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SHORT COMMUNICATION

Dimethomorph activity and its effect on morphology in different oomycete species of economic and veterinary interest

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1 | INTRODUCTION

The class Oomycota comprises species that are pathogenic to plants or animals, including humans, and are able to cause severe economic losses in agriculture and aquaculture industry worldwide.

Among the 4 oomycete orders (Lagenidiales, Leptomitales, Saprolegniales and Peronosporales), *Phytophthora infestans* (Peronosporales, Peronosporaceae) causes losses in potato and tomato crops for more than 6 billion euros per year, as well as damages to ecosystems. The oomycete *Plasmopara viticola* (Peronosporales, Peronosporaceae) is responsible for downy mildew, which is one of the most important diseases of grapevines worldwide. The genus *Pythium* (Peronosporales, Pythiaceae) includes important plant and animal pathogens: particularly, *Pythium insidiosum* was reported to cause disease in humans and in other mammals (Gaastra et al., 2010). Moreover, *Pythium ultimum* is a plant pathogen, the aetiological agent of damping off and root rot disease in a variety of crop and ornamental species (Martin & Loper, 1999) and *Pythium* spp. are reported to grow on eggs and fry of freshwater fish and prawn (Shah et al., 1977). The majority of oomycetes isolated from either naturally or experimentally infected fish, belong to the order Saprolegniales, while the other 3 oomycete orders are rarely isolated in aquatic animals and are more likely opportunistic rather than parasitic, sometimes in association with other oomycetes (Scott, 1964). Among Saprolegniales, members of the genus *Saprolegnia* (Saprolegniaceae) represent a severe problem in freshwater fish farms, where production losses up to 50% are reported (van West, 2006). The species *Saprolegnia parasitica* is among the main agents of disease in aquaculture, while other species (i.e. *Saprolegnia ferax*) are involved in mass mortality outbreaks in wild fish and amphibians (Scott, 1964). Despite the wide distribution and the impact of oomycetes on economic activities and on animal health, there are few effective available molecules against these agents. Following the current classification of malachite green and formalin among carcinogens, there are limited possibilities to control oomycete infections in aquaculture. The selection of alternative treatments to Saprolegniosis has been the focus of recent research founded by the EU (Tedesco et al., 2020; Tedesco et al., 2019).

Current research needs to focus on the identification of novel targets and alternative treatments against fungal pathogens in humans, animals and plants. To overcome the scarcity of active compounds against animal pathogenic oomycetes we carried out a preliminary study utilizing a fungicide largely used in agriculture to control oomycete diseases.

Dimethomorph (DMM) is a cinnamic acid derivative, a mixture of E and Z isomers, but the fungicidal activity resides exclusively in its Z isomer and it shows specific activity against members of the family Peronosporaceae (Albert et al., 1988). DMM is a member of carboxylic acid amides (CAA) fungicides and was the first CAA introduced in 1988, followed by the other members. The mode of action of CAA is linked to the inhibition of the cellulose biosynthesis targeting enzymes regulated by a family of four genes (CesA). In oomycetes, up to four CesA-encoding genes have been identified in Peronosporales, Pythiales and Saprolegniales (Blum et al., 2012).

Among CAA, DMM is a molecule of particular interest because showed differences in the response towards resistant *P. viticola* strains compared to the other member of CAA (Nanni, 2016; Nanni et al., 2016).

Moreover, the photo transformation of dimethomorph in aqueous solution by combining a kinetic study of substrate degradation was evaluated and, interestingly, the detected intermediates were less toxic (Avetta et al., 2014).

The proximate evolutionary relationship between humans, animals and fungi makes it urgent to find new drugs that kill fungi without having toxic effects on humans and animals.

A transdisciplinary angle could encourage the study of molecules, in this case highly effective towards plant pathogenic oomycetes for their potential application against other oomycete species in different contexts. This kind of approach was explored for the management of gastrointestinal pythiosis in dogs, utilizing the agricultural fungicide mefenoxam, with successful results (Hummel et al., 2011). Recently, Cridge et al. (2020) described using mefenoxam with other therapeutic protocols to treat pythiosis in six dogs, five with gastrointestinal pythiosis and one with cutaneous pythiosis. Furthermore, White et al. (2020) used mefenoxam with other therapies to treat cutaneous paralagenidiosis, caused by *Paralagenidium* sp., in one dog.

