Superoxide radical induces sclerotial differentiation in filamentous phytopathogenic fungi: a superoxide dismutase mimetics study

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This study shows that the superoxide radical ($O_2^{\cdot-}$), a direct indicator of oxidative stress, is involved in the differentiation of the phytopathogenic filamentous fungi *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Sclerotinia minor*, shown by using superoxide dismutase (SOD) mimetics to decrease their sclerotial differentiation. The production rate of $O_2^{\cdot-}$ and SOD levels in these fungi, as expected, were significantly lowered by the SOD mimetics, with concomitant decrease of the indirect indicator of oxidative stress, lipid peroxidation.

INTRODUCTION

Sclerotial differentiation is a primitive form of differentiation exhibited by certain phytopathogenic filamentous fungi. It is expressed by four main types of sclerotia (compact bodies of aggregated hyphae), which are loose, terminal, lateral-chained and lateral-simple. These types are represented mainly, but not solely, by *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Sclerotinia minor*, respectively (Chet *et al.*, 1967, 1969; Chet & Henis, 1975; Le Tourneau, 1979; Willetts, 1971, 1978, 1997; Willetts & Wong, 1980). These fungi are of great agricultural interest because they cause stem-rot diseases in a wide variety of economically important agricultural crops. Therefore, elucidating the mechanisms of sclerotium biogenesis of these fungi may lead to the development of non-toxic ways to combat plant diseases, which could be used as alternatives to the traditional toxic fungicides. Moreover, understanding sclerotial differentiation is very important because it will help to understand more complex forms of differentiation in other organisms as well as whether their mechanisms share common biochemical pathways.

It has been proposed that sclerotial differentiation is related to high oxidative stress (Georgiou, 1997), based on the finding that sclerotium biogenesis in *S. rolfsii* was accompanied by the accumulation of high levels of lipid peroxidation products. This finding has been supported by other studies that developed into a theory of oxidative stress as an inducer of sclerotial metamorphosis (Georgiou *et al.*, 2006). Specifically, hydroxyl radical scavengers,

vitamin C and β -carotene (both also acting as endogenous fungal antioxidants), decreased sclerotial differentiation in *S. rolfsii*, *S. minor*, *S. sclerotiorum* and *R. solani*. In more recent studies, we demonstrated a strong relationship between the thiol redox state of these fungi and their sclerotial differentiation (Patsoukis & Georgiou, 2007a, b, 2008a, b, c). Specifically, in *R. solani*, *S. rolfsii* and *S. minor*, there was a decrease in the total amount of reduced thiols during transformation of the undifferentiated mycelium into sclerotium, which, for *S. sclerotiorum* was accompanied by an increase in the total oxidized components. Moreover, the antioxidant thiol redox state modulator *N*-acetylcysteine inhibited sclerotial differentiation in *S. rolfsii*, *R. solani* and *S. sclerotiorum*.

Until now, our studies have associated only indirect indicators of oxidative stress with sclerotial differentiation. However, these indicators cannot conclusively establish such a relationship because they are affected by many metabolic processes that are not directly associated with oxidative stress. This fact necessitated the verification of this association with direct indicators of oxidative stress. Superoxide radical ($O_2^{\cdot-}$) is such an indicator because it is among the main initiators of oxidative stress (Halliwell & Gutteridge, 1999). It is generated by reduction of molecular oxygen (O_2) by a single electron. The next reactive oxygen species produced is hydrogen peroxide (H_2O_2), formed by $O_2^{\cdot-}$ capturing an electron from another $O_2^{\cdot-}$ molecule (dismutation reaction). Finally, the very potent hydroxyl radical is formed from H_2O_2 by capture of an electron from another $O_2^{\cdot-}$ molecule or from free ferrous (Fe^{2+}) or cuprous (Cu^{1+}) ions (released, for example, from proteins oxidatively modified under abnormal conditions) (Halliwell & Gutteridge, 1999). Organisms possess the antioxidant extra- and intracellular superoxide dismutase (SOD) to protect against the deleterious actions of $O_2^{\cdot-}$ by catalysing its dismutation to H_2O_2 and O_2 .

