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pH modulation differs during sunflower cotyledon colonization by the two closely related necrotrophic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*

GENEVIÈVE BILLON-GRAND[1,*], CHRISTINE RASCLE[1], MICHEL DROUX[1], JEFFREY A. ROLLINS[2] AND NATHALIE POUSSEREAU[1]

[1]*Université Lyon 1, Université de Lyon, CNRS, BAYER SAS, UMR 5240 Microbiologie, Adaptation et Pathogénie, 14 impasse Pierre Baizet, BP 99163, F-69263 Lyon cedex 09, France*

[2]*Department of Plant Pathology, University of Florida, 1453 Fifield Hall, Gainesville, FL 32611-0680, USA*

SUMMARY

During pathogenesis on sunflower cotyledons, *Botrytis cinerea* and *Sclerotinia sclerotiorum* show a striking resemblance in symptom development. Based on pH change profiles, the colonization process of both fungi can be divided into two stages. The first stage is associated with a pH decrease, resulting from an accumulation of citric and succinic acids. The second stage is correlated with a pH increase, resulting from an accumulation of ammonia. In this article, we also report that oxalic acid is produced at the late stage of the colonization process and that ammonia accumulation is concomitant with a decrease in free amino acids in decaying tissues. *Sclerotinia sclerotiorum* produces eight-fold more oxalic acid and two-fold less ammonia than *B. cinerea*. Consequently, during sunflower cotyledon colonization by *B. cinerea*, pH dynamics differ significantly from those of *S. sclerotiorum*. *In vitro* assays support the *in planta* results and show that decreases in pH are linked to glucose consumption. At different stages of the colonization process, expression profiles of genes encoding secreted proteases were investigated. This analysis highlights that the expression levels of the *B. cinerea* protease genes are higher than those of *S. sclerotiorum*. This work suggests that the overt similarities of *S. sclerotiorum* and *B. cinerea* symptom development have probably masked our recognition of the dynamic and potentially different metabolic pathways active during host colonization by these two necrotrophic fungi.

INTRODUCTION

Filamentous fungi can be considered as the most successful group of microorganisms causing plant diseases. They can be found in all classes of the Eumycota kingdom and their hosts in all systematic groups of plants. The two closely related Leotiomycetes species *Sclerotinia sclerotiorum* and *Botrytis cinerea* are both necrotrophic and polyphagic plant pathogens provoking white and grey mould diseases, respectively.

It is widely accepted that *S. sclerotiorum* and *B. cinerea* are fungi with similar infection strategies. They directly penetrate the host plant, derive nutrients from dying or dead host tissues, and extensively colonize the host plant. They are known to produce oxalic acid, which is implicated in tissue acidification and plant cell death (Kim *et al*., 2008; Maxwell and Lumdsen, 1970; Verhoeff *et al*., 1988) and they secrete numerous cell wall-degrading enzymes (Choquer *et al*., 2007; van Kan, 2006; van Kan *et al*., 1997). Among these enzymes, proteases can contribute to destabilize the plant cell wall and to provide the pathogen with amino acids, the main source of nitrogen and sulphur (Billon-Grand *et al*., 2002; Gamboa-Melendez *et al*., 2009; ten Have *et al*., 2010). The genome sequences of *B. cinerea* (strain BO5.10) and *S. sclerotiorum* (strain 1980) have been established recently by the Broad Institute (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.htm), and most genes from one species have a corresponding orthologue in the other genome. These genomic resources can contribute to a better comparison and understanding of the infection and colonization processes (Amselem *et al*., 2011).

This article aims to compare the colonization processes of *S. sclerotiorum* and *B. cinerea* during a time course of infection on sunflower cotyledons. pH changes were monitored, organic acids and ammonia were quantified, and the expression of genes encoding secreted proteases was analysed. For both fungi and for the first time, an accumulation of ammonia was observed and oxalic acid was shown to accumulate at a late stage of the colonization process. As *S. sclerotiorum* produces eight-fold more oxalic acid and two-fold less ammonia than *B. cinerea*, pH dynamics differ during sunflower cotyledon colonization by *B. cinerea* and *S. sclerotiorum*. *In vitro* assays support and advance our understanding of the differences observed during the pathogenic processes of these two fungi.

