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Sporulation rate in culture and mycoparasitic activity, but not mycohost specificity, are the key factors for selecting Ampelomyces strains for biocontrol of grapevine powdery mildew (Erysiphe necator)

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	isolated from six different powdery mildew species, including E. necator, did not depend on their mycohost species of origin. Strain RS1-a, isolated from rose powdery mildew, showed, together with three strains from E. necator, the highest rate of parasitism of E. necator chasmothecia. In field experiments, both strains AQ10 and RS1-a, applied twice in autumn, significantly delayed and reduced early-season development of grapevine powdery mildew in the next year. Therefore, instead of mycohost specificity of Ampelomyces presumed in some works, but not confirmed by this study, the high sporulation rate in culture and the mycoparasitic patterns became the key factors for proposing strain RS1-a for further development as a biocontrol agent of E. necator.
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Sporulation rate in culture and mycoparasitic activity, but not mycohost specificity, are the key factors for selecting *Ampelomyces* strains for biocontrol of grapevine powdery mildew (*Erysiphe necator*)

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Abstract

To develop a new biofungicide product against grapevine powdery mildew, caused by *Erysiphe necator*, cultural characteristics and mycoparasitic activities of pre-selected strains of *Ampelomyces* spp. were compared in laboratory tests to the commercial strain AQ10. Then a two-year experiment was performed in five vineyards with a selected strain, RS1-a, and the AQ10 strain. This consisted of autumn sprays in vineyards as the goal was to reduce the number of chasmothecia of *E. necator*, and, thus, the amount of overwintering inocula, instead of targeting the conidial stage of the pathogen during spring and summer. This is a yet little explored strategy to manage *E. necator* in vinyeards. Laboratory tests compared the growth and sporulation of colonies of a total of 33 strains in culture; among these, eight strains with superior characteristics were compared to the commercial product AQ10 Biofungicide® in terms of their intra-hyphal spread, pycnidial production, and reduction of both asexual and sexual reproduction in *E. necator* colonies. Mycoparasitic activities of the eight strains isolated from six different powdery mildew species, including *E. necator*, did not depend on their mycohost species of origin. Strain RS1-a, isolated from rose powdery mildew, showed, together with three strains from *E. necator*, the highest rate of parasitism of *E. necator* chasmothecia. In field experiments, both strains AQ10 and RS1-a, applied twice in autumn, significantly delayed and reduced early-season development of grapevine powdery mildew in the next year. Therefore, instead of mycohost specificity of *Ampelomyces* presumed in some works, but not confirmed by this study, the high sporulation rate in culture and the mycoparasitic patterns became the key factors for proposing strain RS1-a for further development as a biocontrol agent of *E. necator*.

Keywords

Ampelomyces quisqualis; biofungicide; biological control; Erysiphales; mycoparasite; sanitation

Introduction

Pycnidial fungi belonging to the genus *Ampelomyces* are the best-known natural mycoparasites of powdery mildew fungi worldwide. Their hyphae and pycnidia commonly occur inside the hyphae, conidiophores, conidia and ascomata of the Erysiphales in the field (Kiss et al. 2004; Kiss 2008; Tollenaere et al. 2014). Strains of *Ampelomyces* can be isolated from powdery mildew mycelia and maintained on artificial media although their growth is slow in culture (e.g., Szentiványi et al. 2005; Liang et al. 2007; Angeli et al. 2012a) and sporulation may also be restricted or absent in such conditions (Legler et al. 2011a). The species recognition in the genus *Ampelomyces* is still problematic although molecular phylogenetic works based on nrDNA ITS and actin gene (*act1*) sequences have clearly distinguished several lineages (Szentiványi et al. 2005; Liang et al. 2007; Park et al. 2010; Kiss et al. 2011; Pintye et al. 2012). A taxonomic revision has long been proposed for this genus (Sutton 1980) to resolve the nomenclatural problems arising from the use of the binomial '*A. quisqualis*' for all the strains belonging to this group of mycoparasites, but this has not been performed to date.

Since the 1970s, a number of *Ampelomyces* strains have been studied as biocontrol agents (BCAs) of powdery mildew infections of economically important crops such as grapevine and cucumber (Kiss et al. 2004). Among these, strain AQ10 or M-10 (CNCM accession number I-807), isolated from an *Oidium* sp. infecting *Catha edulis* in Israel (Sztejnberg et al. 1989), became the most advanced in terms of commercial development being used as the active agent of the product AQ10 Biofungicide®, registered in the USA and Europe, to be used against powdery mildews of several crops including grapevine. Another strain, designated as 94013, and isolated from *Podosphaera phaseoli* on *Phaseolus angularis* in Korea, was used to develop another biofungicide product, Q-fect®, to be used mainly

against cucumber powdery mildew in Korea (Lee et al. 2004). The two commercially developed strains, M-10 and 94013, are only distantly related based on phylogenetic analyses of their *act1* and ITS sequences (Park et al. 2010).

Biocontrol of powdery mildews on various crops was sometimes reported as poor or inconsistent when using *Ampelomyces* as a BCA in greenhouse or field trials (for reviews, see Paulitz and Bélanger 2001; Kiss et al. 2004) or in laboratory tests (Shishkoff and McGrath 2002; Romero et al. 2003). In particular, the biocontrol activity of AQ10 Biofungicide® was reported to be unsatisfactory, or less efficient than other anti-powdery mildew treatments, in some works (McGrath & Shishkoff 1999; Shishkoff and McGrath 2002; Romero et al. 2003; Pertot et al. 2008; Angeli et al. 2012b), while other studies obtained good results with this biofungicide (Elad et al. 1998; Brand et al. 2009).

A possible reason of the low efficacy of the AQ10 strain and some other *Ampelomyces* strains could be the existence of some degree of mycohost specialization in these mycoparasites suggested by some studies but rejected by others (Sztejnberg et al. 1989; Szentiványi et al. 2005; Liang et al. 2007; Park et al. 2010; Kiss et al. 2011; Angeli et al. 2012a,b; Pintye et al. 2012, 2015). Cross-inoculation experiments showed that *Ampelomyces* strains isolated from different powdery mildew species were able to parasitize different test powdery mildew species *in vitro* (Sztejnberg et al. 1989; Szentiványi et al. 2005; Liang et al. 2007) and also in field experiments (Kiss et al. 2011). However, most of these works did not include a quantification of the mycoparasitic activities in the original and the inoculated species of the *Erysiphales*. When this was done, contrasting results were obtained. Falk et al. (1995) observed a much higher pycnidial production in the mycohost species of origin compared to two other powdery mildew species tested. Angeli et al. (2012b) reported that some, but not all, strains performed better in their mycohosts of origin than in other powdery mildews tested. On the other hand, Kiss et al. (2011) did not detect significant differences in

mycoparasitic activities of genetically distinct *Ampelomyces* strains in two test powdery mildew species which were different from the original mycohosts.

Grapevine powdery mildew was one of the major targets of AQ10® Biofungicide (Paulitz and Bélanger 2001) which was applied against the conidial stage of the pathogen during the vegetation period similar to the use of chemical fungicides. Another, much less exploited strategy to control *E. necator* is to reduce the overwintering inoculum, namely the number of the sexual fruiting bodies, called chasmothecia (Caffi et al. 2013). Chasmothecia are formed on grapevine leaves by the end of the vegetation period, starting from late summer until leaf fall, are mostly washed off the leaves by rain and remain attached to the bark during winter (Rossi et al. 2011). Chasmothecia contain ascospores which initiate the life cycle of *E. necator* next spring by infecting young leaves soon after bud break (Rossi et al. 2010). *Ampelomyces* mycoparasites may be used for this purpose because they destroy powdery mildew chasmothecia completely (Emmons 1930; Speer 1978; Falk et al. 1995; Füzi 2003). In contrast, their mycoparasitic activity in the conidial stage of powdery mildews is less destructive: the initial steps following penetration are apparently biotrophic (Hashioka and Nakai 1980; Kiss 2008) and even powdery mildew conidia parasitized by *Ampelomyces* can germinate and give rise to new, sporulating powdery mildew colonies (Kiss et al. 2010). Thus, *Ampelomyces* strains as BCAs may perform better against chasmothecia compared to their effects on the conidial stages of powdery mildews although this application strategy has not been widely exploited yet (Legler et al. 2011b).