Abbreviations: BHA, butyl hydroxyanisole; HE, hydroethidium; HRP, horseradish peroxidase; MDA, malondialdehyde; SD, sclerotial initiation stage; SM, sclerotial maturation stage; SI, sclerotial development stage; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive species; UD, undifferentiated stage.

Intracellular SOD can be modulated by molecular genetics (introducing multicopy plasmids with a cloned SOD gene) or by certain chemicals (SOD mimetics) that mimic its activity (Day *et al.*, 1995; Gardner *et al.*, 1996; Kubota & Yang, 1984; MacKenzie & Martin, 1998; Mok *et al.*, 1998; Yamada *et al.*, 2003). In this work, we studied the effect of $O_2^{\cdot-}$ in the degree of differentiation of *S. rolfsii*, *S. minor*, *S. sclerotiorum* and *R. solani* by modulating intracellular concentrations of $O_2^{\cdot-}$ and SOD with the SOD mimetics Tiron and TEMPOL in relation to the indirect marker of oxidative stress, lipid peroxidation.

METHODS

Chemicals and reagents. Dihydroethidine (or hydroethidium, HE), horseradish peroxidase (HRP), DNA type III (from salmon testes), dianisidine, SOD, EDTA, butyl hydroxyanisole (BHA), PMSF, Tiron, TEMPOL, thiobarbituric acid (TBA) and Dowex 50X8 (mesh 400) were from Sigma. DMSO, acetone, chloroform, acetonitrile, absolute methanol, ethanol, hydrogen peroxide (H_2O_2), sodium cyanide, isobutanol, riboflavin, TCA and trifluoroacetic acid were from Merck. Hydrophobic Oasis HLB 1 cm^3 (30 mg) extraction cartridges were from Waters Corp. All other reagents and solvents used were of the highest purity.

Fungal strains, growth conditions and developmental stages.

R. solani strain (MUCL-30476) was obtained from the fungi bank of Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; *S. sclerotiorum* (MUCL-38483) was obtained from the Phytopathological Institute Culture Collection of Patras, Patras, Greece; *S. rolfsii* (ATCC-26325) was provided by the American Type Culture Collection; *S. minor* (BPIC-1682) was provided by Benakion Phytopathological Institute Culture Collection, Athens, Greece.

The fungal strains were grown in 25 ml liquid medium (in Petri dishes) consisting of 0.1 M glucose, 15 mM NH_4NO_3 , 2 mM NaCl, 0.5 mM $MgSO_4$, 0.01 mM $FeCl_3$, 0.015 mM $MnCl_2$, 0.07 mM $ZnCl_2$, 0.001 mM thiamine and 0.1% (w/v) yeast extract, in 10 mM potassium phosphate buffer, pH 7. The glucose $10\times$ stock solution was separately sterilized to avoid formation of oxidizing Maillard reaction products (Bridson & Brecker, 1970). Sterilization was performed for 15 min at 120°C and at 2 atm. Petri dishes were inoculated at their centres with an agar plug (0.4 cm diameter) taken from the growing margin of a 2.5 cm diameter fungal colony grown on potato dextrose agar. The inoculation day was counted as growth day 0, and the colonies were grown at 23°C and 65% relative humidity in single dish layers under a 12 h diurnal cycle with illumination ($0.040\text{ mE m}^{-2}\text{ s}^{-1}$) provided by Philips fluorescent lamp TLD 36W/965 with emission range of 400–800 nm.

