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Abstract

We investigated the effect of ivermectin, at environmental concentrations and above, on several strains of filamentous fungi. Ivermectin did not show significant short-term effect on biomass production and germination rate of the spores. By contrast, the spore production was stimulated twofold in *Phanerochaete chrysosporium* and *Mucor racemosus* cultures, whereas that endpoint was reduced in Fusarium cultures. A long- term exposure of *F. oxysporum* to ivermectin reduced both production and germination of fungal spores. Ivermectin was not an inducer of exocellular oxidases.

Keywords

Avermectin; Enzyme induction; Ivermectin; Fungi; Spore germination

Introduction

Avermectins are a class of veterinary medicines with insecticidal and anthelmintic properties. They have been developed for the protection of animals, human and crops. For instance, ivermectin is an avermectin widely used for cattle treatment. After treatment, most ivermectin is excreted in the faeces then is degraded in the dung. The decay process of dung involves the concomitant action of insects, annelids, and microorganisms. During this complex ecological process, no organism can

substitute for another, and the lack of one specific activity induces a strong impact on dung decomposition.

In a previous paper (Mougin et al. 2003), we observed that high levels of ivermectin were poorly degraded in the soil and could thus be stored for long periods. Despite a low bioavailability, ivermectin can alter mesofauna and microorganisms from dung and soil because high amounts of ivermectin enter the environment. Ivermectin reduces the decomposition of dung organic matter in soil (Sommer and Bibby 2002). The effects of anthelmintics on dung-dwelling insects are now well-documented, e.g. by Strong (1992) and Lumaret and Errouissi (2002). In addition, there is a potential risk of ivermectin for springtails and earthworms, namely enchytraeids (Jensen et al. 2003) and *Eisenia foetida* (Halley et al. 1990).

Halley et al. (1990) reported that ivermectin had no effect on soil microbes by studying effects on soil respiration and nitrification. They have also shown that ivermectin had no antifungal or antibacterial effects at environmental concentrations. Nevertheless, there is a lack of data on the potential impact of ivermectin on soil filamentous fungi. Here, we investigated the effect of ivermectin on filamentous fungi. Specifically, we evalu- ated the chemical impact on fungal growth, measured by biomass production, and on reproduction, by observing sporulation and germination of spores.

Experimental

Chemical reagents

High purity ivermectin, named 22,23-dihydroavermectin B_1, and chemical reagents were purchased from Sigma-Aldrich.

Fungi

The fungal strains *Fusarium oxysporum*, a deuteromycete, *Fusarium solani* Martius Saccardo, an ascomycete, and *Mucor racemosus* Fresenius, a zygomycete, were isolated from soil samples. The white-rot basidiomycetes *Phanerochaete chrysosporium* Burdsall and *Trametes versicolor* (L.:Fr.) Pilat were obtained from ATCC, referenced as 24725 and 32745, respectively. They were stored at 4 °C on agar plates.

Liquid cultures, preparation of spore suspensions

Fungal strains for suspensions of spores were grown on a culture medium containing glycerol (*P. chrysosporium*, Mougin et al. 1994) or maltose (all other strains, Lesage- Meesen et al. 1996) and ammonium tartrate as carbon and nitrogen sources. A mycelial mat on 10-mm-diameter agar plugs was inoculated into 10 ml of the culture medium in a 150-mL Erlenmeyer flask. Cultivation was carried out statically in the dark at 25 °C. After 8–12 days of growth, the spores were harvested by shaking the cultures with glass beads, counted and conditioned as liquid suspensions containing $2.5\pm0.5x10^{6}$ spores ml^{-1} for inoculation. Spore counting was achieved by placing 200-µl aliquots of the medium into a Thomas counting chamber.

Short-term toxicity assessment

Culture media were supplemented with ivermectin concentrations ranging from 10^{-3} to 10^{-8} M immediately after inoculation (acetone solutions, 50 µl per Erlenmeyer) to assess the effect on biomass production. Fungal biomass was separated from the medium after 4, 8, and 12 days of culture and dried overnight at 105 °C for dry weight determination. The effect of ivermectin on spore germination was determined in the same experiments after a 24-h exposure by calculating the germinating rate. The effect of ivermectin on sporulation was estimated by counting the spores formed in the cultures treated with ivermectin at 10^{-4} M.

