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5 **Metabolic processes and carbon nutrient exchanges between host and**
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7 ***Sclerotinia sclerotiorum***

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1

2 **Abstract**

3 Interactions between the necrotrophic fungus *Sclerotinia sclerotiorum* and one of its hosts,
4 *Helianthus annuus* L., were analyzed during fungal colonization of plant tissues.
5 Metabolomic analysis, based on ¹³C- and ³¹P-NMR spectroscopy, was used to draw up the
6 profiles of soluble metabolites of the two partners before interaction, and to trace the fate of
7 metabolites specific of each partner during colonization. In sunflower cotyledons, the main
8 soluble carbohydrates were glucose, fructose, sucrose and glutamate. In *S. sclerotiorum*
9 extracts, glucose, trehalose and mannitol were the predominant soluble carbon stores. During
10 infection, a decline in sugars and amino acids was observed in the plant and fungus total
11 content. Sucrose and fructose, initially present almost exclusively in plant, were reduced by
12 85%. We used a biochemical approach to correlate the disappearance of sucrose with the
13 expression and the activity of fungal invertase. The expression of two hexose transporters,
14 *Sshxt1* and *Sshxt2*, was enhanced during infection. A database search for hexose transporters
15 homologues in the *S. sclerotiorum* genome revealed a multigenic sugar transport system.
16 Furthermore, the composition of the pool of reserve sugars and polyols during infection was
17 investigated. Whereas mannitol was produced *in vitro* and accumulated *in planta*, glycerol
18 was exclusively produced in infected tissues and increased during colonization. The
19 hypothesis that the induction of glycerol synthesis in *S. sclerotiorum* exerts a positive effect
20 on osmotic protection of fungal cells and favours fungal growth in plant tissues is discussed.
21 Taken together, our data revealed the importance of carbon-nutrient exchanges during the
22 necrotrophic pathogenesis of *S. sclerotiorum*.

23 **Keywords** Acid invertase . *Helianthus* . Hexose transport . NMR spectroscopy . Polyols .
24 *Sclerotinia*

25 **Abbreviations** GPC: glycerylphosphoryl-choline . GPE: glycerylphosphoryl-ethanolamine .
26 GPG: glycerylphosphoryl-glycerol . GPI: glycerylphosphoryl-inositol . hpi: hours post
27 inoculation . PCA: perchloric acid . PGA: phosphoglyceric acid . Q-PCR: quantitative
28 polymerase chain reaction . UDP-GlcNAc: uridine -5'-diphosphate-N-acetylglucosamine

29

1 **Introduction**

2 As a necrotrophic fungus, *Sclerotinia sclerotiorum* is able to feed on dead cells and is one of
3 the most non-specific and omnivorous plant pathogens (Boland and Hall 1994). Two main
4 pathogenicity factors, secretion of oxalic acid and hydrolytic enzymes, act in concert to
5 macerate plant tissues and generate necrosis. Degradation of plant cell wall components and
6 host tissues is linked to the concerted production of a wide and complex range of extracellular
7 lytic enzymes such as cellulases, hemicellulases, pectinases and proteases. Sequentially
8 secreted by the fungus, lytic enzymes facilitate penetration, colonization and maceration but
9 also generate an important source of nutrients (reviewed by Bolton et al. 2006). Secreted
10 oxalic acid acidifies the apoplastic space, sequesters calcium, interferes with plant defences
11 and appears to be an essential determinant of pathogenicity (Maxwell and Lumdsen 1970;
12 Hegedus and Rimmer 2005). The secretion of oxalic acid by *S. sclerotiorum* results in
13 formation of lesions and water-soaked tissues, in advance of the invading fungal hyphae,
14 rapidly expanding as a frontal zone of hosts cells impaired in their viability (Lumdsen and
15 Dow 1973). To complete their life cycle *in planta*, pathogenic fungi must also be able to gain
16 nutrients from plant cells.

17 Metabolic interactions between plants and fungi have been conducted on biotrophic
18 and mycorrhizal fungi in most cases. Mycorrhizae are characterized by the uptake of minerals
19 from the soil by fungal hyphae, followed by their transfer to the root cells. In return, plant
20 carbohydrates are transferred to the fungal symbiont and their utilization is oriented towards
21 the synthesis of short chain polyols (Martin et al. 1998; Bago et al. 1999). Free amino acids
22 also represent an important sink of absorbed and assimilated carbon (Martin et al. 1998).

23 Biotrophs cause little damage to the host plant, and derive energy from living cells.
24 They produce extensions into plant cells, haustoria, linked to maintain basic compatibility
25 between fungi and their host plants and to nutrient uptake (Mendgen et al. 2000). During a
26 compatible interaction, competition of the parasite with natural sink organs of the host, results
27 in considerable modification of photoassimilate production and alterations in partitioning
28 within host tissues (Scholes et al. 1994; Hall and Williams 2000; Abood and Lösel 2003). A
29 common feature is a reduction in the rate of photosynthesis (Tang et al. 1996; Chou et al.
30 2000). During infection with *Albugo candida*, the decrease in photosynthesis was correlated
31 with an accumulation of carbohydrates in leaves of *Arabidopsis thaliana* (Tang et al. 1996;
32 Chou et al. 2000). Direct analysis of sugar composition of the leaf apoplast of tomato infected
33 by *Cladosporium fulvum* indicated high levels of sucrose accumulated during early stages of
34 infection, that could be linked to the expression of plant or fungal invertases (Joosten et al.

1 1990). The induction of a sink-specific cell-wall invertase at the site of infection appears to be
2 a general response to a biotic stress (Scholes et al. 1994; Fotopoulos et al. 2003; Roitsch et al.
3 2003; Voegelé et al. 2006). Molecular analysis of compatible biotrophic interactions
4 suggested that nutrients were mainly taken up in form of hexoses and amino acids, in
5 accordance with the strong expression of amino acids and hexoses permeases in haustoria
6 (Voegelé et al. 2001; Struck et al. 2004). As shown for the compatible interactions *Uromyces*
7 *fabae-Vicia faba* or *C. fulvum-Lycopersicon esculentum*, much of the carbohydrates supplied
8 to fungal biotrophic pathogens could be converted later in the infection cycle into the C6-
9 polyol mannitol, that could play a pivotal role in suppression of ROS-related defence
10 mechanisms or in carbon storage (Noeldner et al. 1994; Voegelé et al. 2005).

11 The nature of available nutrient supplies metabolized by necrotrophic fungi during
12 infection has received little attention up to now. Studies dedicated to carbohydrate and
13 invertase activity changes during necrotrophic interactions are scarce and mainly focused on
14 plant. Upon infection with *Botrytis cinerea*, photosynthetic gene expression was
15 downregulated in tomato plant tissues and expression of a cell wall invertase was induced by
16 the pathogen (Berger et al. 2004). Accumulation of invertase in the cell walls of tomato plants
17 was induced by *Fusarium oxysporum* in susceptible and resistant hosts (Benhamou 1991). An
18 elicitor preparation of the tomato pathogen *F. oxysporum* also activated invertase gene
19 expression in tomato suspension culture cells (Sinha et al. 2002). In this study, we report a
20 metabolic study of the necrotrophic interaction between *S. sclerotiorum* and cotyledonary
21 leaves of sunflower based on NMR spectroscopy used to monitor cellular metabolism
22 (Roberts and Jardetsky 1981; Shachar-Hill and Pfeffer 1996; Ratcliffe and Shachar-Hill
23 2001). In order to analyze metabolic processes that promote fungal development in plant
24 tissues, we established the profiles of soluble metabolites for each partner and followed the
25 quantitative modifications of these metabolites during the course of infection. Our results
26 indicate a progressive exhaustion of plant carbohydrate stores in favour of the accumulation
27 of glycerol of fungal origin. Fungal elements that could be linked to the decrease of plant
28 sugars have been investigated. Increases of invertase activity and *in planta* expression of
29 fungal hexose transporters are described.

