	ITS group	Intra-hyphal spread of pycnidia [a] ($mm^2 day^{-1} \pm SE$) on		
		P. xanthii	*E. necator*	*P. aphanis*
ATCC 200245	5	43.8 ± 0.2 f	42.7 ± 0.2 g	35.4 ± 0.3 d
ATCC 200246	5	44.0 ± 0.3 f	48.9 ± 0.3 fg	36.1.0 ± 0.4 d
ATCC 200249	5	56.2 ± 0.5 ef	70.1 ± 0.4 ef	38.7 ± 0.4 d
ATCC 200250	5	88.4 ± 0.4 de	77.1 ± 0.5 e	38.8 ± 0.4 d
ITA 1	2A	240.1 ± 1.3 a	245.7 ± 1.7 a	187.7 ± 0.9 a
ITA 2	2A	228.3 ± 1.2 a	232.2 ± 0.8 ab	182.4 ± 0.8 a
ITA 3	5	202.7 ± 1.0 b	207.1 ± 1.3 b	147.4 ± 1.1 b
ITA 4	5	71.7 ± 0.6 e	90.5 ± 0.6 de	59.1 ± 0.3 cd
ITA 5	5	102.8 ± 0.4 d	111.3 ± 0.5 d	78.3 ± 0.3 c
CBS 128.79	1	253.3 ± 0.9 a	261.5 ± 1.1 a	198.5 ± 1.2 a
CBS 129.79	1	224.4 ± 1.4 a	230.6 ± 1.3 ab	184.4 ± 1.3 a
CBS 130.79	1	218.9 ± 1.1 ab	224.0 ± 1.6 ab	188.8 ± 1.2 a
CBS 131.79	1	255.0 ± 1.1 a	262.9 ± 0.9 a	193.0 ± 1.1 a
DSM 2222	2A	194.7 ± 1.3 bc	182.3 ± 0.8 c	138.2 ± 1.0 b
MYA 3389	3	49.4 ± 0.4 ef	55.5 ± 0.3 f	40.1 ± 0.3 d
MYA 3391	3	42.6 ± 0.4 f	30.4 ± 0.3 h	28.1 ± 0.3 d
MYA 3394	3	71.3 ± 0.1 e	78.2 ± 0.1 e	42.2 ± 0.2 d
MYA 3395	3	44.6 ± 0.3 f	54.5 ± 0.3 f	35.9 ± 0.2 d
MYA 3398	3	42.8 ± 0.3 f	38.9 ± 0.3 h	34.4 ± 0.3 d
AQ10	2A	180.0 ± 1.4 c	177.7 ± 1.6 c	132.0 ± 0.8 bc
DSM 2225	2A	188.6 ± 1.0 bc	183.6 ± 0.7 c	145.8 ± 1.0 b
DSM 4624	2A	214.0 ± 1.0 b	205.4 ± 0.8 b	151.3 ± 1.1 b
CABI 272851	2A	205.7 ± 1.5 b	190.4 ± 1.2 bc	172.3 ± 1.2 ab
Mean value [b]				
	1	237.9 ± 1.3 a	244.7 ± 1.4 a	191.2 ± 1.1 a
	2A	205.6 ± 1.2 a	200.1 ± 1.2 a	156.2 ± 1.2 a
	3	50.2 ± 0.3 b	51.5 ± 0.3 b	36.1 ± 0.1 b
	5	87.1 ± 0.5 b	92.5 ± 0.5 b	61.9 ± 0.3 b

Values are means of nine replicates derived from three independent experiments with three replicates per experiment. Standard errors of the means are reported (SE). Values with the same letter within a column are not significantly ($P \leq 0.05$) different according to the Tukey's HSD test.

[a] Powdery mildew mycelial area covered by *A. quisqualis* pycnidia (mm^2 per day).

[b] Average of all *A. quisqualis* strains belonging to an individual ITS group. Values with the same letter within a column are not significantly ($P \leq 0.05$) different according to the Tukey's HSD test.

Assays for evaluating ability to reduce conidiation of powdery mildew and intra-hyphal spread of *A. quisqualis* pycnidia within the host showed that all strains formed pycnidia and reduced conidiation of the three powdery mildew species but with different levels of aggressiveness. A Principal Components Analysis (PCA) based on the interaction between mycoparasitic activities (reduction of conidiation and intra-hyphal growth), ITS group and fungal host of origin showed that the latter two generally corresponded with the grouping based on level of aggressiveness. Three different groups, each comprising strains isolated from the same fungal host, belonging to the same ITS group and with similar levels of aggressiveness could be clearly recognized in the PCA (Fig. 2). In fact, all strains isolated from *P. xanthii* and clustering into ITS group 1 were highly effective against the tested powdery mildews. On the other hand, strains isolated from *P. leuchotricha* and *E. necator* (with exception of ITA 3) and belonging to ITS groups 3 and 5, respectively, were less effective in powdery mildew control. The only ITS group where the strains failed to cluster together in the PCA analysis was group 2A which included strains isolated from different hosts and displaying different levels of aggressiveness. Three strains in this group (ITA 1, ITA 2, and DSM 2222) did not cluster with the other 2A strains.

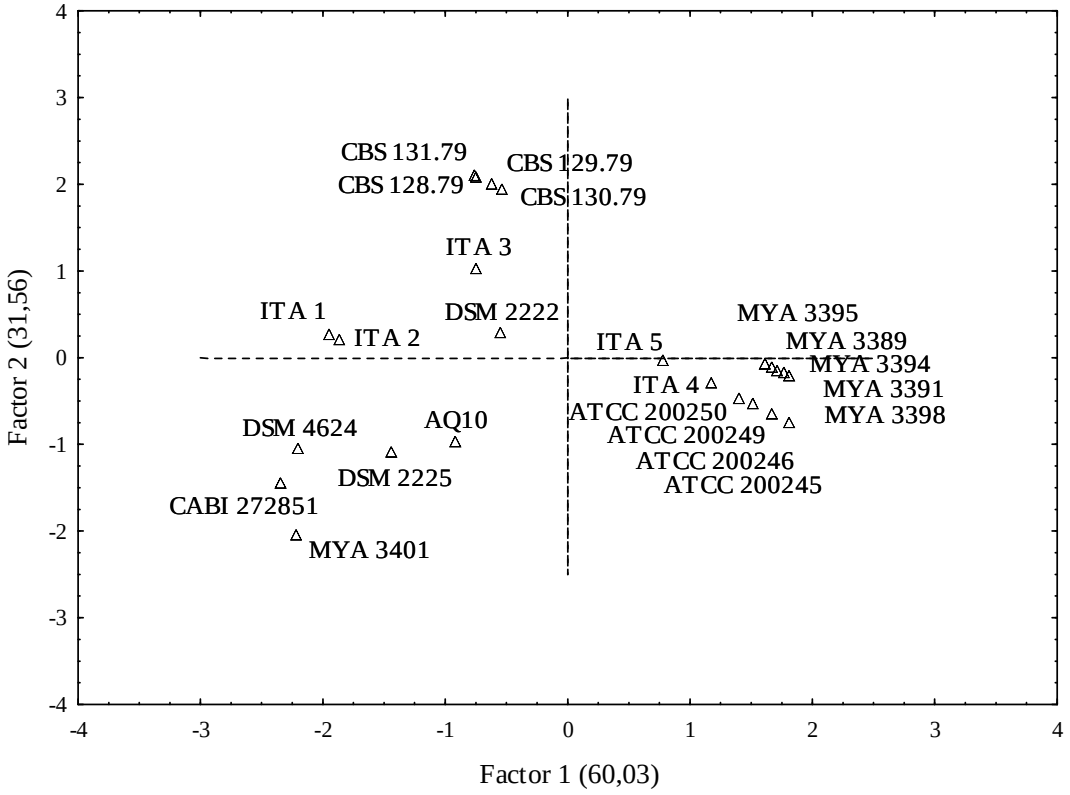


Figure 2. Principal Components Analysis (PCA) of mycoparasitic activities, genotype and fungal host of origin of the *Ampelomyces quisqualis* strains. Four variables were included: ability to reduce powdery mildew conidiation, intra-hyphal spread of pycnidia within the host, genotype (ITS group), and fungal host of origin. Clusters were named according to the ITS rDNA phylogenetic group (Angeli et al., 2012). The first two principal components were plotted to visualize the grouping of samples.

4.3.3 *In vitro* production of CWDEs by *A. quisqualis* culture filtrates

CWDE activities *in vitro* were determined for the 24 *A. quisqualis* strains and compared with those of *T. atroviride* SC1. The levels of chitinase, protease and β-1,3 glucanase secreted by the various *A. quisqualis* strains differed significantly ($P \leq 0.05$) according to a Tukey's multiple range test (Table 3).

None of the *A. quisqualis* strains exhibited endochitinase activity *in vitro* while the greatest chitobiase activity was produced by CBS strains 128.79 (43 mU ml^{-1}) and 131.79 (37 mU ml^{-1}), ATCC 200245 (41 mU ml^{-1}) and DSM 4624 (38 mU ml^{-1}). The activity of these strains was about three times higher than *T. atroviride* SC1. In general, ITS group 1 displayed the greatest

chitobiase activities with an average of 34 mU ml-1. The lowest activities were clearly measured for *A. quisqualis* strains belonging to ITS group 3 (10 mU ml^{-1}). There was a 0.5 to 1-fold decrease in the activities of these strains when compared with the *Trichoderma* control strain.

Strains of *A. quisqualis* generally exhibited lower protease activity compared with *T. atroviride* SC1 under the same conditions. Only the protease activities of ITA 1, ITA 2, ATCC 200249 and CBS 128.79 did not statistically differ from SC1 (10.2 Abs ml^{-1}) whereas the activities of all other strains were significantly lower, with several strains belonging to ITS group 3 and ITS group 5 exhibiting as much as a 5-fold decrease compared with the *Trichoderma* control. In general, ITS groups 1 and 2A displayed higher enzyme proteases activities (Average = 8.1 Abs ml^{-1}) than group 3 (3.8 Abs ml^{-1}) and group 5 (5 Abs ml^{-1}) (Table 3).

Differences in activity levels between individual strains of *A. quisqualis* were also found for β-1,3 glucanase. Maximum activity of β-1,3 glucanase was measured with strains ITA 1, ITA 2, AQ10 and DSM 2225 (405–451 µmol mg^{-1} h^{-1}). These values were about 2 times higher than those for SC1 (199 µmol mg^{-1} h^{-1}). The strains displaying the lowest glucanase activities (e.g. a 2-fold decrease compared with SC1) were ITA 4, MYA 3389 and MYA 3398.

Most of the strains tested exhibiting high chitobiase and protease activities (e.g. strains of ITS groups 1 and 2A) also performed best in parasitizing the powdery mildews and in inhibiting the formation of conidia (Figs. 1 and 3, Tables 2 and 3). Conversely, all the strains belonging to ITS groups 3 and 5, which displayed the lowest chitobiase and protease activities, were also less effective in colonizing powdery mildew *in vivo.* Correlation analysis of the mycoparasitic and enzymatic activities of *A. quisqualis* (Fig. 3; Table 1S) showed the activities of these two CWDEs to be positively correlated with intra-hyphal formation of pycnidia (Figs. 3a,c; Rho = 0.82, R^2 = 0.68; Rho = 0.77, R^2 = 0.67) and inhibition of conidiation *in vivo* (Figs. 3b,d; Rho = 0.70, R^2 = 0.53; Rho = 0.71, R^2 = 0.54). However, no correlation was found between exo-β-1,3 glucanase activity and the two tested mycoparasitic activities of *A. quisqualis* strains belonging to different ITS groups (Figs. 3e,f; Table 1S).

Table 3. Enzymatic activity of different *Ampelomyces quisqualis* strains.