Long-term toxicity assessment

Suspensions of spores were used to inoculate Erlenmeyers containing 10 ml liquid medium supplemented with 10^{-5} M ivermectin, using a previously described protocol (Kollmann et al. 2003). The cultures were then allowed to grow and sporulate in the dark at 25 °C for 10 days. Spores were harvested as described above and the experiments were repeated three times. Fungal biomass was measured on a dry weight basis. Germination and sporulation were expressed as a spore multiplication factor, which reflects the ratio between the amounts of spores produced by a culture versus the amounts of spores used for inoculation.

Peroxidase induction

Cultures of *P. chrysosporium* were carried out as described above in the presence of acetone solutions of ivermectin ranging from 10^{-8} to 10^{-3} M (50 µl solution per Erlenmeyer flask). Aliquots were assayed for peroxidase activity after 4, 8, and 12-day incubations, as previously reported by Mougin et al. (1994).

Experimental design

All experiments were carried out in triplicate. Results are expressed as means ± standard deviation. In several experiments, a Student's t-test was performed to determine significant differences.

Results and discussion

Short-term toxicity of ivermectin on fungi

We studied the dose–response relationship between ivermectin concentration ranging from 10^{-3} to 10^{-8} M and the development of fungi in liquid cultures. It is noteworthy that only three fungal strains, namely *F. oxysporum*, *F. solani*, and *M. racemosus* produced spores in our culture conditions, whereas *T. versicolor* was unable to sporulate. The three former strains were inoculated to media as spore suspensions whereas the latter was used as mycelial mats grown on agar plugs. As shown on Fig. 1, ivermectin has no significant effect on biomass production, measured by weighting control and treated cultures after 4, 8, or 12 days, even at high drug concentrations. Fungal biomass production in liquid cultures was slightly stimulated in the presence of the drug. However, the stimulation was not significant at the 0.05 level. Further, ivermectin did not effect spore germination (data not shown). As a consequence, we were unable to calculate a concentration decreasing the germination of spores by 50% after 24 h (IC_{50}) compared with untreated controls. In order to assess the impact of ivermectin on fungal sporulation, *T. versicolor* was replaced by a spore-producing-basidiomycete, *P. chrysoporium*. Ivermectin at 10^{-4} M altered sporulation at different degrees depending on strain nature (Table 1). For instance, spore production by *P. chrysosporium* and *M. racemosus* was increased twofold, whereas it was reduced in both Fusarium strains.

Long-term toxicity of ivermectin on fungi

We further examined the effect of ivermectin on fungi following four cycles of biological events, namely spore germination, biomass formation and spore production, which may change the size of fungal populations. Two culture conditions were used: controls without ivermectin and assays under constant ivermectin concentration of 10-5 M. We calculated a spore multiplication factor (SMF) reflecting both the production and germination of spores (Kollmann et al. 2003).

The spore multiplication factor usually decreased in control cultures after successive exposure of spores to ivermectin (Fig. 2). The drug modified this endpoint according to the fungal strains. Positive effects of the drug on the spore multiplication factor of *P. chrysosporium* and *M. racemosus* were significant at the end of the experiment. In contrast, a decreasing effect of ivermectin on spore multiplication factor was observed in *F. solani* and *F. oxysporum* cultures, in agreement with the negative effect of the drug noticed during short-term experiments on the sporulation of these strains.

As regards to *F. oxysporum*, the spore multiplication factor values were dramatically decreased in the presence of ivermectin after three and four cycles, suggesting a great sensitivity to the chemical during the reproduction stages. It is noteworthy that this strain responds differently to various chemicals, as the spore multiplication factor was highly stimulated after the treatment of the cultures with nonylphenol (Kollmann et al. 2003).