The aims of this work were: (I) to test in vitro the activity of DMM on different species of *Saprolegnia* and *Pythium* isolated from fish and aquatic environment and (II) to develop a scanning electron microscopy (SEM) protocol assessing the effect of DMM on hyphal morphology of *S. parasitica* reference strain.

2 | MATERIALS AND METHODS

2.1 | Strains tested

Tests were performed using one reference strain of *S. parasitica* (CBS 223.65 provided by CSIC-RJB) isolated from the northern pike *Esox lucius* in the Netherlands, two field strains of *S. parasitica* and *S. delica* isolated from the brown trout (*Salmo trutta*) and the rainbow trout (*Oncorhynchus mykiss*), respectively, one strain of *Pythium pachycaule* isolated from *S. trutta*, one strain of *Pythium dissimile* isolated from the European chub (*Squalius cephalus*), and two strains of *Pythium aquatile* and *Pythium rhizo-oryzae* isolated from water.

2.2 | In vitro assays on *Saprolegnia* spp. and *Pythium* spp.

Saprolegnia and *Pythium* strains were maintained with periodic subcultures on glucose–yeast (GY) agar medium (5 g D-(+)-glucose, 1 g yeast extract, 12 g agar in 1 L deionized water) supplemented with 6 mg/L of penicillin (P) and 10 mg/L of oxolinic acid (ox) (GY + P + ox) and kept at 18°C (Alderman & Polglase, 1986). For the in vitro trials, subcultures of the strains employed were incubated at 18°C until growth covered the full diameter of the dish (48–72 h). Inocula were obtained from the outer 10 mm of the culture, using a sterile 5-mm-diameter glass cannula. In vitro tests were performed following protocol I according to Alderman (1982) modified as previously described (Tedesco et al., 2019) to determine the minimum inhibitory concentration (MIC). Dimethomorph (Sigma–Aldrich) was diluted and different concentrations were added to sterilized liquid GY agar at a temperature of 49°C to reach working concentrations of 0.1, 1, 5, 10, 50 and 100 mg/L in the medium. Mixtures were then distributed in 6-well plates (Ø 35 mm), in triplicate per each strain and each concentration/negative control. Following overnight solidification, a 5-mm-diameter well was excised in the centre of the agar using a sterile glass cannula. The well was then filled with a standard 5-mm inoculum, culture surface uppermost.

Plates were incubated at 18°C and checked after 24, 48, 72 h and 6 days, determining the colony diameter of the growing mycelium as average of two axes measured at 90° from each other. Mycelial growth was then expressed as mean value of the replicates. MIC was defined as the lowest concentration completely inhibiting the growth of the mycelium after 6 days of incubation. The in vitro test was repeated for *S. parasitica* reference strain (CBS 223.65) at the highest tested concentrations (50 and 100 mg/L) and negative control in 24-well plates (Ø 15.6 mm); after 48 h, samples were prepared for observation in scanning electron microscopy.

2.3 | Scanning electron microscopy

Sample were prepared for SEM following the protocol described in Alves et al. (2013), slightly modified. For *S. parasitica*, 5% glutaraldehyde in water was poured into each well for 24 h to fix the samples. Then, the wells were washed three times with 0.1 M phosphate buffer, pH 7.2, then rinsed three times in distilled water and dehydrated in ethanol series (25%, 50%, 75%, 90%, 100%) for 7 min each. At this point, dehydrated samples were taken out from the wells, placed in filter paper bags and transferred to a critical point dryer (Emitech K850) to complete the drying process with carbon dioxide as transition fluid. *S. parasitica* specimen was then mounted on aluminium stubs with double-stick carbon tape, sputter coated with 5 nm gold using a Emitech K500 coater and observed with a Philips SEM 515 at 15 kV.

