The Role of Thiols on Sclerotial Differentiation of Filamentous Phytopathogenic Fungi

Nikolaos Patsoukis and Christos D. Georgiou*

Department of Biology, Section of Genetics, Cell Biology and Development, University of Patras, 26100-Patras, Greece

Abstract: Sclerotial differentiation in filamentous phytopathogenic fungi, represented by four main types of sclerotia, was studied in relation to thiol redox state (TRS) by the TRS modulators glutathione (GSH), N-acetylcysteine [AcCSH, a cysteine (CSH) source and a GSH biosynthesis inducer], and dithiothreitol (DTT, a non specific antioxidant –SH group provider). It was shown that GSH and AcCSH increased either GSH or CSH or both during transition from the undifferentiated to the differentiated state, and, together with DTT, they inhibited sclerotial differentiation. Given the fact that the control thiol DTT had an analogous effect on the unrelated to sclerotial differentiation GSH and CSH levels, these data suggest that the decrease of sclerotial differentiation is related to the antioxidant action of -SH groups and not particularly to GSH and CSH.

Keywords: Glutathione, cysteine, differentiation, sclerotium, thiol redox state.

INTRODUCTION

Sclerotium formation occurs across a wide range of taxonomically and ecological diverse fungi. They comprise plant pathogenic fungi (e.g. *Rhizoctonia, Sclerotinia*, *Aspergillus*, *Botrytis*, *Claviceps*, *Cylindrocladium*, *Macrophomina*, *Phymatotrichum*, *Typhula* and *Verticillium*) as well as non-phytopathogenic fungi (e.g. *Bulbillomyces*, *Collybya*, *Coprinus*, *Elaphomyces*, *Morchella*, *Pleurotus*, *Polyporus*, *Wolfporia*) [1]. Sclerotia in certain filamentous phytopathogenic fungi are compact or loose bodies of hyphae, whose degree of inter-tangling distinguishes four main sclerotium types: loose, terminal, lateral-chained and lateral-simple, represented by *Rhizoctonia solani, Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Sclerotinia minor*, respectively [2-5]. These sclerotiogenic phytopathogenic fungi, besides their great agricultural and economic importance, can be viewed as models of simple differentiation. Understanding sclerotium biogenesis will help to elucidate the mechanisms of more complex forms of differentiation. Moreover, their study may lead to the development of non-toxic fungal pathogen controls *via* inhibition of the generation of sclerotia, which will be alternative to traditional toxic fungicides [6, 7].

At present, it is generally accepted that both nutritional and non-nutritional factors are involved in the induction and the inhibition of sclerotial biogenesis [1]. Concerning the nutritional factors, it has been previously reported that low concentrations of sulfur-containing compounds, such as the amino acids L-cysteine, L-cystine, and L-homocysteine as well as glutathione, inhibit sclerotium biogenesis in *S. rolfsii* [8-10]. Several hypotheses had been advanced in the past for the sclerotiogenesis of filamentous fungi. It has been proposed (a) that sclerotium biogenesis depends on the accumulation of some unspecified metabolite(s) within the hypha, (b) that the formation of a protein-like, morphogenic factor in the mycelium induces sclerotia, and (c) that sclerotium biogenesis follows the inactivation of an unspecified protein containing -SH groups and Cu^{2+} which itself represses this process [1]. Moreover, it has been shown that sclerotium formation is inhibited by cAMP in *S. rolfsii* and *S. sclerotiorum* [11-16], and by calcineurin in *S. sclerotiorum* [17], and that it depends on pH and oxalic acid in *S. sclerotiorum* [14, 18].

Nevertheless, none of these hypotheses proposes a unifying mechanism which would take into account the experimental data on the factors affecting sclerotial biogenesis gathered over the past 50 years. However, we have noticed that these data become meaningful when they are related with oxidative stress [1]. Based on these observations, we hypothesized that sclerotial differentiation is induced by oxidative stress. This theory would predict that any growth factors (nutritional/non-nutritional) that eliminate or promote oxidative stress are expected to inhibit or promote sclerotium biogenesis, respectively [1].

Thiol redox state (TRS) is an essential parameter of prokaryotic and eukaryotic cells and it is associated with all major biological processes [19]. TRS is characterized by the levels of certain thiol components such as glutathione (GSH), cysteine (CSH), and protein thiols (PSH), as well as their contribution to symmetric and mixed disulfides. GSH is considered as the major regulator of the intracellular redox state, and participates in redox reactions *via* the reversible oxidation of its active thiol group [20]. GSH is also related to many physiological processes in which other non-protein (NP) thiols as well as their symmetric and mixed disulfides are involved. PSH and their symmetric and mixed disulfides contribute to the regulation of redox homeostasis, are involved in allosteric, enzymatic and receptor-mediated responses [19] and in cellular signaling cascades [21].

