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

Dario Angeli, Gerardo Puopolo, Monika Maurhofer, Cesare Gessler, Ilaria Pertot

PII: S1049-9644(12)00179-X
DOI: http://dx.doi.org/10.1016/j.biocontrol.2012.08.010
Reference: YBCON 2825

To appear in: *Biological Control*

Received Date: 8 June 2012
Accepted Date: 27 August 2012

Please cite this article as: Angeli, D., Puopolo, G., Maurhofer, M., Gessler, C., Pertot, I., Is the mycoparasitic activity of *Ampelomyces quisqualis* biocontrol strains related to phylogeny and hydrolytic enzyme production?, *Biological Control* (2012), doi: http://dx.doi.org/10.1016/j.biocontrol.2012.08.010

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

Dario Angeli [a,b,*], Gerardo Puopolo [a], Monika Maurhofer [b], Cesare Gessler [b], Ilaria Pertot [a,*]

[a] Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 S. Michele all'Adige (TN), Italy

[b] Department of Environmental Systems Science (D-USYS), Swiss Federal Institute of Technology (ETH), 8092 Zurich (CH), Switzerland

[*] Corresponding author. Phone: +39 0461 615148, Fax: +39 0461 615500.

E-mail address: dario.angeli@fmach.it (D. Angeli).

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 or consumers. *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 powdery mildews and for *in vitro* production of cell wall degrading enzymes (CWDEs). Individual strains differed significantly in elargation of the colonization area by intrahyphal formation of pycnidia within powdery mildew colonies and in inhibition of host sporulation. 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 the production of proteases and chitobiases. 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.

Keywords: *Erysiphales* · Mycoparasitic fungi · Biological control · Enzymatic activity

Abbreviations

CWDEs: Cell wall degrading enzymes

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 mildews 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 and Belanger, 2001; Gilardi et al., 2012). *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 formula 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., while the slow-growing strains (0.5–1.0 mm day^{-1}) have been assigned to *A. quisqualis* sensu stricto (Kiss, 1997; Kiss and 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 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 (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 and Nakai, 1980; Sundheim and Krekling, 1982). Growth of *A. quisqualis* germ-tubes towards powdery mildew hyphae has also been observed (Sundheim and 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 and Ko, 1997). Toxin production has not been detected in *A. quisqualis* (Kiss et al., 2004), in contrast to other pycnidial mycoparasites, such as *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 and 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 and 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 and Chet, 1992; Pozo et al., 2004; Viterbo et al., 2004 Gruber and 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 and 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), 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* (ex *Sphaerotheca fusca*) 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 widely 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: i) whether genetically different *A. quisqualis* strains (isolated from different powdery mildew hosts) have different levels of mycoparasitic activity against powdery mildews; ii) whether the level of mycoparasitic activity is linked to the genotype and/or fungal host of origin; iii) whether 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 sporulation in different powdery mildews 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.

2. Materials and methods

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; D. Angeli, 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 and 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).

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.

2.2. Mycoparasitic activity: ability of A. quisqualis to reduce sporulation of powdery mildews in vivo

The ability of the 24 *A. quisqualis* strains to inhibit sporulation 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 mildews; *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 sporulation 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 sporulation 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 sporulations 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 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.

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 μm^2

247 day^{-1}. Three independent experiments, each with three replicates (leaves with five lesions) per
248 treatment (*A. quisqualis* strain), were carried out.
249
250 *2.4. In vitro production of CWDEs by A. quisqualis culture filtrates*
251
252 The 24 strains of *A. quisqualis* were assessed for chitinase, protease and β-1,3-glucanase
253 activity.
254 Glucanase activity was assessed according to the procedure established by Rotem et al.
255 (1999) to characterize exo-β-1,3-glucanase activity in *A. quisqualis* strain AQ10. The amount
256 of µmol of glucose liberated during the reaction was determined with a glucose hexokinase
257 reagent (Sigma), according to the manufacturer's instructions. Glucanase activity was
258 expressed as µmol of glucose h^{-1} mg^{-1} of dried mycelium.
259 Protease activity was determined using the artificial substrate azocasein according to
260 Girard and Michaud (2002) with some modifications. Briefly, a 6 mm plug of the pycnidia-
261 coated area was excised from 14-day-old *A. quisqualis* PDA cultures. The agar plug was
262 placed in a sterile 1.5 ml tube containing 120 µl of an azocasein solution (1%, w/v in 50mM
263 Tris-HCl, pH 8.8) and incubated for 7 days at 25 °C. The reaction was then stopped and the
264 undigested substrate was precipitated by the addition of 300 µl of cold trichloroacetic acid
265 solution (10%, w/v). After centrifugation for 5 min at 13000 rpm, a volume of 100 µl of
266 supernatant was transferred to 96-multiwell plates and 85 µl of NaOH (1N) was added to each
267 well. Absorbance was then read at 440 nm using a spectrophotometer and protease activity
268 was expressed as the ratio of the absorbance value per ml of supernatant.
269 To induce production of chitinases in *A. quisqualis* strains, 6 mm plugs from pycnidia-
270 coated PDA plates were transferred to sterile tubes containing 200 µl of a carboxymethyl-
271 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 and 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.

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

297 data from the three independent experiments and revealed no significant experiment ×
298 treatment interactions. Data from the three experiments were therefore pooled. Averages of all
299 replicates are presented in Table 2, Table 3 and Figure 1. Statistical significances among
300 treatments were computed with Statistica software 7.0 (Statsoft, Tulsa, OK, USA) and means
301 were separated using the Tukey's HSD ($P < 0.05$).

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

309 Spearman's correlation coefficients (*Rho*) and correlation coefficients (*r*) of the linear
310 regression curves were used to calculate correlations between mycoparasitic and enzymatic
311 activities. The statistical analysis included all tested strains with the exception of ATCC
312 200245 and ATCC 200249 which were identified as outliers and removed from the data. Data
313 were analyzed for significance using Statistica software 7.0. Means were separated using the
314 Tukey's HSD test after significant F-test ($P < 0.01$) with a one-way analysis of variance
315 (ANOVA).