Recently we carried out an extensive preliminary screening of more than 150 *Ampelomyces* strains isolated from many powdery mildew species in different parts of the world to select one or more strains to be further developed as commercial BCA(s) of grapevine powdery mildew. This was done based on a complex screening protocol developed by us for *Ampelomyces* (Legler et al. 2011a). The main goal of the present study was to

continue this work by conducting more detailed investigations on several strains pre-selected during our preliminary screening (Legler et al. 2011a). We characterized the following characteristics of the strains: (i) growth and sporulation rates in culture, (ii) mycoparasitic activity in both asexual and sexual stages of *E. necator* in laboratory experiments, and (iii) control efficacy against *E. necator* in the field. The ultimate objective was to select an *Ampelomyces* strain that grows and sporulates well in culture (an important characteristic for mass-production purposes), parasitizes intensively both asexual and sexual stages of *E. necator*, and can be used as an effective BCA of grapevine powdery mildew when applied in vineyards. Instead of targeting the asexual, conidial stage of *E. necator*, we focused on the reduction of the overwintering inoculum of grapevine powdery mildew by autumn applications of *Ampelomyces* in vineyards against chasmothecia and followed the effects of these treatments in the next season.

Materials and methods

Ampelomyces strains

A total of 32 strains, pre-selected as described in Legler et al. (2011a), together with the commercial strain AQ10 (Table 1), were subcultured on Czapek-Dox agar supplemented with 2% malt (MCzA) by placing a mycelial disk, 5 mm diameter, in the center of a plate, 6 cm diam, containing 15 ml MCzA. The commercial strain AQ10 was included in all the studies as a reference strain. Plastic plates of 6 cm diameter, containing 15 ml MCzA, were used throughout this work unless specified otherwise.

155 Production of coeval colonies of the pre-selected strains

156

157 To measure the growth and sporulation rates of the strains in culture, the first step was to
158 produce coeval cultures, i.e. cultures of exactly the same age, for each strain. Such
159 synchronized cultures, started on the same day, were produced at the beginning of every
160 repetition of each experiment. Plates were inoculated with 1 ml of conidial suspension
161 obtained from sporulating colonies of each strain and spread uniformly on the surface of the
162 culture medium. Conidial suspensions were obtained by pipetting sterile water onto
163 approximately one-month old colonies and then rubbing their surfaces with a sterile brush to
164 liberate as many conidia as possible from pycnidia. The concentrations of the suspensions
165 ranged between 2 to 8 x 10^6 conidia/ml as determined using a Bürker haemocytometer. All
166 *Ampelomyces* conidial suspensions used in different experiments, and described below, were
167 adjusted to these values by dilution. Inoculated plates were kept in complete darkness at 20ºC
168 and 2-week old cultures of each strain were used for further studies.

169

170 Growth and sporulation in culture

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172 To assess mycelium growth, disks of media, 5 mm diam, covered by *Ampelomyces* mycelia,
173 were cut from 2-week old cultures, prepared as described above, and each placed in the
174 middle of another plate in a 5 mm diam hole previously cut out of the medium. The newly
175 inoculated plates were kept at three temperature regimes (15, 20 and 25°C) in complete
176 darkness. Three replicates were used for each temperature regime and the experiment was
177 carried out three times. To measure the radial growth of the mycelial disks, a picture of the
178 plates was taken weekly for four weeks using a digital camera. Images were processed using
179 the ASSESS software (APS, Minnesota, US) to measure the colony area and the area covered

by pycnidia. The average colony growth rate and the sporulating area of the colony were calculated based on these data. The sporulating area of the colony was calculated as the percent ratio between the whole surface of the colony and the sporulating area of the mycelial surface indicated by the presence of pycnidia.

To assess the sporulation rate of the strains which performed well in the previous screenings, plates were inoculated with 1 ml conidial suspension obtained from the coeval 2-week old colonies, and kept at three temperature regimes (15, 20 and 25°C) in complete darkness. Three replicates were used for each temperature value and the experiment was carried out three times. Two disks of medium, 5 mm diam, covered with mycelium, were cut out of the colonies 4, 6, 8 and 10 days post inoculation (dpi). Pycnidia found on the disks were counted under a stereomicroscope and expressed as number of pycnidia per mm^2 colony for each strain. Only the brown, completely mature pycnidia with conidial droplets on their surface were counted. Immediately after this step, the two disks per plate were put in 1 ml sterile water and vortexed for 1 minute to release all conidia from pycnidia. The concentration of each suspension obtained in this way was measured using a Bürker haemocytometer and expressed as number of conidia per mm^2 colony.

Spread and pycnidial production in asexually sporulating powdery mildew colonies

The intra-hyphal spread and the intracellular sporulation rate of *Ampelomyces* strains in the grapevine powdery mildew colonies were determined on detached grapevine leaves. Eight strains selected in the previous steps were included in these screenings (Fig. 1). Instead of the AQ10 strain maintained in culture, the commercial product AQ10 Biofungicide® was used in these, and all the subsequent, experiments as a positive control. We used the commercial product because the sporulation rate of the AQ10 strain maintained in culture was poor or

zero. Potted grapevine plants, *Vitis vinifera* cv. Chardonnay, were kept in a greenhouse and their leaves were inoculated with *E. necator* as described by Legler et al. (2012). Leaves with typical symptoms of powdery mildew infection were detached from plants 10 to 14 dpi, taken to the laboratory, and checked for the presence of freshly sporulating colonies of *E. necator* on their surfaces under a dissecting stereomicroscope. Each sporulating powdery mildew colony, marked with a permanent pen on the leaf surface, was inoculated with a 10 µl droplet of an *Ampelomyces* conidial suspension pipetted in the middle of the powdery mildew colony. Two to four grapevine leaves were used for each strain and each leaf beared one to five inoculated powdery mildew colonies. The inoculated leaves were kept in closed glass plates, 15 cm diam, placed on plastic nets floated on water, with their petioles in water, to ensure leaf survival and also high relative humidity. These plates were placed in a climate chamber with 16 h daily illumination at 20ºC for 10 days. The leaves were then put between paper towels, pressed and dried for 2-3 weeks before examination. These dried leaves, preserved as herbarium materials, were more easily handled during further works than the fresh ones. These experiments were done two or three times for each strain.

To assess the intra-hyphal spread of strains in the grapevine powdery mildew mycelium, each dried grapevine leaf was covered by transparent cellophane sheets and the points of inoculations and the areas around them containing *Ampelomyces* pycnidia in the powdery mildew conidiophores were marked with a fine point permanent marker under a dissecting stereomicroscope. The points of inoculation were markedly different from the rest of the powdery mildew colonies (Fig. 2). The cellophane sheets were then placed on a millimeter paper and scanned to be stored as digital images. To determine the areas of the *E. necator* mycelia parasitized by the different *Ampelomyces* strains, the scanned images were evaluated using the ASSESS software similar to the study of the growth and the sporulation

of *Ampelomyces* strains in culture, as described above. The spread of *Ampelomyces* in powdery mildew colonies was expressed as mm^2 per day.

To assess the sporulation in powdery mildew colonies, the number of *Ampelomyces* pycnidia produced in the *E. necator* conidiophores was determined in the above mentioned area under a dissecting stereomicroscope as described in Kiss et al. (2011). Pycnidia were counted in a single 1.5 mm^2 microscopic field per each powdery mildew colony successfully inoculated with *Ampelomyces* and the value obtained was converted to number of pycnidia per mm^2 powdery mildew-infected leaf surface.

Reduction of *E. necator* conidial production

The potential of each *Ampelomyces* strain to reduce the asexual sporulation of grapevine powdery mildew was also determined using detached grapevine leaves. Powdery mildew-infected leaves of *V. vinifera* cv. Barbera were produced in a greenhouse as described above. Leaf disks, 15 mm diameter, covered by sporulating *E. necator* mycelium were cut out of detached leaves, placed on wet filter paper in a 9 cm diameter Petri dish, and uniformly sprayed with an *Ampelomyces* conidial suspension. Fifteen leaf disks were sprayed with each tested *Ampelomyces* strain and 15 others with sterile distilled water. The latter samples served as untreated control. Leaf discs were kept in a climate chamber at 20°C and 12h daily illumination. At 10 dpi, each leaf disk was placed in an 1.5 ml Eppendorf tube containing 0.5 ml 0.5% Tween 20 solution and vortexed for 10 sec. The number of powdery mildew conidia in the suspensions obtained after vortexing was determined using a Bürker haemocytometer. The experiment was carried out twice with each strain tested.