*Address correspondence to this author at the Department of Biology, Section of Genetics, Cell Biology and Development, University of Patras, 26100-Patras, Greece; Tel: +3061-997227; Fax: +3061-997840;
E-mail: c.georgiou@upatras.gr

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In the present study we investigate the possible involvement of TRS in sclerotial differentiation as expressed by the four main types of sclerotia. In particular, we raise the questions (a) whether GSH and CSH play certain role in sclerotiogenesis or (b) whether their effect is exerted by their additive contribution to the reduced thiol pool *via* their –SH groups. We approached the answer to these questions by studying the effect of certain TRS modulators (GSH, N-acetylcysteine (AcCSH) [20, 22], and the non-specific –SH group provider dithiothreitol (DTT) [20, 23]) on sclerotial differentiation in conjunction with their effect (a) on the reduced TRS components GSH, CSH and PSH, and (b) on the related to them oxidized TRS components oxidized glutathione (GSSG), protein/proteine disulfides (PSSP) and non protein disulfides of CSH (NPSSC).

MATERIALS AND METHODOLOGY

Chemicals

Ethylenediaminetetraacetic acid (EDTA, disodium salt), trichloroacetic acid (TCA), diethyl ether, chlorophorm ($CHCl_3$), sodium borohydride (BH), perchloric acid (PCA), acetonitrile (ACN) and acetone were from Merck-Schuchart (München, Germany). Bromobimane (or monobromobimane, mBrB) was from Fluka Chemie (Steinheim, Switzerland). L-cysteine (CSH) was from Ferak (Berlin, Germany). L-cystine, urea and ninhydrin were from Serva (Heidelberg, Germany). Triton X-100 was from Pierce (Rockford, Illinois, USA). Bovine serum albumin (BSA, fraction V), butylated hydroxyanisole (BHA), Coomassie Brilliant Blue G250 (CBB-G250), glutathione disulfide (GSSG, disodium salt), glutathione (GSH), beta-NADPH (tetrasodium salt), tris-(hydroxymethyl)aminomethane (Tris), 5,5'-dithiobis-(nitrobenzoic acid) (DTNB), phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM), GSSG reductase (from bakers yeast), dithiothreitol (DTT), N-acetylcysteine (AcCSH), acylase and guanidine-HCl were from Sigma Chemical Co. (St. Louis, Missouri, USA). All other chemicals used in this study were of the highest analytical grade.

Fungal Strains, Growth Conditions and Developmental Stages

Rhizoctonia solani (strain MUCL-30476) was from the fungal bank of Mycothéque de l'université Catholique de Louvain, Louvain-la-Neuve, Belgium. *Sclerotinia sclerotiorum* was provided by the Phytopathological Institute Culture collection of Patras, Patras, Greece. *Sclerotium rolfsii* (strain ATCC-26325) was provided by the American Type Culture Collection. *Sclerotinia minor* (strain BPIC-1682) was provided by Benakion Phytopathological Institute Culture collection, Athens, Greece. All fungal strains were grown at least in triplicates in a 25 ml liquid medium (in 9 cm-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 thiamin, 0.1% (w/v) yeast extract, in 10 mM potassium phosphate buffer, pH 7.0. The growth medium and the glucose10x stock solution were separately sterilized to avoid formation of oxidizing Maillard reaction products [24]. Sterilization was performed for 15 min at 120°C and at 1 atm. Petri dishes were inoculated at their centres with an agar plug (0.4 cm diam) taken from the growing margin of a 2.5 cm diameter fungal colony grown on PDA. Inoculation day was counted as zero growth day, 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 mE m^{-2} s^{-1}) provided by Philips fluorescence lamps TLD 36W/965 with emission range 400-800 nm.

Throughout their development, *R. solani*, *S. sclerotiorum*, *S. rolfsii* and *S. minor* undergo specific sclerotial developmental stages each with a characteristic morphology (Fig. **1**). These have been identified as the undifferentiated (UD) state of the mycelium, followed by the transitional to the differentiated state sclerotial initiation (SI) stage, which further develops into the sclerotial development (SD), and sclerotial maturation (SM) stages. UD state is characterized by highly proliferating hyphae, SI stage by the appearance of small distinct sclerotial initials formed from highly proliferating interwoven hyphae, SD stage by the increase of sclerotial size, and SM stage by sclerotial surface delimitation, internal consolidation, melanin pigmentation, and often associated with droplet excretion [25]. For the fungal strains used in this study the developmental periods UD, SI, SD and SM are 3, 4, 5, 6 days for *R. solani*, 3, 4, 7, 9 days for *S. sclerotiorum*, 4, 5, 7, 9 days for *S. rolfsii*, and 3, 4, 6, 7 days for *S. minor*.

The stock solutions of the TRS modulators were prepared fresh in distilled water, filter-sterilized by passing through a cellulose acetate syringe filter (25-mm diam, 0.2-μm pore, Nalgene Co, New York, U.S.A.). GSH stock was 0.1 M GSH (in 0.125 N NaOH), AcCSH stock was 0.1 M (in 0.05 N NaOH), and DTT stock was 0.1 M (its unadjusted pH is near 6.0). They were administered, at least in triplicates, in the growth medium (having pH near 6.0) at various concentrations one day before the day of the UD state. This was done by pipetting appropriate dilutions of the stocks, mixed by mild shaking of the plates. At this day, the net weight of the liquid cultures (including the wet weight of the fungal colony) was measured (after subtracting the dry Petri dish weight) and approximated with its volume, which was then used to determine the appropriate dilution of the stocks of the TRS modulators required to bring the liquid cultures to the tested concentrations of the TRS modulators.

