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Impact of ozone on the viability and antioxidant content of grass seeds is affected by a vertically transmitted symbiotic fungus

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ABSTRACT

Ozone gas is a rising pollutant in the troposphere and is a consequence of human-driven global change. As a novel environmental stressor, interest in the impact of ozone on symbiotic systems is increasing. Focusing on the symbiosis between grasses and *Epichloe* species, we evaluated the effect of ozone exposure on the relative fitness of symbiotic and endophyte free plants and its transgenerational effects including seed performance and endophyte persistence. Endophyte symbiotic and endophyte free *Lolium multiflorum* plants were exposed to high ozone concentration at pre-anthesis. Seed production of symbiotic plants was 23% higher than that of endophyte free plants, being positively correlated with number of spikes and independent of ozone. Seed viability was negatively affected by the endophyte and improved by ozone. A dramatic negative effect of ozone on endophyte viability was manifested only after 25-day seed storage under accelerating ageing conditions. On average, seeds from plants exposed to ozone had higher levels of reduced glutathione (GSH), whilst seeds from symbiotic plants were associated with higher content of glutathione disulfide (GSSG). Consistent with the pattern of seed viability dynamics, the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) was higher (i.e. less negative) in E+ seeds and slightly lower (i.e. more negative) in seeds from plants exposed to high ozone. The relationship between endophyte symbiosis and ozone stress with the levels of tocochromanol antioxidants was unclear, and irrespective of seed or endophyte viability. The concentration of some tocochromanols were not affected, whereas others were positively (β-tocopherol) or negatively (α-tocotrienol and γ-tocopherol) affected by the endophyte, or positively affected (γ-tocopherol) by ozone alone. The fungal symbiont modified the effect of ozone exposure in the maternal environment and thus, grass seed viability and antioxidant content. Although the grass-endophyte symbiosis may promote plant yield under rising ozone levels associated with global change, it may be at the expense of seed viability and endophyte persistence.

1. Introduction

The rising level of air contaminants is one component driving global climate change (IPCC, 2007). There is a general interest from the scientific community to understand the mechanisms by which biological systems, from individuals to ecosystems, will respond to such environmental changes (Kiers et al., 2010; Saikkonen et al., 2012). Specifically, it has been advised that positive interactions will be lost whilst at the same time, there would be an increment of negative ones such as plagues and diseases (Kiers et al., 2010). Symbiotic interactions have attracted attention not only because of their ecological role but also due to their potential use in sustainable agriculture (Tikhonovich and Provorov, 2009; Kiers et al., 2010). The importance of the mutualism between cool-season grasses and *Epichloë* species resides in that they are a potential factor of phenotypic variation, which can be selected to improve quality, persistence and productivity of forage cultivars (Johnson et al., 2013; Gundel et al., 2013). However, there is scarce information on how this symbiosis can cope with the novel factors of climate change.

The grass–endophyte symbiosis is a facultative interaction for plants, but obligate for the fungi. Variation in relative fitness

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between endophyte-symbiotic and endophyte free plants, and in endophyte transmission efficiency determines its frequency and persistence in populations (Clay and Schardl, 2002; Gundel et al., 2008, 2011; Leuchtmann et al., 2014). The endophyte grows systemically in the apoplast of above ground tissues and it colonizes the developing seeds at flowering (i.e. vertical transmission) (Clay and Schardl, 2002; Majewska-Sawka and Nakashima, 2004; Christensen et al., 2008). The fungus remains quiescent in seeds and after germination, it is found in the seedlings (Clay and Schardl, 2002; Gundel et al., 2011; Card et al., 2014). The symbiosis is associated with higher plant resistance to herbivory and higher tolerance to factors of oxidative stress (Malinowski and Belesky, 2000; Clay and Schardl, 2002; Vila-Aiub et al., 2005), although it can often generate costs to the plant (Cheplick et al., 1989; Gundel et al., 2009a, 2010, 2012a). Relative to the vegetative stages, much less is known about the grass–endophyte interaction at the seed stage. The temporal dynamics of the frequency of viable endophyte in seed populations can result from the co-occurrence of two processes: (i) the viability of the fungus relative to host seeds, and (ii) the relative viability of endophyte–symbiotic and endophyte free seeds (Gundel et al., 2008, 2011). Compared to host seeds, the endophyte has shown a higher susceptibility to factors that control its viability (i.e. temperature and humidity), presenting as a consequence, lower longevity (Rolston et al., 1986; Welty et al., 1987; Gundel et al., 2009a, 2010). On the other hand, a negative effect of the endophyte on seed longevity has been observed under harsh storage conditions of high temperature with relative humidity (Gundel et al., 2009a, 2010, 2012a). It is unclear, however, whether seed or endophyte longevity could be more negatively affected under new scenarios of global climate change.

