

Short Communication

High doses of melatonin confer abiotic stress tolerance to phytopathogenic fungi grown *in vitro*

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ABSTRACT

Melatonin is a secondary metabolite produced in all domains of life. Exogenous melatonin triggers defence mechanisms in plants that enhance abiotic stress tolerance. However, knowledge regarding the role of melatonin as a signal or an antioxidant in microbes is lacking. We investigated the *in vitro* growth responses of three phytopathogenic fungi, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Fusarium oxysporum* f.sp. *vasinfectum*, to abiotic stress (2.5% ethanol with/without cold priming) under varying concentrations of melatonin. Melatonin at high concentrations (1000 – 2000 μ M) partially restored fungal growth under stress, compared to controls, suggesting a role for melatonin in alleviating the impacts of stress exposure. Understanding how melatonin impacts fungal growth during stress conditions will be important for future applications using melatonin as a tool for crop protection.

Keywords: melatonin, plant pathogen, abiotic stress, ethanol, cold, antioxidant, reactive oxygen species (ROS).

1. INTRODUCTION

Diseases caused by plant pathogens greatly impact food production globally (1). Vascular wilt disease, attributed to the soil-borne pathogen *Fusarium oxysporum* Schlecht, damages a broad range of cultivated crops (2). Cosmopolitan fungal pathogens such as *Sclerotinia sclerotiorum* (Lib.) de Bary and *Botrytis cinerea* Persoon ex Fries infect many commercially important vegetables and crops (3, 4). Methods to control these fungi are limited, costly and potentially time-consuming, e.g. fungicide applications or development of cultivars with greater pathogen resistance (1-5).

Melatonin (N-acetyl-5-methoxytryptamine) is a secondary metabolite present in all domains of life (6, 7). Biosynthesis of this indoleamine has been described in bacteria as well as in some fungi (6, 8-10). Melatonin can act either as a highly efficient antioxidant, by scavenging excessive stress-induced reactive oxygen species (ROS) (7), or as a signalling molecule

upregulating and/or enhancing activities of ROS-scavenging enzymes (11). Ethanol-induced ROS negatively impacts various aspects of cell functions including cell membrane fluidity, intracellular protein configuration and activities of various glycolytic enzymes (12, 13). Similarly, cold stress applied in the current study (4 °C for 48 hr.) impacts lipid membrane integrity, thus inducing severe oxidative damage due to the excessive accumulation of ROS (14). Melatonin enhances plants' tolerance to various abiotic or biotic stressors including: chemicals, extreme temperatures as well as bacterial and fungal pathogens (15). Endogenous melatonin has been associated with abiotic stress tolerance in the biological control filamentous fungus, *Trichoderma* spp. (16).

We hypothesised that *in vitro* fungal growth under abiotic stress would be improved by exogenous melatonin. To test this, the *in vitro* growth responses were investigated for three different fungi (*S. sclerotiorum*, *B. cinerea* and *F. oxysporum* f. sp. *vasinfectum* [*Fov*]) under highly effective, ROS-producing stressors : i) 2.5% ethanol alone (17, 18) and ii) 2.5% ethanol combined with cold-priming . Levels of melatonin concentrations, from biologically relevant to potentially toxic, were added to the growing media and the radial growths reported as phenotypic responses.

2. MATERIALS & METHODS

2.1. *In vitro* growth of fungal pathogens.

S. sclerotiorum, *B. cinerea* and *Fov* were incubated in darkness at 22 °C in 90 mm Petri dishes containing half strength (½) Potato Dextrose Agar (PDA; 19.5 g/l) (Thermo Fisher Scientific Pty. Ltd., Vic, Australia), supplemented with melatonin dissolved in 50% ethanol (Sigma Aldrich Pty. Ltd., NSW, Australia) at final concentrations 20, 200, 500, 1000, 2000 and 4000 µM . The ethanol concentration was normalised across all treatments and ethanol controls to 2.5% v/v. Cold priming treatment was conducted by incubating the inoculum at 4 °C in the dark for 2 days prior to inoculation onto melatonin/control Petri dishes. Fungal radial growth (mm) was determined 2, 4 and 9 days post inoculation (dpi), respectively.