316

317 **3. Results**

318

319 *3.1. Mycoparasitic activity: ability of A. quisqualis to reduce sporulation of powdery mildews*
320 *in vivo*

321

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 sporulation 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* sporulation, 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 .

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).

Assays for evaluating ability to reduce sporulation of powdery mildew and intra-hyphal spread of *A. quisqualis* pycnidia within the host showed that all strains formed pycnidia and

reduced sporulation 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 sporulation 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.

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 = 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

397 the greatest chitobiase activities with an average of 34 mU ml-1. The lowest activities were
398 clearly measured for *A. quisqualis* strains belonging to ITS group 3 (10 mU ml^{-1}). There was a
399 0.5 to 1-fold decrease in the activities of these strains when compared with the *Trichoderma*
400 control strain.

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

409 Differences in activity levels between individual strains of *A. quisqualis* were also found
410 for β-1,3 glucanase. Maximum activity of β-1,3 glucanase was measured with strains ITA 1,
411 ITA 2, AQ10 and DSM 2225 (405–451 µmol mg^{-1} h^{-1}). These values were about 2 times
412 higher than those for SC1 (199 µmol mg^{-1} h^{-1}). The strains displaying the lowest glucanase
413 activities (e.g. a 2-fold decrease compared with SC1) were ITA 4, MYA 3389 and MYA
414 3398.

415 Most of the strains tested exhibiting high chitobiase and protease activities (e.g. strains of
416 ITS groups 1 and 2A) also performed best in parasitizing the powdery mildews and in
417 inhibiting the formation of conidia (Figs. 1 and 3, Tables 2 and 3). Conversely, all the strains
418 belonging to ITS groups 3 and 5, which displayed the lowest chitobiase and protease
419 activities, were also less effective in colonizing powdery mildew *in vivo*. Correlation analysis
420 of the mycoparasitic and enzymatic activities of *A. quisqualis* (Fig. 3; Table 1S) showed the
421 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 sporulation *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).

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 mycelia by forming intrahyphal pycnidia, expand the parasitized host colony area over time and significantly reduce sporulation 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

447 powdery mildews. Moreover, not all powdery mildew species are equally susceptible to *A.*
448 *quisqualis*: our experiments show that *P. aphanis* is in general less susceptible to all the *A.*
449 *quisqualis* strains than *P. xanthii* and *E. necator*.

450 Recently, studies have shown that the cultural and growth characteristics of these strains
451 are related to ITS group and host fungus and have suggested that a certain degree of mycohost
452 specialization may be present within the *A. quisqualis* species (Angeli et al., 2012; Park et al.,
453 2010). We have demonstrated for the first time that the ability of *A. quisqualis* strains to
454 colonize powdery mildew by forming pycnidia and inhibiting sporulation is completely
455 independent of the geographical origin of the strain, but appears to be related to genotype
456 (ITS rDNA sequence) and the original fungal host, with the exception of strains ITA 1, ITA 2
457 and ITA 3. It is worth noting that the strains belonging to ITS groups 3 and 5 were the least
458 effective in reducing powdery mildews. These strains were isolated from apple and grapevine
459 plants, respectively, the natural hosts of only one powdery mildew species (*M. domestica-P.*
460 *leucotricha*; *V. vinifera-E. necator*). On the other hand, the most aggressive strains belonged
461 to ITS groups 1 and 2A, isolated from plants that, for the most part, are attacked by more than
462 one *Erysiphales*, as in the case of *C. sativus* which is the natural host of *E. cichoracearum* and
463 *P. xanthii* (ITS group 1). In addition, members of the plant species that hosted *A. quisqualis*
464 strains of ITS group 2A are natural hosts of at least two powdery mildews, such as *Daucus* sp.
465 (*E. heraclei* and *E. polygoni*), *H. macrophylla* (*E. polygoni* and *O. hortensiae*) and *Leontodon*
466 sp. (*E. cichoracearum* and *P. xanthii*). Unfortunately, no information is available on the
467 number of powdery mildews occurring on *C. edulis* and *S. molle*. However, it is tempting to
468 hypothesize that co-evolution of *A. quisqualis* strains and plants which are the natural host of
469 more than one powdery mildew drove the selection of more aggressive genotypes. A broader
470 range of strains belonging to different genotypes and originating from different plant hosts
471 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 and Melgarejo, 1996; Lorito et al., 1994a; Oppenheim and 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

496 more efficiently than wild type strains, while the null mutants lost their biocontrol
497 effectiveness (Djonovic et al., 2006, 2007).

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

506 Our results suggest that the ability of some *A. quisqualis* strains to parasitize the host by
507 forming intracellular pycnidia and reducing fungal-host sporulation was associated with their
508 intrinsic ability to secrete proteases and with chitobiase activity in culture filtrates (Fig. 3,
509 Table S1). This was also illustrated by the regression analysis which showed there to be a
510 significant positive correlation (Table S1) between chitobiase and protease activity with intra-
511 hyphal formation of pycnidia and the inhibition of sporulation *in vivo*, whereas exo-glucanase
512 activity was not statistically related to mycoparasitic activities. Therefore, we hypothize that
513 proteases and chitobiases play an important role in *A. quisqualis* mycoparasitism. However,
514 lytic activities alone do not fully explain the level of aggressiveness, although they are
515 probably involved in the biocontrol ability of *A. quisqualis* and of other mycoparasitic fungi
516 (Adams, 2004; Reithner et al., 2011; Vinale et al., 2008).

517 An interesting issue for future research would be to investigate the role played by these
518 enzymatic activities in the antagonistic properties of members of *A. quisqualis* sensu stricto
519 either by creating strains that are defective in these activities or by monitoring expression of
520 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.

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.