Strains	ITS group	Chitobiase activity (mU ml^{-1} ± SE)[a]	Protease activity (Abs ml^{-1} ± SE)[b]	β-1,3-Glucanase activity (µmol mg^{-1} h^{-1} ± SE)[c]
SC1[d]		13.84 ± 0.38 hil	10.22 ± 0.09 a	199.27 ± 4.07 cde
ATCC 200245	5	40.84 ± 1.07 ab	2.96 ± 0.14 hi	134.10 ± 4.46 fgh
ATCC 200246	5	16.67 ± 0.51 ghi	3.21 ± 0.09 hi	160.43 ± 3.73 fgh
ATCC 200249	5	24.25 ± 0.58 efg	9.14 ± 0.07 ab	136.38 ± 3.12 fgh
ATCC 200250	5	10.01 ± 0.31 il	5.16 ± 0.10 g	148.05 ± 3.90 fgh
ITA 1	2A	28.52 ± 0.37 def	9.25 ± 0.12 ab	450.76 ± 5.13 a
ITA 2	2A	33.69 ± 1.09 bcd	9.27 ± 0.20 ab	404.65.28 ± 4.55 a
ITA 3	5	16.76 ± 0.67 ghi	6.42 ± 0.18 ef	173.48 ± 3.74 efg
ITA 4	5	14.17 ± 0.20 hil	2.84 ± 0.20 hi	91.93 ± 2.04 hi
ITA 5	5	13.05 ± 0.27 hil	5.58 ± 0.13 fg	373.28 ± 4.25 ab
CBS 128.79	1	43.19 ± 0.69 a	10.11 ± 0.10 a	276.52 ± 3.89 bcd
CBS 129.79	1	30.76 ± 0.68 cde	6.66 ± 0.12 def	189.76 ± 2.70 def
CBS 130.79	1	23.51 ± 0.64 efg	8.81 ± 0.21 bc	294.28 ± 3.76 bc
CBS 131.79	1	37.32 ± 1.04 abc	7.06 ± 0.08 de	182.46 ± 3.11 def
DSM 2222	2A	29.11 ± 0.46 def	8.46 ± 0.23 bc	147.12 ± 3.35 fgh
MYA 3389	3	7.40 ± 0.15 l	3.54 ± 0.15 h	105.17 ± 2.32 hi
MYA 3391	3	9.48 ± 0.17 il	3.48 ± 0.12 h	115.91 ± 2.49 ghi
MYA 3394	3	13.00 ± 0.41 hil	2.08 ± 0.07 i	352.57 ± 3.84 ab
MYA 3395	3	6.93 ± 0.11 l	7.75 ± 0.13 cd	168.67 ± 3.72 efg
MYA 3398	3	12.70 ± 0.71 hil	2.18 ± 0.08 i	83.59 ± 2.61 i
AQ10	2A	23.85 ± 0.43 efg	8.55 ± 0.17 bc	438.33 ± 5.29 a
DSM 2225	2A	23.74 ± 1.02 efg	8.73 ± 0.10 bc	439.32 ± 5.46 a
DSM 4624	2A	38.14 ± 1.38 abc	7.87 ± 0.15 cd	133.88 ± 2.46 fgh
CABI 272851	2A	21.23 ± 0.95 fgh	5.78 ± 0.10 fg	158.54 ± 4.35 fgh
MYA 3401	2A	18.59 ± 0.99 ghi	6.91 ± 0.18 de	153.65 ± 3.17 fgh
Mean value[e]				
	1	33.70 ± 0.70 a	8.16 ± 0.13 a	235.76 ± 14.82 a
	2A	27.11 ± 0.27 ab	8.10 ± 0.15 a	290.78 ± 26.38 a
	3	9.90 ± 0.19 c	3.81 ± 0.15 c	165.18 ± 12.28 a
	5	19.39 ± 0.49 bc	5.04 ± 0.11 bc	173.95 ± 14.36 a

Values are means of nine replicates derived from three independent experiments with three replicates per experiment. Standard errors of the means are reported (SE). Values with the same letter within a column are not significantly ($P \leq 0.05$) different according to the Tukey's HSD test.

[a] The total chitobiase activity was measured using 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MU-GluNAc) as the substrate. The fluorescence of the liberated 4-MU was measured with excitation at 360 nm and emission at 450 nm. One milliunit of chitobiase activity was defined as the amount of enzyme that liberates 1 nmol 4-MU per min.

[b] The protease activity was measured in absorbance per millilitre of substrate per reaction

[c] The β-1,3-glucanase activity was measured in µmol glucose liberated per h per mg dry weight according to Rotem et al (1999).

[d] Internal positive control (*Trichoderma atroviride* SC1).

[e] Average of all *A. quisqualis* strains belonging to an individual ITS group. Values with the same letter within a column are not significantly ($P \leq 0.05$) different according to the Tukey's HSD test.

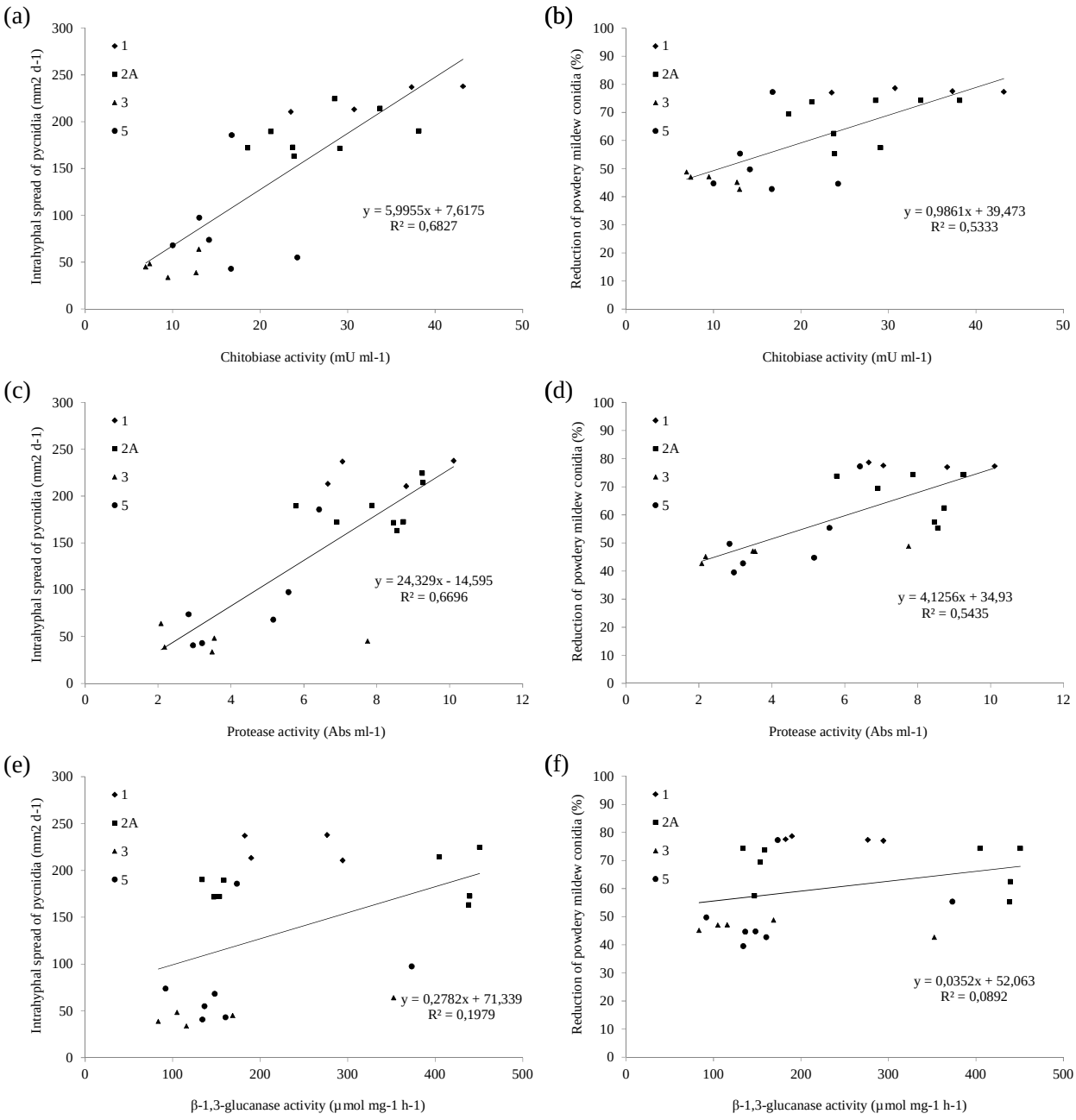


Figure 3. Relationship between CWDE production and mycoparasitic activities in *Ampelomyces quisqualis* strains. Correlation coefficients (*r*) of the linear regression curves were used to calculate correlations between the two mycoparasitic activities tested (intra-hyphal formation of pycnidia and inhibition of powdery mildew conidiation) and chitobiase (a, b), protease (c, d), and β-1,3-glucanase activities (e, f). The four ITS groups (1, 2A, 3, 5) are indicated with different symbols.

Table S1. Estimates between hydrolytic enzyme production and mycoparasitic activities of linear (R^2) and Spearman (Rho) correlation coefficients.

	Intrahyphal spread of pycnidia [a]		Reduction of powdery mildew conidia [a]	
	Rho	R^2	Rho	R^2
Chitobiase activity	0.8231*	0.6827*	0.6996*	0.5333
Protease activity	0.7658*	0.6696*	0.7065*	0.5435*
β-1,3-Glucanase activity	0.4752	0.1979	0.4234	0.0892

* Indicates statistically significant correlations ($P \leq 0.01$).
[a] The mean value of the bioassay tests on cucumber, grapevine and strawberry was considered.

4.4 Discussion

Among the various fungal antagonists of powdery mildews (Kiss, 2003), pycnidial fungi belonging to the genus *Ampelomyces* are the most widespread and the oldest known natural antagonists of *Erysiphales* (Kiss et al., 2004). Since *A. quisqualis* strains are known to parasitize and reduce powdery mildew infections with different degrees of efficacy (Angeli et al., 2009b), we investigated whether there was an association between original mycohost, aggressiveness measured as conidia reduction and expansion of parasitized host area, and *in vitro* production of CWDEs of *A. quisqualis* strains belonging to different ITS groups.

In vivo bioassays showed that all *A. quisqualis* strains tested in our study were able to parasitize powdery mildew mycelium by forming intrahyphal pycnidia, expand the parasitized host colony area over time and significantly reduce conidiation of *E. necator, P. aphanis* and *P. xanthii*. Interestingly, mycoparasitic activity differed according to the powdery mildew species and several of the tested strains were more effective than the commercial strain AQ10. Individual strains differ considerably in aggressiveness, although strains which are highly aggressive against one powdery mildew also perform best against the other two. We identified a group of highly aggressive strains which are more effective than AQ10 and which have a high potential for powdery mildew control. Some Italian strains isolated from grapevine powdery mildew (ITA 1, 2, 3), the CBS strains isolated from cucumber powdery mildew, as well as DSM 4624 and CABI 272851 were very effective in the biological control of all powdery mildew pathogens. Moreover, not all powdery mildew species are equally susceptible to *A. quisqualis*: our

experiments show that *P. aphanis* is in general less susceptible to all the *A. quisqualis* strains than *P. xanthii* and *E. necator*.

Recently, studies have shown that the cultural and growth characteristics of these strains are related to ITS group and host fungus and have suggested that a certain degree of mycohost specialization may be present within the *A. quisqualis* species (Angeli et al., 2012; Park et al., 2010). We have demonstrated for the first time that the ability of *A. quisqualis* strains to colonize powdery mildew by forming pycnidia and inhibiting conidiation is completely independent of the geographical origin of the strain, but appears to be related to genotype (ITS rDNA sequence) and the original fungal host, with the exception of strains ITA 1, ITA 2 and ITA 3. It is worth noting that the strains belonging to ITS groups 3 and 5 were the least effective in reducing powdery mildews. These strains were isolated from apple and grapevine plants, respectively, the natural hosts of only one powdery mildew species (*M. domestica-P. leucotricha*; *V. vinifera-E. necator*). On the other hand, the most aggressive strains belonged to ITS groups 1 and 2A, isolated from plants that, for the most part, are attacked by more than one *Erysiphales*, as in the case of *C. sativus* which is the natural host of *E. cichoracearum* and *P. xanthii* (ITS group 1). In addition, members of the plant species that hosted *A. quisqualis* strains of ITS group 2A are natural hosts of at least two powdery mildews, such as *Daucus* sp. (*E. heraclei* and *E. polygoni*), *H. macrophylla* (*E. polygoni* and *O. hortensiae*) and *Leontodon* sp. (*E. cichoracearum* and *P. xanthii*). Unfortunately, no information is available on the number of powdery mildews occurring on *C. edulis* and *S. molle*. However, it is tempting to hypothesize that co-evolution of *A. quisqualis* strains and plants which are the natural host of more than one powdery mildew drove the selection of more aggressive genotypes. A broader range of strains belonging to different genotypes and originating from different plant hosts will need to be investigated in the future in order to validate this hypothesis.

Another aspect of our work regarded evaluation of the traits associated with strain aggressiveness and testing the hypothesis that CWDEs secreted by *A. quisqualis* strains are an important factor in selecting *A. quisqualis* strains for biocontrol. Although the mycoparasitic activity of *A. quisqualis* against powdery mildews is well documented, information on the role played by CWDEs is in fact scarce (Philipp, 1985; Rotem et al., 1999). These enzymes are produced by mycoparasitic fungi and are involved in lysis of the cell wall through the degradation of chitin, glucan and proteins, the main constituents of the cell walls of higher fungi

(Cao et al., 2009; Larena & Melgarejo, 1996; Lorito et al., 1994a; Oppenheim & Chet, 1992). The importance of the role played by CWDEs in the activity of fungal biocontrol agents has already been demonstrated in several works. For example, the gene *ech42* coding for an endochitinase in *T. harzianum* strain P1 is expressed before contact between the biocontrol fungus and the plant pathogen *Rhizoctonia solani*, while a null mutant lacking a functional copy of this gene was impaired in the control of another plant pathogen *Botrytis cinerea* (Woo et al., 1998; Zeilinger et al., 1999). Szekeres et al. (2004) found that *T. harzianum* strain T334 mutants were able to overproduce proteases and better control *Fusarium culmorum*, *Pythium* and *R. solani in vitro*. In addition, the biocontrol activity of *T. virens* strain GV29-8 against *R. solani* on cotton seeds was much improved when the gene *tvsp1*, coding for an alkaline serine protease, was constitutively expressed, showing that proteolytic activity plays a role in the mycoparasitism of this *T. virens* strain (Pozo et al., 2004). The role played by β-1,3 and β-1,6 glucanases in the same strain has been well documented (Djonovic et al., 2006, 2007). The use of derivatives of the *T. virens* strain GV29-8 lacking glucanolytic activity or overproducing β-1,3 and β-1,6 glucanases in a biocontrol assay on cotton seedlings clearly showed the importance of these enzymes. It was found that GV29-8 derivatives overproducing glucanase were able to control plant pathogens more efficiently than wild type strains, while the null mutants lost their biocontrol effectiveness (Djonovic et al., 2006, 2007).