30

31 **Materials and methods**

32

33 Fungal strain and growth conditions

34

1 *S. sclerotiorum* S5 was initially provided by BayerCropScience, Lyon, France. The strain was
2 maintained on potato dextrose agar. For NMR characterization, mycelia were grown for 48 h
3 on solid minimal medium (Riou et al. 1991) supplemented with 2% glucose. Mycelia were
4 frozen in liquid nitrogen and stored at -80°C .

5

6 Pathogenicity tests

7

8 Phytopathogenicity assays were performed on sunflower cotyledons as hosts. Sunflower
9 plants (Mirasol variety) were purchased from Limagrain (Riom, France). Sunflowers were
10 grown at 25°C with a 14-h light period per day. Cotyledons from one-week-old germlings
11 were infected at the end of a dark period by depositing a 4-mm-mycelium disk at the centre of
12 the adaxial side. At 8 hpi, mycelium discs were tightly attached to the surface of cotyledons,
13 indicating the penetration of fungal hyphae into plant. Necrosis was detectable by the
14 apparition of a brown colour surrounding the starting point of infection. The necrosed and
15 macerated region corresponded to a 2-mm-zone surrounding the mycelium discs 16 hpi. 24
16 hpi, half of the cotyledons were macerated and necrosed. At 48 hpi, the whole cotyledon was
17 infected. Cotyledons were harvested after the different stages of symptoms development (8,
18 16, 24, 36, 48 hpi). For invertase assays and some NMR experiments that were realized on
19 fractions of infected cotyledons, plugs of mycelium were deposited near the tip of the leaves,
20 in order to easily separate two distinct zones at different stages of infection: a non-invaded
21 region and an invaded region. Absence of fungus was confirmed by microscope observation
22 and by the absence of detected UDP-GlcNAc in the spectra. Samples were then frozen in
23 liquid nitrogen.

24

25 NMR spectroscopy

26

27 PCA extracts were prepared from 10 g of *H. annuus* cotyledons, or 10 g of *S. sclerotiorum*
28 mycelia, or 10 g of infected cotyledons, according to the method described by Aubert et al.
29 (1996). Spectra of neutralized PCA extracts were recorded in a Fourier transform NMR
30 spectrometer (model AMX 400, Bruker Billerica MA) equipped with a 10-mm multinuclear
31 probe tuned at 161.9 or 100.6 MHz for ^{31}P - or ^{13}C -NMR studies, respectively. The deuterium
32 resonance of $^2\text{H}_2\text{O}$ (100 μl added per ml of extract) was used as a lock signal. ^{31}P -NMR
33 acquisition conditions: 70° pulses (15- μs) at 3.6-s intervals; spectral width, 8.2 kHz; Waltz-16
34 ^1H decoupling sequence with 1 W decoupling during acquisition and 0.5 W during delay; free

1 induction decays collected as 8,000 data points, zero-filled to 16,000, and processed with a
2 0.2-Hz exponential line broadening. Spectra were referenced to methylene diphosphonic acid,
3 pH 8.9, at 16.38 ppm. Divalent paramagnetic cations were chelated by addition of
4 corresponding amounts of 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA). ¹³C-NMR
5 acquisition conditions: 90° pulses (19-μs) at 6-s intervals; spectral width, 20 kHz; Waltz-16
6 ¹H decoupling sequence with 2.5 W decoupling during acquisition and 0.5 W during the
7 delay; free induction decays collected as 32,000 data points, zero-filled to 64,000, and
8 processed with a 0.2-Hz exponential line broadening. Spectra were referenced to
9 hexamethyldisiloxane at 2.7 ppm. Mn²⁺ ions were chelated by addition of 1mM CDTA.
10 Assignments were made after running series of standard solutions of known compounds at pH
11 7.5 and after the addition of these compounds to PCA extracts as previously described
12 (Aubert et al. 1996). Identified compounds were quantified by comparison of the surface of
13 their resonance peaks to the surface of the resonance peaks of standards added to samples
14 before grinding. Fully relaxed conditions during spectra acquisition (pulses at 20-s intervals)
15 were used for quantification. The standards utilized were methyl phosphonate and maleate for
16 ³¹P- and ¹³C-NMR analyses.

17

18 Preparation of protein extracts and detection of invertase activity

19

20 Frozen healthy plant material and infected plant tissues were ground to a fine powder in liquid
21 nitrogen. Ground tissues were resuspended in cold extraction buffer (100 mM Tris pH 7.5, 2.5
22 mM EDTA, 5 mM DTT, 1 mM PMSF, 5 μg ml⁻¹ pepstatin and 10 mM Chaps) and incubated
23 20 min at 4°C. Extracts were then centrifuged at 13,000g for 30 min at 4°C. Supernatants
24 containing total proteins were collected and stored at -20°C. Secreted proteins from *in vitro*
25 culture filtrates were concentrated by (NH₄)₂SO₄ precipitation (80% saturation) overnight at
26 4°C. After centrifugation at 12,000g for 30 min at 4°C, the proteins present in pellets were
27 dissolved in distilled water and stored at -20°C. Protein concentration was determined using
28 the BioRad protein Assay (BioRad, Marnes la Coquette, France), with BSA as the standard.
29 Proteins (10 μg) extracted from healthy and infected tissues or recovered from culture filtrates
30 were separated by isoelectric focusing (IEF) using ultrathin polyacrylamide gels (Servalyt
31 Precotes, pH 3-10, Serva Heidelberg, Germany). Gels were focused for 1.5 h at 3 W, 1700 V
32 and 1 mA. A part of the slab gel was stained with Coomassie brilliant blue for protein
33 visualization, the other part was used to reveal invertase activities by zymography, according
34 to Chen et al. (1996). After electrophoresis, gels were covered by a 1% agarose overlay

1 containing 500 mM sucrose in 50 mM sodium acetate pH 5.6 or 4 or in 50 mM Hepes buffer
2 pH 7, at 30°C for 30 min. Invertase isoforms were revealed by incubating the gels in 1%
3 2,3,5-triphenyltetrazolium chloride monohydrate (Sigma, St Quentin-Fallavier, France) in
4 0.25 M hot NaOH. Red colour development was stopped with 1% acetic acid.