References

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

Angeli, D., Pellegrini, E., 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., 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., Pertot I., 2012. Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis*. Phytoparasitica 40, 37–51.

545 Benitez, T., Rincon, A.M., Limon, M.C., Codon, A.C., 2004. Bioncontrol mechanisms of
546 *Trichoderma* strains. International Microbiology 4, 249–260.
547 Cao, R., Liu, X., Gao, K., Mendgen, K., Kang, Z., Gao, J., Dai, Y., Wang, X., 2009.
548 Mycoparasistism of endophytic fungi isolated from reed on soilborne phytopathogenic
549 fungi and production of cell wall-degrading enzymews *in vitro*. Current Microbiology 59,
550 584–592.
551 Cook, R.J., Baker, K.F., 1983. The nature and practice of biological control of plant
552 pathogens. American Phytopathological Society, Minnesota p. 539.
553 Djonovic, S., Pozo, M.J., Kenerley, C.M., 2006. Tv-bgn3, a beta-1,6- glucanase from the
554 biocontrol fungus *Trichoderma virens* is involved in mycoparasitism and control of
555 *Pythium ultimum*. Applied Environmental Microbiology 72, 7661–7670.
556 Djonovic, S., Vittone, G., Mendoza Herrera, A., Kenerley, C.M., 2007. Enhanced bicontrol
557 activity of *Trichoderma virens* transformants constitutively coexpressing β-1,3 and β-1,6-
558 glucanase genes. Molecular Plant Pathology 8, 469–480.
559 Elad, Y., Chet, I., Henis, Y., 1982. Degradation of plant pathogenic fungi by *Trichoderma*
560 *harzianum*. Canadian Journal of Microbiology 28, 719–725.
561 Elad, Y., Lifshitz, R., Baker, R., 1985. Enzymatic activity of the mycoparasite *Pythium nunn*
562 during interaction with host and non-host fungi. Physiological Plant Pathology 27, 131–
563 148.
564 Geremia, R.A., Goldman, G.H., Jacobs, D., Ardiles, W., Vila, S.B., Van Montagu, M.,
565 Herrera-Estrella, A., 1993. Molecular characterization of the proteinase-encoding gene,
566 *prb1*, related to mycoparasitism by *Trichoderma harzianum*, Molecular Microbiology 8,
567 603–613.

568 Gilardi, G., Baudino, M., Garibaldi, A., Gullino, M.L., 2012. Efficacy of biocontrol agents
569 and natural compounds against powdery mildew of zucchini. Phytoparasitica 40, 147–
570 155.
571 Girard, C., Michaud, D., 2002. Direct monitoring of extracellular protease activities in
572 microbial cultures. Analytical Biochemistry 308, 388–391.
573 Glawe, D.A., 2008. The powdery mildews: a review of the world's most familiar (yet poorly
574 known) plant pathogens. Annual Review of Phytopathology 46, 27–51.
575 Gruber, S., Seidl-Seiboth, V., 2012. Self versus non-self: fungal cell wall degradation in
576 *Trichoderma*. Microbiology 158, 26–34.
577 Gu, Y.H., Ko, W.H., 1997. Water agarose medium for studying factors affecting germination of
578 conidia of *Ampelomyces quisqualis*. Mycological Research 101, 422–424.
579 Haran, S., Schickler, H., Chet, I., 1996. Molecular mechanisms of lytic enzymes involved in
580 the biocontrol activity of *Trichoderma harzianum*. Microbiology 142, 2321–2331.
581 Hashioka, Y., Nakai, Y., 1980. Ultrastructure of pycnidial development and mycoparasitism
582 of *Ampelomyces quisqualis* parasitic on Erysiphales. Transactions of the Mycological
583 Society of Japan 21, 329–338.
584 Jarvis, W.R., Slingsby, K., 1997. The control of powdery mildew of greenhouse cucumber by
585 water spray and *Ampelomyces quisqualis*. Plant Disease Reporter 61, 728–730.
586 Kiss, L., 1997. Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew
587 fungi, inferred from RFLP analysis of the rDNA ITS region. Mycological Research 101,
588 1073–1080.
589 Kiss, L., 1998. Natural occurrence of *Ampelomyces* intracellular mycoparasites in mycelia of
590 powdery mildew fungi. The New Phytologist 140, 709–714.

591 Kiss, L., Nakasone, K.K., 1998. Ribosomal DNA internal transcribed spacer sequences do not
592 support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew
593 fungi. Current Genetics 33, 362–367.
594 Kiss, L., 2003. A review of fungal antagonists of powdery mildews and their potential as
595 biocontrol agents. Pest Management Science 59, 475–483.
596 Kiss, L., Russell, J.C., Szentivanyi, O., Xu, X., Jeffries, P., 2004. Biology and biocontrol
597 potential of *Ampelomyces* mycoparasites, natural antagonist of powdery mildew fungi.
598 Biocontrol Science and Technology 14, 635–651.
599 Kiss, L., Pintye, A., Kovacs, G.M., Jankovics, T., Fontaine, M.C., Harvey, N., Xu, X., Nicot,
600 P.C., Bardin, M., Shykoff, J.A., Giraud, T., 2011. Temporal isolation explains host-related
601 genetic differentiation in a group of widespread mycoparasitic fungi. Molecular Ecology 20,
602 1492–1507.
603 Kubicek, C.P., Mach, R.L., Peterbauer, C.K., Lorito, M., 2001. *Trichoderma*: from genes to
604 biocontrol. Journal of Plant Pathology 83, 11–23.
605 Larena, I., Melgarejo, P., 1996. Biological control of *Monilinia laxa* and *Fusarium oxysporum* f.
606 sp. *lycopersici* by a lytic enzyme-producing *Penicillium purpurogenum*. Biological Control
607 6, 361–367.
608 Liang, C., Yang, J., Kovacs, G.M., Szentivanyi, O., Li, B., Xu, X.M., Kiss, L., 2007. Genetic
609 diversity of *Ampelomyces* mycoparasite isolated from different powdery mildew species in
610 China inferred from analyses of rDNA ITS sequences. Fungal Diversity 24, 225–240.
611 Lorito, M., Leterbauer, C., Hayes, C.K., Woo, S.L., Harman, G.E., 1994a. Synergistic
612 combination of cell wall degrading enzymes and different antifungal compounds enhances
613 inhibition of spore germination. Microbiology 140, 623–629.