Our enzymatic assays revealed *in vitro* β-1,3-glucanase, protease and chitobiase activity in all tested strains, although the levels of enzymatic activity differed between genetically different *A. quisqualis* strains. This is the first time that *in vitro* protease activity has been reported for members of *A. quisqualis* sensu stricto. It is worth noting that all the strains tested displayed chitobiase activity, but no endochitinase activity was detected using the CM-chitin-RBV method. In agreement with Rotem et al (1999), we found that the commercial strain AQ10 produces a high amount of extracellular β-1,3-glucanase and also displayed good protease and chitobiase activity.

Our results suggest that the ability of some *A. quisqualis* strains to parasitize the host by forming intracellular pycnidia and reducing fungal-host conidiation was associated with their intrinsic ability to secrete proteases and with chitobiase activity in culture filtrates (Fig. 3, Table S1). This was also illustrated by the regression analysis which showed there to be a significant positive

correlation (Table S1) between chitobiase and protease activity with intra-hyphal formation of pycnidia and the inhibition of conidiation *in vivo*, whereas exo-glucanase activity was not statistically related to mycoparasitic activities. Therefore, we hypothize that proteases and chitobiases play an important role in *A. quisqualis* mycoparasitism. However, lytic activities alone do not fully explain the level of aggressiveness, although they are probably involved in the biocontrol ability of *A. quisqualis* and of other mycoparasitic fungi (Adams, 2004; Reithner et al., 2011; Vinale et al., 2008).

An interesting issue for future research would be to investigate the role played by these enzymatic activities in the antagonistic properties of members of *A. quisqualis* sensu stricto either by creating strains that are defective in these activities or by monitoring expression of the genes responsible for lytic activities during the parasitization of powdery mildews. Nevertheless, the biological characterization of the *A. quisqualis* strain collection carried out in this work suggests that mycoparasitism by *A. quisqualis* can be explained in part by the activities of CWDEs. This provides important knowledge for the selection of new, highly effective strains for biocontrol.

4.5 Acknowledgements

The authors thank Dr. Levente Kiss (Plant Protection Institute of the Hungarian Academy of Science, Budapest, Hungary) for providing the fungal strains. We would also like to thank Carmela Sicher and Denise Ress for technical assistance. This work was supported by the AMPELO and ENVIROCHANGE projects funded by the Autonomous Province of Trento.

4.6 Literature

Adams, D.J. (2004). Fungal cell wall chitinases and glucanases. Microbiology 150, 2029-2035.

Angeli, D., Pellegrini, E. and Pertot, I. (2009a). Occurence of *Erysiphe necator* chasmothecia and their natural parasitization by *Ampelomyces quisqualis*. Phytopathology 99, 704-710.

Angeli, D., Pellegrini, E., Micheli, S., Ress, D., Maurhofer, M., Pertot, I. and Gessler, C. (2009b). Molecular characterization of *Ampelomyces* spp. isolates from different host and geographic origin and evaluation of their potential to control powdery mildew of cucumber. IOBC/WPRS bulletin 43, 40-44.

Angeli D., Maurhofer M., Gessler C. and Pertot I. (2012). Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis.* Phytoparasitica 40, 37-51.

Benitez, T., Rincon, A.M., Limon, M.C. and Codon, A.C. (2004). Bioncontrol mechanisms of *Trichoderma* strains. International Microbiology 4, 249-260.

Cao, R., Liu, X., Gao, K., Mendgen, K., Kang, Z., Gao, J., Dai, Y. and Wang, X. (2009). Mycoparasistism of endophytic fungi isolated from reed on soilborne phytopathogenic fungi and production of cell wall-degrading enzymews in vitro. Current Microbiology 59, 584-592.

Cook, R.J. and Baker, K.F. (1983). The nature and practice of biological control of plant pathogens. American Phytopathological Society, Minnesota p. 539.

Djonovic, S., Pozo, M.J. and Kenerley, C.M. (2006). Tv-bgn3, a beta-1,6- glucanase from the biocontrol fungus *Trichoderma virens* is involved in mycoparasitism and control of *Pythium ultimum*. Applied Environmental Microbiology 72, 7661-7670.

Djonovic, S., Vittone, G., Mendoza Herrera, A. and Kenerley, C.M. (2007). Enhanced bicontrol activity of *Trichoderma virens* transformants constitutively coexpressing β-1,3 and β-1,6-glucanase genes. Molecular Plant Pathology 8, 469-480.

Elad, Y., Chet, I. and Henis, Y. (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum.* Canadian Journal of Microbiology 28, 719-725.

Elad, Y., Lifshitz, R. and Baker, R. (1985). Enzymatic activity of the mycoparasite *Pythium nunn* during interaction with host and non-host fungi. Physiological Plant Pathology 27, 131-148.

Elad, Y., Malathrakis, N.E. and Dik, A.J. (1995). Biological control of *Botrytis* incited diseases and powdery mildews in greenhouse crops. Crop Protection 15, 229-240.

Geremia, R.A., Goldman, G.H., Jacobs, D., Ardiles, W., Vila, S.B., Van Montagu, M. and Herrera-Estrella, A. (1993). Molecular characterization of the proteinase-encoding gene, prb1, related to mycoparasitism by *Trichoderma harzianum.* Molecular Microbiology 8, 603-613.

Girard, C. and Michaud, D. (2002). Direct monitoring of extracellular protease activities in microbial cultures. Analytical Biochemistry 308, 388-391.

Glawe, D.A. (2008). The powdery mildews: a review of the world's most familiar (yet poorly known) plant pathogens. Annual Review of Phytopathology 46, 27–51.

Gruber, S. and Seidl-Seiboth, V. (2012). Self versus non-self: fungal cell wall degradation in *Trichoderma.* Microbiology 158, 26-34.

Gu, Y.H. and Ko, W.H. (1997). Water agarose medium for studying factors affecting germination of conidia of *Ampelomyces quisqualis.* Mycological Research 101, 422-424.

Haran, S., Schickler, H. and Chet, I. (1996). Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum.* Microbiology 142, 2321-2331.

Hashioka, Y. and Nakai, Y. (1980). Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. Transactions of the Mycological Society of Japan 21, 329-338.

Jarvis, W.R. and Slingsby, K. (1997). The control of powdery mildew of greenhouse cucumber by water spray and *Ampelomyces quisqualis.* Plant Disease Reporter 61, 728-730.

Kiss, L. (1997). Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. Mycological Research 101, 1073-1080.

Kiss, L. (1998). Natural occurrence of *Ampelomyces* intracellular mycoparasites in mycelia of powdery mildew fungi. The New Phytologist 140, 709-714.

Kiss, L. and Nakasone, K.K. (1998). Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi. Current Genetics 33, 362-367.

Kiss, L. (2003). A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Management Science 59, 475-483.

Kiss, L., Russell, J.C., Szentivanyi, O., Xu, X. and Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonist of powdery mildew fungi. Biocontrol Science and Technology 14, 635–651.

Kiss, L., Pintye, A., Kovacs, G.M., Jankovics, T., Fontaine, M.C., Harvey, N., Xu, X., Nicot, P.C., Bardin, M., Shykoff, J.A. and Giraud, T. (2011). Temporal isolation explains host-related genetic differentiation in a group of widespread mycoparasitic fungi. Molecular Ecology 20, 1492-1507.

Kubicek, C.P., Mach, R.L., Peterbauer, C.K. and Lorito, M. (2001). *Trichoderma*: from genes to biocontrol. Journal of Plant Pathology 83, 11-23.

Larena, I. and Melgarejo, P. (1996). Biological control of *Monilinia laxa* and *Fusarium oxysporum* f. sp. *lycopersici* by a lytic enzyme-producing *Penicillium purpurogenum.* Biological Control 6, 361-367.

Liang, C., Yang, J., Kovacs, G.M., Szentivanyi, O., Li, B., Xu, X.M. and Kiss, L. (2007). Genetic diversity of *Ampelomyces* mycoparasite isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences. Fungal Diversity 24, 225-240.

Lorito, M., Leterbauer, C., Hayes, C.K., Woo, S.L. and Harman, G.E. (1994a). Synergistic combination of cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiology 140, 623-629.

Lorito, M., Hayes, C.K., Di Pietro, A., Woo, S.L. and Harman, G.E. (1994b). Purification, characterization and synergistic activity of a glucan β-1,3-glucosidase and a N-acetyl-β-glucosaminidase from *Trichoderma harzianum*. Phytopathology 84, 398-405.

Machida, K., Trifonov, L.S., Ayer, W.A., Lu, Z.X., Laroche, A., Huang, H.C., Cheng, K.J. and Zantige, J.L. (2001). 3(2H)-Benzofuranones and chromanes from liquid cultures of the mycoparasitic fungus *Coniothyrium minitans*. Phytochemistry 58, 173-177.

McQuilken, M.P., Gemmell, J., Hill, R.A. and Whipps, J.M. (2003). Production of macrosphelide A by the mycoparasite *Coniothyrium minitans*. FEMS Microbiology Letters 219:27-31.

McQuilken, M.P. and Gemmell, J. (2004). Enzyme production by the mycoparasite *Verticillium biguttatum* against *Rhizoctonia solani*. Mycopathologia 157, 201-205.

Monteiro, V., do Nascimento, S.R., Steindorff, A.S., Costa, F.T., Noronha, E.F., Ricart, C.A.O., de Sousa, M.V., Vainstein, M.H. and Ulhoa, C.J. (2010). New insights in *Trichoderma harzianum* antagonism of fungal plant pathogen by secreted protein analysis. Current Microbiology 61, 298-305.

Mucha, J., Dahm, H., Strzelczyk, E. and Werner, A. (2006). Synthesis of enzymes connected with mycoparasitism by ectomycorrhizal fungi. Archives Microbiology 185, 69-77.

Nischwitz, C., Newcombe, G. and Anderson, C.L. (2005). Host specialization of the mycoparasite *Eudarluca caricis* and its evolutionary relationship to *Ampelomyces*. Mycological Research 109, 421-428.

O'Brien, M. and Colwell, R.R. (1987). A rapid test for chitinase activity that uses 4-methylumbelliferyl-N-acetyl-β-D-glucosamininide. Applied and Environmental Microbiology 53, 1718-1720.

Oppenheim, A.B. and Chet, I. (1992). Cloned chitinase in fungal pathogen control strategies. Trends in Biotechnology 10, 392-394.

Park, M.J., Choi, Y.J., Hong, S.B. and Shin, H.D. (2010). Genetic variability and mycohost association of *Ampelomyces quisqualis* isolates inferred from phylogenetic analyses of ITS rDNA and actin gene sequences. Fungal Biology 114, 235-247.

Paulitz, T.C. and Bélanger, R.B. (2001). Biological control in greenhouse systems. Annual Review of Phytopathology, 39, 103–133.

Philipp, W.D. (1985). Extracellular enzymes and nutritional physiology of *Ampelomyces quisqualis* Ces., hyperparasite of powdery mildew, in vitro. Phytopathologische Zeitschrift 114, 274-283.

Pintye, A., Bereczky, Z., Kovács, G.M., Nagy, L.G., Xu, X., Legler, S.E., Váczy, Z., Váczy, K.Z., Caffi, T., Rossi, V. and Kiss, L. (2012). No indication of strict host associations in a widespread mycoparasite: grapevine powdery mildew (*Erysiphe necator*) is attacked by phylogenetically distant *Ampelomyces quisqualis* strains in the field. Phytopathology 102:707-716.

Pozo, M.J., Baek, L.M., Garcia, J.M. and Kenerley, C.M. (2004). Functional analysis of tvspl, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genetics and Biology 41, 336-348.

Quecine, M.C., Araujo, W.L., Marcon, J., Gai, C.S., Azevedo, J.L. and Pizzirani-Kleiner, A.A. (2008). Chitinolytic activity of endophytic *Streptomyces* and potential for biocontrol. Letter in Applied Microbiology 47, 486–491.

Rankovic, B. (1997). Hyperparasites of the genus *Ampelomyces* on powdery mildew fungi in Serbia Mycopathologia 139, 157-164.

Reithner, B., Ibarra-Laclette, E., Mach, R.L. and Herrera-Estrella, A. (2011). Identification of mycoparasistim-related genes in *Trichoderma atroviride.* Applied and Environmental Microbiology 77, 4361-4370.

Rotem, Y., Yarden, O. and Sztejnberg, A. (1999). The mycoparasite *Ampelomyces quisqualis* expresses exgA encoding and exo-β-1,3-glucanase in culture and during mycoparasitism. Phytopathology 89, 631-638.

Samolski, I., de Luis, A., Vizcaino, J., Monte, E. and Suarez, M.B. (2009). Gene expression analysis of the biocontrol fungus *Trichoderma harzianum* in the presence of tomato plants, chitin, or glucose using a high-density oligonucleotide microarray. BMC Microbiology 9, 217.

Sullivan, R.F. and White, J.F. (2000). *Phoma glomerata* as a mycoparasite of powdery mildew. Applied and Environmental Microbiology 66, 425-427.

Sundheim, L. and Krekling, T. (1982). Host-parasite relationships of the hyperparasite *Ampelomyces quisqualis* and its powdery mildew host *Sphaerotheca fuliginea.* I. Scanning electron microscopy. Phytopathology 104, 202-210.

Szekeres, A., Kredics, L., Antal, Z., Kevei, F. and Manczinger, L. (2004). Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum.* FEMS Microbiology Letters 233, 215-222.

Szentiványi, O., Kiss, L., Russell, J.C., Kovács, G.M., Varga, K., Jankovics, T., Lesemann, S., Xu, X.M. and Jeffries, P. (2005). *Ampelomyces* mycoparasites from apple powdery mildew identified as a distinct group based on single-stranded conformation polymorphism analysis of the rDNA ITS region. Mycological Research 109, 429-438.