2.2. Statistical analyses.

Non-parametric analysis (n = 9; three biological replicates, each consisting of three technical replicates per treatment) by pairwise Wilcoxon rank sum tests was applied to determine statistical significance (p < 0.05) between treatments using the statistical software R version 3.5.1 (The R Foundation for Statistical Computing, Boston, USA).

3. RESULTS & DISCUSSION

S. sclerotiorum, *B. cinerea* and *F. oxysporum* are cosmopolitan pathogens with limited control options (2-4). Exogenously applied melatonin had varied ameliorative effects on *in vitro* growth of all three species of fungi across the melatonin concentrations (20 – 4000 µM) under ethanol stress with or without cold priming conditions (Figure 1). In general, high melatonin dose at 1000 - 2000 µM resulted in the greatest radial growth recovery compared to controls. For example, upon exposure to 2.5% v/v ethanol alone, 1000 µM melatonin resulted in relative growth recoveries of 35.1%, 33.6% and 51.8% for *S. sclerotiorum*, *B. cinerea* and *Fov*, respectively (Figure 1). When exposed to the combined abiotic stressors, the growth recoveries of 9.0%, 40.8% and 66.0% were observed at 1000 µM melatonin for *S. sclerotiorum*, *B. cinerea* and *Fov*, respectively compared to the growth reduction caused by exposure to both stressors in control group.

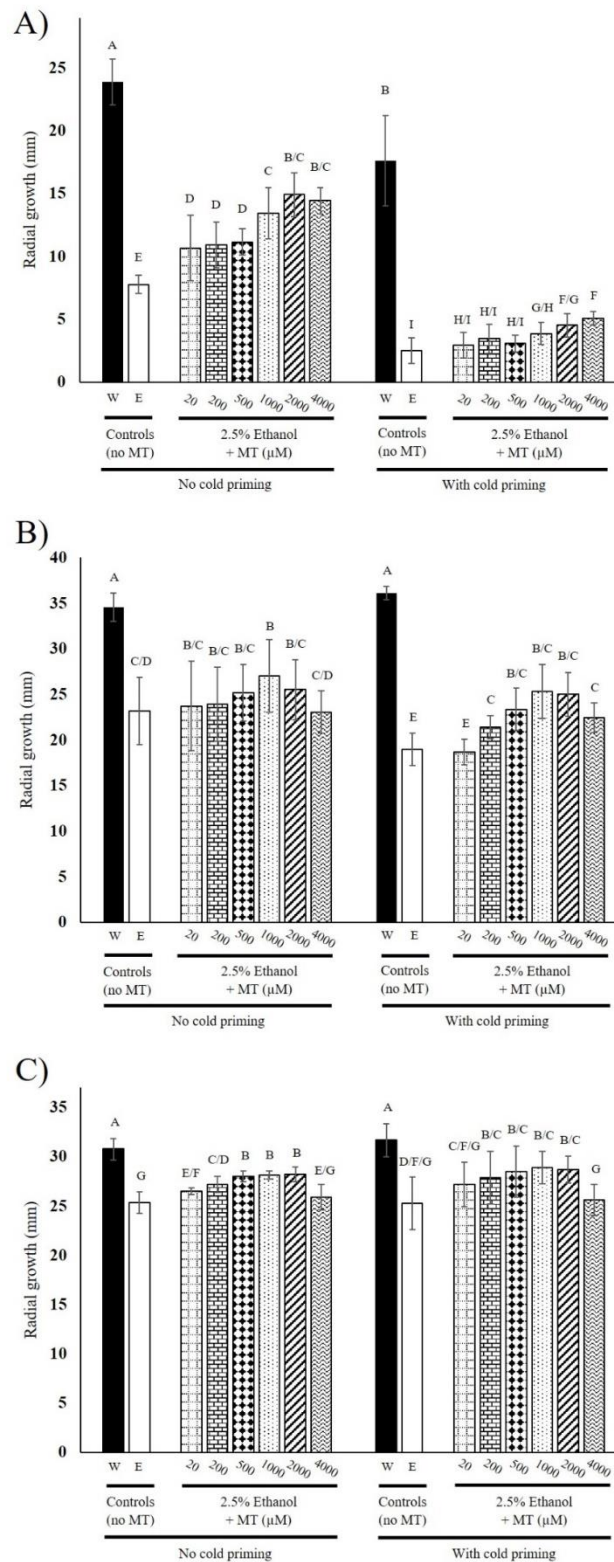


Fig. 1. Radial growth responses of fungi on the abiotic stress with or without melatonin.

a) *S. sclerotiorum*; b) *B. cinerea*; and c) *F. oxysporum f.sp. vasinfectum* (*Fov*) to melatonin (MT) treatments under 2.5% ethanol stress with or without cold priming after 2, 4 and 9 days respectively (Mean \pm SD) ($n = 9$). Controls grew in media without melatonin but containing milli-Q water (W) to replace the melatonin (i.e. no ethanol or melatonin present), and 2.5% v/v ethanol (E). The grouping letters over the bars represent significant differences following Wilcoxon rank sum tests ($p < 0.05$).

