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Review

Fungi Parasitizing Powdery Mildew Fungi: *Ampelomyces* Strains as Biocontrol Agents against Powdery Mildews

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Abstract: Among the mycoparasites, *Ampelomyces* strains are studied in detail, particularly regarding their use as biocontrol agents (BCAs) of powdery mildew (PM) fungi, including their potential to replace conventional agrochemicals. *Ampelomyces* strains are characterized morphologically; their ribosomal DNA internal transcribed spacer (rDNA-ITS) regions and actin gene (*ACT*) fragments were sequenced and their mycoparasitic activity was analyzed. In the interaction between *Ampelomyces* strains and PM fungi, the spores of the mycoparasites germinate on plant leaves, and their hyphae then penetrate the hyphae of PM fungi. *Ampelomyces* hyphae continue their growth internally, initiating the atrophy of PM conidiophores and eventually their complete collapse. Following the successful destruction of PM hyphae by *Ampelomyces*, the mycoparasite produces new intracellular pycnidia in PM conidiophores. The progeny spores released by mature pycnidia become the sources of subsequent infections of intact PM hyphae. As a result, the number of *Ampelomyces*-inoculated PM colonies gradually declines, and the conidial release of PM colonies is inhibited after the first treatment. Almost all conidiophores of 5- and 10-day-old *Ampelomyces*-inoculated PM colonies undergo complete atrophy or collapse. Methodological advances and in-depth analyses of the *Ampelomyces*–PM interaction were recently published. In this review, we summarize the genetic and phylogenetic diversity, the timing of mycoparasitism and pycnidiogenesis, the results of quantitative and visual analyses using electrostatic and digital microscopy technologies, the PM biocontrol potential of *Ampelomyces*, and the potential commercialization of the mycoparasites. The information provided herein can support further biocontrol and ecological studies of *Ampelomyces* mycoparasites.

Keywords: biological control; digital microscopic technique; hyperparasite; hyperparasitism; integrated control; mycoparasite; plant protection

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1. Introduction

Powdery mildew (PM) is a serious disease affecting many crops [1,2]. The leaf damage caused by the fungus significantly reduces crop productivity [3,4]. While fungicides can be sprayed before or after PM colonies appear on host leaves to control the disease, frequent application of commercial fungicides can lead to resistance [5–7]. To avoid fungicide resistance and the environmental problems caused by fungicide residues, new control strategies that are independent of chemical methods are needed to control PM. Biological control offers an alternative method to prevent or suppress PM in crops by exploiting the antagonism between micro-organisms. Mycoparasitic fungi parasitize other fungi and they include a diverse group of parasites. These fungi absorb nutrients from their mycohosts through haustoria or other special interfaces between their cell walls and membranes. Alternatively, they invade the hyphae of their mycohosts, growing from cell to cell in the latter's hyphae, conidia, and conidiophores while absorbing nutrients from the infection structures [8,9].

Kiss [10,11] examined all known fungal antagonists of PM, whether found in the field or tested as potential biocontrol agents (BCAs) of PM infections, including species without any record of a natural antagonistic relationship. More than 40 fungal taxa were shown to suppress the growth and sporulation of PM fungi. Of these, *Aphanocladium album* [12], *Pseudozyma flocculosa*, *Moesziomyces rugulosus* [13,14], *Gjaerumia minor* [15,16], *Lecanicillium lecanii* [14], and *Ampelomyces quisqualis* [17,18] are well known as BCAs against PM.

The mycoparasitic fungus *Ampelomyces quisqualis* Cesati ex Schlechtend (syn. *Cicinnobolus cesatii* de Bary; [17,18]) is a slow-growing pycnidial fungus widely distributed in PM colonies and naturally occurring worldwide [19–21], where it acts as a hyperparasite of strains infecting cultivated and wild plants [10,21–23]. The *Ampelomyces* strains produce progeny spores in mature pycnidia, which develop intracellularly in the hyphae of PM fungi in nature and then suppress mycelial growth, sporulation, and conidial germination of their mycohosts [9–11]. Therefore, this mycoparasitic fungus gained much attention as BCAs for controlling the PMs. The life cycle, mode of action, and biocontrol potential of hyperparasitic fungi were reviewed [9,11,24] with the aim of guiding future research in fungal and plant ecology, as well as in the development of products for the control of plant diseases [25]. However, quantitative data on the impact of hyperparasitism on host fungi are lacking. Thus, in this work, we review (1) the interactions between *Ampelomyces* strains and PM fungi (mycohosts) with respect to the morphological and physiological characteristics and phylogenetic placement of *Ampelomyces* strains; (2) the visualization and impact of fungal hyperparasitism (infection process and pycnidiogenesis) on mycohost survival by using a digital microscopic technique; (3) the quantitative impact of fungal hyperparasitism on the suppression of conidial release from PM colonies infected with *Ampelomyces* strains by incorporating a recent methodological advance; and (4) the practical aspects of using *Ampelomyces* strains as BCAs. Finally, (5) summarizing experimental results, we provide an ideal spray inoculation system for the effective use of *Ampelomyces* as a BCA, as well as in research.