**Correspondence*: Email: genevieve.billon-grand@bayer.com

RESULTS

pH change profile during the colonization process

Pathogenicity assays were performed on 1-week-old sunflower cotyledons infected with mycelial plugs of *S. sclerotiorum* or *B. cinerea*, and cotyledons were harvested at different stages of symptom development. *Sclerotinia sclerotiorum* proved to be more aggressive than *B. cinerea* as necrotrophic lesions (corresponding to 3%–5% of the cotyledon area) appeared at 8 h post-inoculation (hpi) underneath fungal inoculum, whereas this was observed at 16 hpi for *B. cinerea*. Moreover, for *S. sclerotiorum*, 10% and 40% of the area were colonized at 16 and 24 hpi, respectively, whereas these percentages of colonization were observed at 24 and 48 hpi, respectively, for *B. cinerea*. This difference was maintained throughout the colonization process, with *S. sclerotiorum* colonizing the whole cotyledon by 72 hpi and *B. cinerea* by 96 hpi. Furthermore, rotted tissues developed patches of fluffy white mycelium with *S. sclerotiorum*, whereas they were grey in colour with *B. cinerea*, characteristic of the sporulating stage of this fungus (Fig. 1).

pH values of host tissues were followed throughout the infection process. pH values of uninfected cotyledons remained between pH 6.2 and pH 6.5 during the time course experiments.

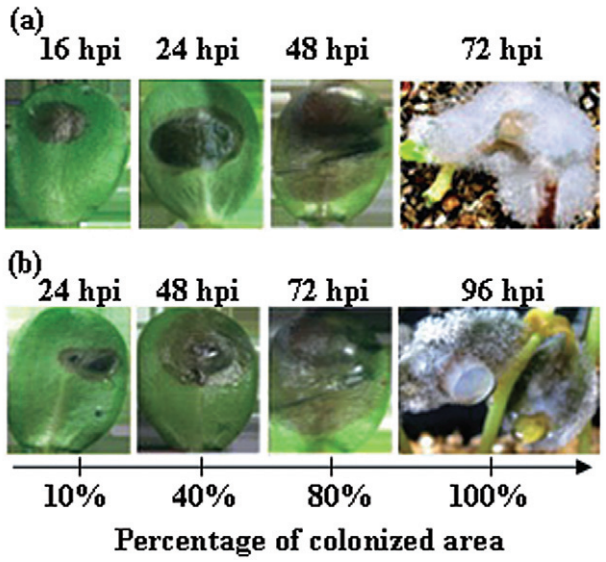

Fig. 1 Comparison of disease severity on sunflower cotyledons colonized with *Sclerotinia sclerotiorum* and *Botrytis cinerea*. (a) With *S. sclerotiorum*, 10%, 40%, 80% and 100% of the cotyledon area exhibited necrosis at 16, 24, 48 and 72 h post-inoculation (hpi). (b) With *B. cinerea*, the same percentage areas exhibited necrosis at 24, 48, 72 and 96 hpi. Cotyledons were inoculated on the adaxial surface and photographs show the area of necrosis on the abaxial surface. Uninfected and infected cotyledon areas were measured and compared using Adobe Photoshop 6.0 software with a resolution of 300 dpi. Results are reported from four biological replications.

For the two fungi, pH values of colonized cotyledons decreased up to the stage corresponding to 40% of the cotyledon area exhibiting necrosis. Acidification of necrotrophic tissues was more evident with *S. sclerotiorum*, where a pH decrease of 2.7 units was measured (final pH 3.7), whereas a pH decrease of 1.7 units was observed with *B. cinerea* (final pH 4.8). However, an unexpected increase in pH values was observed when more than 40% of the cotyledon area was colonized with either fungus. By the end of the colonization process, this increase reached 1.1 units with *S. sclerotiorum* and 2.2 units with *B. cinerea* (Fig. 2a). With *S. sclerotiorum*, the pH value levelled off at pH 4.8 and this fungus remained in an acidic environment. In contrast, the *B. cinerea*-colonized tissue established a final neutral environment with the pH value reaching pH 7.0. This value was higher than that of healthy cotyledon leaves and suggests an alkalization of the host tissues.