Measurement of Sclerotial Differentiation

The effect of the TRS modulators (Fig. **2**) on sclerotial differentiation (sclerotium production) was expressed as % differentiation (derived from at least three plates), where 100% differentiation represents the number of SM sclerotia (approximately 850, 500 and 35 for *S. minor*, *S. rolfsii* and *S. sclerotiorum*, respectively), and for *R. solani* sclerotia dry weight (approx. 0.09 g) formed per fungal colony in the absence of the TRS modulator.

Fungal Tissue Treatment

Mycelial samples, at least in triplicates, from the undifferentiated colony (UD state) and from the sclerotial mycelial substrate SI stage of the differentiated colony were tested. SI-stage mycelia represent a transition between the undifferentiated and differantiated state since they constitute the mycelial substrate on which SI sclerotia are formed.

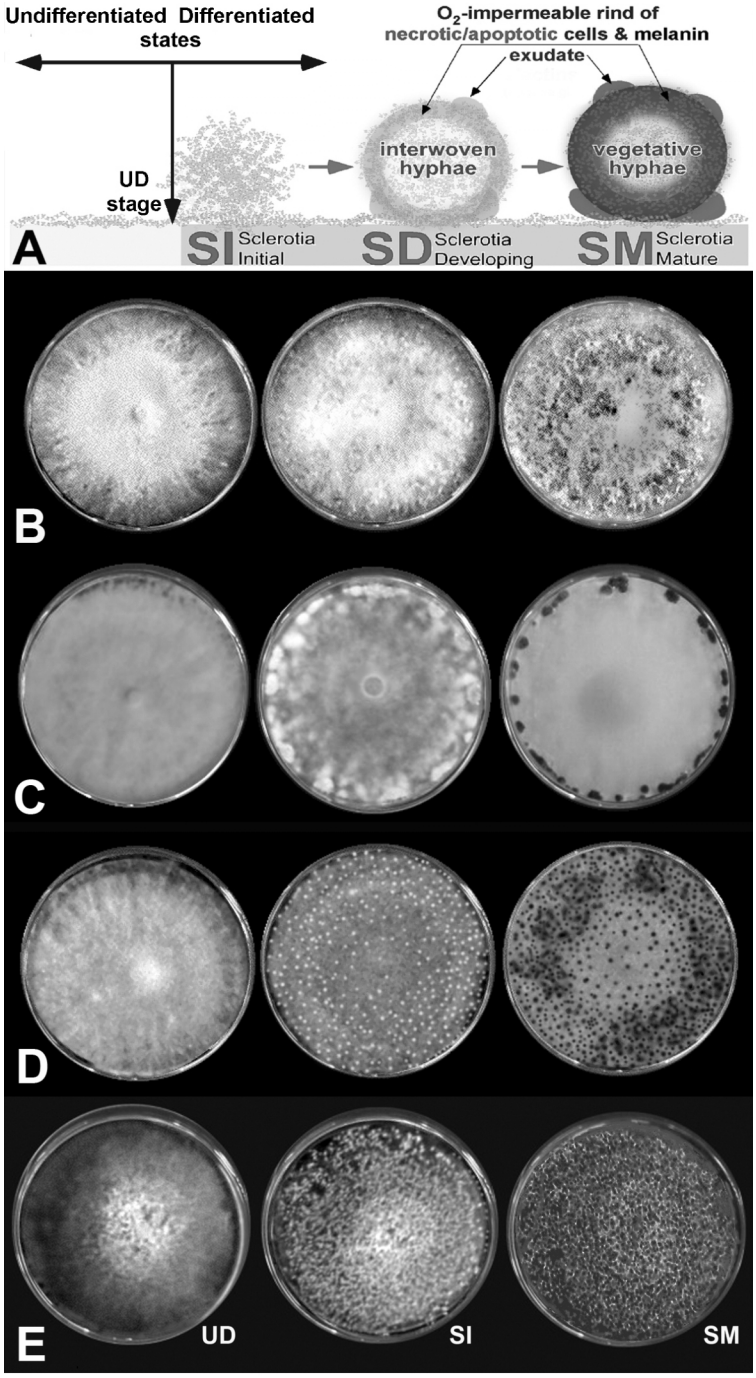

Fig. (1). (**A**) Drawing of sclerotium developmental stages SI, SD and SM, developed from an undifferentiated (UD) colony. Developmental stages (UD, SI and SM) of the phytopathogenic fungi used in this study: (**B**) *R. solani*; (**C**) *S. sclerotiorum*; (**D**) *S. rolfsii*; (**E**) *S. minor*.

Mycelia were collected using forceps. In the case of *S. minor*, the separation of mycelia from sclerotia was not feasible due to the paucity of the surrounding hyphae. Mycelia were washed at least 2 times in 2-3 volumes/gr fungal wet weight ice-cold phosphate-EDTA buffer [10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, 0.5 mM PMSF and 1 mM of the antioxidant BHA (in final 0.3% EtOHl)] by centrifugation at 6000 *g*. The resulting tissue pellets were ground in a porcelain mortar in liquid nitrogen to prevent artificial oxidation of sulfhydryl groups during homogenization. The homogenate was subsequently used for the following assays.