Sztejnberg, A. (1993). *Ampelomyces quisqualis* AQ10, CNCM I-807, for biological control of powdery mildew. US patent no. 5190754.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L. and Lorito, M. (2008). *Trichoderma*-plant-pathogen interactions. Soil Biology and Biochemistry 40, 1-10.

Viterbo, A., Harel, M. and Chet, I. (2004). Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. FEMS Microbiology Letters 238, 151-158.

Wold, S., Geladi, P., Esbesen, K. and Ohman, J. (1987). Multi-way principal components- and PLS-analysis. Journal of Chemometrics 1, 41-56.

Woo, S.L., Donzelli, B., Scala, F., Mach, R.L., Harman, G.E., Kubicek, C.P., Del Sorbo, G.and Lorito, M. (1998). Disruption of ech42 (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* strain P1. Molecular Plant–Microbe Interactions 12, 419-429.

Yarwood, C.E. (1932). *Ampelomyces quisqualis* on clover mildew. Phytopathology 22, 31 (Abstr.).

Zeilinger, S., Galhaup, C., Payer, K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M. and Kubicek, C.P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genetics and Biology 26, 131-140.

Chapter 5

Stimulation of conidial germination as a novel tool to improve the biocontrol potential of *Ampelomyces quisqualis*

To be submitted as

Angeli D., Maurhofer M., Gessler C., Pertot I. 2013. Stimulation of conidial germination as a novel tool to improve the biocontrol potential of *Ampelomyces quisqualis.*

Abstract

Spores of the *Ampelomyces quisqualis* mycoparasitic fungus are unique in their ability to actively infect and kill the causal agents of powdery mildew (*Erysiphales*). The potential of *A. quisqualis* in the biological control of its fungal hosts is often inconsistent under field conditions whereas the most successful biocontrol experiments using *A. quisqualis* were carried out in greenhouses where temperature and relative humidity (RH) are suitable to the mycoparasite. Clearly, the high RH requirement of *A. quisqualis* represents one of the major obstacles in its use as a reliable biocontrol agent. In this study, novel tools for exploring future application of *A. quisqualis* in biological control of powdery mildew fungi were investigated. For this purpose, selection of new highly effective mycoparasitic strains and identification of specific mechanisms to increase the biocontrol efficiency are considered to be the most appropriate method. Thus, some attempts were made to enhance the efficacy of different strains of *A. quisqualis*. Conidia of *A. quisqualis* germinate poorly in sterile, distilled water. A procedure to increase the performance of *A. quisqualis* in the biological control of powdery mildews was developed by increasing the conidial germination rate of the fungus before leaf application. Customized compounds were used to stimulate conidia germination and the efficacy in the control of different powdery mildews was increased by 20–30 %. The method may be useful for improving the efficacy of biological control agents under limiting temperature and RH conditions.

5.1 Introduction

Pycnidial fungi belonging to the genus *Ampelomyces* are the most widespread natural antagonists of *Erysiphales* (Kiss et al., 2004) and are the most studied biocontrol agent of powdery mildews (Kiss, 1997). The wide host range, combined with tolerance to a number of fungicides used against powdery mildews, makes *A. quisqualis* the ideal candidate for use as a biological control agent (Falk et al., 1995a; Sundheim & Tronsmo, 1988; Sztejnberg et al., 1989). Genetically different *A. quisqualis* strains are available from culture collections (Angeli et al., 2012a; Kiss et al., 2011; Liang et al., 2007; Park et al., 2010) and one strain isolated in Israel (CNCM I–807) has been formulated, registered and commercialized under the trade name of AQ10 (Kiss, 1997; Sztejnberg, 1993). In general, when *A. quisqualis* strains are used as biocontrol agents, a certain

level of disease has to be tolerated as they can only attack established infection of powdery mildews (Fokkema, 1993). As they act against powdery mildews through mycoparasitism, without producing antifungal compounds, they destroy the powdery mildew colonies only slowly, taking 5–7 days. Recently, studies on the host-parasite interaction of the mycoparasite and its powdery mildew host showed that the presence of host fungi is recognized by *A. quisqualis*, and a water-soluble substance from powdery mildew conidia was shown to stimulate the germination of their conidia *in vitro* (Gu & Ko, 1997). Directed growth of germ-tubes of *A. quisqualis* towards powdery mildew hyphae has also been observed (Sundheim & Krekling, 1982). *A. quisqualis* invades and grows within powdery mildew hosts. The fungus formes pycnidia within hyphae, conidiophores, conidia and the immature chasmothecia of powdery mildews (Sundheim & Krekling, 1982). Parasitization reduces powdery mildew sporulation, as well as the production of chasmothecia and may eventually kill the entire mildew colony (Hashioka & Nakai, 1980; Sundheim & Krekling, 1982). In order to maintain control, the growth and spread of their fungal hosts must be slower than that of the mycoparasite. As the artificially released mycoparasites, together with the naturally occurring ones, destroyed a part of ascomata of grapevine powdery mildew in autumn, which ensure the perpetuation of the disease (Gadoury & Pearson, 1988), they contributed to a significant delay in the powdery mildew epidemic in the next season (Falk et al., 1995b). Thus, an additional benefit of this biocontrol experiment was the reduction of the overwintering inocula of powdery mildew. Commercial strain AQ10, based on spores of *A. quisqualis*, is widely exploited to control powdery mildew of various crops. Several studies were made to determine the potential of *A. quisqualis* in the biological control of powdery mildews, a group of fungi difficult to control in both greenhouse and field crops. A number of examples of acceptable disease control have been reported for greenhouse and field-grown vegetable crops (Sztejnberg 1993). In most experiments, *A. quisqualis* was sprayed onto infected plants as a conidial suspension and the applications were repeated several times during the season to ensure a high level of control (Sztejnberg et al., 1989). The first significant trial using *A. quisqualis* was reported by Jarvis and Slingsby (1977) who used conidial suspensions of the mycoparasite to control cucumber powdery mildew in greenhouse trials successfully. The control was enhanced when interspersed with water sprays. *A. quisqualis* requires water to germinate and to infect powdery mildew colonies. The high relative humidity (RH) requirement of *A. quisqualis* represents an important limiting factor in its use in biocontrol (Verhaar et al., 1999;

Paulitz & Bélanger, 2001). The dry conditions that usually are suitable for the development of powdery mildews are not conducive to the development of *A. quisqualis* (Kiss et al., 2004; Sundheim & Krekling, 1982). The most successful biocontrol experiments using *A. quisqualis* were carried out in greenhouses where the RH was kept high (Jarvis & Slingsby, 1977; Sundheim, 1982), or in the field where free water was frequently available on the treated leaves (Sztejnberg et al., 1989; Falk et al., 1995b). The efficacy of *A. quisqualis* always decreased rapidly when the RH was below 90–95 % (Kiss et al., 2004; Verhaar et al., 1999). A number of additives, such as an emulsion of 1 % paraffin oil or a 0.3 % mineral oil surfactant, were reported to increase the biocontrol performance of *A. quisqualis* at lower RHs (Hofstein et al., 1996). However, some of these additives can control powdery mildews directly (Verhaar et al., 1996; Pasini et al., 1997). Some trials have been reported where the treatments with *A. quisqualis* were ineffective, and suggested that only very limited control of powdery mildew could be expected in the plant protection practice (Dik et al., 1998; McGrath & Shishkoff, 1999; Verhaar et al., 1999; Shishkoff & McGrath, 2002). Experiments with *A. quisqualis* showed the biocontrol level was unsatisfactory, although parasitism of powdery mildew colonies on the treated crops did occur (Angeli et al., 2009; Gilardi et al., 2012). Recently, biocontrol experiments showed that genetically different *A. quisqualis* strains reduced the sporulation of different powdery mildews under controlled greenhouse conditions and the level of parasitization varies between different strains but the cause of this variation is unknown (Angeli et al., 2012b). However, there is still considerable interest in finding mycoparasitic strains within *A. quisqualis* species that afford more effective plant protection than the existing strains.

This research has focused on the development of new effective strains of *A. quisqualis* which can be used in the organic field to control powdery mildews. Here we aimed at enhancing the performance of *A. quisqualis* in the biological control of powdery mildew under controlled greenhouse conditions. Because of its moisture requirements, a major focus of our research is optimizing nutritional conditions for the culture production of fungal spores focuses on producing spores which will germinate rapidly. Thus, the present study was undertaken to investigate on the existence of external sources affecting some cultural and physiological parameters of the mycoparasitic fungus. The possibility of utilizing different active principles to increase its virulence in the biocontrol of powdery mildews is also investigated.

5.2 Material and methods

5.2.1 Pathogen and microorganism culture

Cucumber **(***Podosphaera xanthii)*, strawberry (*Podosphaera aphanis)* and grapevine (*Erysiphe necator)* powdery mildews were used to evaluate the aggressiveness of mycoparasitic strains of *A. quisqualis.* Fungal hosts *P. xanthii*, *P. aphanis and E. necator* derived from populations collected in Trentino Alto Adige region between 2010 and 2011 on zucchini, strawberry and grapevine plants, respectively. Fresh colonies of powdery mildew were maintained in greenhouse controlled conditions at 25 ± 1 °C and 70 ± 10 % of relative humidity (RH) and natural photoperiod. Subsequent inoculations of powdery mildew were carried out every 30 days by shaking leaves with sporulating mildew on 3-week-old plants having at least five true leaves.

A. quisqualis is known to be a slow-growing fungus with an *in vitro* radial growth rate of 0.5–1.0 mm d^{-1} on Czapek-Dox agar supplemented with 2 % malt extract (MCzA) at 23 °C (Kiss, 1997; Kiss & Nakasone, 1998) and its conidia germinate poorly in sterile, distilled water as previously seen by Angeli et al. 2012a. Three genetically different strains of *A. quisqualis* showing different level of mycoparasitic activities on different powdery mildews in preliminary experiments (Angeli et al. 2012b) were included in this study: strain ITA 3 (HQ108049) isolated from *Erysiphe necator* in Italy, displaying high mycoparastic activity, commercial AQ10 strain (AF035783) isolated from powdery mildew of *Chata Edulis* in Israel displaying intermediate biocontrol activity, and ATCC 200245 (AF126817) isolated from *Erysiphe necator* in USA which is a poor biocontrol agent. Strains were obtained from Ecogen, Langhorne, PA, USA (AQ10), from culture collections (ATCC200245, ATCC, American Type Culture Collection, Rockville, MD, USA) or provided by D. Angeli, FEM, Fondazione Edmund Mach, S. Michele all'Adige, Italy (ITA3). Cultures were grown and maintained on potato dextrose agar (PDA, Oxoid, United Kingdom) solid medium at 25 °C in the dark. For long-term storage, the strains were preserved at -80 °C using microbank vials.

5.2.2 Stimulation of *A. quisqualis* under different conditions

The effect of five different extracts (Table 1) on the cultural behavior and conidial germination of three selected *A. quisqualis* strains was assessed in two different experiments. In order to test

the impact of the extracts on mycelilal growth rate (experiment 1) the three selected *A. quisqualis* strains were evaluated on five different types of media: potato dextrose agar (Oxoid, Hampshire, UK, 39 g l^{-1} twice-distilled water), Water agar (Oxoid, 24 g l^{-1} twice-distilled water), Czapek agar (Oxoid, 33.4 g l^{-1} twice-distilled water), cornmeal agar (Oxoid, 17 g l^{-1} twice-distilled water) and pea agar (12.5 % frozen pea and 1.5 % agar in distilled water). All of them were then modified by adding different extracts listened in table 1 at the concentration of 1 g l^{-1}. The five media were then autoclaved at 121 °C for 30 min and 20 ml were dispersed in 90 mm diameter Petri dishes.

Table 1 Substances used to stimulate the conidia germination of *A. quisqualis* strains

Extract	Active ingredient	Code name	Dosage
Powdery mildew	*P. xanthii* conidia	PM	1 x 10^5 conidia ml^{-1}
Beer yeast	Yeast cells [a]	BY	1 g l^{-1}
Chitoplant	Chitosan	CH	1 g l^{-1}
Mushroom	Mushrooms [b]	MU	1 g l^{-1}
Shrimp shell	Shrimp shell	SS	1 g l^{-1}

[a] *Saccaromyces cerevisiae.*

[b] Mixture of *Agaricus Bisporius, Lentinus Edodes, Pleurotus Ostreatus* and *Boletus Edulis.*

Preparation of extracts: for the preparation of the powdery mildew extract, fresh colonies of cucumber powdery mildew (*P. xanthii*) were collected from leaf surface (10–15 leaves) by using 500 ml of distilled water + the adjuvant Tween 80 0.01 %. Then, we adjusted the spore concentration by Thoma-Zeiss counting chamber to 1 × 10^5 conidia ml^{-1}. The suspension of distilled water + Tween 80 containing 1 × 10^5 conidia ml^{-1} of cucumber powdery mildew was filtered and warmed up (65 °C twice - pasteurization). For the other extracts beer yeast (*S. cerevisiae*), chitoplant, mushrooms *(Agaricus Bisporius, Lentinus Edodes, Pleurotus Ostreatus, Boletus Edulis)* or shrimp shells were homogenized into distilled water at the concentration of 1 g l^{-1}.