Parasitism of *E. necator* chasmothecia

The mycoparasitic activity of the eight strains selected in the previous steps (Fig. 1) was determined in grapevine powdery mildew chasmothecia on potted grapevine plants, cv. Barbera. Plants were kept in a greenhouse and their leaves were inoculated with *E. necator* as described above. The development of powdery mildew colonies was monitored until the appearance of the ascocarps (i.e., chasmothecia) on the leaves. Two cm diameter sampling areas with powdery mildew colonies bearing either immature (i.e. yellow to light brown coloured) or mature (i.e., black) chasmothecia were selected under a dissecting stereomicroscope. Borders of each 2-cm diameter sampling area were marked with a permanent pen on the leaf surface and pictures were taken at the beginning of the experiment using a dissecting stereomicroscope equipped with a camera. Three potted plants, with at least three infected leaves bearing at least two sampling areas for both developmental stages (i.e. two areas with immature and two with mature chasmothecia at the time of inoculation with *Ampelomyces*) were prepared for each *Ampelomyces* strain tested and then uniformly sprayed with *Ampelomyces* conidial suspensions. Three plants with at least six sampling areas for both developmental stages were sprayed with sterile distilled water to serve as untreated controls. The treated plants were put in transparent boxes which ensured high humidity and kept at 18 to 26°C and natural daily illumination. At 12 dpi, each sampling area was examined under a dissecting microscope and compared to the picture taken before treatment. Ten chasmothecia were taken from each sampling area with a glass needle and examined under a light microscope to determine whether these were parasitized by *Ampelomyces*. The mycoparasitic activity of each *Ampelomyces* strain was determined as the percentage of parasitized chasmothecia vs. the total number of chasmothecia studied. These values were determined separately for the two developmental stages of chasmothecia, i.e. for immature and mature

279 stages at the time of inoculation with *Ampelomyces*. The experiment was carried out twice
280 with each *Ampelomyces* strain tested.
281
282 Grapevine powdery mildew control in the field
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284 Based on the results of our laboratory experiments described above, strain RS1-a was pre-
285 selected as a promising new BCA of grapevine powdery mildew. Its biocontrol efficacy was
286 compared to that of the commercial product AQ10® Biofungicide in field experiments, as
287 well. Experiments were carried out in five commercial vineyards in Travazzano, Savarna, and
288 Lugo in Italy, and Eger and Eger-Kőlyuktető in Hungary. Table 2 summarizes the
289 characteristics of these vineyards. In each vineyard, an area consisting of 120 vines was
290 separated from the rest of the plants in early August 2008 and in that area no more fungicide
291 applications were carried out until the end of the season to favour powdery mildew
292 development. In those areas, the following treatments were done according to a complete
293 randomized design with four replicates: i) untreated control, ii) conidial suspension of strain
294 RS1-a, and iii) commercial product AQ10® Biofungicide, both applied twice, until run-off,
295 approximately 1 week before and 1 week post harvest. The harvest took place from late
296 August until mid-October depending on the vineyard. In both treatments the final
297 concentrations of the conidial suspensions were 2 to 8 x 10^6 conidia/ml similar to all the
298 laboratory experiments carried out in this work. Conidial suspensions were applied with a
299 knapsack airblast sprayer SR 420 (Stihl Inc., Virginia Beach, VA, US) that ensured an even
300 distribution of the suspensions over the whole plot. Viability of the *Ampelomyces* inocula by
301 the time of the applications was confirmed by placing 25 to 50 µl suspension on 1.5% water
302 agar in 6 cm diameter plates directly from the sprayer in the vineyard, incubating the plates at

room temperature for 24 hours and then examining the germination of conidia under a light microscope.

Fourteen days after the second application, chasmothecia were randomly sampled on grapevine leaves in each plot. These chasmothecia were squeezed and examined under a light microscope to check the presence of *Ampelomyces* conidia in their bodies. These observations were done to verify the presence of the mycoparasites on the treated powdery mildew-infected leaves.

Starting from bud break in 2009, the vineyards were inspected at least once a week to determine the time of appearance of the first powdery mildew symptoms, such as flag shoots or discrete pale spots on the abaxial surfaces of the basal vine leaves that are typically caused by ascosporic infections (Rossi et al. 2010). Flag shoots were never found in our experimental areas. As soon as the first ascosporic infections were detected in the experimental areas, disease severity was assessed weekly on a sample of 50 randomly collected leaves and 20 bunches for each replicate. The leaves and bunches were not necessarily the same during the assessment times. Leaves and bunches were carefully observed for powdery mildew symptoms and classified as healthy or diseased, and disease incidence was calculated as a percentage of infected leaves/bunches. Disease severity was estimated visually and expressed as a percentage of the total leaf/bunch area (Campbell and Madden 1990). Bunches were not observed in all vineyards. The same treatments were carried out in autumn 2009 in the same five experimental areas and the results were assessed in spring 2010 as described above.

Data analysis

To analyze all the data obtained during laboratory experiments, including growth and sporulation rates in culture and spread and mycoparasitic activities inside the grapevine

328 powdery mildew mycelium, the *Ampelomyces* strains tested were grouped in the following
329 arbitrary groups: (i) those three *Ampelomyces* strains which were isolated from *E. necator*,
330 namely Vitis101, Vitis102 and Vitis109; this was named as the 'Vitis' group; (ii) the strain
331 RS1-a was treated separately because of its superior characteristics; (iii) the AQ10 strain from
332 the commercial product AQ10® Biofungicide was also treated separately for comparative
333 reasons; and (iv) all the other *Ampelomyces* strains isolated from powdery mildew species
334 other than *E. necator* were included in a single group named as 'Others'. The number of
335 strains included in the 'Others' group varied in different studies, being 28 in the initial steps of
336 the screening procedure, i.e. when daily radial growth and % sporulating surface area of the
337 colonies were determined (Table S1), 15 in the subsequent step, when the sporulation rate in
338 culture was assessed (Table S2), and 4 in the last step of the strain selection procedure, when
339 the intra-hyphal spread and reduction of the asexual and sexual sporulation of *E. necator* were
340 determined in detached leaf assays (Table S3).

341 These four groups of strains were created to compare the performances of (a) strains
342 isolated from *E. necator* with those isolated from other powdery mildew species; (b) a
343 particular strain, RS1-a, showing particular characteristics during preliminary experiments
344 with those of all the other strains; and (c) the commercial strain AQ10 with those of all the
345 other strains.

346 The data expressed as percentages (fungal colony area, with and without pycnidia,
347 reduction of powdery mildew conidial production, and parasitized chasmothecia) were arcsine
348 transformed while the values determined as numbers (number of pycnidia produced in
349 culture, powdery mildew conidia from vortexed leaf discs, and intracellular pycnidia
350 produced in powdery mildew colonies) were transformed using the natural logarithm to make
351 variances uniform. Transformed data were subjected to ANOVA for a complete randomized
352 design. Since in a preliminary analysis there was no significant effect of the repeated

experiments, experiments were considered as replicates. For the two groups of *Ampelomyces* strains, 'Vitis' and 'Others', strains were considered as replicates. Disease incidence and severity data determined in field experiments in spring at mid-epidemic on leaves (i.e., when disease incidence was 50% the highest disease incidence) were arcsine transformed and subjected to a 2-factor ANOVA; the factors considered were (i) the vineyards (10 vineyards in total, 5 vineyards each year) and (ii) the *Ampelomyces* strain applied ('untreated', 'RS1-a', and 'AQ10'). The Fisher Protected Least Square Difference test was used at $P = 0.05$ to separate means.

Results

Growth and sporulation in culture

Colony growth was significantly ($P < 0.001$) lower in the 'Vitis' group (0.24 mm/day) than in the 'Others' group and strain AQ10 (0.34 and 0.31 mm/day, respectively), while strain RS1-a was characterized by an intermediate value (0.27 mm/day) (Fig 3A, Table S1). Colony growth significantly ($P < 0.001$) decreased from 25°C (0.38 mm/day in average), to 20°C (0.34 mm/day in average), and to 15°C (0.26 mm/day in average). Interaction Temperature x Population was not significant ($P = 0.38$).

At 28 dpi, the percentage of the colony area covered with pycnidia (i.e., the sporulating area) was significantly ($P = 0.003$) higher in strain AQ10 (71%) than in the 'Vitis' and the 'Others' groups (66% on average) (Fig. 3B, Table S1). The interaction Temperature x Population was significant ($P = 0.013$): for RS1-a the colony area covered by pycnidia was wider at 20 and 25°C (61.5% in average) than at 15°C (54%), while for AQ10 it was wider at 15 and 20°C (64% in average) than at 25°C (54%). Nonetheless, strain AQ10 produced a

significantly ($P < 0.001$) lower number of mature pycnidia on the sporulating area of its colonies (Fig. 3C, Table S2). Consequently, when conidia released from pycnidia were determined, strain AQ10 produced 180 times less conidia per mm^2 of colony than strain RS1-a which produced the highest number of conidia ($9.4 \times 10^4/mm^2$ of colony) ($P < 0.001$) (Fig. 3D, Table S2). Neither Temperature nor interaction Temperature x Population had significant effects on production of pycnidia ($P = 0.11$ and 0.29, respectively) and conidia ($P = 0.058$ and 0.29, respectively).

The strain screening procedure (Fig. 1) started with a total of 33 strains, including AQ10 (Table 1). Nine strains, which did not sporulate at all (Table S1), were not included included in the second step of the screening procedure when the sporulation rates were determined (Table S2). A total of seven other strains had to be excluded from these steps due to a contamination problem.

