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The oxygen concentration in cultures modulates protein expression and enzymatic antioxidant responses in *Metarhizium lepidiotae* conidia

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ABSTRACT

Conidia from *Metarhizium* spp. are used for integrated pest control; however, environmental factors diminish the effectivity of these programs. Several approaches tried to improve conidia resistance to overcome this limitation, although little is known about the mechanisms involved in this effect. Here we measured the activity of antioxidant enzymes and conidia virulence, comparing the proteomic profiles of *Metarhizium lepidiotae* CP-OAX conidia produced under normal (21 % O_2) and high oxygen atmospheres (pulses with 30 % O_2). We detected a higher virulence against *Tenebrio molitor* larvae, in addition to an increase in ultraviolet light tolerance in conidia produced under 30 % O_2, which correlates with increased glutathione reductase activity. Two-dimensional gel electrophoresis (2D SDS-PAGE) of proteins extracted in conidia harvested from both experimental conditions revealed a group of proteins that was observed only in conidia from oxidant atmospheres. Some of those proteins were directly involved in oxidative stress responses, whereas others were involved in conidial virulence, thermo-tolerance, and the central metabolism. Thus, a high atmospheric oxygen concentration (30 %) activates antioxidant defence and general stress response mechanisms involved in conidia resistance to adverse environmental factors, which can ultimately translate into higher effectivity for the use of entomopathogenic fungi conidia in pest control.

Introduction

The genus *Metarhizium* comprises an ubiquitous group of species whose conidia are widely used and commercialized as mycoinsecticides (Zimmermann 1993; Faria & Wraight 2007; Muñiz-Paredes *et al.* 2017). Conidia of entomopathogenic fungi have been used for the biological control of insect pests for decades; however, unlike laboratory tests, where bioassays are

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carried out under optimal conditions (high humidity, constant temperature, and photoperiod) (Butt & Goettel 2000), the effectiveness in field trials is largely affected by environmental stress, such as exposure to ultraviolet light (UV) radiation and extreme temperatures (Tseng et al. 2011).

Insect infection by the entomopathogenic fungi is a multifactorial process that ends with the death of the insect. There are multiple stages of the pathogenic process, including germination, adhesion of conidia to the surface of the insect, penetration, invasive growth, and conidiation (Roberts & St. Leger 2004). At each of these stages, there are multiple stress conditions that affect the success of the invasion, many of which involve oxidative stress: germination represents a change towards a respiratory metabolism that has been associated with the formation of reactive oxygen species (ROS) (Oh et al. 2010). During penetration, high levels of ROS are produced by the degradation of hydrocarbons present on the insect cuticle by peroxisomal β-oxidation reactions (Pedrini et al. 2013). During the invasive growth, the insect response involves wrapping the external agent and placing it in a phagocytic vacuole, where nicotinamide adenine dinucleotide phosphate (NADPH) oxidase catalyses the reduction of molecular oxygen to peroxide and superoxide (Kavanagh & Reeves 2004).

Improvements in the efficacy of mycoinsecticides can be obtained by genetic modification (St. Leger & Wang 2010) or through the modification of physiological factors, such as nutrient deprivation. Examples of the former include increased fungal virulence by overexpression of either the Pr1A protease or the Bbchit1 chitinase (St Leger et al. 1996; Fang et al. 2005). Similarly, UV tolerance is improved in *Beauveria bassiana* by overexpression of a superoxide dismutase (SOD) (Xie et al. 2010) and also in *Metarhizium anisopliae* by heterologous expression of genes from *Alternaria alternata* involved in the synthesis of melanin (Tseng et al. 2014). Likewise, fungal growth under heat stress and tolerance of heat shock-treated conidia to osmotic stress were improved by overexpression of the heat shock protein 25 (HSP25) in *Metarhizium robertsii* (Liao et al. 2014). Similarly, the conidia of *M. anisopliae* produced under nutritive stress (carbon and nitrogen starvation) or under osmotic stress (sodium or potassium chloride added to PDAY) increase heat- and ultraviolet light type B (UV-B) tolerance (Rangel et al. 2008). Higher UV-B tolerance of *M. anisopliae* conidia produced on potato-dextrose agar supplemented with yeast extract (PDAY) under continuous visible light is higher than conidia produced under dark conditions (Rangel et al. 2008; Rangel et al. 2011), and heat shock for *Aspergillus nidulans* caused higher viability and tolerance to subsequent hydrogen peroxide treatment (Couto et al. 1999).