2.4 | Statistical analysis

Upon an initial visual inspection of the data, only the DMM treatments applied within 24 h were considered for statistical analysis because of the clear concentration-related effects of DMM on the radial growth of *Saprolegnia* and *Pythium* species. Then, the Kruskal–Wallis test was separately applied to each species followed by the Dunn test, using pairwise comparisons of each treatment with the control group. The relative *p*-values were finally corrected with the Holm procedure. The threshold *p*-value for determining the statistical significance of both the Kruskal–Wallis and the Dunn tests was 0.05.

3 | RESULTS

In this study, both biological assays and SEM observations were presented.

In the in vitro tests for *Saprolegnia* spp. and *Pythium* spp., no MIC could be determined at tested concentrations, although all strains showed overall reduced radial growth at 50 and 100 mg/L of DMM after 24 h of incubation (Figure 1).

A statistically significant difference in radial growth at 50 mg/L of DMM was only found between the control group and *S. parasitica* ($\chi^2 = 33.36$, adjusted p-value = 0.002), *P. rhizo-oryzae* ($\chi^2 = 17.94$, adjusted p-value = 0.03) and *P. dissimile* ($\chi^2 = 16.15$, adjusted p-value = 0.02). On the other side, a statistically significant difference in radial growth at 100 mg/L of DMM was found between the control group and *S. parasitica* ($\chi^2 = 33.36$, adjusted p-value = $9.3^{*}10^{-6}$), *P. rhizo-oryzae* ($\chi^2 = 17.94$, adjusted p-value = 0.007), *P. aquatile* ($\chi^2 = 14.65$, adjusted p-value = 0.006), *P. dissimile* ($\chi^2 = 16.15$, adjusted p-value = 0.004) and *P. pachycaule* ($\chi^2 = 14.15$, adjusted p-value = 0.01). Therefore, the highest concentration of DMM was more effective at reducing the radial growth of the species tested.

After 6 days, growth of aerial mycelium of *S. parasitica* was inhibited at 50 mg/L (data not shown) while growth of aerial mycelium of *S. delica* was inhibited at 100 mg/L (Figure 2) with respect to the control (K).

In the scanning electron microscopy test *S. parasitica* control culture after 48 h of growth, showed normal mycelium development, with smooth, elongated, branched, cylindrical hyphae forming intricate aggregates (Figure 4a). In the treated samples, mycelial structure has undergone several morphological changes, with an unusual pattern of hyphal growth: the mycelium was more rarefied and shrivelled, and collapsed hyphae were observed (Figure 4b). These changes were more evident at higher concentration of DMM (Figure 4c). At 100 mg/L, in addition to alterations of the hyphae, the presence of gemmae was observed. The gemmae appeared as swelling of the hyphal tips and varied in shape and size (rounded, ovoid or irregular) with wrinkled walls.

4 | DISCUSSION

Members of the class Oomycota encompassing pathogens to plants and animals, including humans, are widespread and able to cause severe economic losses in agriculture and aquaculture; therefore, the identification of alternative and effective treatments for these pathogens is a priority.

The scarcity of molecules available against animal pathogenic oomycetes prompted us to investigate the activity of a fungicide largely used in agriculture to control oomycete diseases.

It is known that fungicides of the class CAA, which includes DMM, inhibit cellulose biosynthesis in the oomycetes by targeting the CesA3 enzyme, involved in cell wall production, given that DMM should be able to inhibit also animal pathogenic oomycetes.