Throughout their development, *R. solani*, *S. sclerotiorum*, *S. minor* and *S. rolfsii* exhibited developmental transition through identical stages, each with a characteristic morphology (Fig. 1). These were identified as the undifferentiated (UD), sclerotial initiation (SI), sclerotial development (SD) and sclerotial maturation (SM) stages. The UD stage is characterized by highly proliferating hyphae, the SI stage by the appearance of small distinct sclerotial initials formed from highly proliferating interwoven hyphae, the SD stage by the increase of sclerotium size and the SM stage is characterized by sclerotium surface delimitation, internal consolidation and melanin pigmentation, and is often associated with droplet excretion (Townsend & Willetts, 1954). Developmental stages UD, SI, SD and SM for *R. solani* were reached in 3, 4, 5 and 6 days, respectively, for *S. sclerotiorum* in 3, 4, 7 and 9 days, respectively, for *S. rolfsii* in 4, 5, 7

and 9 days, respectively, and for *S. minor* in 3, 4, 6 and 7 days, respectively.

SOD mimetics Tiron and TEMPOL, used in this study, were prepared fresh as 1 M (in 0.05 M NaOH) sterile aqueous stock solutions of final pH 6.0. They were administered in the growth medium (also pH ~6.0) of the sclerotogenic strains, 1 day before the end of the UD stage at maximum growth non-inhibiting concentrations. Tiron was administered in *S. rolfsii* and *S. sclerotiorum* at final concentrations of 0.5, 1, 5, 10 and 15 mM; TEMPOL was administered in *R. solani* and *S. minor* at concentrations of 1, 2, 3, 4 and 5 mM and 0.01, 0.03, 0.07, 0.1 and 0.3 mM, respectively. Tiron was used in *S. rolfsii* and *S. sclerotiorum* and TEMPOL in *R. solani* and *S. minor* because they were preferably absorbed by the particular strains, possibly due to strain specificity. Before administration, the net weight (g) of the liquid cultures (including the wet weight of the fungal colony) was measured and equated with their volume (ml), which was then used to determine the appropriate dilution of the stocks of the SOD mimetics required to bring the liquid cultures to the chosen concentrations. These maximum growth non-inhibiting concentrations were determined experimentally so as to not change the growth rate (colony dry weight per growth time) of the strains in the presence of the SOD mimetics (compared with growth in their absence).

Fungal tissue treatment. For *R. solani*, *S. sclerotiorum* and *S. rolfsii*, the assays were performed on the undifferentiated mycelial colony 1 day before the initiation of differentiation (UD stage), and separately on sclerotia representing the SI, SD and SM developmental stages. In the case of *S. minor*, the assays were performed on whole colonies at each of the differentiated sclerotial developmental stages since the separation of sclerotia from the surrounding mycelial matrix was not feasible due to the paucity of hyphae. Sclerotia, whenever possible, were separated from mycelia with sterile forceps and washed at least twice in 2–3 vols ice-cold phosphate–EDTA buffer [1 mM EDTA, 0.5 mM PMSF, 0.3% absolute ethanol and 1 mM BHA (from 0.33 M stock made in 100% absolute ethanol) in 10 mM phosphate buffer, pH 7.2] per g fungal wet weight, and recovered by centrifugation at 6000 g. Tissue pellets were mixed with 1–3 vols ice-cold phosphate–EDTA buffer per g fungal wet weight, and were ground in a porcelain mortar in liquid nitrogen. The resulting homogenate was diluted with 2 vols 50 mM phosphate buffer, pH 7.0, centrifuged at 20 000 g for 10 min, and the resulting clear supernatant (cytoplasmic fraction) was subsequently used for the following assays, apart from the superoxide radical assay, where mycelial tissue and sclerotia were used directly.