TRS Fluorometric Assay

The TRS assay uses the thiol specific probe monobromobimane (mBrB) (which forms –SH/mBrB adduct that fluoresces at 478 nm after excitation at 390 nm) to measure the TRS components protein (P) and non-protein (NP) thiols and disulfides [26]. These are the non-protein thiols glutathione (GSH**)** and cysteine (CSH), the non-protein disulfides of glutathione (GSSG) and cystine (NPSSC), the protein thiols (PSH), and the protein disulfides (PSSP, resulting from oxidation between –SH groups within the same protein and between same or different proteins).

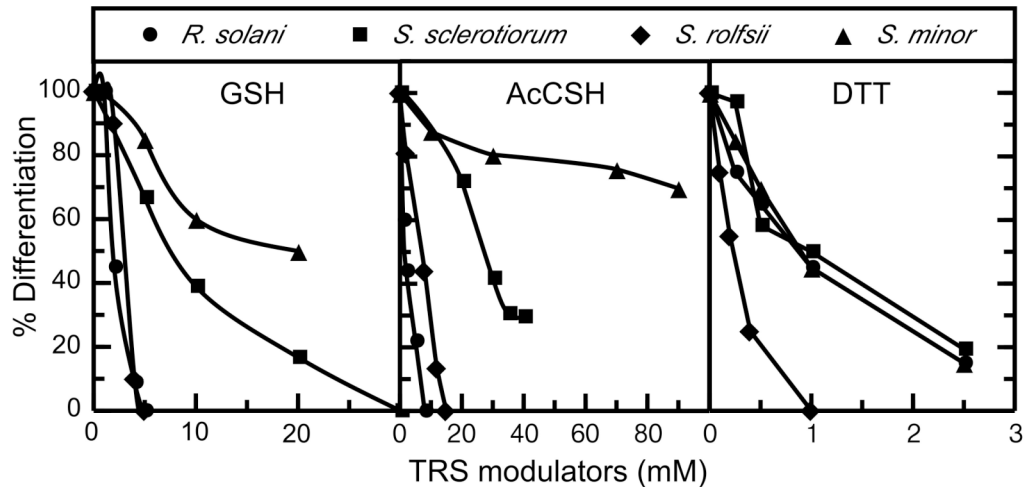

Fig. (2). Effect of various concentrations of the TRS modulators AcCSH, GSH and DTT on the decrease of sclerotial differentiation;100% is the fungal differentiation in the absence of the TRS modulators and corresponds to zero differentiation decrease, while 0% corresponds to 100% differentiation decrease.

These components are isolated and measured alone or in mixtures as certain fractions in the sample homogenate. The CSH/AcCSH-based components of TRS are determined by a photometric procedure of the assay [26, 27], which uses the CSH-specific reagent (at acidic pH) ninhydrin that forms with CSH a purple chromophore absorbing strongly at 560 nm. It measures CSH and its non-protein disulfides NPSSC. In the case of N-acetylcysteine (AcCSH) administration, the use of the enzyme acylase permits the simultaneous measurement of AcCSH and its non-protein disulfides NPSSCAc. GSSG was quantificated by a photometric enzymic assay previously reported [27]. Fluorescence measurements were performed in a quartz micro-cuvette (internal dimensions 4x4x45 mm) using a Shimadzu RF-1501 spectrofluorometer set at 10 nm excitation/emission slit width and at high sensitivity, while photometric measurements were performed in 1-ml cuvettes using a Shimatdzu UV-visible UV-1200 spectrophotometer (both from Shimatzu Co, Kyoto, Japan).

Protein Assay

Protein concentration in fungal homogenate samples was determined by a modification of the CBB-based assay [27]. Specifically, 0.063 ml of various homogenate sample dilutions was mixed with 0.02 ml 0.5% (w/v) Triton X-100 and 0.017 ml 6 N HCl. The resulting mixture was incubated at 100°C for 10 min and after equilibration at room temperature (RT) it was mixed with 0.9 ml 0.033% (w/v) CBB-G250 reagent (prepared in 0.5 N HCl) and incubated for 5 min at RT. The absorbance at 620 nm of the final mixture was converted to mg total protein from a BSA standard curve 0-0.05 mg, using appropriate sample and reagent blanks.

Statistical Analysis of Data

Data were analyzed using the SPSS statistical package (SPSS Inc, 2001, Release 11.0.0, USA), using the one-way ANOVA, followed by Bonferroni's post hoc test when variances across groups were not significantly different or by Dunnette's T3 post hoc test when variances were significantly different. Variance significance was tested by the same software. Comparisons were performed using the T-test. Differences were considered significant when $P<0.05$. Results from three independent experiments (i.e. three experiments, with data derived from at least three different plates per experiment) are expressed as means ± SD (designated as error bars in figures).