Development and pycnidial production of *Ampelomyces* in powdery mildew conidiophores

Strain AQ10 showed a significantly ($P < 0.001$) lower growth rate in powdery mildew colonies (16.5 mm^2/day) than strain RS1-a (30.7 mm^2/day) and than the average growth rate of the strains included in the 'Vitis' (38.8 mm^2/day) and 'Other' groups (29.4 mm^2/day) (Fig. 4A and Table S3). Nonetheless, the number of intracellular pycnidia produced per mm^2 of the area colonized by *Ampelomyces* did not depend on the strain tested ($P = 0.06$), the overall average value being 12.8 ± 0.32 pycnidia per mm^2 parasitized powdery mildew mycelium.

403 Reduction of *E. necator* conidial production

404

405 All the eight *Ampelomyces* strains and the commercial product reduced the number of conidia
406 produced by the parasitized powdery mildew colonies by 74.7% to 91.5% compared to the
407 untreated control, but these differences were not significant among the four groups of strains
408 ($P = 0.43$).

409

410 Parasitism of *E. necator* chasmothecia

411

412 None of the *Ampelomyces* strains tested parasitized chasmothecia which were already mature
413 (i.e., black) at the time of the treatment. On the contrary, 36% of the *E. necator* chasmothecia
414 which were immature when the treatment was done, i.e. being of yellowish to light brown
415 colour at that point, were parasitized by strain RS1-a. Lower percentages of parasitism were
416 found for the commercial product and strains of the 'Others' group (1.7% and 7.8%
417 respectively) ($P = 0.003$). No significant differences were found between parasitism of
418 chasmothecia by the strains of the 'Vitis' group and strain RS1-a (Fig. 4B and Table S3).

419

420 Grapevine powdery mildew control in the field

421

422 In all vineyards, in autumn 2008 and 2009, chasmothecia parasitized by *Ampelomyces* were
423 found 14 days after the second application of both strain RS1-a (Fig. 5) and the commercial
424 product AQ10® Biofungicide. In some places a low number of parasitized chasmothecia were
425 also found in the control plots in autumn 2009.

426 The first symptoms of ascosporic infections occurred from mid-May (at Travazzano,
427 in 2010) to mid-July (at Lugo, in 2009) in the three experimental vineyard plots in Italy, and

at the end of May in both vineyard plots in Hungary. In 60% of vineyards, the first disease symptoms appeared firstly in the untreated plots and then, 1 to 4 weeks later, in the plots treated with either AQ10® Biofungicide or strain RS1-a (data not shown).

After the appearance of first disease symptoms, the number of leaves infected with powdery mildew increased progressively, with different rates in different vineyards. For instance, in the Travazzano vineyard in 2009, 53% of leaves were diseased by the end of May, and almost all leaves were diseased by mid-June (Fig. 6A); at the same time, disease severity has also increased and by mid-July reached approximately 25% of the leaf area in the samples (Fig. 6B). In the Savarna vineyard, the disease developed slowly in 2009 because only 20% of the leaves were infected by mid-July, and practically did not develop at all in 2010 because <1% of the leaves were diseased by mid-June (Table 3). In general, disease development in the plots sprayed with the two *Ampelomyces* strains, RS1-a and AQ10, was lower, and delayed in time, in the early stages of the epidemics. For instance, in the Travazzano vineyard, in 2009, disease incidence was lower in the treated plots compared to the untreated control until mid-June, then approximately 90% of the leaves were diseased in all the plots starting from late June (Fig. 6A); similarly, disease severity was lower in this vineyard until early July in 2009 (Fig. 6B).

At the mid-epidemic stage, both disease incidence and severity were significantly different in the different vineyards ($P < 0.001$) and also in the plots sprayed or not with *Ampelomyces* ($P < 0.001$ and $P = 0.03$ for disease incidence and severity, respectively) (Table 3). Average disease incidence was 19.6% on the untreated leaves, 9.2% and 9.8% on the leaves sprayed with strains RS1-a and AQ10, respectively; disease severity was 1.59, 0.76, and 0.95%, respectively. Therefore, both *Ampelomyces* strains applied twice in autumn reduced both disease incidence and severity at the mid-epidemic stage in the following year by approximately 50%. Since interaction between vineyards and treatments was not

453 significant (P = 0.60 and P = 0.879 for disease incidence and severity, respectively), such a
454 reduction was consistent over the different vineyards (Table 3). In addition to the effect of the
455 disease on leaves, an effect on bunches was observed. For example, in the Savarna vineyard
456 in 2010, the incidence of powdery mildew infection was very low on the leaves (Table 3),
457 however 67.5% of bunches became diseased at mid-July, with 9.81% average disease severity
458 in the control plots. In the plots sprayed with *Ampelomyces* in autumn, average disease
459 incidence on bunches was only 52.5% and disease severity was only 2.19%.
460
461 **Discussion**
462
463 By using our step-by-step screening procedure based on both *in vitro* and *in planta*
464 evaluations (Fig. 1), started in an earlier work (Legler et al. 2011a), we investigated a large
465 culture collection of *Ampelomyces* strains in this work and identified strain RS1-a as a strain
466 that can be developed further as a new BCA for grapevine powdery mildew. In terms of
467 cultural characteristics, i.e., colony growth and sporulation in culture, and mycoparasitic
468 activities in asexual and sexual stages of *E. necator*, RS1-a did not differ significantly from
469 the three strains included in the 'Vitis' group (Figs. 3 and 4), but its overall performance was
470 superior to that of the individual 'Vitis' strains (Tables S1-3). Compared to the commercial
471 AQ10 product, RS1-a showed, faster development in *E. necator* colonies and higher
472 mycoparasitic activity in *E. necator* chasmothecia (Table S3). In vineyard experiments, both
473 strain RS1-a and AQ10 Biofungicide®, applied twice in autumn to reduce the number of
474 overwintering chasmothecia, significantly delayed and reduced early-season development of
475 grapevine powdery mildew in the next year.
476 Late-season application of *Ampelomyces* can be considered a sanitation treatment, i.e.
477 the reduction of the overwintering inoculum that initiates the disease epidemic in the

following year (Legler et al. 2011b; Caffi et al. 2013). The efficacy of our sanitation treatments was confirmed by disease assessments performed in the treated plots in the following seasons: in 60% of the treated plots, the first disease symptoms appeared 1 to 4 weeks later than in the untreated plots. Moreover, both *Ampelomyces* strains, applied twice in late season of the previous year, halved disease incidence and severity at mid-epidemic in the following year. Our studies on detached leaves have shown that *E. necator* chasmothecia were only parasitized by *Ampelomyces* when these were still immature; chasmothecia which have already been mature at the moment of application of *Ampelomyces* were not colonized by the mycoparasite. Similar results were obtained earlier by Falk et al. (1995). This means that timing of the *Ampelomyces* treatments in autumn should be based on the monitoring of the development of chasmothecia in the targeted vineyards. Epidemiological models are helpful in timing plant protection product applications (Carisse et al. 2009; Caffi et al. 2010, 2013) and should be also used to assist the application of *Ampelomyces* as a BCA of *E. necator* in autumn. Recently, a model was developed to predict the time-course of development of the *E. necator* chasmothecia (Rossi et al. 2009; Legler et al. 2012, 2014).

The use of *Ampelomyces* in sanitation treatments can become a part of an integrated disease management strategy of grapevine powdery mildew. By delaying the time of disease onset and reducing disease incidence and severity in spring and early summer, the applications of *Ampelomyces* in the previous year, around harvest in autumn, delay the start of secondary infection cycles that are responsible of the so-called ‘disease explosion’ (Carisse et al. 2009), and facilitate disease control before berries gain ontogenic resistance which occurs rapidly after fruit set (Gadoury et al. 2003).

In this work we developed new methods to study the intra-hyphal spread, intracellular sporulation, and mycoparasitism of chasmothecia by *Ampelomyces* strains. The results did not support the idea of a narrow mycohost specialization in *Ampelomyces* strains and provided the

first evidence that strains isolated from diverse powdery mildew species are able to intensively parasitize the sexual stage of another powdery mildew species, *E. necator*, in addition to heavily parasitizing its asexual stage.