Superoxide radical assay. Superoxide radical ($O_2^{\cdot-}$) was assayed by using a previously reported method (Georgiou *et al.*, 2008), which is based on the reaction between $O_2^{\cdot-}$ and HE that results in the formation of the specific product 2-OH- E^+ , the formation rate of which was measured and converted to superoxide production rate. Fungal tissue (mycelia/sclerotia with wet weight ~0.1 g) was incubated for 20 min in 1 ml phosphate buffer (50 mM Na_2HPO_4 , pH 7.0), supplemented with 20 μM HE, under the same fungal growth conditions (temperature, light). The tissue was thoroughly washed with 10 M HCl and subsequently with ddH $_2$ O. 2-OH- E^+ in the washed tissue was then extracted with 0.9 ml 100% acetone, to which 0.01 ml 10 M NaOH was added. 2-OH- E^+ was further isolated via cation and hydrophobic microcolumn chromatographies and quantified by the use of its fluorescence properties (in the presence and absence of DNA) and its reaction with hydrogen peroxide (catalysed by HRP). Fluorescence measurements were performed in a quartz microcuvette (internal dimensions $4\times 4\times 45\text{ mm}$) with its appropriate holder and a Shimadzu RF-1501 spectrofluorometer set at 10 nm excitation/emission slit width and high sensitivity.

Fig. 1. (a) Schematic of sclerotium developmental stages SI, SD and SM (the differentiated states), starting from a UD colony (undifferentiated state); modified from the paper by Georgiou *et al.* (2006). (b) Developmental stages (UD, SI and SM) of the phytopathogenic fungi used in this study: 1, *R. solani*; 2, *S. sclerotiorum*; 3, *S. rolfsii*; 4, *S. minor*.

SOD assay. The assay is a modification of a previous one that is based on the SOD-inhibited reduction of oxidized dianisidine (Lu *et al.*, 2004; Misra & Fridovich, 1977), which results from the reaction of its reduced form by the photochemically sensitized riboflavin. SOD increases the rate of the photooxidation of dianisidine due to the catalytic scavenging of $O_2^{\cdot-}$, which would otherwise nullify the overall dianisidine photooxidation by reducing an intermediate dianisidine oxidation product (absorbing at 500 nm). Illumination for the photochemical reactions was provided by a custom-made apparatus consisting of a circular fluorescent 22 W lamp (with internal diameter

15 cm, made by FUJI). The 1 ml cuvette used for this assay was centred on a white plastic disk (6 cm diameter) that was mounted on top of a small electric fan ($8 \times 8 \times 2.5$ cm), which was fixed flat in the centre of the lamp (in order to keep a steady temperature, 26°C , around the cuvette).

The assay consists of mixing 0.2 ml supernatant (or 0.2 ml growth medium for measuring the extracellular SOD), at the required fungal developmental stage, with 0.22 ml 0.06 mM riboflavin stock (made fresh in the above phosphate buffer), 0.56 ml phosphate buffer and

0.02 ml 10 mM dianisidine stock (made fresh in absolute ethanol and kept light-protected on ice). The assay mixture was kept in the illumination apparatus and its linear absorbance increase (versus time) was measured at 500 nm after 4 min (sample absorbance). This assay uses two blanks (A and B): blank A consisted of 0.2 ml supernatant (or growth medium) mixed with 0.22 ml 0.06 mM riboflavin stock and 0.56 ml phosphate buffer; blank B consisted of 0.2 ml supernatant (or growth medium) mixed with 0.02 ml 10 mM dianisidine stock and 0.78 ml phosphate buffer. The absorbance of both blanks was then measured at 500 nm after 4 min, added together and then subtracted from the absorbance of the sample; this net absorbance difference rate (corresponding to the increase of oxidized dianisidine due to the presence of SOD in the sample) was converted to SOD units (U) from a standard curve (0–3 U) made with pure bovine erythrocyte SOD. Absorbance measurements were done in a Shimadzu UV-1200 spectrophotometer.