614 Lorito, M., Hayes, C.K., Di Pietro, A., Woo, S.L., Barman, G.E., 1994b. Purification,
615 characterization and synergistic activity of a glucan β-1,3-glucosidase and a N-acetyl-β-
616 glucosaminidase from *Trichoderma harzianum*. Phytopathology 84, 398–405.
617 Machida, K., Trifonov, L.S., Ayer, W.A., Lu, Z.X., Laroche, A., Huang, H.C., Cheng, K.J.,
618 Zantige, J.L., 2001. 3(2H)-Benzofuranones and chromanes from liquid cultures of the
619 mycoparasitic fungus *Coniothyrium minitans*. Phytochemistry 58, 173–177.
620 McQuilken, M.P., Gemmell, J., Hill, R.A., Whipps, J.M., 2003. Production of macrosphelide
621 A by the mycoparasite *Coniothyrium minitans*. FEMS Microbiology Letters 219:27–31.
622 McQuilken, M.P., Gemmell, J., 2004. Enzyme production by the mycoparasite *Verticillium*
623 *biguttatum* against *Rhizoctonia solani*. Mycopathologia 157, 201–205.
624 Monteiro, V., do Nascimento, S.R., Steindorff, A.S., Costa, F.T., Noronha, E.F., Ricart,
625 C.A.O., de Sousa, M.V., Vainstein, M.H., Ulhoa, C.J., 2010. New insights in *Trichoderma*
626 *harzianum* antagonism of fungal plant pathogen by secreted protein analysis. Current
627 Microbiology 61, 298–305.
628 Mucha, J., Dahm, H., Strzelczyk, E., Werner, A., 2006. Synthesis of enzymes connected with
629 mycoparasitism by ectomycorrhizal fungi. Archives Microbiology 185, 69–77.
630 Nischwitz, C., Newcombe, G., Anderson, C.L., 2005. Host specialization of the mycoparasite
631 *Eudarluca caricis* and its evolutionary relationship to *Ampelomyces.* Mycological
632 Research 109, 421–428.
633 O'Brien, M., Colwell, R.R., 1987. A rapid test for chitinase activity that uses 4-
634 methylumbelliferyl-*N*-acetyl-β-D-glucosamininide. Applied and Environmental
635 Microbiology 53, 1718–1720.
636 Oppenheim, A.B., Chet, I., 1992. Cloned chitinase in fungal pathogen control strategies.
637 Trends in Biotechnology 10, 392–394.

638 Park, M.J., Choi, Y.J., Hong, S.B., Shin, H.D., 2010. Genetic variability and mycohost
639 association of *Ampelomyces quisqualis* isolates inferred from phylogenetic analyses of ITS
640 rDNA and actin gene sequences. Fungal Biology 114, 235–247.
641 Paulitz, T.C., Bélanger, R.B., 2001. Biological control in greenhouse systems. Annual Review of
642 Phytopathology, 39, 103–133.
643 Philipp, W.D., 1985. Extracellular enzymes and nutritional physiology of *Ampelomyces*
644 *quisqualis* Ces., hyperparasite of powdery mildew, *in vitro*. Phytopathologische Zeitschrift
645 114, 274–283.
646 Pintye, A., Bereczky, Z., Kovács, G.M., Nagy, L.G., Xu, X., Legler, S.E., Váczy, Z., Váczy,
647 K.Z., Caffi, T., Rossi, V., Kiss, L., 2012. No indication of strict host associations in a
648 widespread mycoparasite: grapevine powdery mildew (*Erysiphe necator*) is attacked by
649 phylogenetically distant *Ampelomyces quisqualis* strains in the field. DOI 10.1094/PHITO-
650 10-11-0270.
651 Pozo, M.J., Baek, L.M., Garcia, J.M., Kenerley, C.M., 2004. Functional analysis of *tvspl*, a
652 serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal
653 Genetics and Biology 41, 336–348.
654 Quecine, M.C., Araujo, W.L., Marcon, J., Gai, C.S., Azevedo, J.L., Pizzirani-Kleiner, A.A.,
655 2008. Chitinolytic activity of endophytic *Streptomyces* and potential for biocontrol. Letter
656 in Applied Microbiology 47, 486–491.
657 Rankovic, B., 1997. Hyperparasites of the genus *Ampelomyces* on powdery mildew fungi in
658 Serbia Mycopathologia 139, 157–164.
659 Reithner, B., Ibarra-Laclette, E., Mach, R.L., Herrera-Estrella, A., 2011. Identification of
660 mycoparasistim-related genes in *Trichoderma atroviride*. Applied and Environmental
661 Microbiology 77, 4361–4370.