If *Ampelomyces* strains were specialized, at least to some extent, to the powdery mildew species which they were isolated from, one would expect a lower mycoparasitic activity in other powdery mildew species during mycoparasitic tests carried out in laboratory conditions or during field applications. However, both our tests done on detached leaves and our vineyard experiments showed the contrary. No significant differences were found in the ability of eight pre-selected strains, isolated from five powdery mildew species, including *E. necator*, to parasitize the conidial stage of *E. necator* because the formation of intracellular pycnidia and reduction of powdery mildew conidial production were similar in these strains (Fig. 4A). Moreover, RS1-a, a strain isolated from *P. pannosa* infecting rose, showed, together with the three strains included in the 'Vitis' group, the highest rate of parasitism of *E. necator* chasmothecia (Fig. 4B). Curiously, the mycoparasitic activity of the commercial strain AQ10 was significantly lower in both asexual and sexual stages of *E. necator* (Figs. 4A and B). This may be a consequence of repeated mass production of this strain at industrial level although no data are available for the partial loss of mycoparasitic activity during subculturing of any *Ampelomyces* strains. The RS1-a strain with superior characteristics concerning both sporulation in culture and mycoparasitic activity was isolated in 2007 (Table 1) and subcultured under laboratory conditions since that time.

No significant differences were found between the results of the vineyard treatments with strains RS1-a and AQ10 (Table 3), although none of these two strains were isolated from *E. necator*. Previous cross-inoculation experiments were somewhat contradictory concerning the mycoparasitic performance of *Ampelomyces* strains in their mycohosts of origin versus other powdery mildew species: Falk et al. (1995) and Angeli et al. (2012b) reported that some

strains perform better in the mycohost species of origin compared to other powdery mildew species tested while such differences were not detected in a comprehensive field experiment (Kiss et al. 2011). On the other hand, Angeli et al. (2009) and Pintye et al. (2012) showed that *E. necator* is naturally parasitized by genetically different *Ampelomyces* strains in the field and the same was demonstrated for *Ampelomyces* strains isolated from *Arthrocladiella mougeotii*, the powdery mildew species infecting a solanaceous weed, *Lycium halimifolium* (Kiss et al. 2011; Pintye et al. 2015).

Based on the results obtained in this work, supplemented with previous data discussed above, there was no reason why to select a strain isolated from *E. necator* as a potential BCA of grapevine powdery mildew. Instead of mycohost specificity, the high sporulation rate in culture, an important factor in mass-production of BCAs, and high mycoparasitic activity became the key factors in proposing strain RS1-a for further development as a new BCA of *E. necator*.

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References

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

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

Brand, M., Messika, Y., Elad, Y., David, D. R., & Sztejnberg, A. (2009). Spray treatments combined with climate modification for the management of *Leveillula taurica* in sweet pepper. *European Journal of Plant Pathology, 124,* 309-329.

Caffi, T., Rossi, V., & Bugiani, R. (2010). Evaluation of a warning system for controlling primary infections of grapevine downy mildew. *Plant Disease, 94,* 709-716.

Caffi, T., Legler, S.E., Rossi, V., & Bugiani, R. (2012). Evaluation of a warning system for early-season control of grapevine powdery mildew. *Plant Disease, 96,* 104-110.

Caffi T., Legler S.E., Buigiani R., & Rossi V. (2013). Combining sanitation and disease modelling for control of grapevine powdery mildew. *European Journal of Plant Pathology, 135,* 817–829.

Campbell, C. L., & Madden, L. V. (1990). Introduction to Plant Disease Epidemiology. Wiley, New York

Carisse, O., Bacon, R., Lefebvre, A., & Lessard, K. (2009). A degree-day model to initiate fungicide spray programs for management of grape powdery mildew (*Erysiphe necator*). *Canadian Journal of Plant Pathology, 31,* 186-194.

Elad, Y., Kirshner, B., & Sztejnberg, A. (1998). Management of powdery mildew and gray mold of cucumber by *Trichoderma harzianum* T39 and *Ampelomyces quisqualis* AQ10. *BioControl, 43,* 241-251.

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

Füzi, I. (2003). Natural parasitism of *Uncinula necator* cleistothecia by *Ampelomyces* hyperparasites in the south-western vineyards of Hungary. *Acta Phytopathologica et Entomologica Hungarica, 38,* 53-60.

Gadoury, D. M., Seem, R. C., Ficke, A., & Wilcox, W. F. (2003). Ontogenic resistance to powdery mildew in grape berries. *Phytopathology, 93,* 547-555.

601 Hashioka, Y., & Nakai, Y. (1980). Ultrastructure of pycnidial development and
602 mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. *Transactions of the*
603 *Mycological Society of Japan, 21,* 329-338.
604
605 Kiss, L. (2008). Intracellular mycoparasites in action: interactions between powdery mildew
606 fungi and *Ampelomyces*. In Avery SV, Stratford M, Van West P (eds) Stress in Yeasts and
607 Filamentous Fungi, Academic Press, Elsevier, London, pp 37-52.
608
609 Kiss, L., Russell, J. C., Szentiványi, O., Xu, X., & Jeffries, P. (2004). Biology and biocontrol
610 potential of *Ampelomyces mycoparasites*, natural antagonists of powdery mildew fungi.
611 *Biocontrol Science and Technology, 14,* 635-651.
612
613 Kiss, L., Pintye, A., Zséli, G., Jankovics, T., Szentiványi, O., Hafez, Y. M., Cook, & R. T. A.
614 (2010). Microcyclic conidiogenesis in powdery mildews and its association with intracellular
615 parasitism by *Ampelomyces*. *European Journal of Plant Pathology, 126,* 445-451.
616
617 Kiss, L., Pintye, A., Kovács, G. M., Jankovics, T., Fontaine, M., Harvey, N., Xu, X., Nicot, P.
618 C., Bardin, M., Shykoff, J. A., & Giraud, T. (2011). Temporal isolation explains host-related
619 genetic differentiation in a group of widespread mycoparasitic fungi. *Molecular Ecology, 20,*
620 1492-1507.
621
622 Lee, S. Y., Lee, S. B., & Kim, C. H. (2004). Biological control of powdery mildew by Q-fect
623 WP (*Ampelomyces quisqualis* 94013) in various crops. *IOBC/wprs Bulletin, 27,* 329-331.
624

625 Legler, S. E., Caffi, T., Kiss, L., Pintye, A., & Rossi, V. (2011a). Methods for screening new
626 *Ampelomyces* strains to be used as biocontrol agents against grapevine powdery mildew.
627 *IOBC/wprs Bulletin, 66,* 149-154.
628
629 Legler, S. E., Caffi, T., & Rossi, V. (2011b). Effect of different plant protection products on
630 the sexual stage of grapevine powdery mildew. In Calonnec A, Thiéry D, Médalin MC (eds).
631 Proceedings of the European Meeting of the Working Group "Integrated Protection and
632 Production in Viticulture", Lacanau, France, pp 2.
633
634 Legler, S. E., Caffi, T., & Rossi, V. (2012). A nonlinear model for temperature-dependent
635 development of *Erysiphe necator* chasmothecia on grapevine leaves. *Plant Pathology, 60,* 96-
636 105.
637
638 Legler, S. E., Caffi, T., & Rossi, V. (2014). A model for the development of *Erysiphe necator*
639 chasmothecia in vineyards. *Plant Pathology, 63,* 911–921.
640
641 Liang, C., Yang, J., Kovács, G. M., Szentiványi, O., Li, B., Xu, X. M., & Kiss, L. (2007).
642 Genetic diversity of *Ampelomyces* mycoparasites isolated from different powdery mildew
643 species in China inferred from analysis of rDNA ITS sequences. *Fungal Diversity, 24,* 225-
644 240.
645
646 McGrath, M. T., & Shishkoff, N. (1999). Evaluation of biocompatible products for managing
647 cucurbit powdery mildew. *Crop Protection, 18,* 471-478.
648

649 Park, M. J., Choi, Y. J., Hong, S. B., & Shin, H. D. (2010). Genetic variability and mycohost
650 association of *Ampelomyces quisqualis* isolates inferred from phylogenetic analyses of ITS
651 rDNA and actin gene sequences. *Fungal Biology, 114,* 235-247.
652
653 Paulitz, T. C., & Bélanger, R. R. (2001). Biological control in greenhouse systems. *Annual*
654 *Review of Phytopathology, 39,* 103-133.
655
656 Pertot, I., Zasso, R., Amsalem, L., Mario Baldessari, M., Angeli, G., & Elad, Y. (2008).
657 Integrating biocontrol agents in strawberry powdery mildew control strategies in high tunnel
658 growing systems. *Crop Protection, 27,* 622-631.
659
660 Pintye, A., Bereczky, Z., Kovács, G. M., Nagy, L. G., Xu, X., Legler, S. E., Váczy, Z., Váczy,
661 K. Z., Caffi, T., Rossi, V., & Kiss, L. (2012). No indication of strict host associations in a
662 widespread mycoparasite: Grapevine powdery mildew (*Erysiphe necator*) is attacked by
663 phylogenetically diverse *Ampelomyces* strains in the field. *Phytopathology, 102,* 707-716.
664
665 Pintye, A., Ropars, J., Harvey, N., Shin, H. D., Leyronas, C., Nicot, P. C., Giraud, T., & Kiss,
666 L. (2015). Host phenology and geography as drivers of differentiation in generalist fungal
667 mycoparasites. PLoS ONE 10(3): e0120703. doi:10.1371/journal.pone.0120703.
668
669 Romero, D., Rivera, M. E., Gazorla, F. M., De Vincente, A., & Pérez-Garcia, A. (2003).
670 Effect of mycoparasitic fungi on the development of *Sphaerotheca fusca* in melon leaves.
671 *Mycological Research, 107,* 64-71.
672