2. Powdery Mildew Fungi

PM fungi (Erysiphaceae) are obligate biotrophic pathogens of more than 10,000 host plant species, including important crops, and are responsible for serious losses in agriculture, horticulture, and forestry [1,26–28]. The sporulation of many PM anamorphs is intense (Figure 1A), and the produced conidia (Figure 1B) spread rapidly [29,30]. While regularly applied fungicides are used to control PM, its frequent and inadequate use can lead to the emergence of fungicide resistance [7,31,32], as demonstrated in cucurbit PM fungi [5,6,33–36]. In addition, plant leaves retain fungicides that are not completely decomposed by microorganisms, and the fungicides may also have negative side effects on plant physiology [37] as well as biodiversity [38]. Thus, to avoid drug resistance and environmental problems, new strategies for the control of PM that are independent of chemical methods are needed.

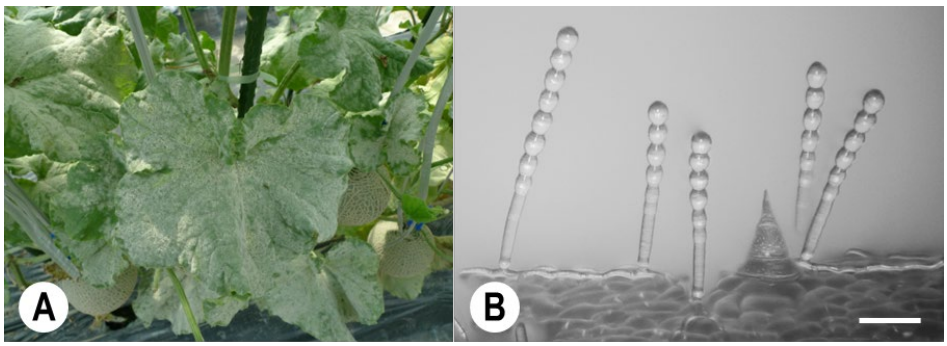

Figure 1. Photograph of powdery mildew (PM) disease caused by *Podosphaera xanthii* on melon leaves, and a micrograph of conidiophores formed in the fungal colonies. (**A**) Melon PM disease involving the whole leaf. (**B**) Melon PM conidiophores observed using a digital microscope (KH-2700 DM). The conidiophores have normal catenate conidia, forming chains. Bar: 60 µm.

3. *Ampelomyces*, a Genus in Need of Taxonomic Revision

Ampelomyces quisqualis was first described from the PM fungus *Erysiphe necator* [17]. However, de Bary [39] used the name *Cicinnobolus cesatii* for the same fungus instead, and the latter became the most commonly used name until the 1970s. However, based on priority, the former name is taxonomically correct [40]. Based on the presumed, but experimentally unproven specialization to host fungi, or based on the host plant of PM infected with *Ampelomyces*, a number of different species were described in the genus. There are more than 40 formally valid descriptions of *Ampelomyces* species in the literature (see [41]). However, as it seems there is no narrow host specificity (see below) in the genus, in recent decades, the name *A. quisqualis* was used for the fungus, hinting that the *Ampelomyces* genus would be monotypic. However, considerable genetic variation characterizes the genus *Ampelomyces*. Between each genetic group, the sequence difference in the ribosomal DNA internal transcribed spacer (rDNA-ITS) region may be as high as 19% [41,42], with even greater variability in actin (*ACT*) sequences [43]. Although lineages that can be separated in the genus are presumably separate species [42,44,45], formally described species do not correspond to the phylogenetic groupings obtained on the basis of DNA sequences. In addition, for some proposed *Ampelomyces* species there are several published names, and a taxonomic revision of the genus is accordingly necessary [42,46]. However, a polyphasic, comprehensive analysis based on colony morphology, micromorphology, and phylogeny is yet to be conducted. In this work, therefore, we do not use the formally existing species names, but rather the terms *Ampelomyces* species or *Ampelomyces* strains, as recommended [47].

4. *Ampelomyces* as an Ecofriendly Biocontrol Agent against PM

Ampelomyces strains were reported in association with more than 65 species (eight genera) of Erysiphaceae from across the world [12,21,22,48–61]. The interactions between mycoparasitic fungi and their mycohosts take place exclusively on the surfaces of aerial plant organs [21,24,62].

In the 19th century, mycologists clearly recognized that some fungi were parasites of PM (e.g., *A. quisqualis* Cesati ex Schlechtend. [17]; *Byssocystis textilis* Riess [63]; and *Cicinnobolus cesatii* [39]). The first study of *A. quisqualis* was carried out by De Bary [39], who identified the fungus as an intracellular parasite of Erysiphaceae. De Bary [39] also showed that *Ampelomyces* hyphae grow within the mycelia of PM, spreading from cell to cell through septal pores, with pycnidia produced in one or two cells of the hyphae, conidiophores, and conidia of their mycohosts. Emmons [49] later conducted an extensive cytological study, describing in detail the penetration, growth, and sporulation of *Ampelomyces* in the ascomata of PM. Shortly thereafter, Yarwood [64] described the treatment of PM-infected plants using a conidial suspension of *Ampelomyces*, the first experiment demonstrating the biocontrol of a plant pathogenic fungus. Since then, hyperparasitic fungi of the genus *Ampelomyces* began to be applied as BCAs against PM fungi in various crops worldwide [9,10,23,44,46,65,66], thus demonstrating their utility as an ecofriendly method of PM disease management.