662 Rotem, Y., Yarden, O., Sztejnberg, A., 1999. The mycoparasite *Ampelomyces quisqualis*
663 expresses *exgA* encoding and *exo*-β-1,3-glucanase in culture and during mycoparasitism.
664 Phytopathology 89, 631–638.
665 Samolski, I., de Luis, A., Vizcaino, J., Monte, E., Suarez, M.B., 2009. Gene expression analysis
666 of the biocontrol fungus *Trichoderma harzianum* in the presence of tomato plants, chitin, or
667 glucose using a high-density oligonucleotide microarray. BMC Microbiology 9, 217.
668 Sullivan, R.F., White, J.F., 2000. *Phoma glomerata* as a mycoparasite of powdery mildew.
669 Applied and Environmental Microbiology 66, 425–427.
670 Sundheim, L., Krekling, T., 1982. Host-parasite relationships of the hyperparasite *Ampelomyces*
671 *quisqualis* and its powdery mildew host *Sphaerotheca fuliginea*. I. Scanning electron
672 microscopy. Phytopathology 104, 202–210.
673 Szekeres, A., Kredics, L., Antal, Z., Kevei, F., Manczinger, L., 2004. Isolation and
674 characterization of protease overproducing mutants of *Trichoderma harzianum*. FEMS
675 Microbiology Letters 233, 215–222.
676 Szentiványi, O., Kiss, L., Russell, J.C., Kovács, G.M., Varga, K., Jankovics, T., Lesemann, S.,
677 Xu, X.M., Jeffries, P., 2005. *Ampelomyces* mycoparasites from apple powdery mildew
678 identified as a distinct group based on single-stranded conformation polymorphism analysis
679 of the rDNA ITS region. Mycological Research 109, 429–438.
680 Sztejnberg, A., 1993. *Ampelomyces quisqualis* AQ10, CNCM I-807, for biological control of
681 powdery mildew. US patent no. 5190754.
682 Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L., Lorito, M., 2008.
683 *Trichoderma*-plant-pathogen interactions. Soil Biology and Biochemistry 40, 1–10.
684 Viterbo, A., Harel, M., Chet, I., 2004. Isolation of two aspartyl proteases from *Trichoderma*
685 *asperellum* expressed during colonization of cucumber roots. FEMS Microbiology Letters
686 238, 151–158.

Wold, S., Geladi, P., Esbesen, K., 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., 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., Kubicek, C.P., 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genetics and Biology 26, 131–140.

Figure captions

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.

712

713 Figure 2

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

720

721 Figure 3

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

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 yr o f 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
ITA4	*E. necator*	*V. vinifera*	5	Italy, 2007	D. Angeli (FEM)	HQ108050
ITA5	*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), ? [d]	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
MYA 3398	*P. leucotricha*	*M. domestica*	3	United Kingdom (GB), 2002	L. Kiss (PPI)	HQ108045
AQ10	Powdery mildew [d]	*Catha edulis*	2A	Israel (IL), ? [d]	Ecogen Italia	AF035783
DSM 2225	*Erysiphe heraclei*	*Daucus* sp.	2A	Germany (D), ? [d]	DSMZ	HQ108042
DSM 4624	*Sphaerotheca fuliginea*	*Leontodon* sp.	2A	Germany (D), ? [d]	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.

10 **Table 2**

11 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
ITA4	5	71.7 ± 0.6 e	90.5 ± 0.6 de	59.1 ± 0.3 cd
ITA5	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 > 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 > 0.05) different according to the Tukey's HSD test.

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
ITA4	5	14.17 ± 0.20 hil	2.84 ± 0.20 hi	91.93 ± 2.04 hi
ITA5	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 ($P > 0.05$) different according to the Tukey's HSD test.

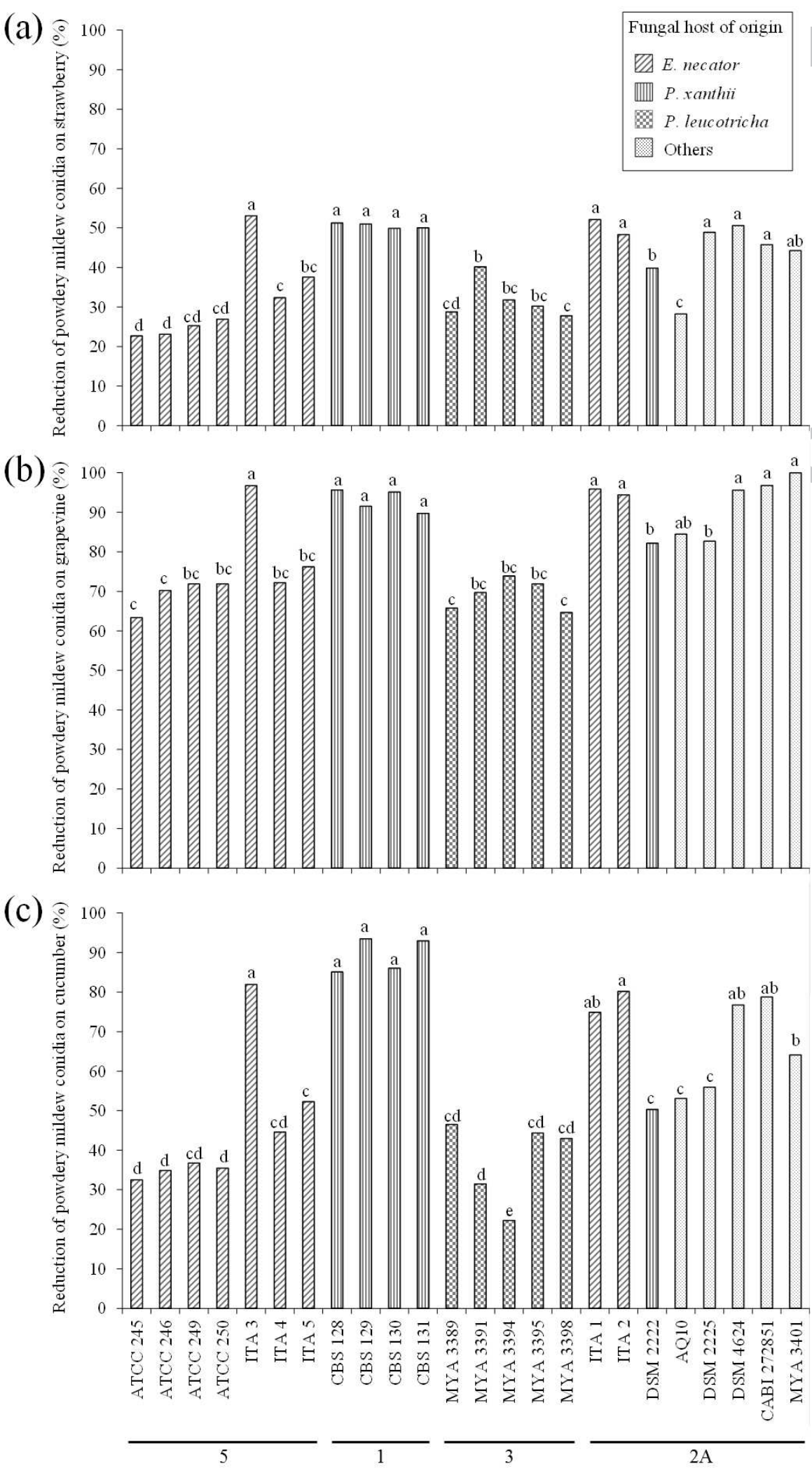
[a] The total chitobiase activity was measured using 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MU-GluNAc) as the substrate. The fluorescence of the liberated 4-MU was measured with excitation at 360 nm and emission at 450 nm. One milliunit of chitobiase activity was defined as the amount of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