Biomass production in the control cultures of the four strains showed a slight decrease as the number of biological cycles increased (data not shown). There was no significant effect of ivermectin on that endpoint in *P. chrysosporium*, *F. solani*, and *M. racemosus* cultures. By contrast, the presence of ivermectin decreased the biomass production of *F. oxysporum* by 45–50% after three and four cycles. Concerning *F. oxysporum*, it appears that ivermectin, which was strongly affected the fungal reproduction after a long-term exposure, also decreased the production of biomass and then the size of the population.

Induction of exocellular oxidases by ivermectin

Various exocellular oxidases, namely lignin-peroxidases, manganese-dependent peroxidases and laccases, are produced by white-rot fungi. Here, the effect of ivermectin was assessed by measuring

the activities of peroxidases in cultures of *P. chrysosporium*. At concentrations ranging from 10^{-8} to 10^{-3} M, ivermectin did not modify the activities of these oxidases in comparison with untreated controls after 4, 8, and 12 days of exposure (data not shown).

The effect of ivermectin on laccases produced by *T. versicolor* has already been studied by Mougin et al. (2002). We showed that their activity was stimulated twofold after 3 days of exposure to the drug at $0.5x10^{-3}$ M. In a similar way, treatment of fungal cultures with 8,9-Z and 10,11-Z isomers of ivermectin, formed through phototransformation processes at the soil surface (Mougin et al. 2003), enhanced laccase activity by two and three, respectively. In the present study, higher concentrations (10-5 M) of these ivermectin isomers did not show additional effects.

Conclusions

Using fungal strains isolated from soil samples or originating from our collection, we showed that ivermectin affects the reproduction and growth of several strains of fungi. Nevertheless, these effects were observed after fungal exposure to amounts of the drug much higher than those bioavailable in the soil or in the dung. For that reason, we conclude that ivermectin at environmental concentration is without effect on the fungal endpoints studied here.

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Table 1. Short-term effect of ivermectin on fungal sporulation

Fungal strain	Spores produced (% of the untreated control)
P. chrysosporium	212±51
F. solani	83±46
M. racemosus	220±83
F. oxysporum	42±16

Fig. 1. Short-term effects of ivermectin on fungal biomass production in liquid cultures. Filled circle: *Trametes versicolor*; empty circle: *Fusarium solani*; inverted filled triangle: *Mucor racemosus*; empty triangle: *Fusarium oxysporum*

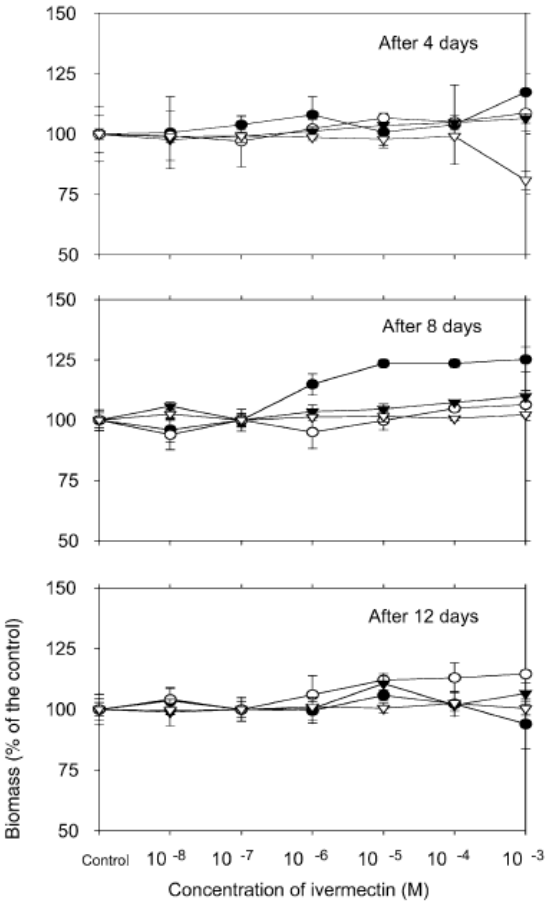

Fig. 2. Effect of ivermectin on the spore multiplication factor during long-term exposure of fungal liquid cultures. Black bars refer to untreated controls and grey bars refer to cultures treated with 10^{-5} M ivermectin