There is little on the physiological, biochemical, and molecular interactions between *Ampelomyces* strains and their host fungi; therefore, overall, little is known about the molecular mechanism of the mycoparasitism exerted by *Ampelomyces* [65,67]. A few studies reported that enzymatic, and also mechanical processes play a role during penetration into PM structures. Appressorium-like structures were observed at the penetration sites [68]. Five to ten days later, the mycoparasite degrades the cytoplasm of the host [49,69]. This suggests that the interaction becomes necrotrophic at a later stage. The activity of several hydrolytic enzymes (such as chitobiases, proteases [70], β-glucosidase, β-N-acetylglucosaminidase, acid phosphatase, ribonuclease, β-1,3-glucanase, and α-1,4-glucanase [71,72]) was demonstrated in *Ampelomyces* strains. It was suggested [72] that *Ampelomyces* probably interferes with the energy metabolism and protein and cell wall synthesis of the host. Based on transcription studies, several other genes are differentially expressed during mycoparasitism, including lipases, oxygenases, and peptidases [65]. In addition to enzymatic

processes, another underexplored mechanism, toxin biosynthesis, may also take place during mycoparasitism [65].

5. Morphological and Molecular Analyses, and the Identification of *Ampelomyces* Strains

Based on morphological and molecular phylogenetic analyses, Németh et al. [61] recently identified hyperparasites isolated from different PM species in Japan as *Ampelomyces* Cesati ex Schlechtend. Spores of Japanese hyperparasitic strains were produced in pycnidia, which develop intracellularly in the mycelia of PM fungi. The spores are unicellular, hyaline, ellipsoid–ovoid to doliiform (size range: 5.7–9.2 × 2.6–5.0 µm), mostly guttulate, and embedded in a mucilaginous matrix within swollen ampulliform or pyriform pycnidia [61]. The spores germinate *ca*. 15–20 h after their inoculation, forming elongated hyphae that branch under conditions of high relative humidity (RH). Hyphae formed from the spores reach a length of 6.2–78.2 µm 48 h after inoculation. Fungal colonies slowly and concentrically spread after the inoculation of a single mature pycnidium in the centre of Czapek–Dox agar medium supplemented with 2% malt extract. The colony area reaches 148.4–391.3 mm^2 at 20 days post-inoculation (dpi). Isolates significantly differ in their germination rate and hyphal length, but not in their colony area. The strains grow slowly, with an *in vitro* radial growth rate of 0.5–1.0 mm day^{-1}. Thus, the morphological and physiological characteristics of the Japanese strains clearly resemble those of *A. quisqualis* isolates [41,59,60,69,73].

As noted above, molecular analyses based on the rDNA-ITS region and *ACT* fragment revealed considerable genetic diversity among *Ampelomyces* strains [42–45,62,74,75]. Using sequences from these two loci, Németh et al. [61] confirmed the existence of at least five different phylogenetic lineages within the genus *Ampelomyces*, and showed that the newly isolated Japanese strains belong to three major clades. The authors analyzed the phenotypic characteristics of *Ampelomyces* strains isolated from four different PM samples, and four different strains isolated from the same PM sample. There were no morphological characteristics that could clearly be associated with a given genotype or clade. The four strains isolated from the same PM sample, however, differed significantly in their measured hyphal lengths, germination rates, and the number of spores that developed in single pycnidia, as well as strong evidence of strain-level differences, as reported in other studies [46,70]. Whether the differences in the phenotypic characteristics of different strains of *Ampelomyces* are related to an as-yet unrevealed genetic diversity or are simply caused by phenotypic plasticity is currently unknown. The possibility of strain-level differences, however, needs to be considered in studies aimed at the development of *Ampelomyces* as BCAs.

6. *Ampelomyces* Strains May Be Associated with, but Are Not Specific to, Their Host PM Species

The specificity of *Ampelomyces* was investigated using two fundamentally different approaches: by isolating *Ampelomyces* from a diverse range of PM fungi and then investigating possible associations between the interacting partners and via cross-inoculation experiments.

"Some degree of mycohost specialization" and "evidence for narrow host specialization" were reported for *Ampelomyces* based on the genetic clustering of strains according to the mycohost [44,75,76]. However, other studies that employed a similar methodology obtained different results. Several *Ampelomyces* strains, all isolated from grapevine PM naturally infected by *Ampelomyces*, belong to four different genetic clades [43]. After a similar sampling, *Ampelomyces* strains isolated solely from *Arthrocladiella mougeotii* were assigned to three different clades [77]. These studies suggest that *Ampelomyces* strains isolated from a given PM fungal species can belong to genetically different groups, and isolates from different host fungi can belong to the same genetic group [41–44,47,77,78]. Taken together, these results support the lack of host specificity of *Ampelomyces*.

Host specificity was also experimentally investigated in other studies. In cross-inoculation experiments carried out by De Bary [39], *Ampelomyces* mycoparasites col-

lected from a given PM species were shown to also produce intracellular pycnidia in the mycelia of other PM species. In other cross-inoculation experiments, including *in vitro* studies [42,70,74,79] and field experiments [46,77,80] involving different *Ampelomyces* strains and several PM species, these mycoparasitic strains did not show strict host specificity; instead, they were capable of infecting many host species irrespective of the original host, producing intracellular pycnidia in the mycelia of other species of Erysiphaceae [39,42,46,47,70,74,77,79–81]. Following inoculation tests with Japanese isolates and five PM species, Németh et al. [61] observed the degeneration and constriction of parasitized hyphae of all five PM species tested, as well as pycnidial formation in the hyphae and conidiophores of four PM fungi. These results show that Japanese *Ampelomyces* strains can infect PM hyphae irrespective of the original host, as they produced intracellular pycnidia in the mycelia of four out of the five tested mycohosts. Additional experiments showed that *Ampelomyces* strains from apple PM naturally infect *Golovinomyces orontii* (s. l.), the tobacco PM fungus, and *P. xanthii* causing cucumber PM [77]. These results and those of several other studies [39,70,74,77,82] support the lack of host specificity with the tested *Ampelomyces* strains.

