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Responsiveness of entomopathogenic fungi to menadione-induced oxidative stress

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ABSTRACT

Entomopathogenic fungi are predisposed to ROS induced by heat and UV–A radiation when outside the insect host. When inside the host, they are subject to phagocytic cells that generate ROS to eliminate invading pathogens. The oxidative stress tolerance of the entomopathogenic fungi *Aschersonia aleyrodis* (ARSEF 430 and 10276), *Aschersonia placenta* (ARSEF 7637), *Beauveria bassiana* (ARSEF 252), *Isaria fumosorosea* (ARSEF 3889), *Lecanicillium aphanocladii* (ARSEF 6433), *Metarhizium acridum* (ARSEF 324), *Metarhizium anisopliae* (ARSEF 5749), *Metarhizium brunneum* (ARSEF 1187 and ARSEF 5626), *Metarhizium robertsii* (ARSEF 2575), *Tolypocladium cylindrosporum* (ARSEF 3392), *Tolypocladium inflatum* (ARSEF 4877), and *Simplicillium lanosoniveum* (ARSEF 6430 and ARSEF 6651) was studied based on conidial germination on a medium supplemented with menadione. Conidial germination was evaluated 24 h after inoculation on potato dextrose agar (PDA) (control) or PDA supplemented with menadione. The two *Aschersonia* species (ARSEF 430, 7637, and 10276) were the most susceptible fungi, followed by the two *Tolypocladium* species (ARSEF 3392 and 4877) and the *M. acridum* (ARSEF 324). *Metarhizium brunneum* (ARSEF 5626) and *M. anisopliae* (ARSEF 5749) were the most tolerant isolates with MIC 0.28 mM. All fungal isolates, except ARSEF 5626 and ARSEF 5749, were not able to germinate at 0.20 mM.

Introduction

Insect-pathogenic fungi are important biological control agents used in several countries as an alternative to chemical insecticides (Alston et al. 2005). When used as mycoinsecticides on crops, fungi are subject to oxidative stress caused by solar UV-A radiation (Griffiths et al. 1998), thermal solar radiation (Fridovich 1978), and starvation (Jakubowski et al. 2000). However, when living inside the host, they may be inactivated by reactive oxygen species (ROS) produced by phagocytic cells of the infected insect's immune system (Lavine & Strand 2002). Oxidative stress caused by ROS is an immune response to eliminate invading microorganisms (Lavine & Strand 2002).

The first line of defense against oxidative stress is the enzyme superoxide dismutase (SOD), which takes superoxide, which is a harmful ROS, and converts it to hydrogen peroxide, another ROS (Missall et al. 2004). Catalase and peroxidase also protect the cell by degrading hydrogen peroxide into oxygen and water. Once in cells, these enzymes limit ROS levels and minimize its damage (Missall et al. 2004). Cellular antioxidants

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are also represented by such diverse molecules as the non-enzymatic vitamins C and E (Halliwell & Whiteman 2004), melanin (Nosanchuk & Casadevall 2003), and carotenoids (Luque *et al.* 2012). In addition, fungal mitochondria contain a constitutive enzyme alternative oxidase (AOX), which reduces oxygen to water by accepting electrons directly from ubiquinol (Uribe & Khachatourians 2008).

Oxidative stress induces several changes in biomolecules with subsequent inactivation of their functions. Several classes of biomolecules that can be damaged by ROS include proteins, lipids, and DNA (Halliwell & Whiteman 2004).

Chemicals, such as menadione, diamide, and paraquat are also potent oxidizing agents (Gulshan *et al.* 2011). Menadione is a quinone that produces superoxide radicals by redox cycling in the cell (Mutoh *et al.* 2005). The reduction of menadione is catalyzed by flavoenzymes such as NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, or NADH-ubiquinone oxidoreductase, which cause the formation of semiquinones. Semiquinones are very unstable substances that react rapidly with oxygen and lead to the formation of highly ROS, such as hydroxyl and superoxide radicals. Menadione can be reduced to the corresponding hydroquinones by DT-diaphorase without the formation of semiquinone free radical intermediates. This reduction process is considered to be a detoxification reaction, because hydroquinones are more stable and are also readily excreted. Thus, DT-diaphorase has been proposed to protect cells against carcinogenicity, mutagenicity, and other toxic effects caused by oxidant compounds (Rao *et al.* 2006). In fungi, hydrogen peroxide is generated by menadione from the oxidation of hydroquinones (Watanabe & Forman 2003).