Manteau *et al*. (2003) have shown that some ripe fruits exhibit acidic values (from pH 3.3 to pH 4.4), whereas leaves, vegetables, stems and roots show near-neutral values (pH 5.8–6.3). As *S. sclerotiorum* and *B. cinerea* are able to infect a wide variety of host plants which differ in their pH values, we monitored the pH changes on different host tissues, including chicory leaf (pH 6.0), carrot root (pH 6.2) and apple fruit var. 'Golden' (pH 4.4). When colonized with *S. sclerotiorum*, pH values levelled off at pH 4.0 on apple fruit, whereas pH values decreased from pH 6.0/6.2 to pH 3.0 on carrot root and chicory leaf (Fig. 2b). No increase in pH values was measured by the end of the colonization process, as observed on sunflower cotyledons. With *B. cinerea*, two different pH profiles were observed depending on the infected host tissues. Although pH profiles similar to those of *S. sclerotiorum* were found on apple fruit and carrot root, pH values decreased on chicory leaf from pH 6.0 to pH 5.2, and then increased from pH 5.2 to pH 7.5, this latter pH value being higher than that of the healthy leaf (Fig. 2c). Therefore, significant differences were revealed between the two fungi depending on the host tissue. In all the examined interactions, *S. sclerotiorum* maintained or further developed an acidic environment. *Botrytis cinerea* did the same on apple fruit and carrot root, but was also able to initially acidify and then alkalinize both sunflower cotyledons and chicory leaf, leading to the neutralization of these tissues.

Quantification of organic acids

During the early stages of the colonization process on sunflower cotyledons, a pH value decrease was observed for both fungi. To decipher the role of fungal organic acids in this tissue acidification, their accumulation was assayed during the colonization process on sunflower cotyledons (Fig. 3). With both fungi, the level of oxalic acid remained similar to that of uninfected cotyledons (i.e. 1.0 μM/g dry matter) until 10% of the cotyledon area was colonized. Oxalic acid production increased significantly once 40% of the cotyledon area had become necrotic, but, at the end of the