RESULTS AND DISCUSSION

In this study the effect of GSH-specific and general TRS modulators was studied at the undifferentiated and differentiated states of fungal strains representing the main four types of sclerotia in phytopathogenic fungi (Fig. 1). The TRS modulators used in this study were (a) GSH, (b) the indirect GSH biosynthesis inducer AcCSH (the antioxidant non-cytotoxic form of CSH, which is also a precursor of GSH), and as control the non-specific –SH group provider DTT (source of –SH reducing groups). Specifically, their effect was studied on the degree of differentiation and on the levels of the main reduced (GSH, CSH, AcCSH, PSH) and oxidized (GSSG, NPSSC, NPSSCAc, PSSP) TRS components during the transition of the undifferentiated colony (UD) to the sclerotial initiation SI stage of the differentiated state (Figs. 2-5). Reduced TRS components are related with low oxidative stress while oxidized TRS components with high oxidative stress [20]. The aim of this study was to establish a causal relationships between TRS-associated -SH groups and sclerotial differentiation in relation to the reduced and oxidized TRS components.

Loose Type Sclerotiogenesis (*R. solani*)

When GSH and AcCSH were administered to *R. solani*, the reduced TRS components (GSH or CSH) increased with concomitant decrease of sclerotiogenesis by 100% and 78%, respectively (Figs. **2-4**). In general, GSH is not transferred in cells as intact molecule. Instead, gamma-glutamyl-transpeptidase (gamma-GT) is responsible for extracellular GSH decomposition to glutamic acid and cysteinyl-glycine, which can then be used by other GSH biosynthesis enzymes to generate GSH in mixture with CSH [20]. Thus, this

biosynthetic scheme may explain the increase of the reduced TRS component CSH throughout development of this fungus upon GSH administration (Fig. **3**). Similarly, AcCSH may be converted to CSH and then to GSH, as indicated by the observed increase of endogenous CSH and GSH upon AcCSH administration, and by the relatively small levels of accumulated AcCSH (Fig. **4**). These results suggest that the observed decrease of sclerotial differentiation by GSH and AcCSH is related to the antioxidant effects mediated by the general thiol reserves of the cell and not specifically by GSH. This conclusion is supported by the observation that the general disulfide reductant and antioxidant DTT also caused a decrease of sclerotial differentiation (by 55%, Fig. **2**), with concomitant increase of GSH and CSH throughout development (Fig. **5**). Furthermore, the reduced TRS component PSH decreased in the UD state in the presence of the TRS-modulators (Figs. **3**-**5**), and, in conjunction with their sclerotial differentiation inhibiting effect (Fig. **2**), it may be suggested that the reduced thiol proteins (PSH) are directly related to the formation of sclerotia. Concerning TRS components, it was found that GSSG and NPSSC levels were significantly elevated upon GSH and AcCSH administration (Figs. **3**,**4**), especially at the UD state, possibly because of the reaction of GSH and CSH with free radicals [20]. This is also supported by the increased the levels of GSSG and NPSSC in the UD state by the antioxidant thiol DTT (Fig. **5**), which strongly suggests that this antioxidant is not utilized to protect GSH and CSH from oxidation. Concerning the TRS component PSSP, it was not traced in the UD and in the SI colony in the absence or presence of the TRS modulators, suggesting that protein thiols in this fungus are protected from oxidative stress.

Terminal Type Sclerotiogenesis (*S. sclerotiorum*)

As in the case of loose type sclerotial differentiation, GSH addition in the growth medium of *S. sclerotiorum* resulted in total inhibition of sclerotial differentiation, with concomitant increase of the endogenous reduced TRS component GSH at the UD state, and increase of CSH throughout development (Figs. **2**,**3**). In contrast, AcCSH administration decreased GSH levels and increased CSH levels as expected (Fig. **4**). In addition, significant amount of intracellular AcCSH was detected, suggesting that a large amount of the administered AcCSH was available to exert its known antioxidant properties [20], providing a low oxidative stress reduced thiol environment associated with the observed decrease in sclerotial differentiation (by 69%, Fig. **2**). Thus, it could be suggested that the decrease of sclerotial differentiation in this fungus may be also related to the antioxidant properties of the general cellular thiol reserves and not particularly to GSH or CSH. This hypothesis is supported by the finding that DTT decreased sclerotial differentiation by 50% and increased endogenous GSH throughout development, possibly at the expense of its precursor CSH -since the latter was not traced (Figs. **2**,**5**). As for the reduced TRS component PSH, it either remained unchanged or decreased at the UD state by the tested TRS modulators (Figs. **3**-**5**), suggesting once more a role of the reduced thiol proteins to the formation of sclerotia.