Seemingly contradictory results were obtained with *B. graminis*. A previous study reported the lack of pycnidial production of a strain isolated from *E. artemisiae* in *B. graminis* on barley [60], which is similar to the findings of Németh et al. [61]. Other studies reported typical mycoparasitism, including the formation of intracellular pycnidia, in *B. graminis* conidiophores on cereals (wheat and barley) by *Ampelomyces* strains isolated from PM infecting dicots [59,62,79,83,84]. The contradictory results might be due to unfavourable experimental conditions, as described by Kiss [10], and not to the inability of *Ampelomyces* strains to infect *B. graminis*. It should be noted, however, that *Ampelomyces* strains seem to parasitize PM fungi less commonly, such as *B. graminis* infecting monocot plants, than PM species on dicotyledonous plants [59].

However, even in the absence of a strict host association between *Ampelomyces* and PM fungi, i.e., no species specificity, qualitative differences between *Ampelomyces* strains in their ability to infect different PM fungi cannot be ruled out. In a previous study, *Ampelomyces* mycoparasites formed more pycnidia in colonies of the original host than in those of other PM fungi [82]. In other studies, the opposite was observed, namely that *Ampelomyces* strains isolated from different PM species were similarly capable of parasitizing colonies of other PM species, regardless of the original host, both *in vitro* and in field experiments [46,74,77]. In their mycoparasitic tests with Japanese *Ampelomyces* strains, Németh et al. [61] used five PM species maintained in the greenhouse: *B. graminis* f. sp. *hordei* race 1 KBP-01 (on barley *Hordeum vulgare* L. cv. 'Kobinkatagi'), *E. neolycopersici* (=*Pseudoidium neolycopersici*) KTP-03 (on tomato *Solanum lycopersicum* Mill. cv. 'Moneymaker'), *E. trifoliorum* KRCP-4N (on red clover *Trifolium pratense* L., cv. 'Megium'), *P. aphanis* KSP-7N (on strawberry *Fragaria* × *ananassa* Duchesne cv. 'Sagahonoka'), and *P. xanthii* KMP-6N (on melon *Cucumis melo* L., cv. 'Earl's Favourite'). Then, PM-infected plants were spray-inoculated with spore suspensions and then the mycoparasitic activity was scored. Japanese *Ampelomyces* strains successfully infected all five PM isolates and formed mature pycnidia in four out of five mycohost colonies (*E. trifoliorum*, *E. neolycopersici*, *P. aphanis,* and *P. xanthii*). The tested strains infected melon PM more heavily than the other hosts, as reflected by the formation of a larger number of pycnidia at 14 dpi. However, there were no significant differences in the mycoparasitic activity of the eight Japanese *Ampelomyces* strains based on three-level scoring.

Understanding host specificity is complicated by the existence of strain-level differences between *Ampelomyces* strains, as in laboratory experiments, strong differences in the mycoparasitic ability of different *Ampelomyces* strains were observed [46,70], including with respect to the PM species [70]. However, in general, the most effective *Ampelomyces* strains are very effective not only against the original host, but also against other PM species [46,70]. Those observations imply that the degree of mycoparasitism does not depend on the original host fungus [46], nor is it a general characteristic of individual genetic clades; rather, it reflects differences at the strain level. Indeed, the contradictory

results obtained in experimental work might be partially explained by differences at the strain level.

A summary of the available data leads to the conclusion that they do not contradict the possibility of a "certain degree of host specialization" [44] among these mycoparasites. However, as this conclusion conflicts with the experimental evidence, strict (exclusive) host specialization can in fact be ruled out, and it instead suggests structural specificity [85], defined as the ability of a given parasite to parasitize different host fungi but in different proportions or with different abundances depending on the host [85]. This holds true for *Ampelomyces*. Structural specificity can also result in an apparent association with host fungi without implying a narrow host specialization. This is well demonstrated by *Ampelomyces* strains associated with the causal agent of apple PM (*P. leucotricha*): while these strains are mostly found in *P. leucotricha*, they easily colonize other mycohosts as well [74,77].

From a practical point of view, the lack of strict host specificity [24] allows a single *Ampelomyces* strain to be applied as a BCA against a wide range of PM species. Several studies demonstrated the biocontrol potential of *Ampelomyces* species against PM on various crops, such as *E. trifoliorum* on red clover [64], *P. leucotricha* on apple [84,86], *P. xanthii* on cucumber [19,20,66,76,79,86–92] and melon [93–95], *E. necator* on grapevine [22,76,82,92,96,97], *B. hordei* on barley [59,83], and *B. graminis* on wheat [83], and several other species as well [64,76,79,86,89,98–102].