5 6 Immunological methods

7
8 Proteins (10 µg) were separated by SDS polyacrylamide gel electrophoresis (SDS PAGE)
9 according to Laemmli (1970), using the miniprotein-2D system (BioRad, Marnes la Coquette,
10 France). After migration in a 7% acrylamide gel, proteins were blotted onto nitrocellulose
11 (Schleicher and Schuell GmbH, Dassel, Germany) according to Towbin et al. (1979).
12 Nitrocellulose membranes were incubated for 2 h at room temperature in 5% non fat dry milk,
13 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.01% Tween 20, rinsed in 150 mM NaCl, 50 mM
14 Tris-Hcl pH 7.4, 0.05% Tween 20 and then incubated 1 h in the presence of antisera. Primary
15 antisera were used at a dilution of 1:5000. Antisera raised against tobacco cell wall invertase
16 were kindly provided by Dr S. Greiner (Heidelberger Institut für Pflanzenwissenschaften,
17 Germany). Antisera raised against *Candida albicans* invertase were purchased from
18 USBiological (Euromedex, Mundolsheim, France). Incubations with anti-rabbit IgGs (anti-
19 tobacco invertase, 1:20000 and anti-*C. albicans* invertase, 1:2000) was followed by detection
20 using enhanced chemiluminescent substrate (Pierce Super Signal Substrate, Pierce Perbio-
21 France, Brebières, France). Cross reactivity of the antisera was tested. No proteins from
22 healthy plant tissues extracts were detected by anti fungal antisera. As well, antiplant antisera
23 did not detect any protein extracted from the mycelium of *S. sclerotiorum* or secreted by the
24 fungus.

25 26 Cloning *Sshxt1* and *Sshxt2* genes

27
28 EST sequences (BfCon0401 and BfCon0411) from the related necrotrophic fungus *Botrytis*
29 *cinerea*, deposited in the public databases (COGEME phytopathogen EST databases,
30 <http://cogeme.ex.ac.uk/>) as putative hexose transporters (Soanes et al. 2002), were used as
31 probes to screen at low stringency the genomic EMBL3 library of *S. sclerotiorum*. For *Sshxt1*,
32 a 205-bp *B. cinerea* genomic DNA fragment was amplified using the sense primer 5'-
33 GAATTGTCCTTTGCTTGCCTC-3' and the antisense primer 5'-
34 TGGGGTGAAGAATGCAAG-3'. For *Sshxt2*, the sense primer 5'-

1 GATCTTGGGTCTGCGATGAC-3' and the antisense primer 5'-
2 CTGGTGCCGTTCTTATCTG-3' were used to amplify a 300-bp *B. cinerea* genomic DNA.
3 25 ng of *B. cinerea* genomic DNA were used as template. PCR conditions were as follows:
4 after denaturation at 94°C for 5 min, annealing of the primers was done at 54°C to amplify the
5 probe used to clone *Sshxt1* and at 60°C to amplify the probe used to clone *sshxt2*. After an
6 extension step at 72°C for 1 min, a final elongation step at 73°C for 8 min was added for 30
7 cycles. Each amplified fragment was sequenced and used to screen the library at low
8 stringency (37°C, 50% formamide, 5[×] SSC). One recombinant phage giving the strongest
9 hybridization signal for each gene was chosen for further studies. Subcloning and routine
10 procedures were performed with standard protocols (Sambrook 1989). Sequences of *Sshxt1*
11 and *Sshxt2* are available in GenBank under the following accession no.: AY647267 and
12 AY647268 respectively.

13

14 RNA isolation and Q-PCR

15

16 RNA was extracted from plant material frozen in liquid nitrogen and kept at -80°C. Samples
17 were ground in liquid nitrogen, and total RNA was purified by using RNeasy mini spin
18 column as described by manufacturers (Qiagen, Courtabeuf, France). Transcripts of *Sshxt1*
19 were amplified using the sense primer 5'-GGTGTCGAAGAATCCCATCCA-3' and the
20 antisense primer 5'-GTGCTGGCAAACCGACGAT-3'. Transcripts of *Sshxt2* were
21 amplified using the sense primer 5'-ACTACTATGTGCTTGTCTTTGC-3' and the antisense
22 primer 5'-GATGCTGCTTCCCAAACGCCATTA-3'. To detect transcripts of the *S.*
23 *sclerotiorum* actin gene *Ssact1*, the sense primer 5'-CTTCGTGTAGCACCAGAGGA-3' and
24 the antisense primer 5'-ATGTTACCATACAAATCCTTA-3' were used. For Q-PCR
25 analysis, total RNA was DNaseI treated (RQ1 RNase free DNase, Promega, Charbonnières,
26 France) to remove genomic DNA. Absence of DNA was analyzed by performing a PCR
27 reaction similar to the real-time PCR program, on the DNaseI-treated RNA using Taq-DNA
28 polymerase (Promega, Charbonnières, France). Q-PCR experiments were performed using the
29 one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Courtabeuf, France) according to the
30 instructions of the manufacturer. Reactions were performed in a final volume of 20 µl, using 1
31 µg of total RNA, 1 µM of each primer, 10 µl of QuantiTect SYBR Green RT-PCR Master Mix
32 (containing Hot Start Taq DNA polymerase, QuantiTect SYBR Green RT-PCR buffer, dNTP
33 mix, SYBR Green and 5mM MgCl₂). The amplification was effected in the LightCycler
34 (Roche, Meylan, France). The following amplification program was used: 20 min at 50°C for

1 cDNA synthesis, 15 min at 95°C to activate the Hot Start DNA polymerase and 45 cycles of
2 amplification as follows: 15 s at 94°C, 30 s at 60°C for *Sshxt1*, 54°C for *Sshxt2* and 60°C for
3 *Ssact1* and 30 s at 72°C. Relative quantification was based on the C_T method using *Ssact1* as
4 a calibrator reference. Amplifications were done in triplicate.

5

6 **Results**

7

8 Metabolic characterization of plant and fungal pathogen

9

10 Perchloric acid extracts of healthy cotyledonary leaves collected from 8-d-old *H. annuus*
11 germlings and of *S. sclerotiorum* mycelia collected after saprophytic growth on minimal
12 glucose medium were analyzed using ¹³C- and ³¹P-NMR spectroscopy. Representative spectra
13 are shown in figures 1 and 2, and comparative data are given in Table 1.

14 In *H. annuus* cotyledon extracts, the main stores of soluble carbohydrates detected by
15 ¹³C-NMR (Fig. 1) were glucose, fructose and sucrose (63, 40 and 11 μmol g⁻¹ FW of plant
16 tissues, respectively). Plants do not accumulate trehalose. Inositol (10 μmol g⁻¹ FW) was the
17 only detected polyol. The most abundant Krebs cycle intermediates were fumarate, malate,
18 succinate and citrate. Glutamate (18 μmol g⁻¹ FW) was the main amino acid store. The
19 concentration of alanine, the second unambiguously identified amino acid, was 18 times
20 lower. The most abundant compound measured by ³¹P-NMR (Fig. 2b) was inorganic
21 phosphate (2.5 μmol g⁻¹ FW). Among identified P-compounds (from upfield to downfield):
22 glucose-6-P, glycerol-3-P, PGA, P-choline, two phosphodiesteres, GPG and GPI were
23 detected. Nucleotides (mainly ATP), pyridine nucleotides (NAD and NADP), UDP-glucose
24 and UDP-galactose were also detected. The abundance of GPG and GPI may be related to
25 membrane traffic accompanying the first steps of growth of the germlings (Aubert et al.
26 1996).