Preparation of *A. quisqualis* conidia: cultures of the three *A. quisqualis* strains, approximately 2 weeks old, were flooded with sterile, distilled water and left for about 1 hour for the conidia to ooze out of the pycnidia. The resulting conidial suspension was harvested and adjusted with a Thoma-Zeiss counting chamber to 1×10^3 conidia ml^{-1} (experiment 1) or 1×10^5 conidia ml^{-1} (experiment 2).

For the first experiment Petri-dish cultures of the three fungal strains on the five media amended with the different extracts were prepared (75 plates per strain, 15 plates per media and 3 plates per extract) with 100 µl of a suspension of sterile distilled water containing 10^3 conidia ml^{-1}. Cultures were incubated in the dark at 25 °C for 30 days. The radial growth rate of the colonies was estimated by measuring the diameter of each colony after 30 days. All of the experiments were conducted with three replicates (plates) per strain. The experiment was conducted twice.

In the second experiment each strain was paired with the five selected extracts. Sterile distilled water suspensions of the substances (1 g l^{-1}) together with each fungal strain (1×10^5 conidia ml^{-1}) were prepared. Suspension of only *A. quisqualis* conidia in sterile distilled water were used as controls. Four drops (10 µl each) per combination (four replicates) were put onto a glass slide, which was placed in a Petri dish (RH = 100 %) and stored in a dark incubator kept at 25 °C, which is the temperature that Gu and Ko (1997) used in their study of the factors affecting the germination of *A. quisqualis* conidia. The germination rates and germ-tube elongation of 200 conidia per strain (four replicates, 50 conidia per replicate) were evaluated under a light microscope (Hund Wetzlar H 600LL, Wetzlar, Germany) after 12 hours. The experiment was conducted twice

5.2.3 Biocontrol activity after stimulation of conidial spores of *A. quisqualis*

Cucumber (*Podosphaera xanthii*), strawberry (*Podosphaera aphanis)* and grapevine (*Erysiphe necator*) powdery mildew were used as fungal hosts to evaluate the ability of *A. quisqualis* in reducing mildew infections under controlled conditions. Two weeks old plants were inoculated with powdery mildew spraying a suspension of distilled water containing 1×10^5 conidia ml^{-1}. Once powdery mildew cultures were sporulating (four/seven days later) a suspension of distilled water containing 1×10^6 conidia ml^{-1} of *A. quisqualis* and some soluble substances was prepared.

Fungal cultures, approximately 2 weeks old, were flooded with 20 ml of sterile, distilled water + Tween 80 and left for about 1 hour for the conidia to ooze out of the pycnidia. Conidia of *A. quisqualis* were collected from 5 plates and adjusting the concentration to 1×10^6 conidia ml^{-1} under a light microscope (Hund Wetzlar H 600LL, Wetzlar, Germany) by using Thoma-Zeiss counting chamber. Then, soluble extracts of powdery mildew, shrimp shell, mushroom, beer yeast, chitoplant were prepared as described above and mixed with *A. quisqualis* conidia suspensions to finally obtain 250 ml of a suspension containing 1×10^6 conidia ml^{-1} and 1 g extract ml^{-1}. The extracts were added to the conidia suspensions 12 hours before inoculation time. Plants bearing sporulating colonies of *P. xanthii*, *E. necator* and *P. aphanis* were homogeneously treated with the *A. quisqualis*/extract suspensions using a hand air-sprayer. Only two basal leaves per plant previously infected with the specific powdery mildew were treated (10 ml of suspension per leaf). Plants with powdery mildew and *A. quisqualis* infections were kept at 25 °C and 95 % of RH for 48 hours and then stored at the same temperature and 70 ± 10 % of RH. Untreated control plants were infected with powdery mildew and then sprayed with the natural extract dissolved into distilled water and Tween 80 only. The reduction of mycelial fungal infection of powdery mildews was evaluated ten days after *A. quisqualis* inoculation. Powdery mildew infected leaf area (severity) was evaluate by observing the presence of powdery mildew spots on the upper leaf surface of the two basal leaves. Results were expressed as percentage reduction of the powdery mildew infected leaf area in comparison with the powdery mildew control.

5.2.4 Data evaluation and statistical analysis

Assays were performed on two independent experiments arranged in a randomized complete block design with three replications. Data were first tested for the homogeneity of variances and then subjected to the analysis of variance (ANOVA). Two-way ANOVA was performed on log-transformed data from the two independent experiments and revealed no significant experiment × treatment interaction. Therefore, data from the two experiments were pooled. Averages of all replicates are presented in Table 2, Figure 1 and 2. Statistical significances among treatments were computed with Statistica software 7.0 (Statsoft, Tulsa, OK, USA) and means were separated using the Tukey's HSD ($P \leq 0.05$).

5.3 Results

5.3.1 Stimulation of *A. quisqualis* under different conditions

The radial growth rate of the three selected *A. quisqualis* strains were different on the five tested media (Tab. 2). The highest growth rates were observed on potato dextrose and pea agar plates for all strains whereas strains grew slowest on water agar. The growth rate of commercial AQ10 strain (0.12–0.79 mm d^{-1}) was higher than that of ITA 3 (0.11–0.59 mm d^{-1}) and ATCC 200245 (0.09–0.54 mm d^{-1}) on different media. Interestingly, our results indicate that the presence of some natural extracts into different media increased the growth of *A. quisqualis* strains. Significant differences in the induction of growth rate were detected among the different extracts. *A. quisqualis* responded to mushroom and shrimp shell supply with increased growth rate on different media. The effect is higher on water agar and czapek media where the growth rate of strains was increased by 80–250 % and 50–160 %, respectively. Powdery mildew extract significantly enhanced the growth of *A. quisqualis* strains by 40–120 % on all considered media. However, the other two extracts (chitoplant and beer yeast) did not stimulate the growth on these media.

Table 2. Effect of the stimulatory extracts on the growth rate of *Ampelomyces quisqualis*

Strain	Stimulator extract [b]	Radial growth rate of *A. quisqualis* on different media ($mm^2 day^{-1} \pm SE$) [a]				
		PDA	WA	Czapek	CMA	PEA
AQ10	Control [c]	0.76 ± 0.05 c	0.12 ± 0.02 b	0.39 ± 0.03 c	0.44 ± 0.04 d	0.79 ± 0.04 d
	PM	1.18 ± 0.08 b	0.20 ± 0.04 a	0.54 ± 0.06 b	0.61 ± 0.05 bc	1.16 ± 0.09 b
	BY	0.76 ± 0.03 c	0.13 ± 0.03 b	0.30 ± 0.04 d	0.24 ± 0.04 e	0.79 ± 0.05 d
	CH	0.61 ± 0.04 d	0.17 ± 0.04 ab	0.41 ± 0.05 c	0.56 ± 0.04 c	0.91 ± 0.06 c
	MU	1.37 ± 0.11 a	0.22 ± 0.05 a	0.77 ± 0.08 a	0.84 ± 0.07 a	1.40 ± 0.12 a
	SS	1.21 ± 0.07 b	0.23 ± 0.04 a	0.58 ± 0.06 b	0.70 ± 0.06 b	1.26 ± 0.09 b
ITA 3	Control	0.53 ± 0.03 c	0.11 ± 0.01 c	0.26 ± 0.03 c	0.31 ± 0.05 c	0.59 ± 0.03 c
	PM	0.89 ± 0.07 a	0.22 ± 0.03 b	0.41 ± 0.04 b	0.51 ± 0.05 a	0.81 ± 0.05 b
	BY	0.48 ± 0.04 c	0.09 ± 0.02 c	0.27 ± 0.05 c	0.25 ± 0.03 c	0.57 ± 0.05 c
	CH	0.62 ± 0.04 b	0.11 ± 0.02 c	0.29 ± 0.03 c	0.30 ± 0.04 c	0.66 ± 0.05 c
	MU	0.99 ± 0.07 a	0.36 ± 0.05 a	0.67 ± 0.04 a	0.59 ± 0.07 a	1.06 ± 0.10 a
	SS	1.03 ± 0.08 a	0.39 ± 0.05 a	0.61 ± 0.06 a	0.62 ± 0.05 a	0.96 ± 0.07 a
ATCC 200245	Control	0.54 ± 0.02 d	0.09 ± 0.01 c	0.25 ± 0.03 c	0.30 ± 0.02 c	0.49 ± 0.03 c
	PM	0.85 ± 0.05 b	0.20 ± 0.02 b	0.49 ± 0.05 b	0.50 ± 0.05 b	0.67 ± 0.04 b
	BY	0.52 ± 0.03 d	0.10 ± 0.02 c	0.28 ± 0.01 c	0.27 ± 0.03 c	0.44 ± 0.02 c
	CH	0.60 ± 0.03 c	0.13 ± 0.03 c	0.33 ± 0.03 c	0.31 ± 0.02 c	0.51 ± 0.03 c
	MU	0.97 ± 0.06 a	0.30 ± 0.02 a	0.65 ± 0.03 a	0.60 ± 0.04 a	0.89 ± 0.07 a
	SS	0.84 ± 0.03 b	0.27 ± 0.03 a	0.56 ± 0.04 b	0.51 ± 0.03 b	0.87 ± 0.06 a

Values are means of six replicates derived from two independent experiments with three replicates per experiment. Standard errors of the means are reported (SE). Values with the same letter within a column are not significantly ($P \leq 0.05$) different according to the Tukey's HSD test.

[a] Radial growth of the colonies was measured once a week for 30 days.

[b] Stimulatory extracts added to the media are beer yeast (BY), Chitoplant (CH), mushroom (MU), powdery mildew extract (PM) and shrimp sheels (SS).

[c] Conidia of *A. quisqualis* were used as positive control.

The experiment in which we investigated the effect of stimulatory extracts on conidial germination of *A. quisqualis* strains showed that germination rates and germ-tube elongation of strains AQ10, ITA 3 and ATCC 200245 were very poor in the absence of stimulators (Figs. 1a–b). In sterile, distilled water only a small fraction (less than 12 %) of the conidia of *A. quisqualis* had germinated at the end of 12 hours incubation and the lenght of their germ tubes was less than 5 µm. Both parameters were strongly enhanced when 1 g l^{-1} of different extracts inducing fungal growth on artificial media (Tab. 2) were added to the distilled water. However, significant differences in the induction of germination were detected among the different extracts. Similarly to the mycelial growth the conidial germination and tube elongation of all strains were stimulated most by shrimp shell (60–87 % and 19–30 um) and mushroom extract (60–69 % and 17–25 um). Germination was much less, but still significantly induced by powdery mildew conidia and chitoplant extracts whilst treatments with beer yeast did not statistically differ from untreated control, with the exception of f strain ATCC 200245 which was slightly stimulated by yeast extract. Furthermore, we observed differences among the conidial germination rates of the different strains, independent of the extract treatments. With the exception of beer yeast, the germination rate and tube elongation of ITA 3 the most aggressive of the tested strains were strongly induced by all of the tested extracts (33–87 % and 7–31 um) whereas the lower aggressive strains were generally less responsive.

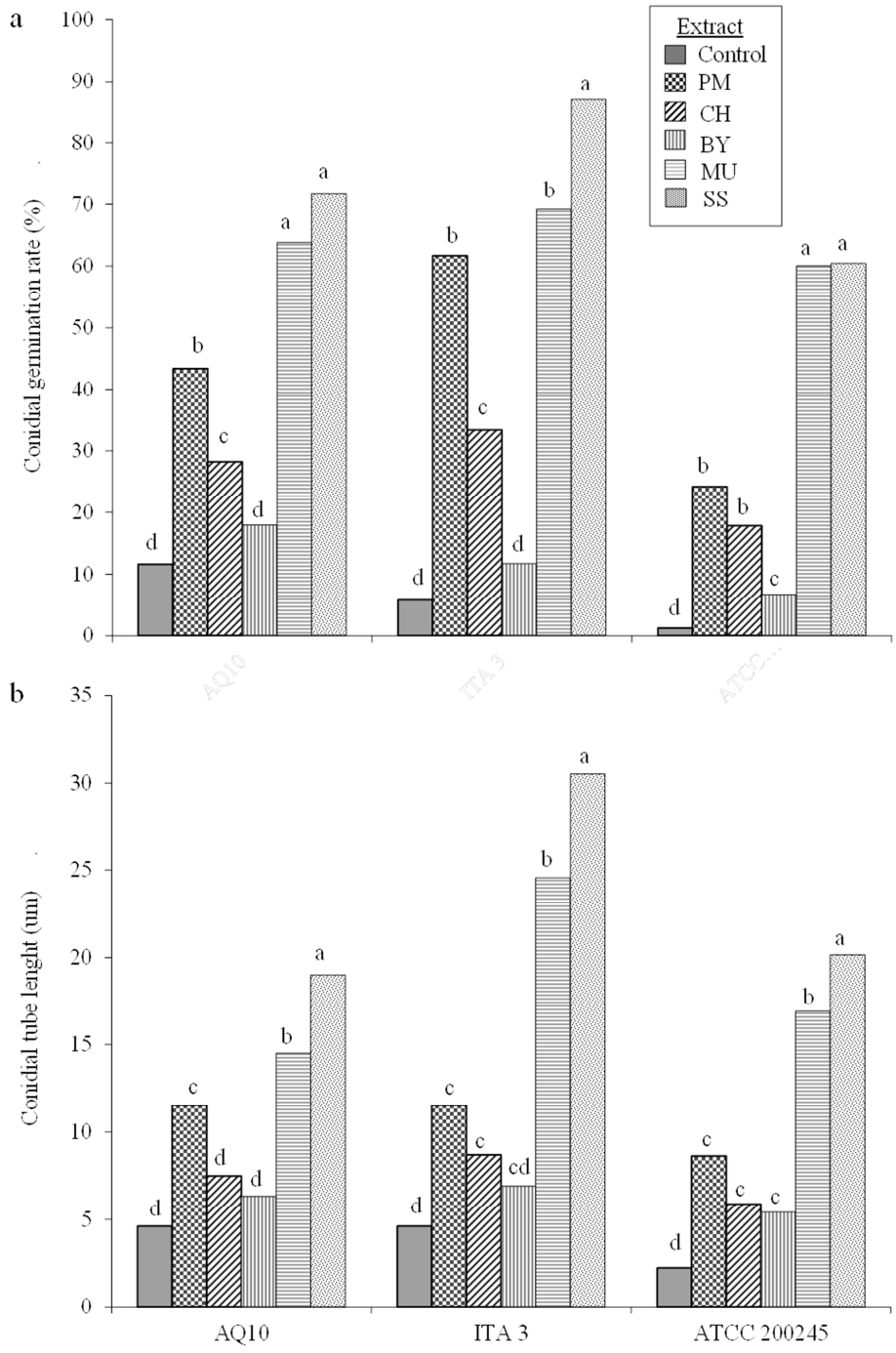


Figure 1 Effects of beer yeast (BY), Chitoplant (CH), mushroom (MU), powdery mildew extract (PM) and shrimp sheels (SS) on the conidial germination (%) (a) and germ tube elongation (µm) (b) of AQ10, ITA 3 and ATCC 200245. Measurements were taken in aqueous suspensions containing mixtures of *A. quisqualis* conidia and extracts that had been incubated together for 12 hours at 25 °C. Untreated conidia

were used as control. Columns with the same design represent the same stimulator extract. Columns labeled with the same letter (a-d) are not significantly different ($P \leq 0.05$) according to Tukey's test. Four replicates (50 conidia per replicate) of each strain were evaluated. Presented values are means of two independent experiments with four replicates per experiment.