7. Latest Results on *Ampelomyces*—PM Interaction

7.1. Methodological Considerations of Spray Inoculation of Ampelomyces Spores onto PM Colonies

The effective control of PM using mycoparasitic strains requires a method for inoculating hyperparasite spores onto PM fungal colonies. *Ampelomyces* is usually spray-inoculated onto PM-infected plants as a spore suspension, with the applications repeated several times during the season to ensure a high level of control [79,103]. Gu and Ko [104] reported that the concentration of hyperparasite spores is an important factor affecting their germination and infection in pathogens, as spore germination decreases rapidly at spore concentrations $>10^6$ spores mL^{-1}, due to the production of self-inhibitory compounds. In our spray inoculation system, spore suspensions of *Ampelomyces* are diluted to 5×10^5 spores mL^{-1}, and polyoxyethylene sorbitan monolaurate (Tween 20) is added to a final concentration of 0.05%. With this method, spores of *Ampelomyces* germinate successfully 15–20 h after spray inoculation onto PM-inoculated plant leaves at high RH [61,95].

7.2. Infection Processes of Ampelomyces Strains in PM Fungi

In Németh et al. [62], *A. quisqualis* transformants expressing an integrated green fluorescent protein (GFP) gene could be visualized in PM fungi and PM-infected leaves, which allowed for the localization of mycoparasitic fungi in PM hyphae. The method described by Suzuki et al. [105] was used to visualize tri-trophic interactions among mycoparasites, mycohosts, and plant cells. Further insights into mycoparasitism, including direct observations of the infection process of *Ampelomyces* strains, were obtained in real-time using high-fidelity digital microscopy (KH-2700 DM; Hirox, Tokyo, Japan) to monitor mycoparasite–mycohost interactions and thus determine how and when mycoparasites invade PM structures. The infection process of *Ampelomyces* strains in tomato PM *E. neolycopersici* on leaf type I trichomes of common tomato (*S. lycopersicum* Mill. cv. 'Moneymaker') and in melon PM colonies was also observed using digital microscopy (KH-2700 DM). Németh et al. [61] visually followed the infection of tomato PM colonies and subsequent conidiogenesis of an *Ampelomyces* strain. Foot cells and generative cells (GCs) of PM conidiophores began to atrophy at 5–6 dpi, with the formation of intracellular pycnidia of the hyperparasite strain initiated in basal cells of the conidiophores at 6–8 dpi, followed by the complete collapse of the conidiophores at 10–14 dpi. Kimura et al. [95] observed the degeneration and constriction of hyphae in melon PM *P. xanthii* prior to intracellular pycnidial formation in the hyphae (ex. conidiophores). Infection and conidiogenesis by the tested hyperparasitic *Ampelomyces* strain were very similar in melon PM fungus and in

tomato PM fungus, as reported by Németh et al. [61]. Interestingly, almost all intracellular pycnidia were produced in conidiophores of the mycohost.

Based on earlier work and our detailed microscopic analysis, the approximate time course of infection, the events that take place in the mycoparasites once they entered the mycohosts (PM fungi), and the morphological changes in a mycoparasite-infected mycohost can be summarized as follows: *Ampelomyces* hyphae within parasitized PM conidia are spread by wind [20,106,107] and spores are dispersed, e.g., by rain splash [68,108]. The processes that, after spore germination, allow *Ampelomyces* to penetrate and parasitize hyphae of PM fungi may be mechanical [68] or enzymatic [70,71]. Penetration of mycohost structures by the hyperparasite *Ampelomyces* can occur within 24 h [68,93]. The mycoparasite hyphae continue their growth in PM structures, extending from cell to cell through the septal pores, and further ramifying throughout the mycohost hyphae [68,69]. The mycohost invasion by *Ampelomyces* leads to atrophy in 5–6 dpi and then to complete disruption of the mycohost conidiophores at 7 dpi. Disruption of the cytoplasm of the fungal hosts causes the reduced growth and eventually the death of the host fungus [22,69,82]. During the course of infection, *Ampelomyces* produces intracellular pycnidia in the hyphae or conidiophores of their mycohosts at 5–10 dpi [19,68,81]. In contrast to other pycnidial mycoparasites, such as *Coniothyrium minitans* Campbell [109–111], toxin production by *Ampelomyces* was not detected [112].

7.3. Pycnidial Development of Ampelomyces Strains in PM Fungi

Spores of *Ampelomyces* strains are produced in pycnidia that develop intracellularly in the mycelia of PM fungi [92]. The pycnidia of *Ampelomyces* are formed ubiquitously in PM colonies (Figure 2A), with a change in colour from pale yellow (immature) to black (mature) over time [95]. The number of pycnidia of *Ampelomyces* per melon PM colony was shown to increase with the age of the PM colony [95]. Mature pycnidia have a size range of 40.2–84.2 × 22.6–48.1 µm, and a single mature pycnidium produces 199.4–1492.7 spores by 14 dpi [61]. Both the number of spores developed in a single pycnidium and the sizes of pycnidia among strains can significantly differ [61].