673 Rossi, V., Caffi, T., & Legler, S. E. (2010). Dynamics of ascospore maturation and discharge
674 in *Erysiphe necator*, the causal agent of grape powdery mildew. *Phytopathology, 100,* 1321-
675 1329.
676
677 Rossi, V., Caffi, T., Legler, S.E., Bugiani, R., & Frisullo, P. (2011). Dispersal of the sexual
678 stage of Erysiphe necator in northern Italy. *IOBC/WPRS Bulletin 67*, 115-121.
679
680 Shishkoff, N., & McGrath, M. T. (2002). AQ10 biofungicide combined with chemical
681 fungicides or AddQ spray adjuvant for control of cucurbit powdery mildew in detached leaf
682 culture. *Plant Disease, 86,* 915-918.
683
684 Speer, E. O. (1978). Beitrag zur Morphologie von *Ampelomyces quisqualis* Ces. *Sydowia, 31,*
685 242-246.
686
687 Sutton, B. C. (1980). The Coelomycetes. Fungi imperfecti with pycnidia, acervuli and
688 stromata. Commonwealth Mycological Institute, Kew, UK
689
690 Szentiványi, O., Kiss, L., Russell, J. C., Kovács, G. M., Varga, K., Jankovics, T., Lesemann,
691 S., Xu, X., & Jeffries, P. (2005). *Ampelomyces* mycoparasites from apple powdery mildew
692 identified as a distinct group based on single-stranded conformation polymorphism analysis
693 of the rDNA ITS region. *Mycological Research, 109,* 429-438.
694
695 Sztejnberg, A., Galper, S., Mazar, S., & Lisker, N. (1989). *Ampelomyces quisqualis* for
696 biological and integrated control of powdery mildew in Israel. *Journal of Phytopathology,*
697 *124*, 285-295.

Tollenaere, C., Pernechele, B., Mäkinen, H. S., Parratt, S. R., Németh, M. Z., Kovács, G. M., Kiss, L., Tack, A. J. M., Laine, A. L. (2014) A hyperparasite affects the population dynamics of a wild plant pathogen. *Molecular Ecology, 23*, 5877-5887.

Figure captions

Fig. 1 Flow chart indicating the strain screening procedure applied in this work based on the procedure developed by Legler et al. (2011a). Note that the commercial strain AQ10 was included in every step of the screening procedure as a control.

Fig. 2 Mycelium of *Erysiphe necator* on a grapevine leaf, cv. Chardonnay, two weeks after inoculation with a 10 µl droplet of an *Ampelomyces* conidial suspension, 2 to 8 x 10^6 conidia/ml. The dried area surrounded by a red line shows the point of inoculation while the area surrounded by a blue line indicates the powdery mildew mycelium where intracellular pycnidia of *Ampelomyces* were produced in powdery mildew conidiophores following inoculation.

Fig. 3 Growth and sporulation of *Ampelomyces* strains on Czapek-Dox agar with 2% malt (MCzA). The values determined for strains RS1-a and Aq10 are shown separately; those for the three strains isolated from *Erysiphe necator* are included in the 'Vitis' group; and those for the rest of the strains form the 'Others' group (Tables S1 and S2). (A) Growth of colonies (mm/day) on MCzA, (B) percentage of colony areas covered by pycnidia, (C) number of pycnidia, and (D) number of conidia produced per mm^2 colony. Columns show averages of three experiments. Bars represent the standard error. Values followed by different letters are significantly different according to the LSD test at $P = 0.05$.

Fig. 4 Mycoparasitic activities of *Ampelomyces* strains inside the asexually sporulating mycelium (A) and the chasmothecia of *Erysiphe necator* colonies (B). Strains were grouped as in Fig. 2. (A) Intra-hyphal growth rate (mm^2/day) determined based on the powdery

mildew areas containing pycnidia 14 dpi as shown in Fig. 1. (B) Percentage of parasitized young *E. necator* chasmothecia over the total number of chasmothecia observed. Growth rates in (A) are averages of two or three experiments, depending on the strain; percentages in (B) are averages of two experiments for each strain (Table S3). Bars represent the standard error. Values followed by different letters are significantly different according to the LSD test at $P = 0.05$.

Fig. 5 A sample of chasmothecia collected from a grapevine leaf treated with a conidial suspension of the *Ampelomyces* strain RS1-a in an experimental vineyard in autumn. Most chasmothecia, representing different stages of maturation, were parasitized by *Ampelomyces*, containing masses of conidia of the mycoparasite (see the enlarged picture on the right). A mature chasmothecium, shown by a yellow arrow, escaped the treatment.

Fig. 6 Dynamics of powdery mildew epidemics in the Travazzano vineyard (North Italy) in 2009, in unsprayed plots (●) and in plots sprayed with *Ampelomyces* strain AQ10 (♦) and RS1-a (■). (A) average disease incidence; (B) average disease severity. Disease incidence and severity were assessed weekly after symptoms onset on a sample of 50 random leaves of two plants placed in the center of each plot. Bars represent the standard error.

Table 1 Designations, host fungi and plants, and country and year of isolation for the 33 *Ampelomyces* strains included in this study. During data analysis four arbitrary groups of strains were defined: 'RS1-a', 'AQ10', 'Vitis' and 'Others'. The strains are listed below according to this grouping.

Strain designation*	Host fungal species	Host plant species	Country and year of isolation
GROUP 1:			
RS1-a	*Podosphaera pannosa*	*Rosa* sp.	Hungary, 2007
GROUP 2:			
AQ10	*Oidium* sp.	*Catha edulis*	Israel, ?**
GROUP 3: 'Vitis'			
Vitis101 (CBS 132221)	*Erysiphe necator*	*Vitis vinifera*	Hungary, 2009
Vitis102 (CBS 132222)	*E. necator*	*V. vinifera*	Hungary, 2009
Vitis109 (CBS 132225)	*E. necator*	*V. vinifera*	Hungary, 2009
GROUP 4: 'Others'			
A1 (ATCC 201056)	*Arthrocladiella mougeotii*	*Lycium halimifolium*	Hungary, 1990
B2 (MYA-3389)	*P. leucotricha*	*Malus domestica*	Hungary, 1995
B4 (MYA-3390)	*P. leucotricha*	*M. domestica*	Hungary, 2000
B22	*P. leucotricha*	*M. domestica*	Czech Republic,

(MYA-3392)			2002
B34	*P. leucotricha*	*M. domestica*	UK, 2002
(MYA-3396)			
B40	*P. leucotricha*	*M. domestica*	UK, 2002
(MYA-3397)			
B61	*P. leucotricha*	*M. robusta*	France, 2008
B190	*P. leucotricha*	*M. domestica*	Hungary, 2008
B221	*P. leucotricha*	*M. domestica*	France, 2008
CBS 130.79	*P. xanthii*	*Cucurbita pepo*	Canada, 1975
CBS 131.79	*P. xanthii*	*C. pepo*	Canada, 1975
DSM2222	*P. xanthii*	*Cucumis* sp.	Germany, ?
G2	*E. polygoni*	*Rumex patientia*	Hungary, 1992
HMLAC226	*E. polygoni*	*Polygonum aviculare*	China, 2003
MA3-a	*E. berberidis*	*Mahonia aquifolium*	Hungary, 2007
D2	*Golovinomyces*	*Aster salignus*	UK, 1999
(MYA-3399)	*cichoracearum*		
D4	*G. cichoracearum*	*A. salignus*	UK, 1999
(MYA-3400)			
263	*G. cichoracearum*	*Artemisia absinthium*	Canada, 1974
TP1	*E. trifolii*	*Trifolium pratense*	France, 2007
LV2-b	*Erysiphe* sp.	*Ligustrum vulgare*	Hungary, 2007
A8	*A. mougeotii*	*L. halimifolium*	Hungary, 2007
A62-b	*A. mougeotii*	*L. halimifolium*	Hungary, 2007
A98	*A. mougeotii*	*L. halimifolium*	Hungary, 2007
MA6-b	*E. berberidis*	*Ma. aquifolium*	Hungary, 2007

MA8	*E. berberidis*	*Ma. aquifolium*	Hungary, 2007
AqW	*P. pannosa*	*Rosa* sp.	Netherlands, ?
GYER	*E. arcuata*	*Carpinus betulus*	Hungary, 2008