Ozone is a gaseous molecule (O_3) whose level is rising in the troposphere due to anthropic action and often, above the damage threshold for living organisms (i.e. 40 ppb, U.S.E.P.A., 2006). Ozone is a secondary contaminant produced via reactions catalysed by solar radiation and primary pollutants such as nitrogen oxides, sulphate oxides, carbon monoxide, hydrocarbons and volatile organic compounds. Therefore, it exhibits daily and seasonal dynamics with peaks at noon and during the summer (Booker et al., 2009; Schnell et al., 2009). Ozone enters the plant through the stomata and diffuses into the apoplast reacting with cellular components and producing reactive oxygen species (ROS) (Fiscus et al., 2005). Although ozone plays a role in signalling at the cellular level, it can generate different levels of oxidative stress depending on the intensity and frequency of exposure. Episodic exposure to ozone can elicit the activation of the antioxidant machinery that regulates oxidative stress, while chronic exposures to high ozone concentration may scale up from damage at cellular and tissue to the individual and crop level (Kanofsky and Sima, 1995; Sandermann et al., 1998; Kangasjärvi et al., 1994, 2005; Booker et al., 2009).

The antioxidant machinery which regulates oxidative stress involves ROS scavenging enzymes and molecules (Bailly, 2004; Kranner et al., 2006). In orthodox seeds (i.e. tolerant to desiccation; Roberts and Ellis, 1989), the antioxidants glutathione and tocochromanols have been associated with seed ageing and other vital quality parameters (germination rate, viability, among others) (Kranner et al., 2006; Seal et al., 2010). Glutathione is a water soluble antioxidant found in the cell cytoplasm in the reduced form (γ-glutamyl-cysteinyl-glycine or GSH) in unstressed conditions. With the increment of ROS, 2GSH can donate electrons and form glutathione disulphide (GSSG), a reversible reaction which is catalysed by the enzyme glutathione reductase (Kranner et al., 2006; Seal et al., 2010). A negative linear relationship has been found between the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) and the viability of orthodox seeds (Kranner et al., 2006). The relationship of tocopherols, which are lipid-soluble antioxidants associated to cell membranes, and seed viability has been more erratic with some studies finding a positive correlation between seed viability loss and a reduction in α-tocopherol content (Senaratna et al., 1988; Sattler et al., 2004; Seal et al., 2010).

Recently, it has been proposed that ROS may play a role in regulating the symbiotic interaction between plants and fungi (Rodriguez and Redman, 2005; White and Torres, 2010; Hamilton et al., 2012; Hamilton and Bauerle, 2012). Molecular studies suggested that the redox balance in plants would regulate the stability of the mutualistic symbiosis between grasses and fungal endophytes (Tanaka et al., 2006; Eaton et al., 2011). A screen to identify symbiotic genes isolated a fungal mutant that altered the interaction from mutualistic to antagonistic (Tanaka et al., 2006). This mutant has a single-copy plasmid insertion in the coding region of a NADPH oxidase gene, noxA. The fungal biomass in these associations is increased dramatically while the plants infected with the noxA mutant become severely stunted, show precocious senescence, and eventually die. ROS accumulation was detected in the endophyte extracellular matrix in wild-type but not in noxA mutant associations. This led to the hypothesis of a dual role for ROS in the grass–endophyte interaction. ROS could increase the permeability of the plant membranes favouring nutrient leaching to the apoplast and could also be involved in controlling the endophyte growth within the host (White and Torres, 2010; Eaton et al., 2011). Both roles would in turn lead to the activation of the plant's anti-stress defence system by increasing the antioxidant content (White and Torres, 2010; Hamilton et al., 2012; Hamilton and Bauerle, 2012). The antioxidant hypothesis proposes that there would be a higher production of antioxidant compounds, some of them of fungal origin (e.g. mannitol, proline; Richardson et al., 1992; Rasmussen et al., 2008), that would explain the higher stress tolerance usually found in symbiotic plants compared to endophyte free ones (Rodriguez and Redman, 2005; Malinowski and Belesky, 2000; White and Torres, 2010; Hamilton and Bauerle, 2012). However, information relating the endophyte-symbiosis to plant stress tolerance and antioxidant content is certainly scarce (Hamilton et al., 2012) and there is much less in relation to the endophyte effects on seed viability (Gundel et al., 2012b).

Here, we studied the effect of ozone on the persistence of the symbiosis between a grass and an endophyte of the *Epichloë* species. Specifically, we evaluated the effect of ozone exposure on the relative fitness of symbiotic and endophyte free plants and endophyte transmission efficiency. Using this framework, we explored the consequences of ozone exposure at pre-anthesis on the plant vegetative and reproductive stages. We hypothesized that (1) exposure of plants to ozone at pre-anthesis would negatively affect seed production, with lower impact on endophyte symbiotic plants than on endophyte free plants, but it would not affect the endophyte transmission to seeds because the fungus is found in the ovaries before seed formation (Philipson and Christey, 1986; Majewska-Sawka and Nakashima, 2004; Sugawara et al., 2004); (2) oxidative stress caused by ozone at pre-anthesis would stimulate the production of antioxidants promoting seed longevity only in non-symbiotic plants since the fungus increases stress tolerance mediated by antioxidants; and (3) the stress triggered by ozone in the apoplast of plants might adversely affect the endophyte and thus, its own longevity in the seeds.

2. Materials and methods

2.1. Study system and biological material

We worked with the symbiosis between the annual grass *Lolium multiflorum* L. and its natural endophyte fungus, *Epichloë occultans* (≡*Neotyphodium occultans* C.D. Moon, B. Scott and M.J. Chr. Mycologia 92:1113. 2000; Leuchtmann et al., 2014). Naturalized populations in Argentine Pampa grasslands present high