Modifying the atmospheric oxygen concentration in cultures of entomopathogenic fungi was proposed as a strategy to improve conidia production (Tlecuitl-Beristain et al. 2010), under the rationale that high atmospheric oxygen would increase the intracellular ROS concentration and trigger mycelium cell differentiation to conidia (Aguirre et al. 2005). Several studies have reported higher yields in conidia production in different conidia from *Isaria fumosorosea* (Miranda-Hernández et al. 2014) and *Metarhizium lepidiotae* stat. nov. (= *M. anisopliae* var. *lepidiotae*) (Garcia-Ortiz et al. 2015) cultured under high oxygen atmospheres; this increase in yield is usually associated with changes in parameters that define the quality of the produced conidia. Higher thermotolerance and germination rates were improved in *I. fumosorosea* ARSEF 3302 conidia produced under 26 % O_2 (Miranda-Hernández et al. 2014) and increased thermotolerance in *M. lepidiotae* CP-OAX conidia produced under 30 % O_2 (Garcia-Ortiz et al. 2015). These authors also defined the period of mycelium competence as the time when a change in the atmospheric oxygen concentration has the highest effect (60 h) on the yield and quality of conidia and obtained the maximum conidia production at 132 h. Higher yields of conidia production after elevation of the concentration of oxygen in the culture generate oxidative stress (Garza-López et al. 2012), which increases the concentration of ROS (Turrens 2003) that play a crucial role in various aspects of cellular physiology, cellular differentiation, signalling, and pathogen defence (Kawasaki & Aguirre 2001). However, the improved virulence and thermotolerance as a response to an oxygen-enriched atmosphere may be the result of a more general stress response, which is a phenomenon referred to as 'cross-protection' (Rangel 2011).

In this study, we exposed a competent mycelium of *M. lepidiotae* to an oxygen-rich atmosphere and determined the effect on conidia quality measured as virulence and resistance to UV radiation. The analysis comprised the determination of changes in the antioxidant enzymes, protein expression and fungal general stress responses, aimed at further understanding the mechanisms involved in the increase of quality in conidia from *M. lepidiotae* cultured under oxygen-enriched atmospheres.

Materials and methods

Microorganism and culture conditions

The fungal strain *Metarhizium lepidiotae* stat. nov. (= *Metarhizium anisopliae* var. *lepidiotae*, after the multigene phylogenetic approach by Bischoff et al. 2009) was obtained from the culture collection of the Colegio de Posgraduados (Texcoco, Mexico) and was identified by Tlecuitl-Beristain et al. (2010). This strain was deposited into the Culture Collection ENCB-IPN WDCM449 with the identification number ENCB-MG-81. The initial inoculum was obtained by propagating the fungus in Petri dishes containing oat-peptone agar, as reported previously (Garcia-Ortiz et al. 2015). Incubations were carried out at 28 ± 1 °C with a 12/12 h light/dark photoperiod. Conidia obtained after seven incubation days were used as inoculum during the conidial production.

Modified atmospheres

Two atmospheric conditions were used: normal (21 % O_2) and oxygen-enriched (30 % O_2) atmospheres. The oxygen-enriched gas mixture (or atmosphere) was manufactured and standardized by Praxair Company (Monterrey, Mexico). Replacing normal oxygen levels with an oxygen-enriched atmosphere was carried out as described previously (Garcia-Ortiz et al. 2015). Briefly, all bottles were capped with cotton plugs to allow continuous gas exchange with the external environment and were incubated for 132 h at 28 ± 1 °C. After 60 h of culture,

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