27 In *S. sclerotiorum* extracts, main stores of soluble carbohydrates (Fig. 1) were glucose
28 (115 μmol g⁻¹ FW) and trehalose (26 μmol g⁻¹ FW). Contrary to *H. annuus*, fructose and
29 sucrose stores were negligible in *S. sclerotiorum*. Mannitol (27 μmol g⁻¹ FW) was the only
30 abundant polyol, while glycerol, arabitol and erythritol, commonly found in other fungi
31 (Jennings 1984) were below the threshold of ¹³C-NMR detection. Malate and fumarate were
32 the two main stores of Krebs cycle intermediates. Like in *H. annuus* cotyledons, glutamate
33 (17 μmol g⁻¹ FW) was the main store of amino acid. Alanine (8.0 μmol g⁻¹ FW) was 8 times
34 more abundant than in plant. Here again, the most abundant compound measured by ³¹P-

1 NMR (Fig. 2a) was inorganic phosphate ($2.7 \mu\text{mol g}^{-1}$ FW). Glucose-6-P, PGA, ATP, UDP-
2 glucose and UDP-galactose were equally detected in mycelium and in plant tissues. In
3 contrast, there were striking differences concerning UDP-GlcNAc, trehalose-6-P, gluconate-
4 6-P, P-choline and phosphodiester. UDP-GlcNAc was the major P-compound in mycelium
5 ($1.4 \mu\text{mol g}^{-1}$ FW) whereas it was only present as a trace in cotyledons. In fungi, UDP-
6 GlcNAc is predominantly involved in the synthesis of chitin, a structural constituent
7 carbohydrate polymer of cell wall and septum with glucans (Cabib et al. 1991). Similarly,
8 trehalose-6-P, which was not detected in cotyledons, was relatively abundant in mycelium
9 ($0.19 \mu\text{mol g}^{-1}$ FW), in accordance with the presence of trehalose. GPC and GPE were the
10 most abundant P-diester. Interestingly, though glycerol-3-P was present in both partners
11 grown *in vitro*, glycerol was detected in none of them.

12

13 Metabolic profiling during infection

14

15 As control experiments, we first verified that the NMR profiles of non infected cotyledons,
16 maintained in the same growth conditions as inoculated cotyledons for a 48 h period of time
17 equivalent to the course of infection, did not change significantly. Moreover, NMR spectra of
18 samples collected 0 hpi were realized. At the initial stage of infection, the major components
19 from fungal origin (UDP-GlcNAc, mannitol, trehalose) were not detectable. Analysis of the
20 spectra revealed profiles identical to that of healthy cotyledons profiles. On the contrary, ^{13}C -
21 and ^{31}P -NMR spectra of *H. annuus* cotyledons infected by *S. sclerotiorum* revealed that the
22 interaction induced changes in the composition of the pool of soluble metabolites (Fig. 1, Fig.
23 2 and Table 1). A decrease in sugars and amino acids was observed. The total carbohydrate
24 level was only 59 and $57 \mu\text{mol g}^{-1}$ FW of infected tissues, 24 and 48 hpi, whereas it was 125
25 and $179 \mu\text{mol g}^{-1}$ FW in plant and fungal tissues, respectively. More specifically, fructose and
26 sucrose, initially present almost exclusively in plant, were reduced by 85%. These results
27 suggest that plant carbohydrate stores were utilized for fungal growth.

28 Analyses of ^{13}C -NMR spectra also revealed changes in the composition of the pool of
29 storage carbohydrates: inositol, the plant polyol marker, decreased from 10 to $2.1 \mu\text{mol g}^{-1}$
30 FW of infected tissues 48 hpi (Fig. 1, Table 1). Trehalose, a specific fungal carbohydrate
31 remained constant during infection, whereas the level of mannitol, the main fungal polyol,
32 revealed a 4-fold increase from 24 to 48 hpi. This may suggest that trehalose did not
33 constitute the main carbohydrate endogenous store in fungus, whereas mannitol was actively
34 produced. The accumulation of glycerol in infected cotyledons was more surprising since this

1 polyol was detected neither in plant nor in fungus cultivated separately. During infection, free
2 glycerol increased steadily, reaching 23-25 $\mu\text{mol g}^{-1}$ FW 48 hpi. Additional experiments
3 indicated that *S. sclerotiorum* accumulated glycerol but not mannitol as compatible osmolyte,
4 when cultivated for 24 h in a hyper-osmotic medium containing 0.4 M NaCl (data not shown).
5 Therefore, glycerol synthesis could occur in reaction to osmolarity changes possibly
6 associated to the release of metabolites by dying plant cells or contribute to the osmotic
7 stabilization of the hyphae required for invasive growth through tissues of living plants.

8 Glutamate and alanine, the two main free amino acids of plant or fungal origin, were
9 detected in infected cotyledons at 24 and 48 hpi. Glutamate, initially abundant both in plant
10 and mycelium, decreased strongly during the first hours of infection (data not shown),
11 reaching less than 10% of the initial values 24 hpi, then increasing to 22% 48 hpi. Alanine,
12 initially much more abundant in mycelium, accumulated moderately throughout the
13 development of infection (Fig. 1, Table 1).

14 Carbohydrate and amino acid storage in cotyledons was also affected at distance
15 during fungal infection. For example, 40 to 50% decrease in glucose, fructose, sucrose and
16 glutamate was observed 24 hpi in the non-invaded region of leaves, beyond the infected area
17 (data not shown). This suggests that the fungus behaves as a sink towards the metabolites
18 required for its own growth, at the expense of plant stores.

19 ^{31}P -NMR spectra first showed the appearance of tre-6-P and a dramatic increase of
20 UDP-GlcNAc, reflecting the rapid proliferation of fungal hyphae in infected cotyledons
21 tissues. The concentration of GPC in infected tissues increased simultaneously, reaching the
22 one of GPI (Fig. 2). Accumulation of GPC in plant cells often reveals a stress, leading to
23 partial hydrolysis of phospholipids (Aubert et al. 1996). Thus, its increase in infected tissues
24 could indicate that fungal invasion gave rise to plant membrane systems hydrolysis and to
25 subsequent release of metabolites in the apoplast. As GPC is the most abundant
26 phosphodiester in *S. sclerotiorum* mycelium (Table 1), its presence in infected cotyledons
27 may also reflect fungal growth in plant tissues.

28 29 Detection of invertase in infected cotyledons

30
31 Analysis of ^{13}C -NMR spectra revealed that sucrose, present exclusively in plant, decreased
32 from 11 to 2.1 $\mu\text{mol g}^{-1}$ during the first 24 hpi and was not detectable 48 hpi. The
33 disappearance of sucrose from infected sunflower cotyledons could likely be correlated with
34 an increase of invertase activities. In order to discriminate between the induction of fungal