*If applicable, accession numbers in international public culture collections are shown in parentheses: ATCC, American Type Culture Collection, Manassas, VA, USA (strains with accession numbers starting with 'MYA' were also deposited at ATCC); CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; DSM: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

**Missing data

1 **Table 2** Characteristics of the experimental vineyards

Vineyard	Geographic coordinates	Cultivar	Trellis system	Spacing (within / between)	Year of plantation
Travazzano (IT)	44°51’53” N, 9°47’53” E	Trebbiano Romagnolo	Duplex Casarsa	1.8 m / 2.5 m	1993
Savarna (IT)	44°30’05” N, 11°58’28” E	Pinot blanc	spurred cordon system	1.5 m / 4 m	2002
Lugo (IT)	44°24’35” N, 11°53’19” E	Chardonnay	GDC	0.8 m / 4 m	2003
Eger (HU)	47° 54’42” N, 20° 20’59” E	Oporto	permanent cordon system	1.2 m / 2 m	1992
Eger-Kolyukteto (HU)	47°52’01” N, 20°22’57” E	Kékfrankos	spurred cordon system	1 m / 2 m	1993

2

Table 3 Incidence and severity of powdery mildew determined on leaves in the experimental vineyards during this study

Vineyard	Date	Disease incidence (%)[a]			Disease severity (%)[b]		
		Untreated	RS1-a	AQ10	Untreated	RS1-a	AQ10
Travazzano (IT)	26 May 2009	53.0±9.00	13.0±1.00	18.0±6.00	1.66±1.24	0.07±0.01	0.22±0.09
Travazzano (IT)	01 June 2010	32.0±8.00	12.0±4.00	28.0±1.00	2.60±0.80	0.90±0.70	1.60±0.30
Savarna (IT)	15 July 2009	20.0±7.00	8.5±3.50	11.5±4.50	2.73±0.23	3.02±0.52	3.69±1.19
Savarna (IT)	16 June 2010	0.5±0.10	0	0	0.02±0.01	0	0
Lugo (IT)	15 July 2009	9.5±4.50	0.5±0.25	5.0±2.00	5.10±2.60	1.25±0.70	2.50±1.3
Lugo (IT)	16 June 2010	5.0±1.00	6.0±1.00	4.0±1.00	0.13±0.02	0.16±0.02	0.11±0.02
Eger (HU)	27 May 2009	3.9±1.17	0	0	1.51±0.73	0	0
Eger (HU)	25 May 2010	53.8±7.16	48.8±11.2	26.0±14.0	3.32±0.28	2.12±1.68	1.25±0.95
Eger-Kőlyuktető (HU)	27 May 2009	7.7±2.33	0	1.0±0.64	0.07±0.02	0	0.01±0.01
Eger-Kőlyuktető (HU)	25 May 2010	11.2±1.50	2.7±0.18	4.1±0.08	0.28±0.03	0.07±0.01	0.10±0.01
Mean		19.6 a[c]	9.2 b	9.8 b	1.59 a	0.76 b	0.95 b

[a]Mean values and standard error values of disease incidence calculated as a percentage of infected leaves.

[b]Mean values and standard error values of disease severity estimated visually and expressed as a percentage of the total leaf area.

[c] Numbers followed by different letters are significantly different according to the LSD test at $P = 0.05$. Interaction vineyard x treatment was not significant.

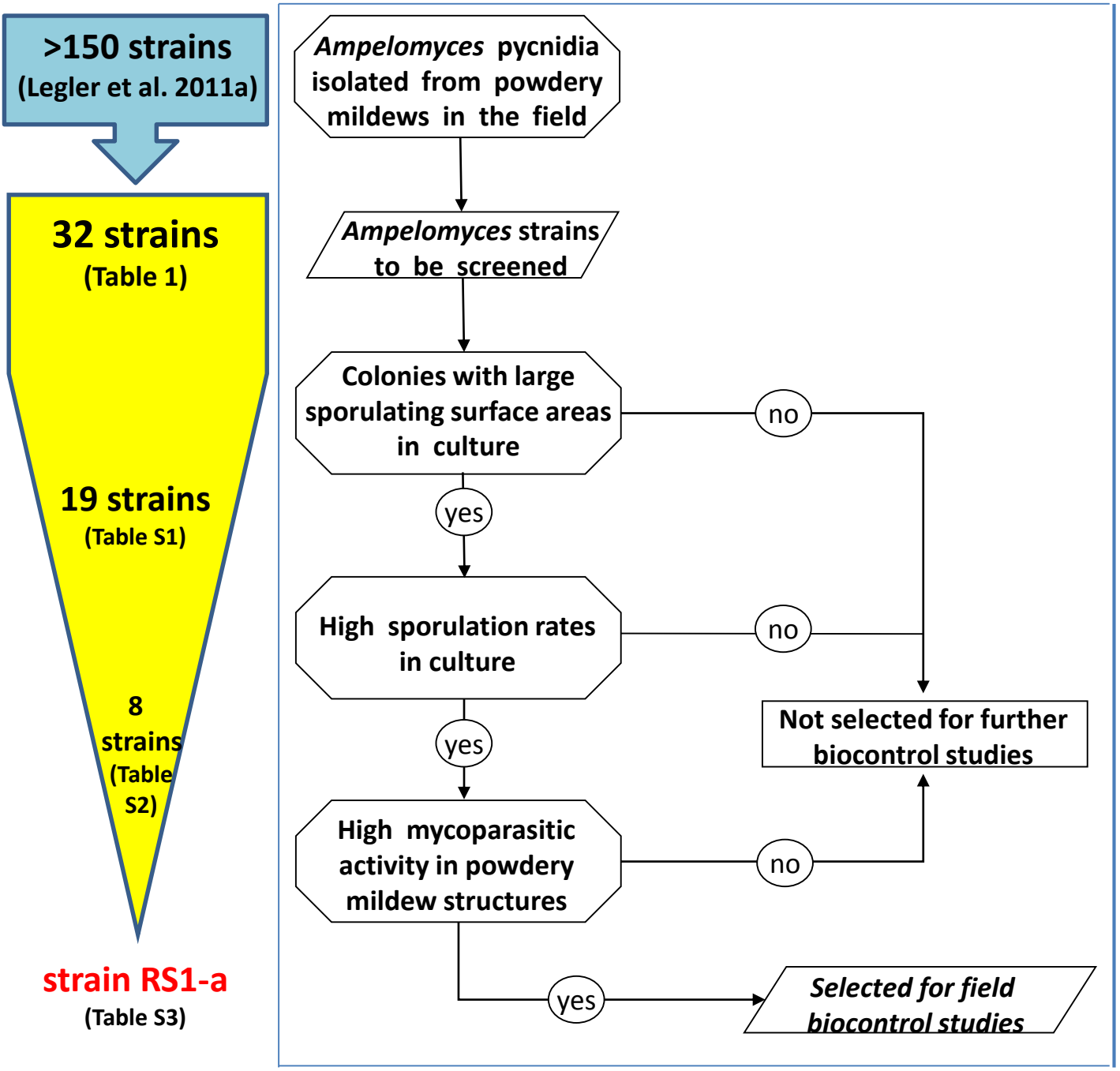
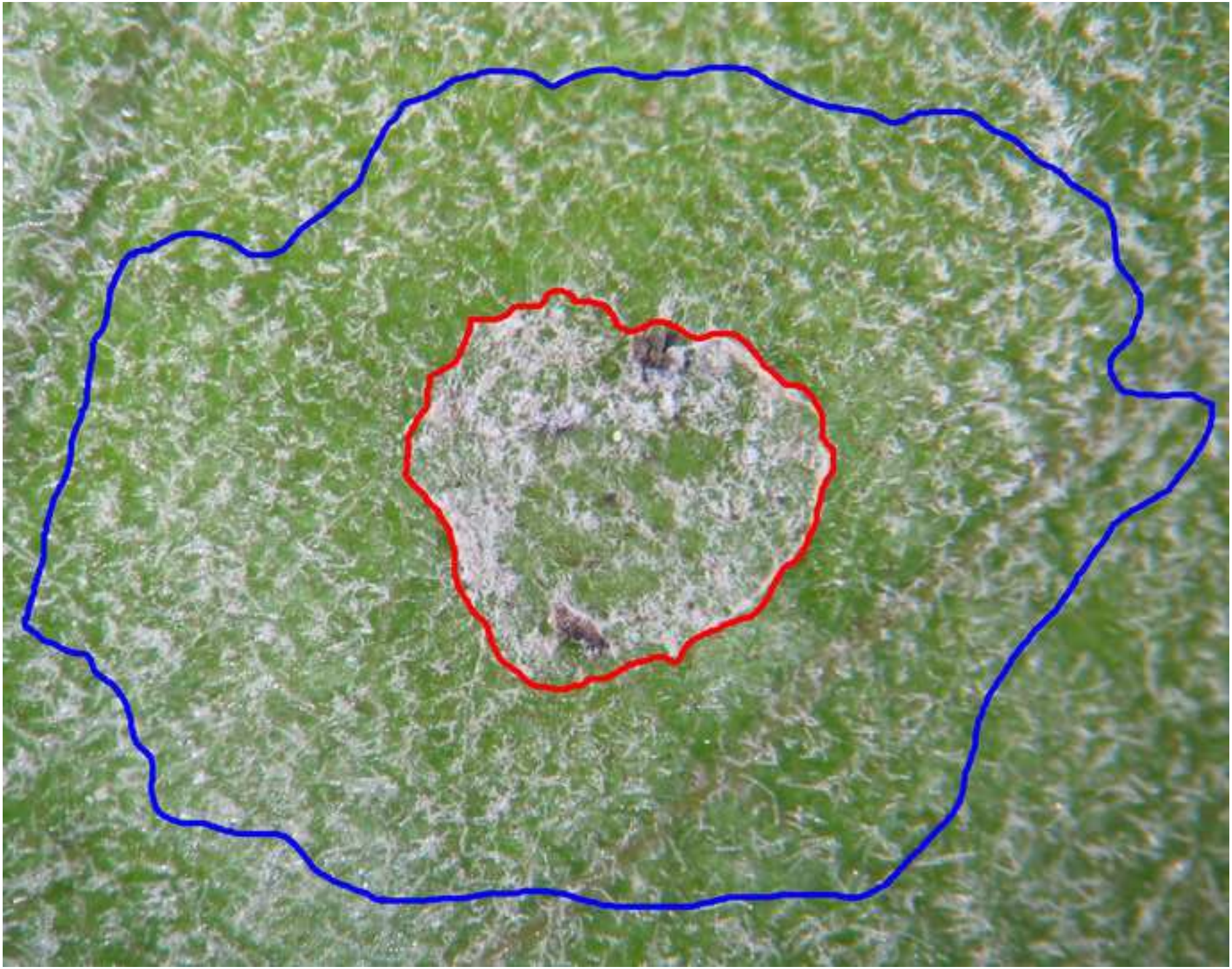