Previous reports showed that melatonin application had no significant effect on *in vitro* growth for several plant pathogenic filamentous fungi including *Mycosphaerella arachidicola*, *B. cinerea*, *Physalospora piricola* and *F. oxysporum* (18, 19); however, these fungi were not exposed to abiotic stress, such as did in the current study. It is possible that the way by which exogenous melatonin permeates into the fungi, its utilisation, and mode of action once internalised could differ according to the type of genera and therefore melatonin might become a stressor for some fungi at very high concentrations (20). Collectively, these studies, coupled with our data, suggest that fungi may benefit from the presence and/or bioavailability of melatonin at certain concentrations when exposed to particularly stressful environments. Therefore, it would be appropriate to investigate the effect of exogenous melatonin in non-pathogenic fungi capable of interacting with plants such as mycorrhizae, endophytes (e.g. *Trichoderma* spp.), and saprobes.

Ethanol (21, 22) and cold (23-25) stressors can negatively impact fungal growth to varying degrees, with both stressors increasing cellular ROS production in microbes (17, 18, 26, 27). Melatonin has been shown to act as a highly efficient antioxidant, scavenging up to 10 ROS per molecule (7), and as a signalling molecule, related to the ROS-scavenging activity of enzymes such as superoxide dismutase, catalase and peroxidase (28). In the current study, the increased tolerance of phytopathogenic fungi to abiotic stress may relate to the reduced cellular ROS levels caused by the melatonin. Indeed, exogenous melatonin application increased catalase activity in *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts, and decreased ROS accumulation under oxidative stress (H₂O₂), resulting in enhanced growth viability under abiotic stress, thus complementing the findings in our study with filamentous fungi (29). The ability of melatonin to ameliorate the negative impacts of cold and chemical stressors has already been well documented in plants (8, 9). Melatonin has also been found to improve resistance for plants against pathogen attack, for example, a root drench of apple [*Malus prunifolia* (Willd.) Borkh. cv. Donghongguo] with 100 µM melatonin resulted in enhanced immunity to the foliar fungal pathogen, *Diplocarpon mali*, in which melatonin was applied as a spore suspension of the leaves (30). Overall, there is a potentially future use of melatonin in agriculture, such as via seed-coatings (31), soil treatments (32), or foliar sprays (33). Therefore, understanding how soil pathogens respond to exogenous melatonin is an essential component in determining the viability of this possibility (34).

To our knowledge, our study is the first demonstrating that exogenous melatonin enhances tolerance to abiotic stresses for the three phytopathogenic filamentous fungi. However, additional analyses, such as determining the antioxidant levels and ROS scavenging enzyme activities under such stresses, could help to further explain the growth phenotype of the phytopathogenic filamentous fungi. Additional research is also needed to understand the effect of melatonin on fungal growth and development under stress, using *in planta* or *in vitro* experiments, such as the *in vitro* sexual reproduction of *Stagonospora nodorum* altered by the indoleamine serotonin (35). Currently there is no study regarding the molecular mechanisms underlying the effect of exogenous melatonin and the genetic determinants for its endogenous synthesis, achievable using transcriptome analysis and functional gene characterization, for example. Finally, fungal plant pathogen establishment and growth in mixed microbial communities in regard to microbial interaction is also of interest, for example in the context of contaminated agricultural soils using stressed and non-stressed conditions (36). All of these should be the goals of the future studies.

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AUTHORSHIP

APM, FB and KMP conceived the study. APM and CH carried out the experiments with APM performing the data analysis. FB and KMP supervised and administered the study. APM prepared the original draft. APM, FB, AEF and KMP wrote, edit and reviewed the manuscript. FB, AEF and KMP acquired the funding. All co-authors reviewed and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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