Concerning TRS components, GSSG and NPSSC levels were also significantly elevated in this fungus throughout

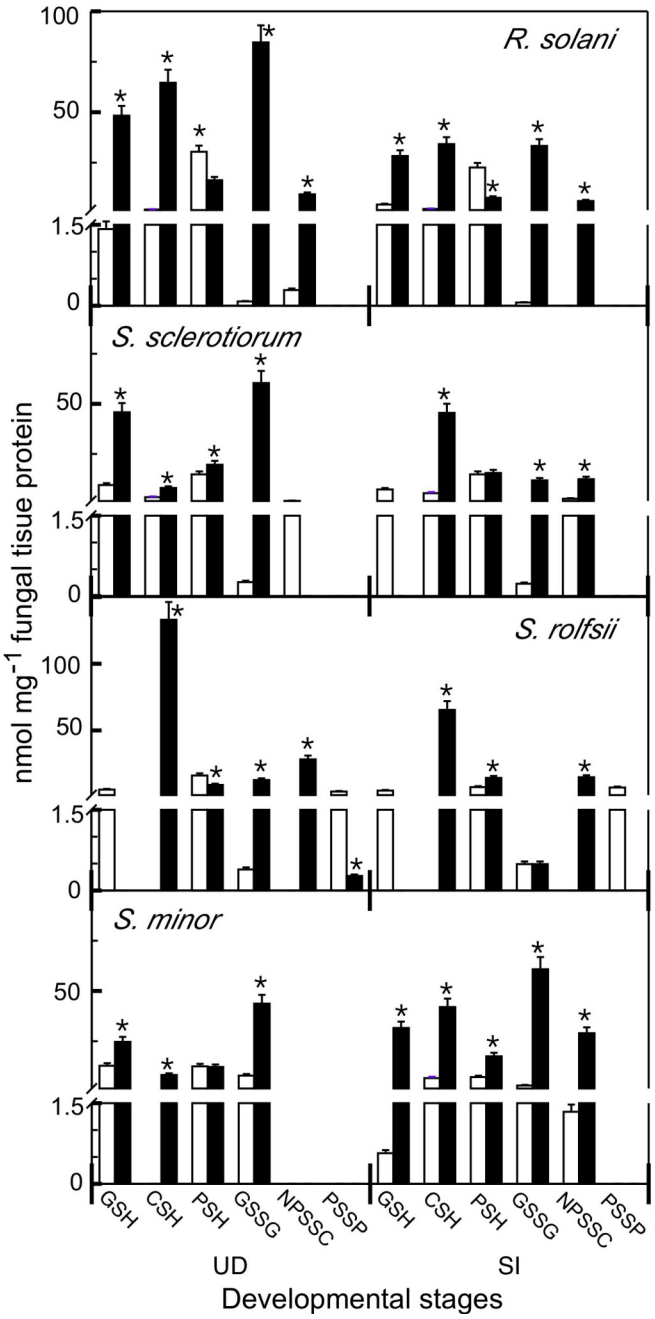

Fig. (3). Profiles of reduced and oxidized TRS components in the undifferentiated colony (UD) and in the SI differentiation stage mycelial substrate of the tested fungi in the absence (open bars) and presence (filled bars) of the TRS modulator GSH. GSH was added to the growth medium of *R. solani*, *S. sclerotiorum*, *S. rolfsii* and *S. minor* at growth non-inhibiting concentrations 10, 20, 5 and 10 mM, respectively. Asterisks over the filled bars show that their value is statistically different to the value of their corresponding open bars, for $P<0.05$.

development upon GSH and AcCSH administration (Figs. **3**,**4**), possibly due to GSH and CSH oxidation during their free radical scavenging reactions. In the case of AcCSH administration, significant quantities of its oxidized mixed disulfide NPSSCAc were formed (Fig. **4**), possibly due to the oxidation of the intracellular AcCSH by free radicals. As for the oxidized TRS component PSSP, it was not traced in the UD and SI colony of the in the presence or absence of

the TRS modulators, suggesting again that protein thiols in this fungus are protected from oxidative stress.