1 activity or plant enzymes activated in response to the fungal attack, we used a biochemical
2 approach to investigate the presence of invertase during infection. Plants contain different
3 isoforms of invertases, which can be distinguished by their subcellular location and
4 biochemical properties (Godt and Roitsch 1997). To assess an invertase enzymatic activity *in*
5 *planta*, proteins were extracted from healthy and infected sunflower cotyledons. Except for 0
6 and 48 hpi, infected tissues were separated in two regions: healthy tissues not colonized
7 (region a) and invaded tissues (region b). Proteins were separated by IEF over a pH range of
8 3-10. Invertase activity was revealed at pH 5.6 and visualized by staining reducing sugars in
9 the gel. Two isoforms (pI 4 and 4.5) were detected (Fig. 3a). Invertase activity associated to
10 the protein with pI of 4.5 was detected in healthy sunflower cotyledons and remained constant
11 until 24 hpi. Thereafter, plant tissues were totally macerated. On the contrary, the presence of
12 the invertase isoform with a pI of 4 was correlated with the presence of the fungus in the
13 infected plant. Invertase activity was hardly detectable 8 hpi (region 8b), then increased
14 strongly at 24 hpi (region 24b) and 48 hpi. Detection of invertase activity was also performed
15 at pH 4 and 7 and revealed, at a lower level, the same pattern of activity for the protein with a
16 pI of 4, while no activity was detected at pH 4 and 7 for the isoform with a pI of 4.5 (data not
17 shown). This indicates that the activities of both enzymes were detected using favourable pH
18 conditions. In order to reveal the fungal or plant origin of the major invertase activity detected
19 at a pI of 4, we used immuno specific detection. Western blot analyses (Fig. 3b, c) revealed
20 two isoforms, differing in their molecular weight and separately detected by antibodies raised
21 against a fungal invertase (Fig. 3b) and a plant cell wall invertase (Fig. 3c). A 81 kDa isoform
22 was abundantly detected by anti-fungal invertase antibodies and its presence was limited to
23 regions severely colonized by *S. sclerotiorum* (24b and 48 hpi). The 40 kDa isoform, detected
24 by anti-plant invertase antibodies exhibited a different pattern and was present in healthy
25 tissues and during infection during the first 24 hpi. This isoform was not detected 48 hpi when
26 sunflower cotyledons were completely invaded and macerated. This protein was consequently
27 not linked to the fungus but rather correlated with the presence of healthy plant tissues. The
28 presence of the 81 kDa isoform paralleled the invertase activity detected at at pI 4 and could
29 likely be attributed to the fungus. Analyses of biochemical properties of the fungal invertase
30 expressed during *in vitro* growth in the presence of 10 mM sucrose, confirmed our
31 suggestions. Figure 3d revealed an unique active isoform at pI 4, released in the culture
32 medium by *S. sclerotiorum* during 36 h growth in the presence of sucrose, that corresponds to
33 the isoform detected in plant tissues (Fig. 3a). Moreover, western blot analyses conducted
34 with proteins of the same origin and anti-fungal invertase antibodies (data not shown),

1 revealed a band of 81 kDa, corresponding to the isoform detected *in planta* (Fig. 3b). These
2 results strongly suggested that the major acid invertase activity detected during infection was
3 mainly of fungal origin.

4 5 Expression of fungal hexose transporters during infection

6
7 Pathogenic fungi must feed on their hosts. During pathogenesis of sunflower cotyledons, plant
8 stores and particularly carbohydrates are likely transferred in the fungal mycelium, which
9 suggests that the fungal plasma membrane is equipped with corresponding transporters. Thus,
10 two genes *Sshxt1* and *Sshxt2*, encoding hexose transporters from *S. sclerotiorum*, have been
11 isolated. For this purpose, EST sequences from the related necrotrophic fungus *B. cinerea*,
12 identified in public databases (Soanes et al. 2002) and showing several convincing matches to
13 fungal hexose transporters were used as probes to screen at low stringency the genomic
14 EMBL3 library of *S. sclerotiorum*. Two recombinant phages giving the strongest
15 hybridization signals were chosen for further studies. Restriction and Southern analyses
16 allowed to clone and to characterize two sequences of 2133 and 2099 bp containing the
17 coding sequences of *Sshxt1* and *Sshxt2*, respectively. Each sequence had two introns. SsHXT1
18 and SsHXT2 possess 12 membrane-spanning domains (Fig. 4) characteristic for members of
19 the major facilitator super family (Marger and Saier 1993). The GRR or GRK conserved
20 regions are implicated in the membrane topology of this group of transporter proteins (Sato
21 and Mueckler, 1999). Sequences analyses and databases searches revealed that SsHXT1 and
22 SsHXT2 contain a five-element fingerprint that provides a signature for the sugar transporter
23 family of membrane proteins (InterProScan, Zdobnov and Apweiler 2001). The presence of a
24 conserved Phe residue, situated in the transmembrane domain X, implicated in the specificity
25 of the transport, was also detected in all sequences (Özcan and Johnston 1999). Highly
26 conserved regions identified within the fructose transporters as fungal fructose-proton
27 symporter signatures and found in BcFRT1, a fructose transporter present in *B. cinerea*
28 (Doehlemann et al. 2005), were not detected in SsHXT1 and SsHXT2 sequences. Thus,
29 SsHXT1 and SsHXT2 contain the main sugar transport signatures but are probably not sole
30 fructose transporters.

31 Recently, the release of the genome sequence of *S. sclerotiorum*
32 (<http://www.broad.mit.edu>) offered new opportunities to identify sugar transporters
33 sequences. A Blast search for possible homologies with SsHXT1 revealed, for the first hits, at
34 least six additional sequences. These proteins belong to the family of membrane proteins

1 responsible for the transport of sugars, as revealed by the InterProScan database (Zdobnov
2 and Apweiler 2001). To illustrate the relatedness of these and other sequences, a dendrogram
3 was generated (Fig. 5). Sugar transport sequences do not form a uniform group. It clearly
4 showed that SsHXT1 and SsHXT2 clustered together (46% identity to each other) and were
5 related to ApHXT1, an *A. parasiticus* monosaccharide transporter (Yu et al. 2000) with
6 respectively 30.3 and 34.3 % identity. Five other sequences were more related to the *N.*
7 *crassa* glucose sensors NcRCO3 (Madi et al. 1997) and the monosaccharide transporter
8 AmMSTA (Nehls et al. 1998), a candidate gene encoding an homologue to the glucose
9 sensors Rgt2 and Snf3 in *Saccharomyces cerevisiae* (Wei et al. 2004). Among these five
10 sequences, three (SsIG 028441, SsIG 066201 and SsIG 084251) were predicted to be high
11 affinity transporters by the databases (InterProscan, Zdobnov and Apweiler 2001), whereas a
12 separated branch contained a *B. cinerea* fructose transporter and one *S. sclerotiorum* putative
13 hexose transport sequence (Ss1G 030921).

14 *In planta* expression of *Sshxt1* and *Sshxt2* genes was analyzed. Relative levels of
15 *Sshxt1* and *Sshxt2* mRNAs were determined using real-time Q-PCR (Fig. 6). In these
16 experiments, mRNA level for the stably expressed fungal gene encoding actin *Ssact1*, was
17 evaluated as control gene for Q-PCR analyses. Total mRNAs were extracted from infected
18 tissues collected 8, 12, 24, 36 and 48 h after inoculation. Analysis of the expression profiles of
19 *Sshxt1* and *Sshxt2* revealed wave expression patterns with a maximum of transcripts reached
20 36 hpi. Expression levels of *Sshxt1* and *Sshxt2* were not similar. *Sshxt1*, contrary to *Sshxt2*,
21 was highly expressed during infection. The high level of expression of *Sshxt1* suggested that
22 this gene is predominantly implicated in hexose transport during pathogenesis of sunflower
23 cotyledons.

24

25 **Discussion**

26

27 This study reports on metabolic profiles of a necrotrophic interaction between the widespread
28 pathogen *S. sclerotiorum* and sunflower cotyledons as host plant. For this purpose, the NMR
29 spectroscopy offered an elegant way to build up a foundation of metabolic informations about
30 nutrition of a necrotroph fungal pathogen during infection.