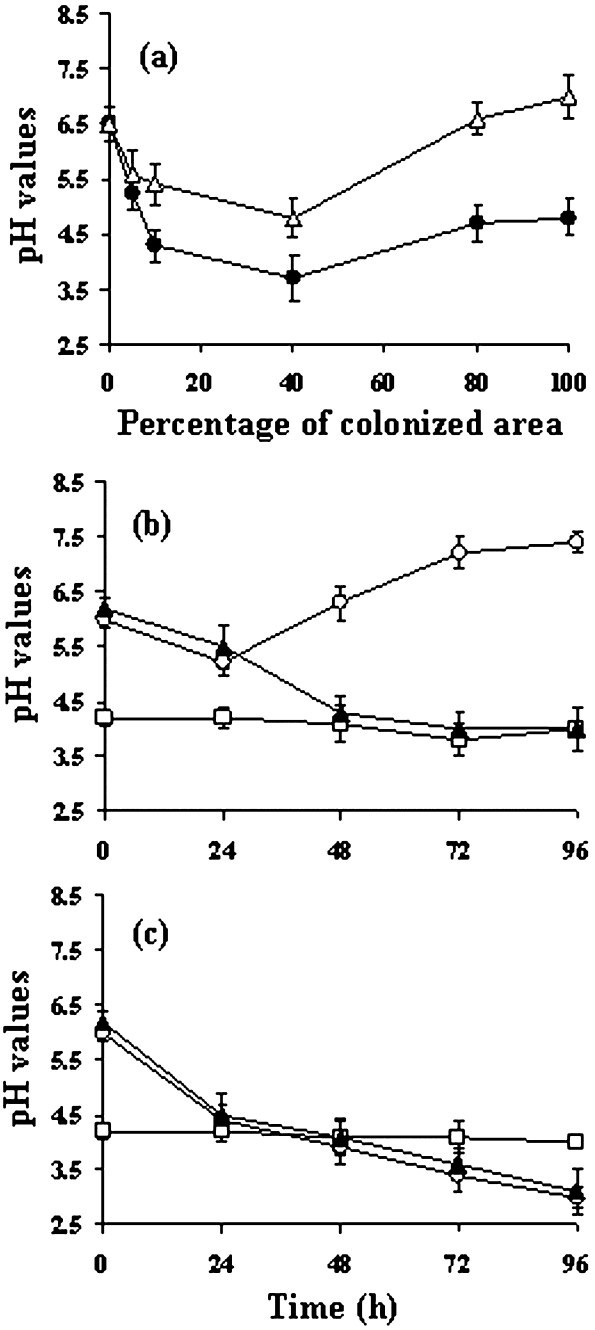

Fig. 2 pH changes in different tissues colonized with *Sclerotinia sclerotiorum* or *Botrytis cinerea*. pH values were measured with a flat electrode positioned on the lesion centre. pH values of uninfected cotyledons were measured in the same way on the cotyledon centre. Each pH value was the average of 10–15 measurements and four biological replications were carried out. (a) Comparison of pH changes in sunflower cotyledons colonized with *S. sclerotiorum* (●) or *B. cinerea* (△). pH changes in tissues infected with *S. sclerotiorum* (b) or *B. cinerea* (c) for apple fruit (□), carrot root (▲) and chicory leaf (○).

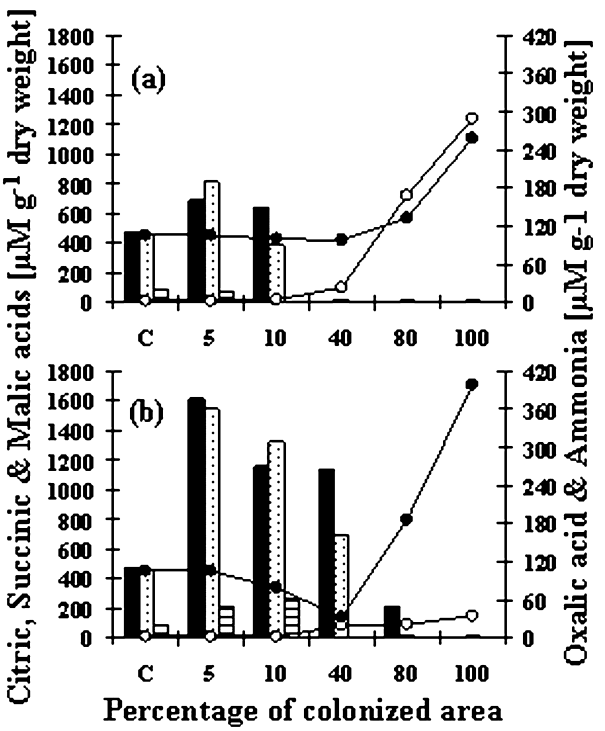

Fig. 3 Comparison of organic acids and ammonia production in sunflower cotyledons colonized with *Sclerotinia sclerotiorum* (a) or *Botrytis cinerea* (b). Organic acids and ammonia were quantified from infected sunflower cotyledons over time. All values given are the average of four biological replications, but error bars are not visible as they are too low compared with the scale. C, values of uninfected sunflower cotyledons used as controls; ▬, citric acid; ⁙, succinic acid; ▤, malic acid; ○, oxalic acid; ●, ammonia.

colonization process, its level was eight-fold higher with *S. sclerotiorum* than with *B. cinerea*. The level of citric and succinic acids, close to 460 µM/g dry matter in uninfected sunflower cotyledons, increased significantly as 5% of the cotyledon area became colonized. With *B. cinerea*, contrary to *S. sclerotiorum*, citric and succinic acids accumulated until 40% of the area was colonized, and their levels were two-fold higher than in uninfected cotyledons. However, for both fungi, citric and succinic acids disappeared once oxalic acid showed a significant increase. Malic acid was found only with *B. cinerea*, until 10% of the area had been colonized, but its level was insignificant compared with the levels of succinic and citric acids. The profiles of organic acid accumulation suggest that succinic and citric acids are responsible for the pH decrease, as oxalic acid was only produced in significant amounts after 40% of the leaf area had been colonized.