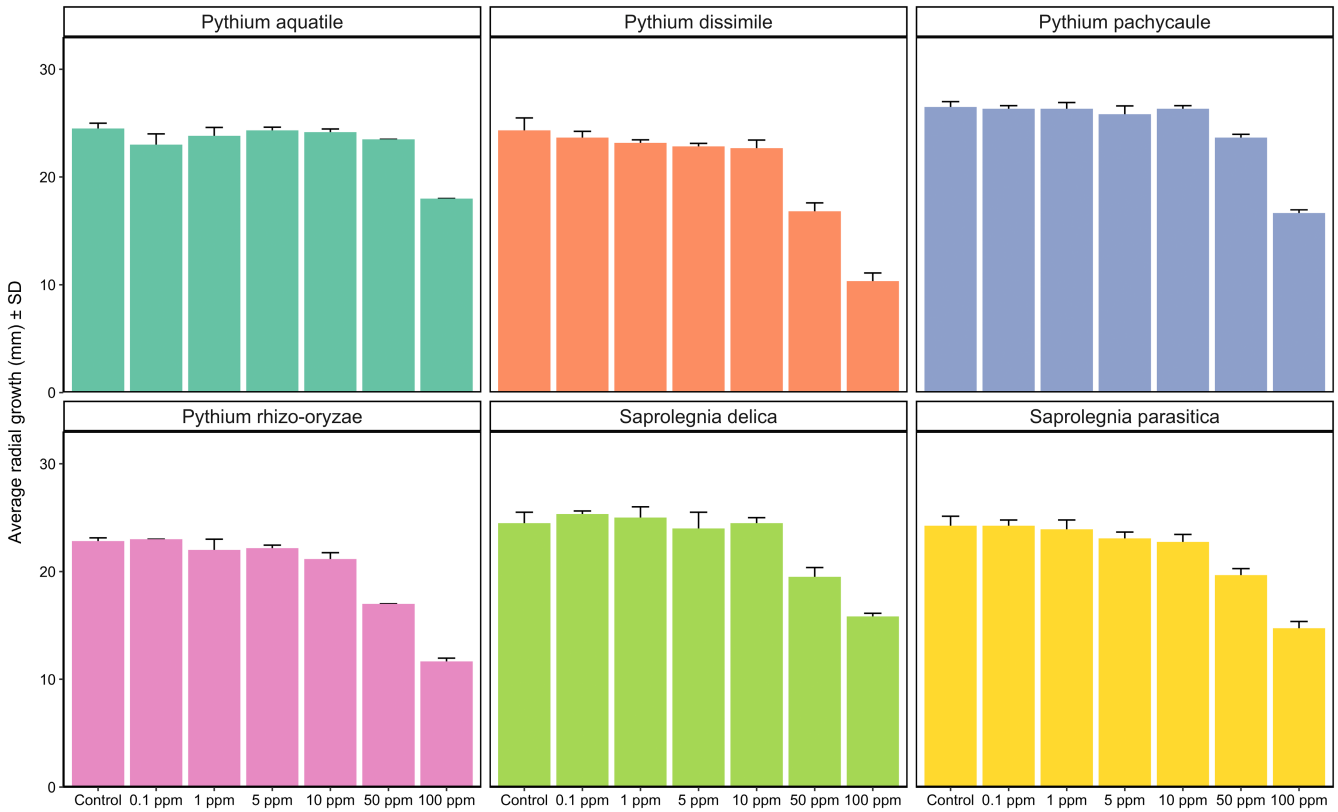

FIGURE 1 Average of radial growth (mm) ± standard deviation (SD) of *Saprolegnia* spp. and *Pythium* after 24 h.

In the present work, we tested the activity in vitro of DMM on different species of *Saprolegnia* and *Pythium* isolated from fish and aquatic environments. Although it was not possible to observe a MIC at 6 days of incubation, the product slowed down mycelial growth of all the tested strains at the highest concentrations (50 and 100 mg/L); moreover, an inhibitory effect on the aerial mycelium was observed at 50 mg/L for *S. parasitica* and at 100 mg/L for *S. delica*. The inhibitory effect on aerial mycelium of *Saprolegnia* was also observed during some in vitro tests with other chemicals (Tedesco et al., 2020) and in previous work (Tedesco et al., 2019) where this effect was hypothesized to be caused by chemically induced morphological changes in the hyphae.

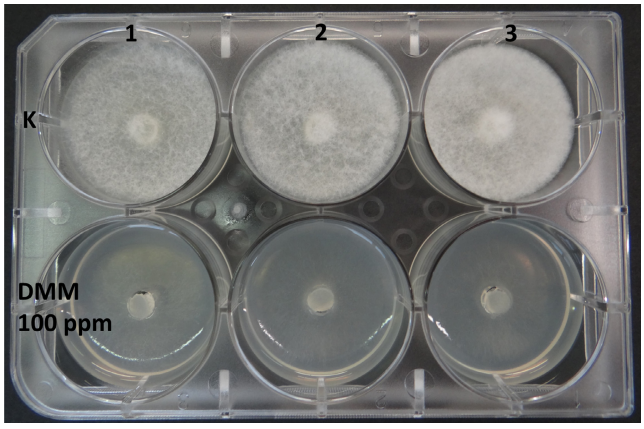

FIGURE 2 *S. delica* after 6 days of incubation with DMM at 100 mg/L. With respect to the *Pythium* strains, *P. dissimile* and *P. rhizo-oryzae* showed the highest sensitivity, and particularly with 100 mg/L DMM, radial growth after 6 days had not reached the diameter of the control (Figure 3).