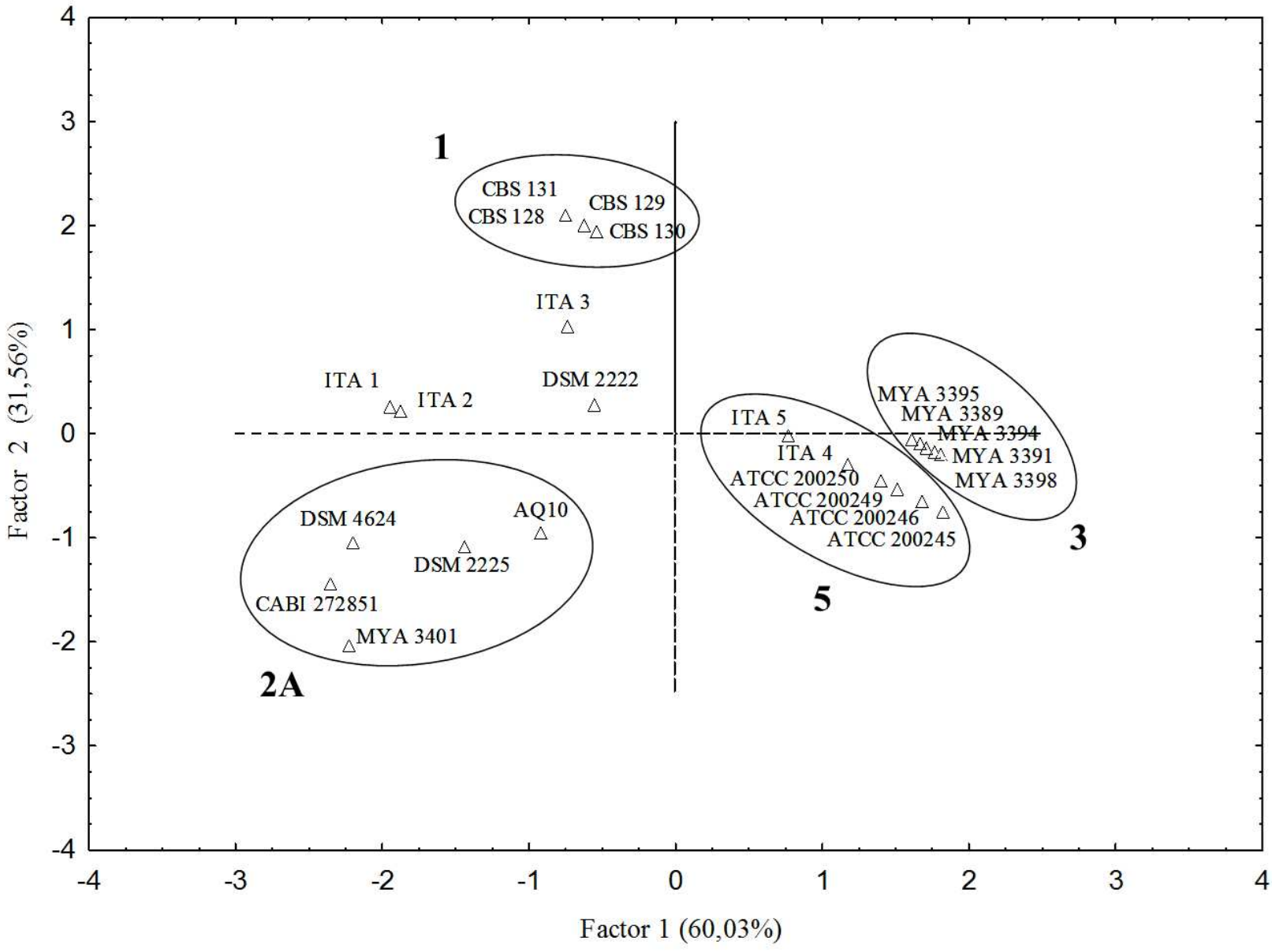
[b] The protease activity was measured in absorbance per millilitre of substrate per reaction

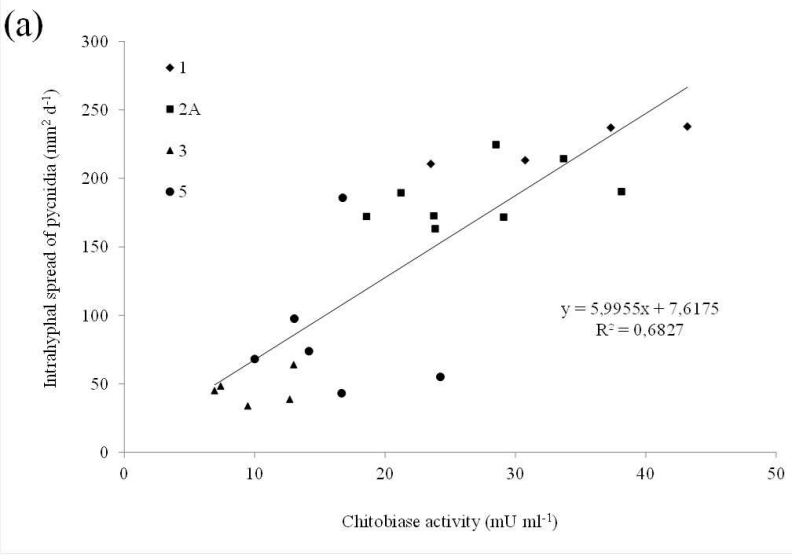
[c] The β-1,3-glucanase activity was measured in µmol glucose liberated per h per mg dry weight according to Rotem et al (1999).