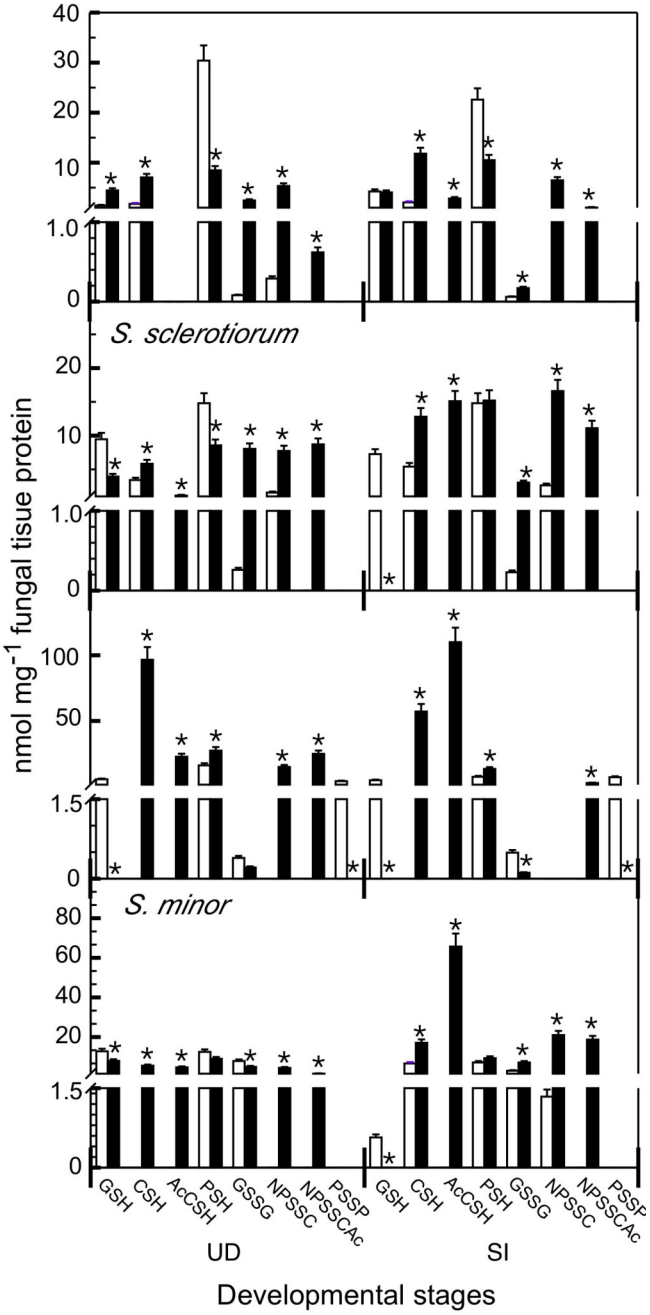

Fig. (4). Profiles of reduced and oxidized TRS components in the undifferentiated colony (UD) and in the SI differentiation stage mycelial substrate of the tested fungi in the absence (open bars) and presence (filled bars) of the TRS modulator AcCSH. AcCSH was added to the growth medium of *R. solani*, *S. sclerotiorum*, *S. rolfsi* and *S. minor* at growth non-inhibiting concentrations 5, 40, 15 and 20 mM, respectively.

Lateral-Chained Sclerotiogenesis (*S. rolfsii*)

When *S. rolfsii* was exposed to exogenous GSH its sclerotial differentiation decreased by 100% (Fig. **2**), with a concomitant increase of CSH and the disappearance of GSH throughout development (Fig. **3**). This suggests that the endogenous GSH does not have direct metabolic role in differentiation, and that the exogenous GSH may act as general thiol antioxidant by being converted to CSH. Similarly, AcCSH administration resulted in a decrease of sclerotial differentiation (by 100%, Fig. **2**), which concurred with disappearance of GSH and increase of CSH and of the intracellular AcCSH throughout development (Fig. **4**). The decrease of GSH by AcCSH suggests again no direct role for GSH on differentiation, while the AcCSH differentiation inhibiting effect may be related to its known antioxidant action. The relation of sclerotial differentiation with the antioxidant action of -SH group reserves was also shown by its 45% decrease by DTT (Fig. **2**), which concurred with unaltered GSH levels and absence of CSH (Fig. **5**). This, also, can be attributed to the strong antioxidant effect of DTT –SH groups. Furthermore, PSH increased by GSH and AcCSH (Figs. **3**,**4**) possibly reflecting the -SS- bond reducing properties of these thiols at the concentrations used. Concerning TRS components, GSH administration significantly increased GSSG only at the UD stage (Fig. **3**), suggesting that GSH was oxidized to GSSG as result of its direct free radical scavenging reactions [20]. Furthermore, AcCSH administration decreased GSSG mainly at the SM stage (Fig. **4**), which can be explained by the antioxidant function of AcCSH in relation to its high intracellular concentration throughout development. This is also supported by the accumulation of the oxidation product of AcCSH, NPSSCAc, particularly at the UD state, which can be attributed to the antioxidant function of AcCSH. As in the case of *S. sclerotiorum*, the observed in *S. rolfsii* accumulation of NPSSCAc by AcCSH administration, in relation to its sclerotium inhibiting effect, suggests that the accumulation of the oxidized TRS components GSSG and NPSSC may not be necessarily cell damaging indicators. They may rather serve as a potential non-protein thiol pool *via* the reversible oxidation of non-protein thiols [20]. Furthermore, DTT administration did not change GSSG levels (Fig. **5**), which suggests that DTT in this fungus is not an efficient disulfide reductant at the concentrations used (and internally accumulated). Finally, the oxidized TRS component PSSP decreased throughout development upon administration of all tested TRS modulators, possibly reflecting the protein disulfide formation inhibiting effect of the tested TRS modulators.

Lateral-Simple Type Sclerotiogenesis (*S. minor*)

Administration of GSH in the growth medium of *S. minor* caused a substantial decrease (50%) of sclerotial differentiation (Fig. **2**) and increase of GSH and CSH throughout development (Fig. **3**). Moreover, AcCSH administration decreased sclerotial differentiation only by 16% (Fig. **2**) and decreased endogenous GSH possibly due to the fact that the AcCSH-derived CSH remained unused for GSH formation as indicated by the disappearance of CSH at the SI stage (Fig. **4**). In addition, DTT decreased differentiation by 85%, which concurred with a significant decrease of GSH at the UD state and increase at the SI stage (Fig. **5**). These data suggest that sclerotial differentiation is affected by –SH groups and not by those of GSH and CSH. Concerning TRS proteins PSH, they increased in the SI stage upon GSH administration (Fig. **3**), possibly as result of their participation in disulfide exchange reactions, while in the