Fungi have developed several defense mechanisms against oxidative stress to ensure their environmental survival and virulence against the insect hosts. The oxidative stress tolerances caused by the strong superoxide-generating agent menadione sodium bisulphate were studied in several species of insect-pathogenic fungi. This study shows positive and negative correlations among the menadione tolerances and the tolerances to other stress conditions that also induce oxidative stress as published elsewhere (Braga *et al.* 2001; Rangel *et al.* 2005b).

Materials and methods

Fungal isolates and culture conditions

All isolates were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, Robert W. Holley Center for Agriculture and Health, Ithaca, NY, USA) (Table 1). Stock cultures were maintained at 4 °C in test tubes on slants of potato dextrose agar (Difco Laboratories, Sparks, MD, USA) (PDA) adjusted to pH 6.9.

Conidia of each isolate were produced on 23 ml of the PDA medium on polystyrene Petri dishes (95 × 15 mm). A conidial suspension (100 μl of 10^7 conidia ml^{-1}) was inoculated evenly with a glass rod spreader onto the agar media. The cultures were incubated in the dark at 26 ± 1 °C for 14 d. Each treatment was repeated four times, with a new batch of conidia produced for each repetition.

Menadione survival curve

To prepare inoculum for oxidative stress experiments, aerial conidia were removed with a microbiological loop without touching the substrate and suspended in 10 ml of sterile Tween 80 solution (0.01 % v/v) (Sigma–Aldrich Corporation, USA) using Pyrex tubes (20 × 125 mm) (Corning, Corning, NY, USA). The suspensions (10^5 conidia ml^{-1}) were vigorously agitated (vortex), and 40 μl of the suspensions were inoculated (dropped, but not spread) on the center of the PDA medium (control = 0.00 mM) or PDA supplemented with the strong superoxide-generating agent menadione sodium bisulfate (2-methyl-1,4-naphthoquinone) (Sigma–Aldrich Corporation, USA) in polystyrene Petri dishes (60 × 15 mm).

For all isolates, 20 concentrations of menadione were used from 0.01 to 0.20 mM, with increments of 0.01 mM. The

Table 1 – Insect-pathogenic fungi isolates.

Isolate	Species	Substrate/host	Geographic origin	Year
ARSEF 252	*Beauveria bassiana*	*Leptinotarsa decemlineata* [Coleoptera: Chrysomelidae]	Orono, Maine, USA	1978
ARSEF 324	*Metarhizium acridum*	*Austracris guttulosa* [Orthoptera: Acrididae]	Queensland, Australia	1979
ARSEF 430	*Aschersonia aleyrodis*	[Homoptera: Aleyrodidae] on citrus leaves	Lake Alfred, Florida, USA	1980
ARSEF 1187	*Metarhizium brunneum*	*Oxycanus* sp. [Lepidoptera: Hepialidae]	Palmerston North, New Zealand	1966
ARSEF 2575	*Metarhizium robertsii*	*Curculio caryae* [Coleoptera: Curculionidae]	South Carolina, USA	1988
ARSEF 3392	*Tolypocladium cylindrosporum*	Soil	Nepal	1991
ARSEF 3889	*Isaria fumosorosea*	*Bemisia tabaci* [Homoptera: Aleyrodidae]	Hawaii, USA	1993
ARSEF 4877	*Tolypocladium inflatum*	[Coleoptera: Scarabaeidae: Aphodiinae]	Danby, New York, USA	1994
ARSEF 5626	*Metarhizium brunneum*	*Tenebrio molitor* [Coleoptera: Tenebrionidae] bait from soil	Pälkäne, Hämeen Lääni, Finland	1986
ARSEF 5749	*Metarhizium anisopliae* s.l.	*Schistocerca piceifrons* [Orthoptera: Acrididae]	Cerro de Ortega, Colima, Mexico	1992
ARSEF 6430	*Simplicillium lanosoniveum*	*Leptopharsa heveae* [Hemiptera: Tingidae]	French Guiana	2000
ARSEF 6433	*Lecanicillium aphanocladii*	Triangulo Agroindustrial S.A.	Pontes e Lacerda, Mato Grosso, Brazil	2001
ARSEF 6651	*Simplicillium lanosoniveum*	*Leptopharsa heveae* [Hemiptera: Tingidae]	French Guiana	2000
ARSEF 7637	*Aschersonia placenta*	Wet semi-deciduous forest	Kakum National Park, Jukua, Ghana	2003
ARSEF 10276	*Aschersonia aleyrodis*	Scale insects in guava tree	São José dos Campos, SP, Brazil	2011