Lipid peroxidation assay. Lipid peroxidation was assayed as TBA reactive species (TBARS) by a modified TBA-based method (Buege & Aust, 1978; Zamora *et al.*, 1997). Specifically, up to 0.15 ml supernatant was mixed with 0.15 ml TBA reagent (made as 0.5 % w/v TBA in 20 % w/v TCA and 0.33 M HCl) and BHA [2 µl of 2 % (w/v) made in absolute ethanol] was added to prevent artificial lipid peroxidation during the assay. The mixture was incubated at 100 °C for 15 min, brought to room temperature, mixed with 0.3 ml isobutanol (by vigorous vortexing) and centrifuged at 15 000 g for 3 min. Then, the fluorescence of the upper isobutanol layer was measured at excitation/emission 535/550 nm against the isobutanol-treated sample [0.15 ml sample plus 0.15 ml 20 % TCA containing 0.33 M HCl and 0.02 % (w/v) BHA] and reagent blank [0.15 ml phosphate buffer plus 0.15 ml TBH reagent containing 0.02 % (w/v) BHA]. Fluorescence was converted to malondialdehyde (MDA) equivalents (nM MDA in cytoplasm) from a standard curve using malonaldehyde bis(dimethyl acetal) (0–2 nM). Measurements were done in a Shimadzu RF-1501 spectrofluorophotometer set at low sensitivity and excitation/emission bandwidth of 10 nm.

Measurement of sclerotial differentiation. The effect of the SOD mimetics on sclerotial differentiation (sclerotium production) was expressed as per cent differentiation, where 100 % differentiation represents the number of SM sclerotia (approx. 850, 500 and 35 for *S. minor*, *S. rolfsii* and *S. sclerotiorum*, respectively) and dry weight for *R. solani* sclerotia (approx. 0.09 g) formed per fungal colony in the absence of the SOD mimetics.

Statistical analysis. All data were reported as mean $\pm$ SEM from at least triplicate experiments. The significance was determined using Student's unpaired *t* test, with a value of $P < 0.05$ considered to be significant.

RESULTS AND DISCUSSION

These data show that the direct indicator of oxidative stress, $O_2^{\cdot-}$, is directly involved in the differentiation of *S. rolfsii*, *S. minor*, *S. sclerotiorum* and *R. solani* throughout their developmental stages (Fig. 1) because radical sclerotogenesis of these fungi is decreased in the presence of SOD mimetic scavengers. Specifically, sclerotial differentiation in *S. rolfsii* and *S. sclerotiorum* was decreased to nearly 50 % by Tiron, while for *S. minor* and *R. solani*, sclerotial differentiation was decreased to 70 and 80 %, respectively, by TEMPOL (Fig. 2). The profiles of the SOD mimetic effect in the presence and absence of Tiron and TEMPOL on the decreased sclerotial differentiation showed that it is associated with the endogenous concentration of $O_2^{\cdot-}$. The levels of $O_2^{\cdot-}$, especially at the UD stage and during the transition stage (SI) between the undifferentiated and differentiated state, were decreased by 10–20 % in the presence of the SOD mimetics. This decrease was less than the corresponding differentiation decrease (Fig. 3), suggesting that $O_2^{\cdot-}$ may trigger additional molecular processes (e.g. involving its dismutation product H_2O_2) that may add up to its partial inhibition of sclerotial differentiation. The effectiveness of SOD mimetics in decreasing the levels of $O_2^{\cdot-}$ was shown by the concomitant decrease (10–50 %) they caused in the activity of the intracellular SOD at the UD and SI developmental stages of these fungi, although this was not significant in all species (Fig. 4). The decreased oxidative stress (i.e. decreased $O_2^{\cdot-}$) that was caused by the SOD mimetics during these stages was supported by the similar level of decrease they also caused in lipid peroxidation (TBARS), which, in some but not all cases, extended into the SD and SM stages (Fig. 5).