DMM is a molecule of particular interest because was able to control resistant *P. viticola* strains differently, compared to the other members of CAA.

In the present study, the lowest concentration considered for SEM analysis was 50 mg/L, since it was the minimum concentration able to reduce the radial growth of *S. parasitica*.

Scanning electron microscopy represents a useful tool to examine morphological changes produced after fungicide treatment. Here, the observation with SEM allowed a high-magnification study of hyphal morphology of *S. parasitica*. Particularly, SEM analysis showed that DMM causes inhibition of hyphal growth and defects in hyphal morphology.

With respect to *Saprolegnia*, morphological alteration due to chemicals was reported in the past by Kaminskyj and Heath (1992). Another work describes alterations in hyphal morphology following treatment with two different chitosans, which produced various degrees of damage in the cell wall, plasma membrane and internal organelles (Muzzarelli et al., 2001).

In our study, changes in the production and morphology of *Saprolegnia* hyphae are likely due to alterations in the formation of the cell wall. In addition to hyphal alterations, we observed the production of 'gemmae' at a concentration of 100 mg/L. Gemmae, or Chlamydospores, are distended densely cytoplasmic regions sometimes delimited from parent hyphae, single or in a catenulate fashion, their function is sometimes obscure. The observed production of gemmae in *Saprolegnia* may be attributed to the occurrence of unfavourable conditions, as already hypothesized in previous work (Ali, 2009). In true fungi, Chlamydospores are considered as forms of resistance, able to survive for a long time with unfavourable conditions (Badillet et al., 1987). Ali (2009) observed the production of gemmae following exposure to non-lethal