5.3.2 Biocontrol activity after stimulation of conidial spores of *A. quisqualis*

Experiments conducted on three different fungal hosts showed that the ability to reduce mildew infections of *A. quisqualis* strains can be increased by the presence of some of the tested extracts (Fig. 2). As observed for mycelial growth and conidia germination (Table 2, Fig. 1) the stimulating effect of mushroom and shrim shell extracts was most pronounced. In the presence of these substances all tested strains showed a stronger reduction of infected leaf area of all tested powdery mildews. Bear yeast and Chitoplant extracts did not have any impact on biocontrol activity. Extracts of mildew conidia stimulated only the activity *A. quisqualis* on *P. aphani* (Fig. 2 A). However, individual *A. quisqualis* strains differ in their aggressiveness and ITA 3 was more effective than ATCC 200245 and the commercial strain AQ10. Best results were obtained with ITA 3 stimulated by shrimp shell and mushroom extracts which reduced the infected leaf area on strawberry, grapevine and cucumber (Fig 2). Values ranged from 62 to 91 % reduction of powdery mildew infected leaf area. The improving effects of the extracts on obtained with AQ10, ITA 3 and ATCC 200245 were clearly higher on strawberry powdery mildew which is less susceptible versus *A. quisqualis* than cucumber and grapevine. For example, mushroom and shrimp shell extracts increased the reduction of mildew infection by more than 20 % in comparison to ITA 3 and AQ10 treatments without extract addition (Fig. 2a).

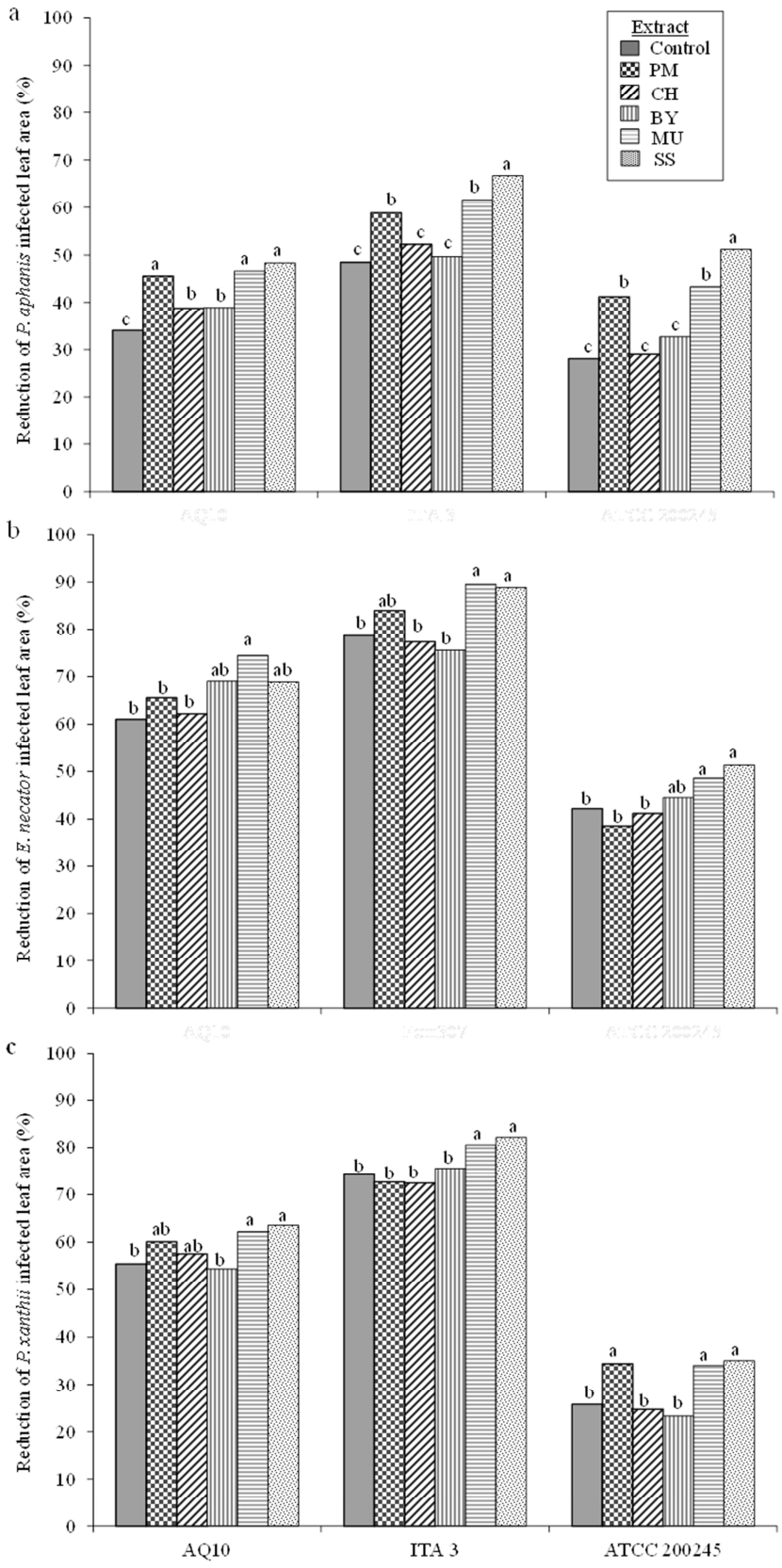

a
b
c
Extract
Control
PM
CH
BY
MU
SS
Reduction of P. aphanis infected leaf area (%)
Reduction of E. necator infected leaf area (%)
Reduction of P. xanthii infected leaf area (%)
AQ10
ITA 3
ATCC 200245

Figure 2 *P. aphanis* (a), *E. necator* (b) and *P. xanthii* (c) infected area on leaves sprayed with AQ10, ITA 3 and ATCC 200245 stimulated by beer yeast (BY), Chitoplant (CH), mushroom (MU), powdery mildew extract (PM) and shrimp sheels (SS). Untreated conidia were used as positive control. Columns with the same design represent the same stimulator extract. Columns with the same letters (a-c) do not significantly differ ($P \leq 0.05$, Tukey's test). Presented values are means of two independent experiments with three replicates per experiment.

5.4 Discussion

Biological control of powdery mildews remains a challenge for future research and development. The results obtained so far showed some promises of practical biocontrol of a number of powdery mildew diseases but more efforts are needed to prove the efficacy of these methods in agricultural and horticultural practice. The major constraint to the use of fungal pathogens as biological control agents is consistent performance under field conditions. Powdery mildews are biotrophic pathogens infecting the aerial parts of their host plants and biocontrol agents have to perform their actions in the phyllosphere, a high dynamic environment, where biological control of different plant pathogenic fungi is, in general, less successful. Moreover, the sporulation capacity of powdery mildews is high and their spread very fast making their control extremely difficult even with chemicals. AQ10, is the first commercialized biocontrol product based on spores *of A. quisqualis* against powdery mildews but it seems that it cannot provide satisfactory control in a number of crops including grape, rose and vegetables (Angeli et al., 2009; Gilardi et al., 2012). Inconsistent performance is often attributed to a lack of free moisture which is required by the fungal spores during process of germination and penetration of the host (Jackson, 1997). The requirement for free moisture is a major environmental constraint of *A. quisqualis* to effective control under field conditions. The slow growth rate and relatively low conidial germination and concentration is another disadvantage to its use as biocontrol agents.

Because of these constraints, one of our primary goals in producing fungal spores for use as a biocontrol agent is to reduce the impact of the free moisture requirement by producing spores which germinate more rapidly and form an appressorium more frequently. Here different media containing sufficient nutrients to support microbial growth were compared to a medium essentially free of nutrients (water agar), an approach that is useful for the detection of

stimulators or inhibitors (Gu & Ko, 1997). The mycelium of *A. quisqualis* grows slowly on artificial media *in vitro*. On tested media growth of colonies of *A. quisqualis* was greatly enhanced by including mushroom and shrimp shell extracts in the media. These extracts seem to have a general stimulating effect on *A. quisqualis* since they stimulated the growth of all three tested strains.

Only a few studies examined the impact of added nutrients on the conidial germination of this hyperparasite whereas there are numerous reports of the stimulatory effects of nutrients on the germination of spores of other fungal species. Leachate from aerial plant surfaces contains nutrients that stimulate the germination of fungal propagules of *Alternaria alternata* and *Epicoccum purpurascens* (Tukey, 1971). Blakeman (1975) found that sugars promoted germination of *Botrytis cinerea* conidia. Some aminoacids were stimulatory while others inhibited germination. The amount of sugars and aminoacids present on leaves were sufficient to stimulate germination of *B. cinerea* conidia.

Cochrane 1966 differentiated between fungi which require external nutrients for germination and those that germinate without the addition of nutrients. Conidia of *A. quisqualis* fall into the first category. Some organic molecules present in the hyphae of the host fungus and other fungi are needed for germination. However, the addition of simple carbohydrates or aminoacids did not promote germination of *A. quisqualis* (Cochrane, 1966). Recently, Gu & Ko (1997) showed that *A. quisqualis* recognizes the presence of the host and a water-soluble substance from powdery mildew conidia has been shown to stimulate germination of *A. quisqualis* conidia *in vitro*. The stimulatory substance in powdery mildew was thermostable and its molecular weight was in the range of 1200–1500 dalton. However, the identity of the fungal substance that stimulates germination of *A. quisqualis* conidia remains to be elucidate.

The results obtained in this study confirm that the mildew host stimulates the germination of *A. quisqualis* conidia but show that supplementation with external sources of nutrient precursors present in shrimp shell and mushroom extracts even had a stonger positive effect on the germination of conidia. We identified new extracts based on different active ingredients able to stimulate the growth rate on plate and conidial germination of *A. quisqualis* and their stimulatory effect is often higher than that of the fungal host (*P. xanthii* conidia). Of the tested extracts mushroom, shrimp shell and mildew conidia extracts, but not bear yeast and Chitoplant extracts

stimulated both growth and conidial germination of all tested *Ampelomyces* strains. Interestingly conidia of ITA3 the strain displaying the highest level of biocontrol were stimulated the most by mushroom and shrimp shell extracts.

In this work conidia of the three tested strains germinated poorly or not at all in sterile, distilled water. Cook (1977) hypothesized that self-inhibitors in fungal spores are common and that failure of spores to germinate is due to substances within the spores. Nutrients may cause germination by deactivating the self-inhibitors in the spores. Other studies showed that spores of some fungi germinate readily under dispersed conditions but when crowded they germinate poorly due to the presence of a self-inhibitor (Allen, 1976; Lax et al., 1985). A few years later Gu & Ko (1997) developed an *in vitro* method for laboratory observation of the interaction between conidia of powdery mildews and conidia of their mycoparasite and they reported on the presence of self-inhibitor in conidia of *A. quisqualis.* On water agarose media germination of *A. quisqualis* conidia decreased as their concentration increased. In light of this discovery, we identified some extracts which seem to easly overcome the self-inhibition of germination of *A. quisqualis* conidia. Whether indeed, some compounds in the extracts we used interact with self-inhibitors would be an interesting issue for investigation in future research.

Furthermore, we report on improvement of *in vivo* biocontrol acitivity of *A. quisqualis* in three different pathosystems by the addition of stimulants. Our results show that performance of different *A. quisqualis* strains isolated from different plants and fungal hosts can be increased by the presence of extracts stimulating growth and conidial germination. Protection against powdery mildew was clearly higher on plants treated with a suspesion of *A. quisqualis* ITA 3 strain stimulated through mushroom and shrimp shell. The greatest stimulating effect was observed on plants infected with strawberry powdery mildew which is less susceptible to *A. quisqualis* than cucumber and grapevine.