This study dealt with two kinds of fungal developmental differences in relation to $O_2^{\cdot-}$ -induced sclerotial differentiation: (i) those referring to the transition from the UD to the differentiated state (the initiation of which is designated SI), and these are the main focus of the paper; (ii) those referring to transitions within the differentiated state, designated SI, SD and SM stages. Differences between SI, SD and SM among the four strains studied seem to be

Fig. 2. Effects of the SOD mimetics Tiron and TEMPOL, tested up to concentrations not inhibiting fungal growth, on the per cent sclerotial differentiation of (a) *S. rolfsii* (●), *S. sclerotiorum* (■), (b) *R. solani* and (c) *S. minor* (see Methods for definition). Bars, SEM.

Fig. 3. Profiles of the rate of formation of $O_2^{\cdot-}$ in the cytoplasm (equal to wet minus dry weight) in the end of the undifferentiated colony (UD) and in the sclerotium sub-stages SI, SD and SM of the differentiated colonies of (a) *S. rolfii* and (b) *S. sclerotiorum* in the absence/presence of Tiron (15 mM) and (c) *R. solani* and (d) *S. minor* in the absence/presence of TEMPOL (0.3 and 5 mM, respectively). Filled and open bars show the absence/presence of SOD mimetics, respectively. Asterisks indicate that the difference between the $O_2^{\cdot-}$ rate of accumulation in the absence and presence of the SOD mimetic is statistically significant; $P < 0.05$.

more difficult to explain because they may be also related to the structural and metabolic differences that exist in their different sclerotial types. The SOD mimetics employed in this study shed light on the pro-oxidant role of $O_2^{\cdot-}$ in the induction of sclerotial differentiation, although they do not simulate the $O_2^{\cdot-}$ -scavenging of the native SOD to the full extent, due to their unknown intracellular concentration and site of action in the studied strains.

Fig. 4. Profiles of SOD activity in the cytoplasm (wet minus dry weight) in the end of the undifferentiated colony (UD) and in the sclerotium sub-stages SI, SD and SM of the differentiated colonies of (a) *S. rolfii* and (b) *S. sclerotiorum* in the absence/presence of Tiron (15 mM) and (c) *R. solani* and (d) *S. minor* in the absence/presence of TEMPOL (0.3 and 5 mM, respectively). Filled and open bars show the absence/presence of SOD mimetics, respectively. Asterisks indicate that the difference between SOD activity in the absence and presence of the SOD mimetic is statistically significant; $P < 0.05$.

The relationship of $O_2^{\cdot-}$ and SOD with differentiation has also been shown in *Neurospora crassa*, where $O_2^{\cdot-}$ and other reactive oxygen species act as signal molecules for cytodifferentiation at certain stages of its development (Belozerskaia & Gessler, 2006). Moreover, it was found that a manganese-type SOD-encoding gene is upregulated during conidiogenesis of the plant-pathogenic fungus *Colletotrichum graminicola* (Fang *et al.*, 2002), and

Fig. 5. Profiles of lipid peroxidation (expressed as TBARS concentration in the cytoplasm, equal to wet minus dry weight) in the end of the undifferentiated colony (UD) and in the sclerotium sub-stages SI, SD and SM of the differentiated colonies of (a) *S. rolfii* and (b) *S. sclerotiorum* in the absence/presence of Tiron (15 mM) and (c) *R. solani* and (d) *S. minor* in the absence/presence of TEMPOL (0.3 and 5 mM, respectively). Filled and open bars show the absence/presence of SOD mimetics, respectively. Asterisks indicate that the difference between lipid peroxidation in the absence and presence of the SOD mimetic is statistically significant; $P < 0.05$.

Candida albicans, lacking the SOD gene, showed delayed hyphal growth necessary for the full virulence of the organism to be expressed (Hwang *et al.*, 2002).

The present study shows that $O_2^{\cdot -}$ in association with SOD regulates the sclerotial differentiation of *S. rolfii*, *S. minor*, *S. sclerotiorum* and *R. solani*. Moreover, SOD mimetics can be used as potent fungicides against sclerotigenic phytopathogenic fungi by causing a decrease in sclerotial

production and sustaining fungi in their undifferentiated hyphal stage, which is more vulnerable to degradation by soil microorganisms.

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