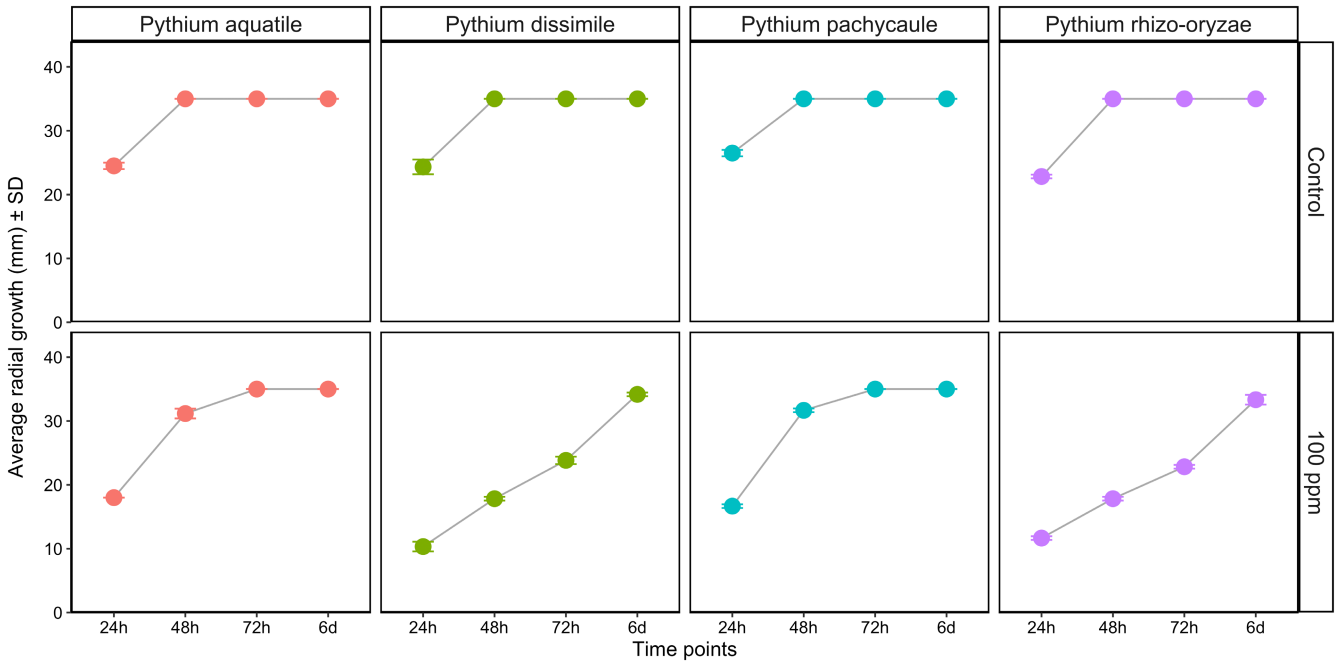

FIGURE 3 Average of radial growth (mm) ± standard deviation (SD) of *Pythium* species at 100 mg/L at different time points.

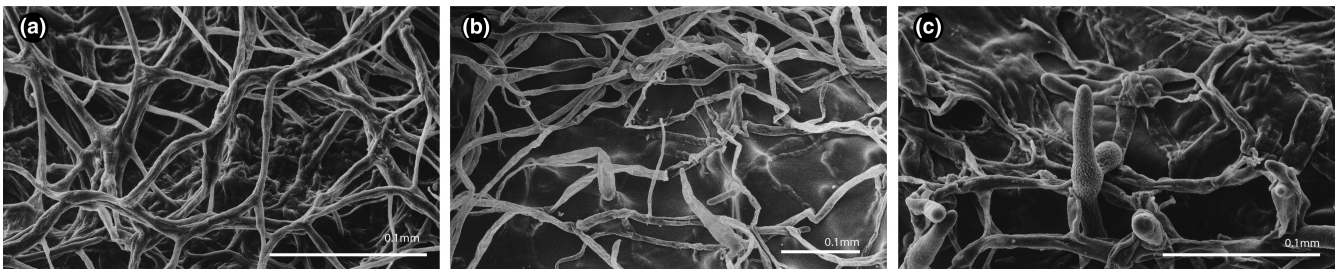

FIGURE 4 SEM observations of *Saprolegnia parasitica*: (a) control; (b) 50 mg/L; (c) 100 mg/L.

concentrations of NaCl. Therefore, we cannot exclude that at a concentration of 100 mg/L of DMM, which does not completely inhibit *Saprolegnia* responds to the fungicide producing these resistant structures until environmental conditions become favourable again. The methodology adopted in the present work allowed to process the mycelium of *Saprolegnia* for SEM without damaging the mycelium integrity. *Saprolegnia* is characterized by the presence of coenocytic hyphae which normally collapse when the mycelium is cut. For this reason, in the present study, the protocol developed by Alderman (1982) for in vitro testing of chemicals against *Saprolegnia* was modified to allow direct fixation of treated and untreated samples. On the basis of our preliminary results, although DMM does not completely inhibit the growth of *Saprolegnia*, it causes morphological changes in the mycelium, indicating a level of distress in this parasite and suggesting the need to conduct further investigations to understand the molecular mechanisms involved. Antifungal compounds against plant pathogens are well studied and several modes of action are available to control these pathogens; for this reason, the plant pathology field could provide useful knowledge to develop successful control strategies against oomycete infections of aquatic animals. This approach was already applied for the management of gastrointestinal pythiosis in dogs, utilizing the agricultural fungicide mefenoxam, with promising results (Cridge et al., 2020; Hummel et al., 2011) and for the treatment of cutaneous paralagenidiosis in dog (White et al., 2020).

In this context, our research highlights the importance to carry out further investigations on the mode of action and on the activity of already available anti-oomycete compounds to initiate cross-kingdom studies, without forgetting the eco-toxicological effects.

AUTHOR CONTRIBUTIONS

Irene Maja Nanni: Conceptualization; investigation; methodology; writing – original draft; supervision. **Perla Tedesco:** Conceptualization; investigation; methodology; writing – original draft; supervision. **David Baldo:** Methodology; investigation; writing – review and editing; supervision. **Roberta Galuppi:** Writing – review and editing; supervision. **Marina Collina:** Supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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