In this study we demonstrated there is considerable potential for improving the efficiency of the fungus *A. quisqualis* in the control of powdery mildew pathogen. These findings indicate that the nutritional environment during culture growth can have an effect on hyphal growth pattern and, even more relevant on spore attributes such as germination and germ tube elongation. Our studies have demonstrated that the conidial germination ability of *A. quisqualis* can be positively related to virulence against powdery mildew and can, therefore, be considered as relevant factor

in the selection of new good biocontrol agents. We feel that by optimizing the spore attributes we can improve the biocontrol efficacy of this agent under field conditions. This provides important knowledge for the selection of new, highly effective mycoparasitic fungal strains for biocontrol potential and its exploitation in sustainable agriculture.

5.5 Acknowledgements

We would like to thank Franca Micheli, Carmela Sicher and Denise Ress for technical assistance. This work was supported by the AMPELO and ENVIROCHANGE projects funded by the Autonomous Province of Trento.

5.6 Literature

Allen, P. J. (1976) Spore germination and its regulation. Control of spore germination and infection structure formation in the fungi. Encyclopedia of Plant Physiology New Series Volume 4:51-85.

Angeli, D., Pellegrini, E., Micheli, S., Ress, D., Maurhofer, M., Pertot, I. and Gessler, C. (2009). Molecular characterization of *Ampelomyces* spp. strains from different hosts and geographic origins and evaluation of their potential to control powdery mildew of cucumber. IOBC/WPRS Bulletin 43:40-44.

Angeli, D., Maurhofer M., Gessler C. and Pertot I. (2012a). Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis*. Phytoparasitica 40:37-51.

Angeli, D., Puopolo, G., Maurhofer M., Gessler C. and Pertot I. (2012b). Is the mycoparasitic activity of *Ampelomyces quisqualis* biocontrol strains related to phylogeny and hydrolytic enzyme production?. Biological control 63(3):348-358.

Blakeman, J.P. (1975). Germination of *Botrytis cinerea* conidia in vitro in relation to nutrient conditions on leaf surfaces. Trans. Br. Mycol. Soc. 65:239-247.

Cochrane, V.W. (1966). Respiration and spore germination. In “The fungus spore” ed. M.F. Madelin, pp. 201-213. Butterwortiis, London. 338 pp.

Cook, R.J. (1977). Management of the Associated Microbiota. In “Plant Disease” ed. J.G. Horsefall and E.B. Cowling, vol. 1, pp. 145-166. Academic Press, London and New York. 465 pp.

Dik, A.J., Verhaar, M.A. and Bélanger, R.R. (1998). Comparison of three biological control agents against cucumber powdery mildew (*Sphaerotheca fuliginea*) in semi-commercial-scale glasshouse trials. European Journal of Plant Pathology 104:413-423.

Falk, S. P., Gadoury, D. M., Cortesi, P., Pearson, R. C., and Seem R. C. (1995a). Parasitism of *Uncinula necator* cleistothecia by the mycoparasite *Ampelomyces quisqualis.* Phytopathology 85:794-800.

Falk, S. P., Gadoury, D. M., Pearson, R. C., and Seem, R. C. (1995b). Partial control of grape powdery mildew by the mycoparasite *Ampelomyces quisqualis.* Plant Dis 79:483-490.

Fokkema, N.J. (1993). Opportunities and problems of control of foliar pathogens with micro-organisms. Pesticide Science 37:411-416.

Gadoury, D. M. and Pearson, R. C. (1988). Initiation, development, dispersal, and survival of cleistothecia of *Uncinula necator* in New York vineyards. Phytopathology 78:1413-1421.

Gilardi, G., Baudino, M., Garibaldi, A., Gullino, M.L. (2012). Efficacy of biocontrol agents and natural compounds against powdery mildew of zucchini. Phytoparasitica 40:147-155.

Gu, Y.H. and Ko, W.H. (1997). Water agarose medium for studying factors affecting germination of conidia of *Ampelomyces quisqualis.* Mycological Research 101:422-424.

Hashioka, Y. and Nakai, Y. (1980). Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. Trans. Mycol. Soc. Jpn 21:329-338.

Hofstein, R., Daoust, R.A. and Aeschlimann, J.P. (1996). Constrains to the development of biofungicides: the example of ‘‘AQ10’’, a new product for controlling powdery mildews. Entomophaga 41:455-460.

Jackson, M.A. (1997). Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. Journal of Industrial Microbiology & Biotechnology 19:180-187.

Jarvis, W.R. and Slingsby, K. (1977). The control of powdery mildew of greenhouse cucumber by water sprays and *Ampelomyces quisqualis.* Plant Dis Rep 61:728-730.

Kiss, L. (1997). Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. Mycological Research 101:1073-1080.

Kiss, L. and Nakasone, K. K. (1998). Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi. Curr. Gen. 33:362-367.

Kiss, L., Russell, J.C., Szentivanyi, O., Xu, X. and Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonist of powdery mildew fungi. Biocontrol Science and Technology 14:635-651.

Kiss, L., Pintye, A., Kovacs, G.M., Jankovics, T., Fontaine, M.C., Harvey, N., Xu, X., Nicot, P.C., Bardin, M., Shykoff, J.A. and Giraud, T. (2011). Temporal isolation explains host-related genetic differentiation in a group of widespread mycoparasitic fungi. Molecular Ecology 20:1492-1507.

Lax, A. R., Templeton, G. E. and Meyer, W. L. (1985). Isolation, purification, and biological activity of a self-inhibitor from conidia of *Colletotrichum gloeosporioides*. Phytopathology 75(4):386-390.

Liang, C., Yang, J., Kovacs, G.M., Szentivanyi, O., Li, B., Xu, X.M. and Kiss, L. (2007). Genetic diversity of *Ampelomyces* mycoparasite isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences. Fungal Diversity 24:225-240.

McGrath, M.T. and Shishkoff, N. (1999). Evaluation of biocompatible products for managing cucurbit powdery mildew. Crop Protection 18:471-478.

Park, M.J., Choi, Y.J., Hong, S.B. and Shin, H.D. (2010). Genetic variability and mycohost association of *Ampelomyces quisqualis* isolates inferred from phylogenetic analyses of ITS rDNA and actin gene sequences. Fungal Biology 114:235-247.

Pasini, C., D'Aquila, F., Curir, P. and Gullino, M.L. (1997). Effectiveness of antifungal compounds against rose powdery mildew (*Sphaerotheca pannosa* var *rosae*) in glasshouses. Crop Protect 16:251-256.

Paulitz, T.C. and Bélanger, R.R. (2001). Biological control in greenhouse systems. Annu Rev Phytopathol 39:103-133.

Shishkoff, N. and McGrath, M.T. (2002). AQ10 biofungicide combined with chemical fungicides or AddQ spray adjuvant for control of cucurbit powdery mildew in detached leaf culture. Plant Disease 86:915-918.

Sundheim, L. (1982). Control of cucumber powdery mildew by the hyperparasite *Ampelomyces quisqualis* and fungicides. Plant Pathol 31:209-214.

Sundheim, L., Krekling, T. (1982). Host-parasite relationships of the hyperparasite *Ampelomyces quisqualis* and its powdery mildew host *Sphaerotheca fuliginea*. I. Scanning electron microscopy. Phytopathology 104:202-210.

Sundheim, L. and Tronsmo, A. (1988). Hyperparasites in biological control. Pages 53-69 in: Biocontrol of Plant Diseases. Vol. I. CRC Press, Boca Raton, FL.

Sztejnberg, A., Galper, S., Mazar, S., and Lisker, N. (1989). *Ampelomyces quisqualis* for biological and integrated control of powdery mildews in Israel. J Phytopath 124:285-295.

Sztejnberg, A. (1993). *Ampelomyces quisqualis* AQ10, CNCM I-807, for biological control of powdery mildew. US patent no. 5190754.

Tukey, H.B. (1971). Leaching of substances from plants. In "Ecology of leaf surface micro-organisms" ed. T.F Preece. and C.H. Dickinson, pp. 67-80. Academic Press, London and New York. 640 pp.

Verhaar, M.A., Huwegen, T. and Zadoks, J.C. (1996). Glasshouse experiments on biocontrol of cucumber powdery mildew (*Sphaerotheca fuliginea*) by the mycoparasites *Verticillium lecanii* and *Sporothrix rugulosa*. Biological Control 6:353-360.

Verhaar, M.A., Kerssies, A. and Huwegem, T. (1999). Effect of RH on mycoparasitism of rose powdery mildew with and without treatments with mycoparasites. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 106:158-165.

Chapter 6

General conclusions

6.1 General conclusions

Biological control of powdery mildews remains a challenge for future research and development. The results obtained so far show some promises of practical biocontrol of a number of powdery mildew diseases, especially on cucumber and rose, but more efforts are needed to prove the efficacy of these methods in horticultural practice. A product based on *P. flocculosa* (Sporodex), the most promising biocontrol alternative for use against powdery mildews, has received a temporary registration in Canada only since May 2002, and no data about its commercial success, are available yet. Unfortunately, it seems that AQ10 biofungicide based on spores of *A. quisqualis*, the first commercialized biocontrol product against powdery mildews, cannot provide satisfactory control in a number of crops including grape, rose and vegetables (Bélanger & Labbé, 2002; Dik et al., 1998; McGrath & Shishkoff, 1999; Verhaar et al., 1996). Currently, its use is recommended as a part of an integrated programme and as a preventive measure only (Bélanger & Labbé, 2002; Paulitz & Bélanger, 2001). Another formulated *A. quisqualis* product was registered in Australia in the late 1980s, but did not meet the expectations of the plant protection practice. However, a number of possibilities for the use of *A. quisqualis* as an effective biocontrol agent have yet to be exploited. Some powdery mildews can be controlled efficiently with these mycoparasites (Kiss L., 2004). Moreover, there is a high genetic diversity in *A. quisqualis* strains that offers the not yet exploited possibility of screening a large number of different types of strains (Angeli et al., 2009b; Angeli et al., 2012a; Kiss, 1997; Kiss & Nakasone, 1998; Kiss et al., 2011; Liang et al., 2007). Strains of *V. lecanii* and *Tilletiopsis* spp. represent another promising source of successful biocontrol agents of powdery mildews. However, in spite of intensive studies on their biology and biocontrol potential there have been no initiatives to develop commercial products based on these fungi (Askary et al., 1998; Dik et al., 1998; Urquhart et al., 1994; Urquhart & Punja, 1997; Urquhart & Punja, 2002; Verhaar et al., 1996; Verhaar et al., 1999;). This thesis shows that there are still many unexplored possibilities for research and development of biocontrol agents. Well-studied and little known fungal antagonists constitute together a valuable source that will surely be used to develop biocontrol or integrated programs to reduce the use of chemicals against powdery mildews in the foreseeable future. Powdery mildew caused by *E. necator* is an important disease of grapevine in the Trentino-Alto Adige region.

The first objective of the present work was to investigate the natural occurrence of overwintering inoculum of powdery mildew in a wide viticulture area in Northern Italy (Trentino-Alto Adige region) and evaluate the presence of *A. quisqualis* on grapes (Chapter 2). Here we demonstrated that the initiation and development of chasmothecia can occur in any year in which conditions are favorable for disease development (Angeli et al., 2009a). The high incidence of chasmothecia in the vineyards of the Trentino-Alto Adige region, which was associated with the absence of flag shoots in the monitored vineyards, could imply that chasmothecia are the primary inoculum and confirm previous results, which had indicated that chasmothecia represent the main source of inoculum in the spring (Cortesi et al., 1995). The many studies on *E. necator* chasmothecia have reported unclear and sometimes contradictory effects of environmental factors on the development of chasmothecia (Gadoury & Pearson, 1988). Our results suggest that the concentration of chasmothecia was mainly related to the level of disease in the vineyard, which is usually the result of complex interactions among the amount of initial inoculum, the efficacy of disease control measures, cultivar susceptibility, the physiology of the crop, and the weather conditions during the growing season. Reports have stated that most of the chasmothecia die during the winter without having any opportunity to release ascospores (Cortesi et al., 1995), but a reduction in the number of overwintering chasmothecia by hyperparasites may nevertheless be useful for reducing the amount of primary inoculum (Falk et al., 1995a,b). *A. quisqualis* is one of the most successful commercialized biocontrol agents and it acts directly by invading and destroying the viable chasmothecia (Hashioka & Nakai, 1980). The present study is the first extensive investigation of quantitative aspects of *A. quisqualis* on grapevine powdery mildew in Italy. *A. quisqualis* was observed parasitizing chasmothecia of *E. necator* suggesting that *A. quisqualis* was naturally present in the region. More than 200 strains of *A. quisqualis* were identified and some of them were genetically different from the commercial strain (AQ10). They may represent strains better adapted to local environmental conditions than AQ10 and can be developed as new biocontrol agents to control powdery mildew under the environmental conditions found in Northern countries.

Further analyses (chapter 3) were performed to characterize the isolated strains and other *A. quisqualis* strains derived from different regions and hosts and to investigate their environmental requirements for development and parasitism. These investigations aimed also at the identification of phenotypic markers that can be used to differentiate genetically distinct groups

within *A. quisqualis*. Data on the morphology and cultural patterns of *A. quisqualis* strains found in the literature are controversial and incomplete (Kiss, 1997; Park et al., 2010). We revealed some significant variation among the selected strains, which provides evidence for the existence of different physiological forms within the *A. quisqualis* species. Two groups that display differential growth on artificial media were identified. These groups also differ in the morphology of their mycelium, but not in the morphology of their pycnidia and conidia. Temperature greatly affected the *in vitro* growth of the *A. quisqualis* strains and growth rate was closely correlated to colony color. Differences in the conidial germination of distinct strains were observed during the recognition phase of the parasitic relationship. Different powdery mildew agents strongly affect the germination of *A. quisqualis* conidia but specialization is indistinct. Phylogenetic analysis of the ITS rDNA sequence revealed a high level of genetic diversity among *A. quisqualis* strains and suggested that ITS groups could be related to the fungal host of origin and morphological and growth characteristics. It appears that powdery mildew fungi and *A. quisqualis* might have coevolved through the processes of specialization and adaptation. Thus, a degree of mycohost specialization was identified within the *A. quisqualis* species.