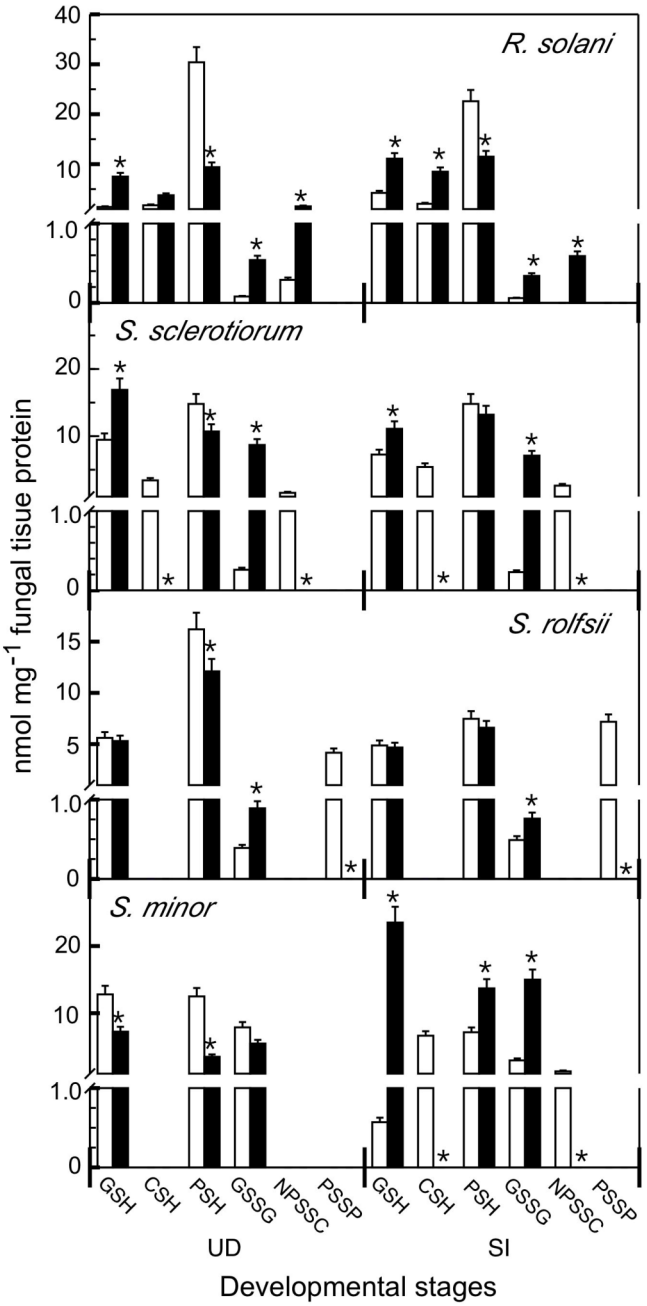

Fig. (5). Profiles of reduced and oxidized TRS components in the undifferentiated colony (UD) and in the SI differentiation stage mycelial substrate of the tested fungi in the absence (open bars) and presence (filled bars) of the TRS modulator DTT. DTT was added to the growth medium of *R. solani*, *S. sclerotiorum*, *S. rolfsi* and *S. minor* at growth non-inhibiting concentrations 1, 1, 0.2 and 1 mM, respectively.

case of AcCSH administration PSH levels remain unchanged (Fig. **4**), suggesting that the antioxidant action of AcCSH was rather ineffective on this fungus. Concerning TRS components, GSSG increased significantly throughout development, and NPSSC increased at the SI stage upon GSH administration (Fig. **3**), possibly because of GSH and CSH reacting with free radicals. As in the case of the other three fungi, the accumulation of these disulfides in *S. minor* may not be cell damaging but rather serve as a potential thiol pool *via* the reversible oxidation of non-protein thiols. This is supported by the accumulation of NPSSCAc by AcCSH administration (Fig. **4**). Furthermore, DTT administration resulted in an increase of GSSG at the SI stage, suggesting that this effect might be attributed to the direct antioxidant action of the –SH groups of DTT. Furthermore, PSSP was generally absent throughout development and unaffected by the TRS modulators (Figs. **3-5**).

CONCLUSIONS

This study showed that the main four types of sclerotial differentiation are related with TRS. It was shown that exogenous GSH and AcCSH increased either GSH or CSH or both during transition of the tested fungi from the undifferentiated state (UD) to the differentiated state (SI stage). These GSH biosynthesis inducers TRS modulators and the non specific –SH group provider antioxidant thiol DTT also caused inhibition of sclerotial differentiation. Given the fact that the –SH group provider DTT had an analogous effect on intracellular GSH and CSH levels, strongly suggests that the decrease of sclerotial differentiation is related to the antioxidant action of -SH groups and not particularly to the –SH groups of GSH and CSH. Moreover, these TRS modulators decreased PSH and PSSP, and increased GSSG, suggesting a role of these TRS components in sclerotial differentiation. It should be noted that the increase of the widely accepted high oxidative stress indicator GSSG [20] by the known antioxidants AcCSH and DTT strongly suggests that GSSG is not a reliable indicator of high oxidative stress, an observation previously reported [27]. Instead, it may also serve as a GSH pool functioning as such *via* its oxidation to GSSG by glutathione reductase [20]. The data of this study are also in accordance with our hypothesis that sclerotial differentiation is induced by high oxidative stress [1].

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