31 Analyses of the natural abundance spectra revealed the progressive exhaustion of plant
32 carbohydrates stores like sucrose and fructose. Simultaneously, fungal activities implicated in
33 sucrose degradation and hexose transport were expressed. Upon compatible interactions of
34 plants with pathogenic fungi, plant carbohydrate metabolism is affected. An increase in

1 extracellular invertase activity, the inverse regulation of photosynthesis and carbohydrates
2 withdraw from the plant by the pathogen, creates a sink which reflects the switch from normal
3 metabolism to defence metabolism (Roitsch et al. 2003). The precise origin of the increase in
4 invertase activity remains controversial, as both host and pathogen possess soluble and
5 insoluble invertases. Therefore, it is difficult to establish whether the invertase activity
6 stimulation is due to activation or to an increase in the amount of host proteins, or to a fungal
7 invertase. The major plant invertases activated during a compatible interaction with biotrophic
8 fungi are described as acid insoluble (cell wall-bound) enzymes (Benhamou et al. 1991;
9 Scholes et al. 1994; Fotopoulos et al. 2003; Voegelé et al. 2006). Acid soluble (vacuolar) and
10 cytoplasmic invertase isoforms do not exhibit a major increase of their expression during
11 biotrophic interactions, but are thought to be implicated in the metabolism of stored plant
12 sucrose (Roitsch et al. 2003; Voegelé et al. 2006). Our study elucidated the origin of the
13 invertase activity detected in tissues infected by *S. sclerotiorum*. The use of specific
14 antibodies directed against plant or fungal invertases revealed that much of the increase in
15 activity could be attributed to a fungal isoform. In contrast to biotrophs, *S. sclerotiorum*
16 triggers cell death (Dickman et al. 2001) and subsequently feeds as a saprophyte, which
17 suggests that hexoses transported to the fungus are more likely the product of a fungal than a
18 plant invertase. During infection, the acid invertase activity from plant origin did not support
19 any increase and disappeared at the final stage of colonization, but a rise in fungal invertase
20 expression was observed. During *in vitro* growth in the presence of sucrose, the fungal
21 invertase was also produced and secreted in the culture medium. Voegelé et al. (2006)
22 reported on the appearance of a secreted fungal invertase during the biotrophic interaction of
23 *U. fabae* with *V. faba*. While sink soluble plant invertases seem to be cell wall bound, this
24 might not be the case for the respective enzymes provided by the pathogen. Involvement of
25 fungal invertase in pathogenesis has only been demonstrated in the compatible biotrophic *V.*
26 *faba/U. fabae* interaction (Voegelé et al. 2006) and for the necrotrophic parasite *B. cinerea*
27 (Ruiz and Ruffner 2002). Thus, our results contribute to establish that fungal invertase
28 expression takes part to fungal plant pathogenesis and to the necrotrophic strategy of *S.*
29 *sclerotiorum*.

30 Sucrose degradation and hexose sugar consumption were coordinated, as the expression
31 of the hexose transporters genes *Sshxt1* and *Sshxt2* reached maximal values 36 hpi, before
32 decreasing at the latter stage of infection. Interestingly, levels of expression of *Sshxt1* and
33 *Sshxt2* differed. *Sshxt2* was weakly expressed and was quantified as twenty times lower than
34 *Sshxt1*. The wave patterns of expression exhibited by both transporters during infection could

1 be related to specific ambient parameters created during infection. By lowering the ambient
2 environmental pH, oxalic acid may affect the transcriptional regulation of pH-regulated genes
3 necessary for pathogenesis and developmental life cycle of *S. sclerotiorum* (Cotton et al.
4 2004; Bolton et al. 2006). Extracellular hexose concentration is also a non-negligible control
5 element. Glucose level reached 63 mM in healthy cotyledons, then 50 mM 48 hpi, which may
6 favour the expression of low affinity hexose transporters. However, soluble intracellular
7 sugars and hexoses released from cell wall and storage polymers degradation could be
8 present, but inaccessible during the first hours of infection. As revealed by detection of a
9 fungal invertase activity, sucrose degradation is actively performed until the end of infection
10 and, consequently, should be correlated with a transport activity. The decreasing level of
11 expression observed for both transporters 36 hpi, strongly suggests that additional transporters
12 are necessary to support growth and development during the late stage of infection. Thus, a
13 multiple transport system is likely to be expressed in *S. sclerotiorum* during pathogenesis. A
14 screening for putative transporters-encoding genes revealed at least six additional sequences
15 in the *S. sclerotiorum* genome. Candidate genes encoding, among others, homologues to sugar
16 sensors (predicted to be high affinity transporters) or fructose transporters were revealed by
17 the dendrogram. Therefore, *S. sclerotiorum* possesses a multigenic sugar transport system that
18 could provide an efficient and flexible tool to this broad host range pathogen. Transcriptional
19 analyses conducted during *in vitro* growth and functional characterizations of transporters
20 should provide new insights on regulation, specificity and affinity of those transport proteins.
21 The disappearance of sucrose in infected sunflower cotyledons, the expression of an invertase
22 activity together with the expression of fungal hexose transporters are consistent with glucose
23 and fructose being the sugars transferred from the host tissues to the pathogen. Even healthy
24 parts of leaves also exhibited a loss of sugars, indicating that *S. sclerotiorum* was able to
25 uptake nutrients from a distance.

26 During infection, strong modifications of NMR profiles reflecting the hydrolytic
27 activity of the necrotroph were detected. The invasion of plant tissues was marked by an
28 important rise of GPC and GPE levels, probably triggered by the attack of membrane polar
29 lipids (Aubert et al. 1996). These data suggest that autolysis of the host membrane occurred.
30 Fungal enzymes, such as phospholipases, are expressed during pathogenesis and could
31 contribute to the degradation of these molecules (Lumdsen 1970). Moreover, these data are
32 consistent with the behaviour of *S. sclerotiorum* which is able to elicit host cell death and the
33 fact that necrotrophic pathogens may need to trigger plant apoptotic pathway for successful
34 colonization and subsequent disease development (Dickman et al. 2001).

1 The evolution in fungal trehalose and polyol contents was also followed during the
2 course of infection. Trehalose, the second most abundant sugar accumulated in *S.*
3 *sclerotiorum* mycelium, is a common storage product within microbial cell and especially in
4 spores. Increased trehalose levels in fungi also have been correlated with cell survival under
5 adverse conditions (Arguelles 1997). Trehalose was not accumulated during sunflower
6 cotyledon infection and remained at a very low level. Infection-related development and
7 colonization of host tissues could require the pathogen to mobilize storage carbohydrates.
8 Trehalose mobilization has been involved in virulence-associated functions that follow host
9 colonization in the pathogenic fungus *Magnaporthe grisea* (Foster et al. 2003). The absence
10 of stored trehalose may also reflect a deviation of the fungal metabolites oriented toward an
11 increased production of protective polyols such as glycerol. In filamentous fungi, polyols such
12 as mannitol, glycerol, arabinitol and erythritol are widely distributed and can be accumulated
13 to a high concentration (Jennings 1984). Their intracellular concentrations depend on growth
14 conditions and developmental stages, suggesting that polyols have important functions in
15 fungal physiology. Mannitol was the only polyol detected in the mycelium of *S. sclerotiorum*
16 cultivated *in vitro*. It was produced by the fungus *in planta*. A 4-fold increase was noticed in
17 infected tissues from 24 to 48 hpi, whereas the amount of UDP-GlcNAc, that could reflect the
18 evolution of the fungal biomass only doubled. Mannitol production, through the mannitol
19 cycle (Jennings 1984) could be supplied by the degradation of sucrose from plant origin,
20 particularly by an active conversion of fructose. Mannitol is considered as an important
21 intermediate in the physiology of fungi (Jennings 1984). This hexilitol can be stored in fungal
22 hyphae, or further metabolized in order to store reducing power or constitute a reserve carbon
23 source (Ruijter et al. 2003). Secretion of mannitol is thought to directly protect invading
24 pathogens by quenching host-produced reactive oxygen species (Jennings et al. 2002). In
25 ectomycorrhizas, NMR spectroscopy investigations, to characterize carbohydrate metabolism
26 during symbiotic state, revealed greater allocation of glucose to the synthesis of short chain
27 polyols, whereas sucrose decreased in colonized roots (Martin et al. 1998). In the necrotrophic
28 fungal pathogen *Stagonospora nodorum*, mannitol has been implicated in fungal plant
29 interaction by revealing the incapacity of the mutant to sporulate *in planta* (Solomon et al.
30 2006). Levels of mannitol found in apoplastic fluids of infected leaves and in extracts of
31 spores were observed to rise dramatically in the biotrophic interaction of the rust *U. fabae*
32 with its host plant (Voegelé et al. 2005). Thus, symbiotic or pathogenic interactions trigger
33 similar metabolic responses, like an increase in mannitol production that could be a key
34 regulatory component of carbon flow.