Detailed observations on pycnidial development were obtained using tomato and melon PM colonies infected with *Ampelomyces* strains following spray inoculation [61,95]. Almost all pycnidia were produced in conidiophores of the mycohost (Figure 2B). Following infection of tomato PM fungi, the first signs of atrophy were seen in foot cells and GCs of the conidiophores (normal noncatenate conidia) at 5–6 dpi. Intracellular pycnidia of *Ampelomyces* were initially produced mostly in the basal cells of the conidiophores at 6–8 dpi, during which time *Ampelomyces* hyphae and pycnidia continued to elongate in the host hyphae. The conidiophores completely collapsed at 10–14 dpi. In melon PM fungus, intracellular pycnidia of *Ampelomyces* initiated within GCs of the conidiophores (normal catenate conidia, forming chains) at 6–8 dpi. Single conidia formed at the top of the conidiophores and began to atrophy at 7–9 dpi, with complete atrophy at 10–11 dpi and complete collapse of the conidiophores at 11–12 dpi. PM hyphae containing conidiophores on melon leaves also underwent complete collapse. Melon PM colonies were therefore unable to scatter their asexual progeny conidia from the conidiophores. Pycnidia of *Ampelomyces* matured within 12–14 days. In the presence of water, *Ampelomyces* spores were released from intracellular pycnidia by the rupture of both the pycnidial and the PM cell walls (Figure 2C). The released mature spores served as sources of subsequent infections for PM hyphae.

7.4. Quantitative Analysis of PM Conidia Released from Ampelomyces-Parasitized PM Colonies under Greenhouse Conditions

In the natural environment, the asexual conidia produced by PM fungi on conidiophores (Figure 1B) are dispersed by wind over large areas and are the source of host plant infection [113–116]. *Ampelomyces* mycoparasites suppress both asexual and sexual sporulation of the attacked PM mycelia by colonizing and destroying conidiophores [24].

Philipp et al. [88] observed that parasitized PM colonies can continue their radial growth, but their sporulation is stopped soon after *Ampelomyces* penetrates their mycelia. Similarly, Shishkoff, and McGrath [91] showed that *Ampelomyces* could not prevent the spread of PM colonies *in vitro*, but the parasite caused a reduction in the inoculum produced by each colony. In addition, if *Ampelomyces* is to be used as a BCA, its growth and spread must outpace that of PM fungi (mycohosts). The conidiation rate of PM colonies depends on several factors, including the inoculum density, physiological patterns of the host plant, and abiotic factors [29,117,118]. Intense conidiation and spread of PM fungi will prevent their successful control by *Ampelomyces* mycoparasites applied as BCAs. In these cases, the effect of *Ampelomyces* will be limited to a reduction in disease severity and a milder impact of the PM fungus on the infected plants.

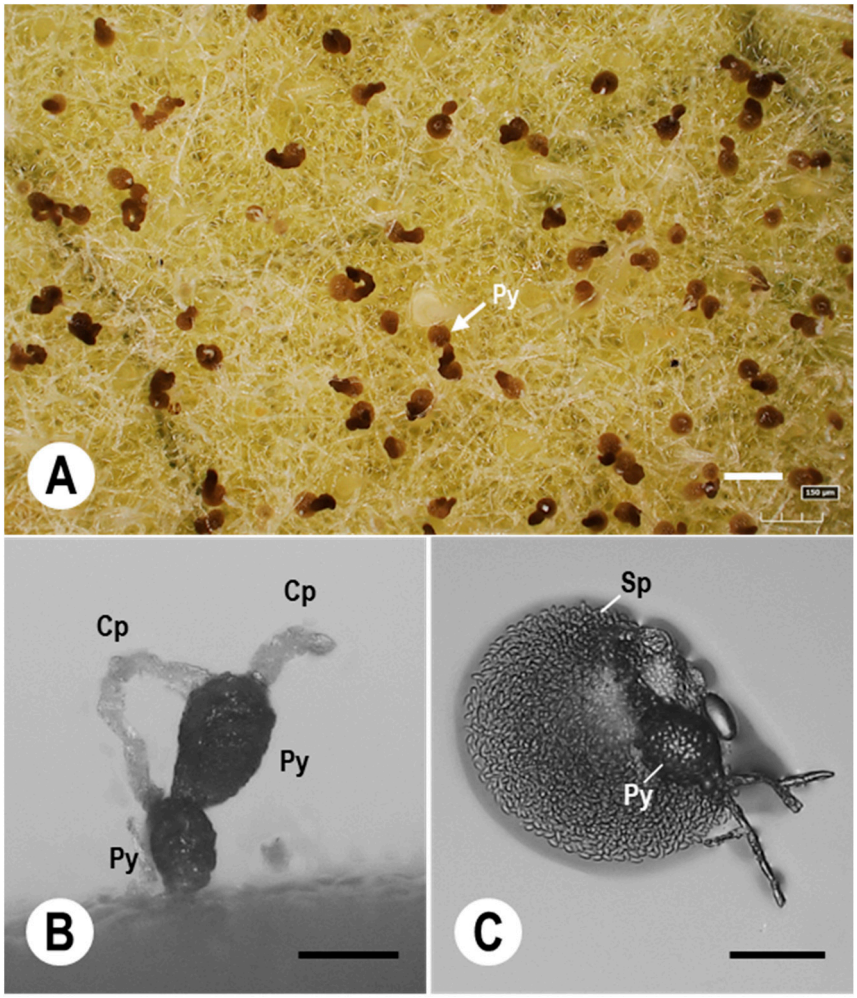

Figure 2. Digital micrographs of *P. xanthii* colonies and conidiophores on melon leaves spray-inoculated with spores of a Japanese *Ampelomyces* strain. (**A**) Digital microscopy images of pycnidia (Py) of an *Ampelomyces* strain cultivated in plastic boxes at 70–80% relative humidity (RH) and under growth chamber conditions; the images were taken at 10 days post-inoculation (dpi) of 15-day-old melon PM colonies. (**B**) Pycnidia (Py) of the *Ampelomyces* strain that developed in melon PM conidiophores (Cp). The pycnidia were successfully produced in generative cells of the conidiophores at high RH (70–80%). (**C**) *Ampelomyces* spores released from a pycnidium after treatment with a 10 µL drop of distilled water. Mature pycnidia (Py) released abundant progeny spores (Sp). Bars: 150 µm (**A**,**B**), and 60 µm (**C**).