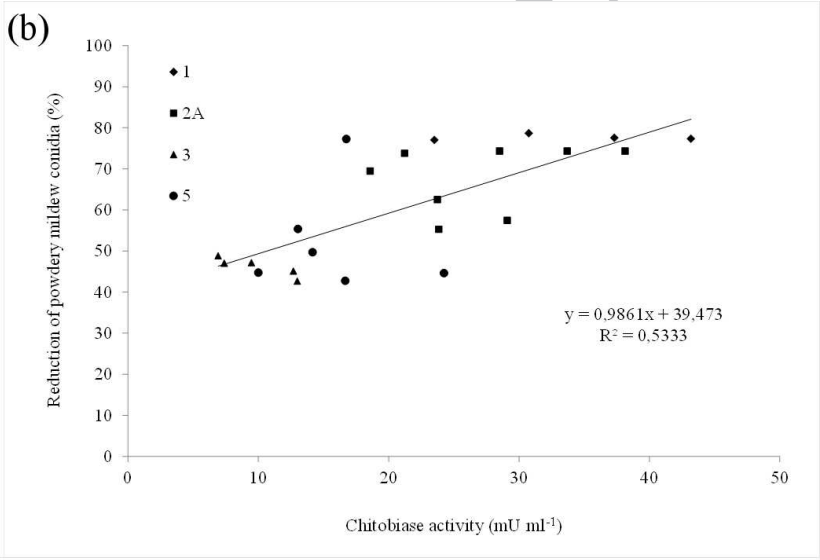
[d] Internal positive control (*Trichoderma atroviride* SC1).

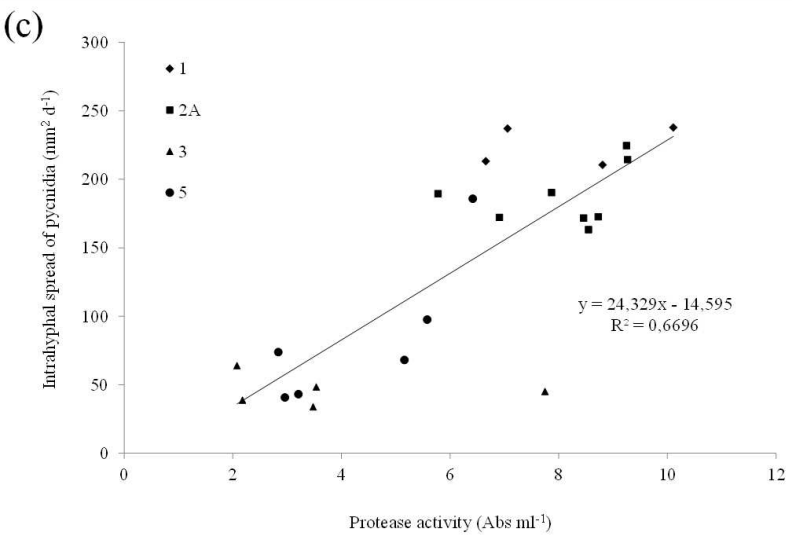
[e] Average of all *A. quisqualis* strains belonging to an individual ITS group. Values with the same letter within a column are not significantly ($P > 0.05$) different according to the Tukey's HSD test.

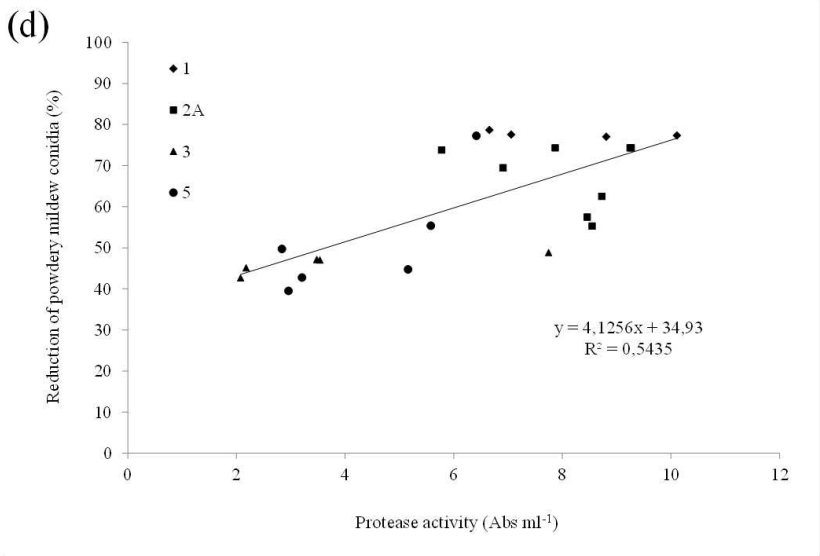

(a)
Reduction of powdery mildew conidia on strawberry (%)
(b)
Reduction of powdery mildew conidia on grapevine (%)
(c)
Reduction of powdery mildew conidia on cucumber (%)
Fungal host of origin
E. necator
P. xanthii
P. leucotricha
Others
ATCC 245
ATCC 246
ATCC 249
ATCC 250
ITA 3
ITA 4
ITA 5
CBS 128
CBS 129
CBS 130
CBS 131
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
5
1
3
2A