1 Contrary to mannitol, glycerol was not detected in *S. sclerotiorum* mycelium
2 during *in vitro* growth, but it appeared in tissues from the first hours of infection. During the
3 invasion process, glycerol increased by a factor of 5.4 from 24 to 48 hpi. By contrast,
4 glycerol-3-P initially present in fungus and fairly abundant in the host plant, decreased during
5 mycelium proliferation. Previous studies showed that, in plants, glycerol permeates all cell
6 compartments and is phosphorylated very efficiently in the cytoplasm (Aubert et al. 1994).
7 We have verified that sunflower cotyledons (healthy as well as infected) also phosphorylated
8 an exogenous source of glycerol in a very efficient manner (data not shown). We could
9 therefore suggest that the glycerol observed in infected tissues was not localized in plant but
10 was in the fungal hyphae during host plant invasion, where it was very likely synthesized and
11 where it accumulated without permeating outside fungal hyphae. Subsequent modification of
12 the fungal wall, like melanization, should render the hyphae non permeable to glycerol. For
13 *M. grisea*, mechanical pressure derived from elevated osmotic pressure within melanized
14 appressoria, through the accumulation of glycerol (Howard and Ferrari, 1989). Melanization,
15 which has been described in the sclerotial stroma of *S. sclerotiorum* (Bolton et al., 2006),
16 could also be implicated in the modification of the infection hyphae. The amount of glycerol
17 accumulated during infection was 4 times higher than that of mannitol, which characterized
18 the mycelium during *in vitro* growth. During infection a metabolic switch could occur.
19 Mannitol could be replaced by glycerol. However, the origin of this metabolic response
20 remains to be elucidated. During *M. grisea* appressorium turgor generation, glycerol
21 accumulation is a consequence of lipolysis (Wang et al. 2005). During the infection of
22 sunflower cotyledons, glycerol could be a by-product of the degradation of lipids stored in
23 germinations and subsequently accumulated by the fungus as a carbon storage compound.
24 Alternatively, glycerol has also been reported as a compatible solute assuming osmotic stress
25 protection necessary to maintain fungal cell expansion (Han and Prade 2002). We have
26 observed that glycerol can abundantly accumulate in *S. sclerotiorum* under an osmotic stress
27 provoked by addition of sodium chloride to the culture medium, while the mannitol content
28 remained constant. Thus, glycerol could play a prominent role in osmostress adaptation.
29 Osmoregulation during the course of pathogenesis has been demonstrated in the
30 phytopathogenic fungus *C. fulvum*. In that case, arabinitol was the main polyol to respond to
31 reduced water availability *in planta* and *in vitro* (Clark et al. 2003). Glycerol could play the
32 same role in *S. sclerotiorum* where its accumulation could generate a turgor pressure essential
33 for penetration of the fungus. As suggested by Voegelé et al. (2005), conversion of
34 carbohydrates taken up by the fungus into polyols would also maintain a gradient of

1 metabolites toward the pathogen to support fungal development. Our results argue that
2 metabolism and transport of soluble carbohydrates are of significance during plant pathogen
3 interactions. During infection, the necrotrophic pathogen, *S. sclerotiorum* produces a drastic
4 depletion of nutrients in plant tissues. The strong carbohydrate sink capacity of the fungus is
5 linked to the presence of a multigenic hexose transport system and the expression of a fungal
6 invertase during infection. Once transferred to the parasite, plant carbohydrates are likely to
7 be converted in polyols. By enhancing penetration and draining capacities, accumulation of
8 mannitol and glycerol *in planta* are likely to sustain the degradative strategy of *S.*
9 *sclerotiorum*. The present study revealed the involvement of a fungal invertase during the
10 necrotrophic pathogenesis of *S. sclerotiorum*, and the exclusive production of glycerol *in*
11 *planta*. Molecular and biochemical analyses in these directions may help for developing new
12 knowledge about the pathogenesis of necrotrophic fungi.

13

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19

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1 **Table 1** Metabolic profiling of *S. sclerotiorum* mycelium, *H. annuus* healthy cotyledons and
 2 infected cotyledons collected 24 and 48 hpi. Metabolites are identified and quantified from a
 3 series of experiments, using maleate and methylphosphonate as internal standards for ¹³C- and
 4 ³¹P-NMR, respectively, as indicated in Materials and methods. Values are given as $\mu\text{mol g}^{-1}$
 5 FW. nd: not detected. Results are given as mean \pm SD ($n=3$)

Metabolite	Healthy cotyledons	Infected cotyledons (24 hpi)	Infected cotyledons (48 hpi)	<i>S. sclerotiorum</i> mycelium
Total carbohydrate	125 \pm 10	59 \pm 5	57 \pm 5	179 \pm 12
Glucose	63 \pm 5	35 \pm 3	50 \pm 4	115 \pm 12
Fructose	40 \pm 4	20 \pm 2	7.0 \pm 0.6	2.3 \pm 0.2
Sucrose	11 \pm 1	2.1 \pm 2	nd	< 1.0
Trehalose	nd	1.3 \pm 0.2	1.0 \pm 0.02	26 \pm 3
Glycerol	nd	4.4 \pm 0.3	24 \pm 2	nd
Inositol	10 \pm 1	3.3 \pm 0.3	2.1 \pm 0.2	nd
Mannitol	nd	1.3 \pm 0.2	5.6 \pm 0.6	27 \pm 3
Malate	6.8 \pm 0.5	7.2 \pm 0.4	2.4 \pm 0.2	12 \pm 1
Succinate	3.5 \pm 0.3	3.7 \pm 0.3	1.2 \pm 0.2	1.0 \pm 1
Citrate	2.5 \pm 0.2	3.5 \pm 0.3	1.2 \pm 0.2	nd
Fumarate	23 \pm 2	27 \pm 2	23 \pm 2	6.0 \pm 1
Glutamate	18 \pm 2	1.9 \pm 0.2	4.0 \pm 3	17 \pm 2
Alanine	1.0 \pm 0.2	< 1.0	2.4 \pm 0.2	8.0 \pm 1
Pi	2.5 \pm 0.2	2.1 \pm 0.2	2.90 \pm 0.3	2.7 \pm 0.3
Glucose-6-P	0.86 \pm 0.06	0.78 \pm 0.6	0.63 \pm 0.5	1.2 \pm 0.1
Trehalose-6-P	nd	0.07 \pm 0.02	0.14 \pm 0.015	0.55 \pm 0.05
Glycerol-3-P	0.67 \pm 0.05	0.12 \pm 0.01	0.16 \pm 0.015	0.19 \pm 0.02
PGA	0.13 \pm 0.02	0.2 \pm 0.02	0.2 \pm 0.02	0.23 \pm 0.02
P-choline	0.50 \pm 0.04	0.39 \pm 0.04	0.23 \pm 0.03	nd
GPG	0.54 \pm 0.04	0.45 \pm 0.04	0.38 \pm 0.04	< 0.04
GPE	< 0.04	0.06 \pm 0.01	0.21 \pm 0.02	0.37 \pm 0.04
GPI	0.98 \pm 0.07	0.72 \pm 0.06	0.68 \pm 0.06	0.18 \pm 0.015
GPC	0.07 \pm 0.01	0.31 \pm 0.03	0.68 \pm 0.06	0.90 \pm 0.07
ATP	0.16 \pm 0.015	0.18 \pm 0.02	0.18 \pm 0.02	0.35 \pm 0.03
NAD	0.08 \pm 0.01	nd	nd	0.04 \pm 0.01
NADP	0.055 \pm 0.006	nd	nd	0.12 \pm 0.01