Recent methodological advances and the use of an electrostatic spore collector system facilitated the quantification of conidial release from PM colonies. In a study in which an electrostatic rotational spore collector consisting of a dielectrically polarized insulator

drum was used for this purpose, a single melon and strawberry PM colony maintained under greenhouse conditions was found to release an average of 12.6×10^4 conidia and 6.7×10^4 conidia throughout its lifespan, respectively [119,120]. The collection device had no detrimental effect on the survival of the fungus; the electrostatically collected conidia produced normally elongated hyphae and formed conidiophores that produce living progeny conidia [119,120].

The same methodology was employed to study *Ampelomyces*–PM interactions. Using electrostatic and digital microscopy techniques, Kimura et al. [95] estimated the control effects and infection efficiency of an *Ampelomyces* strain against a melon PM fungus. The aim of their study was to determine whether asexual PM progeny conidia, which are a source of host plant infection, are released from *Ampelomyces*-inoculated melon PM colonies at different developmental stages (5-, 10-, and 15-day-old colonies). The authors found that the number of conidia released from 5- to 10-day-old melon PM colonies after spray inoculation with mycoparasite spores decreased gradually, with no release by 3–5 dpi and 4–11 dpi, respectively. Thus, conidial release from melon PM colonies was suppressed completely by the application of an *Ampelomyces* strain under greenhouse conditions. However, the complete prevention of progeny conidial release from 15-day-old melon PM colonies required two spray inoculations with the mycoparasite; a single treatment was insufficient. In response to a single inoculation, 5-, 10-, and 15-day-old melon PM colonies ceased their expansion at 4, 5, and 2 dpi, respectively. After electrostatic spore collection, conidiophores in uninoculated melon PM colonies had a normal morphology, forming conidial chains under greenhouse conditions, whereas conidiophores and hyphae in inoculated melon PM colonies either atrophied or collapsed, with a clear decrease in the number of normal conidiophores. There were no normal melon PM conidiophores per single 5- and 10-day-old melon PM colonies following a single spray inoculation of hyperparasite spores, unlike in 15-day-old colonies. Based on these results, for successful disease control, PM colonies should be spray-inoculated with hyperparasitic fungal spores during early developmental stages (e.g., when the colonies are 5–10 days old, or as soon as PM is detected on host leaves). If older colonies (e.g., >15 days old) are spray-inoculated with mycoparasite spores, a few normal conidiophores will persist due to suboptimal control by *Ampelomyces* strains, allowing PM fungi to scatter progeny conidia from the colonies. During that period, some of the conidiophores of the invaded mycelium will continue to produce fresh conidia, although they might already be infected, and will thus contain intracellular hyphae of *Ampelomyces* [107].

8. Practical Application of *Ampelomyces* Strains as Biocontrol Agents of PM

Yarwood [64] first showed the potential of *Ampelomyces* as a BCA by demonstrating the control of clover PM (*E. polygoni*) in a basic experiment that reproduced the events of a natural epidemic recorded in the previous year. On the other hand, there was also a problem with the emergence of fungicide resistance to chemical control agents. Therefore, from the 1970s, interest in the biological controls of PMs increased. Kiss [121] determined that potential BCAs need to be active in the phyllosphere because PM fungi are biotrophic pathogens infecting the aerial parts of their host plants. The first significant trial of *Ampelomyces* was reported by Jarvis and Slingsby [19], who used conidial suspensions of the mycoparasite to successfully control cucumber PM in greenhouse trials. Control was enhanced when application was interspersed with water sprays. The many other positive examples in which *Ampelomyces* was subsequently used to control PM on several crops paved the way for commercialization [24,66,96,97,122]. In addition, Sundheim and Tronsmo [123] recommended *Ampelomyces*-based fungal biocontrol products in plant protection practice as they can be used without any risk to human health. The absence of nontarget effects of *Ampelomyces* biocontrol procedures was reported as well [62,124].

Ampelomyces-based BCAs can also be applied prophylactically [125–127], as the mycoparasite can survive on leaves without immediate contact with the targeted PM fungus, as demonstrated experimentally [62].

The most successful biocontrol experiments using *Ampelomyces* were carried out in greenhouses, under high RH [19,20,103], and in the field, where free water was frequently available on the treated leaves [79,82]. However, the efficacy of *Ampelomyces* was shown to decrease rapidly at a RH < 85–90% [125,126] or <90–95% [88,128,129]. This high RH requirement of *Ampelomyces* is a major obstacle to its use as a reliable BCA. Our tested *Ampelomyces* strain also did not produce intracellular pycnidia in PM hyphae under greenhouse conditions that included a low RH, but it did produce them in PM hyphae in growth chambers with a high RH [95]. These results, as well as those of previous studies [19,20,22,24,61,81,82], demonstrate the importance of high-RH conditions for hyperparasitic *Ampelomyces* strains to produce intracellular pycnidia in mycohost hyphae. The inability to form pycnidia may explain the suboptimal biocontrol of *Ampelomyces* at low RH.