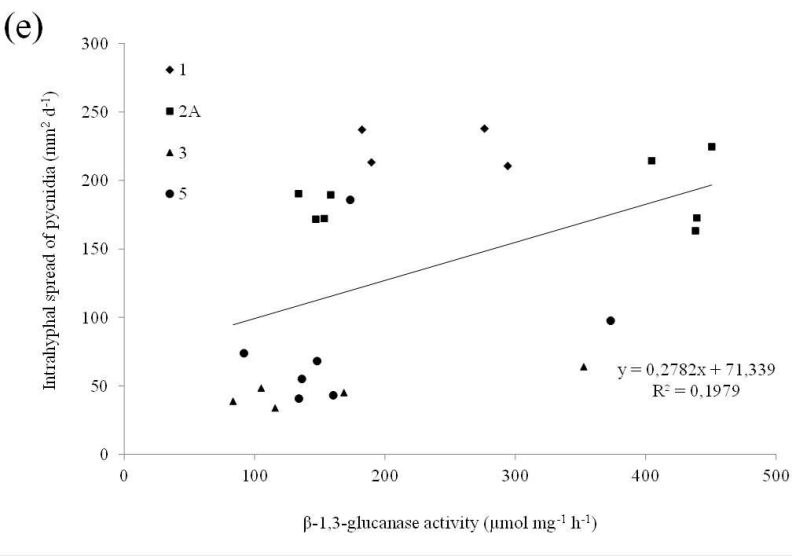


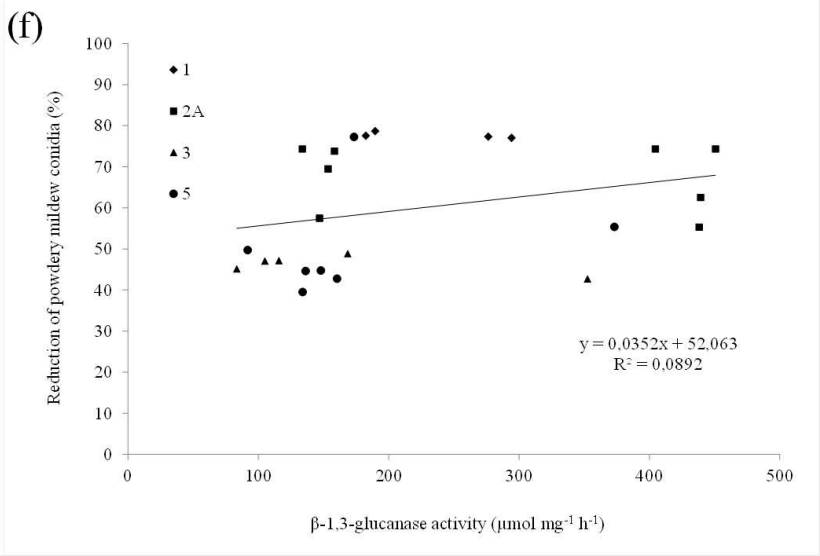


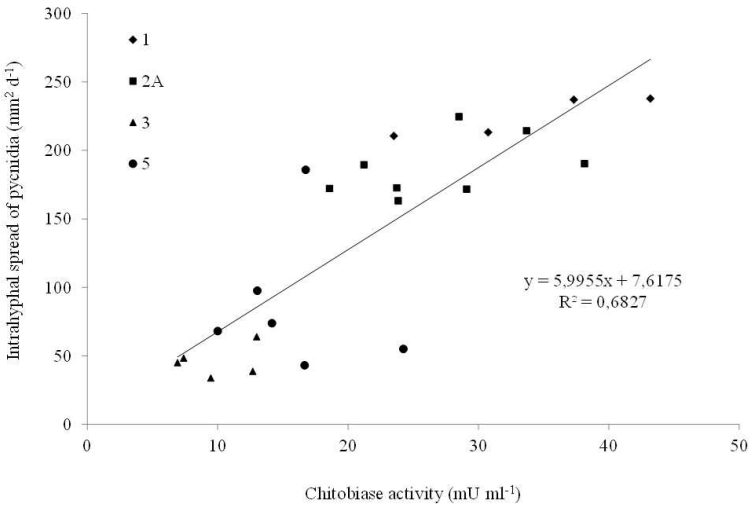


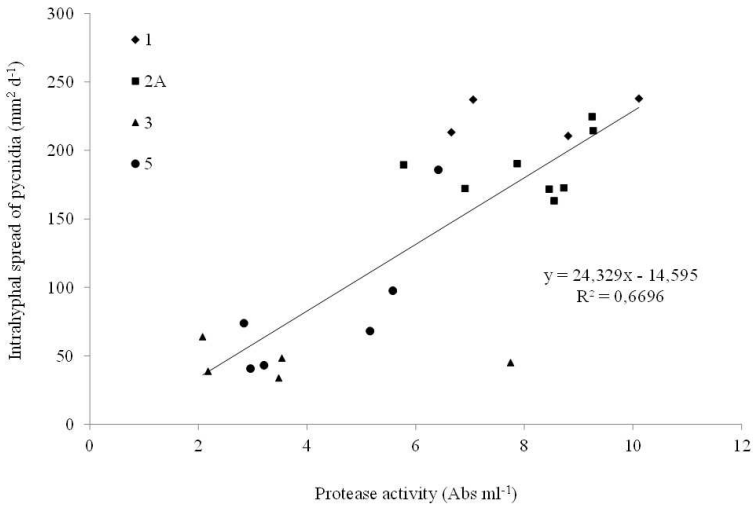












Highlights

- We report on biocontrol of *Ampelomyces quisqualis* against powdery mildews.
- Strains of *A. quisqualis* exibited a different level of mycoparasitic activity.
- Mycoparasitic activity may be explained by the genotype and fungal host.
- *A. quisqualis* strains differed for production of cell wall degrading enzymes.
- Chitobiase and protease activity can be associated with the mycoparasitic activity.