UDP-Glc	0.25 ± 0.03	0.22 ± 0.03	0.35 ± 0.04	0.89 ± 0.07
UDP-GlcNAc	< 0.04	0.29 ± 0.03	0.57 ± 0.05	1.4 ± 0.12

1

2

3 Figure legends

4 **Fig. 1a-c.** ¹³C-NMR spectra of *S. sclerotiorum* mycelium (a), *H. annuus* cotyledon (b) and
5 sunflower cotyledons infected by *S. sclerotiorum* 48 hpi (c). Perchloric extracts were prepared
6 from 10 g fresh material as described in “Materials and methods”. ¹³C-NMR spectra (100.6
7 MHz), recorded at 20°C, were the result of 900 transients (90 min). Peak assignments are as
8 follows: Ala, alanine; cit, citrate; fru, fructose; fum, fumarate; glc, glucose ;Glu, glutamate;
9 gly, glycerol; ins, inositol; mal, malate; mnt, mannitol; n.i., not identified; scn, succinate; suc,
10 sucrose; tre, trehalose. Prominent fungal compounds are indicated in bold in panel c. Panels
11 on the right show a focused region of each spectra

12

13 **Fig. 2a-c** Proton-Decoupled *in vitro* ³¹P-NMR spectra of *S. sclerotiorum* mycelium (a), *H.*
14 *annuus* cotyledon (b) and sunflower cotyledons infected by *S. sclerotiorum* 48 hpi (c).
15 Perchloric extracts were prepared from 10 g fresh material as described in “Materials and
16 Methods”. Spectra (161.9 MHz) recorded at 20°C were the result of 1024 transients (60 min).
17 Peak assignments are as follows: fru-6-P, fructose-6-phosphate; glcn-6-P, gluconate-6-P; glc-
18 6-P, glucose-6-P; gly-3-P, glycerol-3-P; man-6-P, mannose-6-phosphate; P-cho,
19 phosphorylcholine; PEP, phosphoenolpyruvate; P-eth, phosphorylethanolamine; phy, phytate;
20 Pi, inorganic phosphate; poly-P, polyphosphates; tre-6-P, trehalose-6-P; UDP-glc, UDP-
21 glucose; UDP-gal, UDP-galactose. The internal reference is not shown. Spectra are
22 representative of three independent experiments. Prominent fungal compounds are indicated
23 in bold in panel c

24

25 **Fig. 3a-d** Detection of soluble invertase *in planta* and *in vitro*. All lanes were loaded with 10
26 µg. For lanes 8a, 8b, 24a, and 24b infected cotyledons were cut in half. Lanes 8a and 24a
27 correspond respectively to healthy regions of infected cotyledons 8 and 24 hpi, lanes 8b and
28 24b correspond to regions colonized by *S. sclerotiorum* 8 and 24 hpi. Samples were prepared
29 on an equal leaf area. **a** Isoelectrofocusing pattern of invertase produced during the time
30 course of infection of sunflower cotyledons by *S. sclerotiorum*. Invertase activity was
31 visualized by staining reducing sugars with TTC after incubation at pH 5.6. **b** and **c**
32 Immunospecific detection of plant and fungal invertases in infected sunflower cotyledons

1 extracts revealed respectively with anti-*C. albicans* invertase and anti-tobacco invertase. **d**
2 Isoelectrofocusing pattern of *S. sclerotiorum* invertase produced after 36 h of growth on 10
3 mM sucrose medium. Lane 1, protein standards stained with Coomassie blue ; lane 2,
4 invertase activity revealed as in section a. Western blots and IEFs were repeated at least twice
5
6 **Fig. 4** Conserved amino acid stretches for fungal monosaccharide transporters. Locations of
7 the conserved sequences, in SsHXT1 and SsHXT2, are indicated by numbering above the
8 SsHXT1 sequence. Residues conserved in all seven transporters homologues are shaded in
9 grey. Black bars indicate transmembrane domains of SsHXT1. Conserved domains were
10 deduced from the alignment of the following protein sequences obtained by using the
11 CLUSTALW algorithm and are positioned by arrows on the SsHXT1 sequence.
12 Abbreviations and accession no. are as follows: *S. sclerotiorum* SsHXT1 (AY647267), *S.*
13 *sclerotiorum* SsHXT2 (AY647268), *U. fabae* UfHXT1 (AJ310209), *T. harzianum* ThGTT1
14 (AJ269534), *A. muscaria* AmMST1 (ZZ83828), *A. parasiticus* ApHXT1 (AF010145), *B.*
15 *cinerea* BcFRT1 (AY738713)

16
17 **Fig. 5** Phylogram of hexose transporters-related proteins from *S. sclerotiorum* and other
18 fungi. Consensus tree prediction was performed by using multiple sequence alignment, by
19 cluster algorithms with the TreeTop-Phylogenetic Tree prediction program (GenBee).
20 Numbers represent the percentage of occurrence obtained after bootstrap analysis (1000
21 random samples) of the phylogenetic tree. Abbreviated species names are as indicated in Fig.
22 4 and as follows: *N. Crassa* RCO3 (accession no. U54768), *A. nidulans* AnMSTA (accession
23 no. AJ535663). Other putative *S. sclerotiorum* sequences (Ss1G_084251, Ss1G_066201,
24 Ss1G_054561, Ss1G_060321, Ss1G_028441, Ss1G_030921) are predicted proteins obtained
25 from the *Sclerotinia sclerotiorum* Sequencing Project, Broad Institute of Harvard and MIT
26 (<http://www.broad.mit.edu>) according to InterProScan databases (Zdobonov and Apweiler,
27 2001). Sequences from *S. sclerotiorum* are underlined

28
29 **Fig. 6** Relative *Sshxt1* and *Sshxt2* expression analyzed by Q-PCR during time course of
30 sunflower cotyledon infection. Relative expression levels were normalized with respect to
31 *Ssact1* expression levels

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