The high RH requirement of *Ampelomyces* must be addressed before the mycoparasite can be used in biocontrol [125,129]. In attempts to enhance the efficacy of *Ampelomyces* at lower RHs, Epton and Hamed El Nil [130] selected isolates that were able to germinate at a higher vapor deficit than wild types. In an alternative approach, a number of additives, such as an emulsion of 1% paraffin oil [128], a 0.3% mineral oil surfactant [97], 0.5% Tween 20 [46], or 0.01% Tween 80 [92], were shown to increase the biocontrol performance of *Ampelomyces* at lower RHs, although some of these additives can control PM directly [14,98,131,132]. To observe the effects of the BCA alone requires the use of surfactants at concentrations that do not affect the development of PM hyphae [61,95].

9. Formulation and Commercialization of *Ampelomyces* as BCAs

The scale-up of *Ampelomyces* inoculum for biocontrol purposes was one of the crucial steps towards its commercialization and practical application in plant protection. Sztejnberg et al. [133] developed and patented (European Patent Office, publ. no. 0353662/1988) a simple, inexpensive method for the production of large amounts of *Ampelomyces* spores in fermenters. The different formulations were tested in various crops, particularly grapevine. An improved product (AQ10^{TM} Biofungicide) registered in 1995 for use in the control of grapevine PM was subsequently also registered for use in other fruits and vegetables in conjunction with the wetting agent (formulated as water-dispersible granules) Add-Q, a spray adjuvant recommended for use together with AQ10 biofungicides [125,126]. However, Shishkoff and McGrath [91] found that in the control of cucumber PM, Add-Q was as effective alone as when combined with AQ10. Therefore, the effect of the additives should be clearly distinguished from that of *Ampelomyces* when assessing the efficacy of a BCA [125].

Other studies likewise showed that the efficacy of biocontrol achieved with commercial anti-PM biofungicide products, including AQ10® (Ecogen Incorporated, Langhome, PA, USA) [97], Q-fect® (Green Biotech, Paju, South Korea), Powderycare® (AgriLife, Medak, India) [10,44,65], and Bio-Dewcon 2.00 WP (India) [66], varies significantly. Some trials reported that *Ampelomyces* treatment was ineffective, others suggested only very limited control of PM [90,91,129,132], others reported suboptimal control [90,134], and still others reported satisfactory results [95,102,135,136], including a level of control comparable to that using conventional fungicides [122]. These contradictions might result from experimental differences, such as humidity [10], differences in the mycoparasitic activities of individual *Ampelomyces* strains [10,70], and/or from physiological differences between genetically similar or uniform strains of *Ampelomyces* [46,70,76].

Recently, the large-scale production of a new strain, CPA-9 was reported [136]. Formulation was also developed, and the efficiency of the formulated product was demonstrated [102].

10. An Ideal Spray Inoculation System for the Effective Use of *Ampelomyces* as a BCA

An efficient inoculation method of *Ampelomyces* spores to PM colonies is needed for *in vitro* experimental studies of the fungus, as well as for its successful use as a BCA. Based on the experimental results and the studies conducted to date, a list of criteria for the

delivery of *Ampelomyces* as a BCA in an optimal spray inoculation system can be compiled as follows: (1) The selected strains should have exceptional mycoparasitic abilities [46,70]. (2) Adding a surfactant as a wetting agent [46,95,97,122,128] increases efficacy. (3) Spraying should be conducted at high RH, such as in the early morning or late afternoon [122]. (4) Alternatively, although technically less feasible, PM colonies can be inoculated with mycoparasite spores at night, when conidiophores do not release progeny conidia, in contrast with during the day, when progeny conidia are actively released [95,119,120]. (5) For effective control, young colonies should be targeted, i.e., as soon as PM fungi are observable on host leaves (e.g., in 5- to 10-day-old colonies), when the leaf incidence of PM is still low (<10%) [95,122]. (6) Efficiency can be further improved by adding interspersed water sprays [19] and by repeated applications [79,95,103]. In demonstration trials, spraying with *Ampelomyces* was as effective as conventional fungicides [122] when conditions were optimized. In addition, because *A. quisqualis* tolerates a number of pesticides applied in plant protection [20,81,87,93,102], it can be included in integrated plant protection programs [102].

11. Conclusions

Ampelomyces strains were demonstrated to be able to suppress PM development. The lack of their strict host species specificity enables the use of *Ampelomyces* strains as a BCA against a wide range of PM fungi. There are, however, problems associated with the taxonomy of the genus, and occasional difficulties with their practical use. Considerable knowledge gaps concerning *Ampelomyces* include the molecular and biochemical processes during mycoparasitism, which are largely understudied. For the study these, genomic [23] and transcriptomic [65] resources are available, and an efficient transformation system [62], as well as a toolbox for gene knockout [67] were developed. These provide a basis for future studies aimed at deciphering the molecular background of mycoparasitism.

On the other hand, there were some recent advances in the study of these mycoparasites, facilitating the use of *Ampelomyces* strains for the effective control of PM fungi. These findings and the experimental results reported in this review lead to the development of an ideal spray inoculation system for the delivery of hyperparasitic fungi to control PM pathogens. The spray inoculation system should aid experimental research on *Ampelomyces*, and also its practical use as a BCA. Due to the recent methodological advancements and newest results on the biology of the fungus, *Ampelomyces* strains have the potential to be used as effective BCAs against PM fungi as an eco-friendly alternative to conventional fungicides.

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