The second main objective of the thesis was to assess the mycoparasitic activity of the *A. quisqualis* strains in terms of their ability to inhibit conidiation in different powdery mildew pathogens and intra-hyphal formation of their intracellular pycnidia within powdery mildews *in vivo*. At the same time, production of cell wall degrading enzymes (CWDEs) was measured under *in vitro* conditions and factors important in effective mycoparasitism of *A. quisqualis* were detected (Chapter 4). Results showed that individual strains differed significantly in enlargement of the colonization area by intrahyphal formation of pycnidia within powdery mildew colonies and in inhibition of host conidiation. Pronounced differences in the activity of CWDEs were also observed between strains. The ability of some *A. quisqualis* strains to parasitize the host by forming intracellular pycnidia and reducing fungal-host conidiation was associated with their intrinsic ability to secrete proteases and with chitobiase activity. Finally, we have demonstrated that the ability of *A. quisqualis* strains to colonize powdery mildew by forming pycnidia and inhibiting conidiation is completely independent of the geographical origin of the strain, but appears to be related to genotype (ITS rDNA sequence) and the original fungal host. The biological characterization of the *A. quisqualis* strains carried out in this work suggests that mycoparasitism can be explained by the genotype and in part by the activities of CWDEs. This

provides important knowledge for the development of highly effective strains for biocontrol of powdery mildews. However, the level of parasitization varies between different strains, and the cause of this variation is unknown (Angeli et al., 2012b). There is still considerable interest in finding mycoparasitic strains within *A. quisqualis* species that afford more effective plant protection than the existing strains.

In the last chapter we aimed at enhancing the performance of *A. quisqualis* in the biological control of powdery mildew. A major focus of this part of the thesis was to optimize nutritional conditions for the culture production of fungal spores focusing on producing spores which will germinate rapidly, form an appressorium more frequently and can, therefore, increase the virulence of the fungus in the biocontrol of powdery mildews. Inconsistent performance is often attributed to a lack of free moisture which is required by the fungal spores during process of germination and penetration of the host (Gu & Ko, 1997). The requirement for free moisture is a major environmental constraint of *A. quisqualis* to effective control under field conditions. Conidia of *A. quisqualis* germinate poorly in sterile, distilled water but a water-soluble substance from the powdery mildew host was shown to stimulate the germination of the conidia. Here we identified new extracts based on different active ingredients which stimulate the growth rate and conidial germination of *A. quisqualis* conidia and the stimulatory effect seem to be extract species-specific (Chapter 5). Biocontrol assays showed that the performance of different *A. quisqualis* strains can be increased by the presence of extracts stimulating growth and conidial germination. These findings revealed that the conidial germination ability of *A. quisqualis* could be positively related to virulence against powdery mildew and can, therefore, be considered as relevant factor in the selection of new good biocontrol agents.

In conclusion, the major constraint to the use of this fungal pathogen as biological control agents is consistent performance under field conditions. Inconsistent performance is often attributed to a lack of free moisture which is required by the fungal spores during process of germination and penetration of the host. These studies demonstrates that there is considerable potential for improving powdery mildew control by *A. quisqualis* for example by improving germination characteristics of conidia. We further provide important knowledge on this fungus that is not only useful for improving the efficacy of biological control agents under limiting conditions but also for development of new screening strategies enabling the detection of highly effective mycoparasitic fungal strains for on optimized biocontrol in sustainable agriculture.

6.2 Literature

Angeli, D., Pellegrini, E. and Pertot, I. (2009a). Occurrence of *Erysiphe necator* chasmothecia and their natural parasitization by *Ampelomyces quisqualis*. Phytopathology, 99, 704-710.

Angeli, D., Pellegrini, E., Micheli, S., Ress, D., Maurhofer, M., Pertot, I. and Gessler, C. (2009b). Molecular characterization of *Ampelomyces* spp. strains from different hosts and geographic origins and evaluation of their potential to control powdery mildew of cucumber. IOBC/WPRS Bulletin, 43, 40-44.

Angeli D., Maurhofer M., Gessler C. and Pertot I. (2012a). Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis*. Phytoparasitica 40, 37-51.

Angeli, D., Puopolo, G., Maurhofer M., Gessler C. and Pertot I. (2012b). Is the mycoparasitic activity of *Ampelomyces quisqualis* biocontrol strains related to phylogeny and hydrolytic enzyme production?. Biological control 63(3):348-358.

Askary, H., Carriere, Y., Bélanger, R.R. and Brodeur, J. (1998). Pathogenicity of the fungus *Verticillium lecanii* to aphids and powdery mildew. Biocontrol Sci Technol 8:23-32.

Bélanger, R.R. and Labbé, C. (2002). Control of powdery mildews without chemicals: prophylactic and biological alternatives for horticultural crops, in The powdery mildews: a comprehensive treatise, ed by Bélanger, R.R., Bushnell, W.R., Dik, A.J. and Carver, T.L.W., American Phytopathological Society, St Paul, USA, pp 256-267.

Cortesi, P., Gadoury, D. M., Seem, R. C. and Pearson, R. C. (1995). Distribution and retention of cleistothecia of *Uncinula necator* on the bark of grapevines. Plant Dis. 79:15-19.

Dik, A.J., Verhaar, M.A. and Bélanger, R.R. (1998). Comparison of three biological control agents against cucumber powdery mildew (*Sphaerotheca fuliginea*) in semi-commercial-scale glasshouse trials. European Journal of Plant Pathology 104:413-423.

Falk, S. P., Gadoury, D. M., Cortesi, P., Pearson, R. C. and Seem R. C. (1995a). Parasitism of *Uncinula necator* cleistothecia by the mycoparasite *Ampelomyces quisqualis*. Phytopathology 85:794-800.

Falk, S. P., Gadoury, D. M., Pearson, R. C. and Seem, R. C. (1995b). Partial control of grape powdery mildew by the mycoparasite *Ampelomyces quisqualis.* Plant Dis. 79:483-490.

Gadoury, D. M. and Pearson, R. C. (1988). Initiation, development, dispersal, and survival of cleistothecia of *Uncinula necator* in New York vineyards. Phytopathology 78:1413-1421.

Gu, Y.H. and Ko, W.H. (1997). Water agarose medium for studying factors affecting germination of conidia of *Ampelomyces quisqualis*. Mycological Research 101, 422-424.

Hashioka, Y. and Nakai, Y. 1980. Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. Trans. Mycol. Soc. Jpn 21:329-338.

Kiss, L. (1997). Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. Mycol. Res. 101:1073-1080.

Kiss, L. and Nakasone, K. K. (1998). Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi. Curr. Gen. 33:362-367.

Kiss, L., Russell, J.C., Szentivanyi, O., Xu, X., Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonist of powdery mildew fungi. Biocontrol Science and Technology 14, 635-651.

Kiss, L., Pintye A., Kovacs G.M., Jankovics T., Fontaine M.C., Harvey N., Xu X., Nicot P.C., Bardin M., Shykoff J.A. and Giraud T. (2011). Temporal isolation explains host-related genetic differentiation in a group of widespread mycoparasitic fungi. Molecular Ecology, 20, 1492-1507.

Liang C., Yang J., Kovacs G.M., Szentivanyi O., Li B., Xu X.M. & Kiss L. (2007). Genetic diversity of *Ampelomyces* mycoparasite isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences. Fungal Diversity, 24, 225-240.

McGrath, M.T. and Shishkoff, N. (1999). Evaluation of biocompatible products for managing cucurbit powdery mildew. Crop Protect m18:471-478.

Paulitz, T.C. and Bélanger, R.R. (2001). Biological control in greenhouse systems. Annu Rev Phytopathol 39:103-133.

Park M.J., Choi Y.J., Hong S.B. & Shin H.D. (2010). Genetic variability and mycohost association of *Ampelomyces quisqualis* isolates inferred from phylogenetic analyses of ITS rDNA and actin gene sequences. Fungal Biology, 114, 235-247.

Urquhart, E.J., Menzies, J.G. and Punja, Z.K. (1994). Growth and biological control activity of *Tilletiopsis* species against powdery mildew (*Sphaerotheca fuliginea*) on greenhouse cucumber. Phytopathology 84:341-351.

Urquhart, E.J. and Punja, Z.K. (1997). Epiphytic growth and survival of *Tilletiopsis pallescens*, a potential biological control agent of *Sphaerotheca fuliginea*, on cucumber leaves. Canad J Bot 75:892-901.

Urquhart, E.J. and Punja, Z.K. (2002). Hydrolytic enzymes and antifungal compounds produced by *Tilletiopsis* species, phyllosphere yeasts that are antagonists of powdery mildew fungi. Canad J Microbiol 48:219-229.

Verhaar, M.A., Huwegen, T. and Zadoks, J.C. (1996). Glasshouse experiments on biocontrol of cucumber powdery mildew (*Sphaerotheca fuliginea*) by the mycoparasites *Verticillium lecanii* and *Sporothrix rugulosa*. Biological Control 6:353-360.

Verhaar, M.A., Kerssies, A. and Huwegem, T. (1999). Effect of RH on mycoparasitism of rose powdery mildew with and without treatments with mycoparasites. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 106:158-16.

Acknowledgements

I am grateful to:

Cesare Gessler.

Ilaria Pertot.

Sustainable Agro-Ecosystems and Bioresources Department (DASB).

AMPELO Group.

La mia famiglia.

Tutti quelli che mi sono stati vicini sopportandomi in questi anni.

Curriculum Vitae

Personal Data

Name	Dario
Surname	Angeli
Date of birth	08.02.1976
Place of birth	Mezzolombardo, Trento
Nationality	Italy

Education

2008-2013	PhD at the Institute of Integrative Biology Swiss Federal Institute of Technology (ETH), Zürich, Switzerland Thesis: *"Opening new frontiers in plant protection by understanding the interaction between a plant pathogen and its mycoparasite."*
2003-2007	Research assistant at the SafeCrop Centre Fondazione Edmund Mach (IASMA), S. Michele all'Adige, Trento, Italy Innovative research oriented toward the technologies for reducing chemical inputs in agriculture and using low impact plant protection methods.
1996-2002	Bachelor degree in "Agronomic Science and Technology" School of Agricultural Science, University of Padua Thesis: *"Efficacy of Benzyladenine as thinning and sizing agent in relation to bloom intensity"*.

Publications

Angeli, D., Pellegrini, E. and Pertot, I. (2009). Occurrence of *Erysiphe necator* chasmothecia and their natural parasitization by *Ampelomyces quisqualis*. Phytopathology, 99, 704-710

Angeli D., Maurhofer M., Gessler C. and Pertot I. (2012). Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis*. Phytoparasitica 40, 37-51.

Angeli, D., Puopolo, G., Maurhofer M., Gessler C. and Pertot I. (2012). Is the mycoparasitic activity of *Ampelomyces quisqualis* biocontrol strains related to phylogeny and hydrolytic enzyme production?. Biological control 63(3):348-358.

Angeli, D., Maurhofer M., Gessler C. and Pertot I. (2013). Stimulation of conidial germination as a novel tool to improve the biocontrol potential of *Ampelomyces quisqualis*. To be submitted to BioControl.

Conferences attended

Sept 2008 IOBC/WPRS Working Group "Biological control of fungal and bacterial plant pathogens entitled "Molecular Tools for Understanding and Improving Biocontrol", September 9-12, 2008, Interlaken (Switzerland).

Talk: "*Molecular characterization of Ampelomyces spp. isolates from different host and geographic origin and evaluation of their potential to control powdery mildew of cucumber*".

Jan 2010 Mycology Symposium, Research Station Agroscope, January 22, 2010, Wadenswil (Switzerland).

Talk: "*Characterization of several Ampelomyces quisqualis isolates and potential to control cucumber and strawberry powdery mildew"*

Jun 2010 IOBC/WPRS Working Group "Biological control of fungal and bacterial plant pathogens entitled "Climate change: Challenge or Threat to Biocontrol", June 7-10, 2010, Graz (Austria).

Talk: *"Selecting highly effective strains of Ampelomyces quisqualis for the control of powdery mildews"*.

Poster: *"Influence of temperature on morphology and physiology of different isolates of Ampelomyces quisqualis"*.

Jul 2010 International Workshop on Grapevine Downy & Powdery Mildew. INRA, July 4-9, 2010, Bordeaux (France).

Talk: *"Can different isolates of the mycoparasite Ampelomyces quisqualis efficiently control powdery mildew infections?"*

Mar 2011 Symposium "Biocontrol of grapevine diseases", May 26-27, 2011, Toulouse-Castanet-Tolosan, (France).

Talk: *"Enhancing the aggressiveness of the powdery mildew mycoparasite Ampelomyces quisqualis"*.

Mar 2012 IOBC/WPRS Working Group "Biological control of fungal and bacterial plant pathogens entitled "Biocontrol of plant pathogens in sustainable agricultural", June 24-27, 2012, Reims (France).

Poster: *"Increasing efficacy of Ampelomyces quisqualis against powdery mildews"*.