Fig. 1 Flow chart indicating the strain screening procedure applied in this work based on the procedure developed by Legler et al. (2011a). Note that the commercial strain AQ10 was included in every step of the screening procedure as a control.

colour figure

Click here to download colour figure Fig2 strain H14 AMPELOMYCES.jpg



line figure

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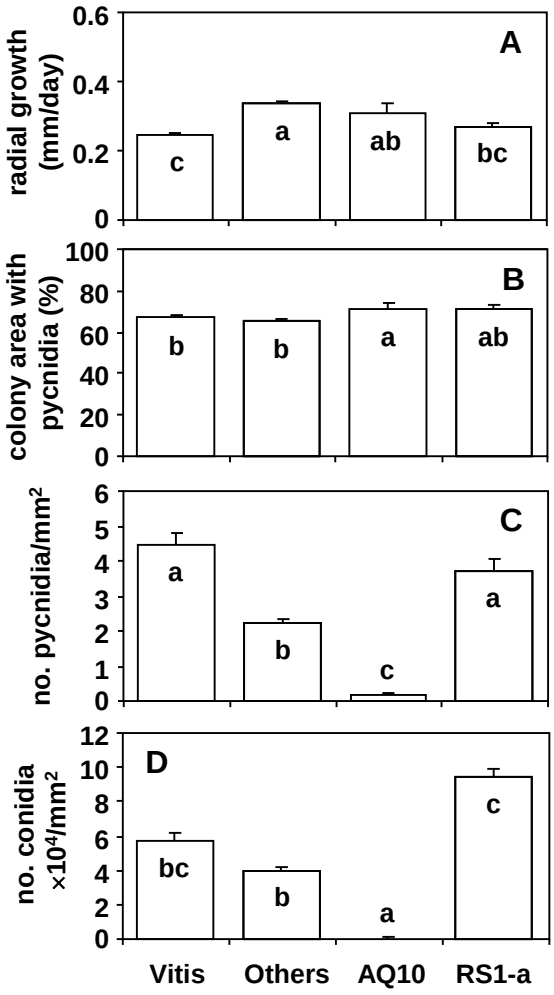

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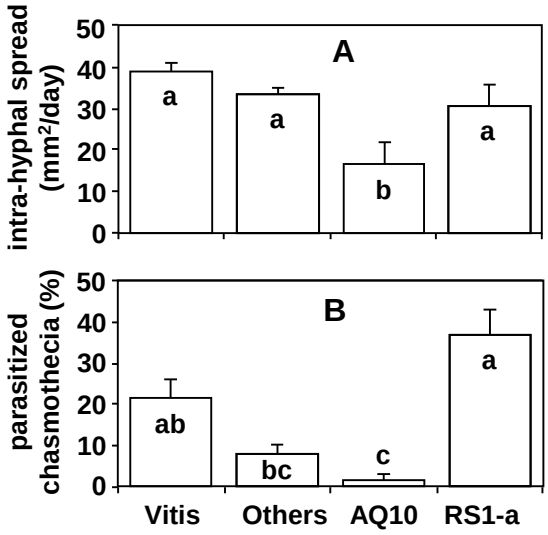

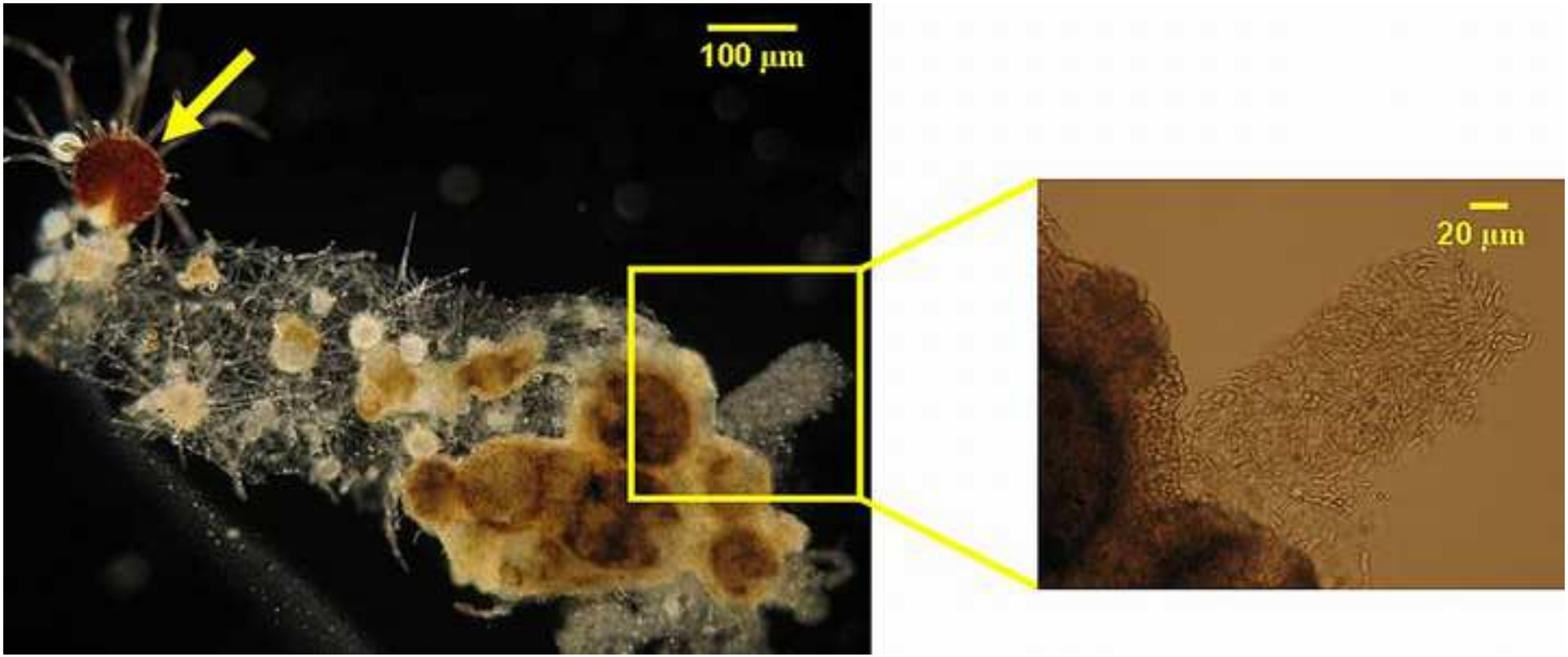

line figure

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