Quantification of ammonia and amino acids

Neither *S. sclerotiorum* nor *B. cinerea* has been reported to alkalinize plant tissues, but an increase in pH values was measured

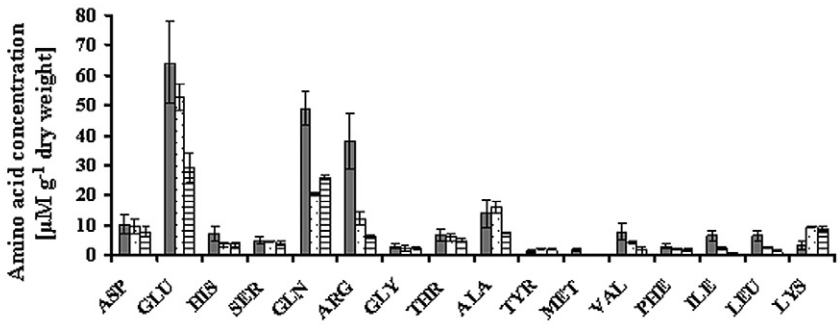

Fig. 4 Amino acid concentrations in sunflower cotyledons colonized with *Sclerotinia sclerotiorum* or *Botrytis cinerea*. Amino acids were separated and quantified by reversed phase chromatography by high-performance liquid chromatography (HPLC) (Waters HPLC system) after labelling with *ortho*-phthadialdehyde. For each amino acid, the concentration was evaluated as the necrosis area reached 100% and results were compared with those obtained for uninoculated sunflower cotyledons. All values are the average of four biological replications and three runs of HPLC per replication with: ⬚, *S. sclerotiorum*; ▤, *B. cinerea*; ■, uninoculated cotyledon. ALA, alanine; ARG, arginine; ASP, aspartic acid; GLN, glutamine; GLU, glutamic acid; GLY, glycine; HIS, histidine; ILE, isoleucine; LEU, leucine; LYS, lysine; MET, methionine; PHE, phenylalanine; SER, serine; THR, threonine; TYR, tyrosine; VAL, valine.

during the colonization process on sunflower cotyledons with both fungi (Fig. 2a). This pH increase could be caused by ammonia, as some fungi, including *Colletotrichum gloeosporioides* (Alkhan *et al*., 2008) and *Metarhizium anisopliae* (St Leger *et al*., 1999), are known to produce ammonia. On this basis, ammonia quantification was performed during the colonization process with healthy cotyledons used as controls. With *S. sclerotiorum*, the level of ammonia remained stable until 40% of the cotyledon area had been invaded, whereas it decreased by 70% with *B. cinerea*. Subsequently, the level of ammonia increased 2.5-fold with *S. sclerotiorum* and 8.0-fold with *B. cinerea* to reach 250 and 400 µM/g dry matter, respectively, once the whole plant was colonized (Fig. 3). These results were associated with a pH value increase measured at the same stage.

Ammonia production is an end product of the oxidative deamination of amino acids (Voet and Voet, 2005), and the concentration of some amino acids has been shown to fluctuate throughout the process of sunflower cotyledon colonization (Dulermo *et al*., 2009; Jobic *et al*., 2007). In this study, the quantification of 16 amino acids was performed to determine the fate of free amino acids during host colonization. As the highest pH values were reached when 100% of the cotyledon area had been colonized, we focused on this stage. The concentration of amino acids decreased significantly for both fungi during colonization, except for serine, tyrosine and lysine, and this decrease appeared to be higher with *B. cinerea* (Fig. 4). Therefore, the decrease in free amino acids appears to be well associated with ammonia production and, consequently, with the pH value increase.

Protease gene expression

During the colonization process on sunflower cotyledons, the expression of genes encoding secreted proteases was analysed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using specific primers (Table 1). All genes chosen for *B. cinerea* have orthologues in the *S. sclerotiorum* genome, except for BC1G_01794, BC1G_00545 and BC1G_03070 encoding three aspartic proteases (*Bcap5*, *Bcap7* and *Bcap8*). As it has been shown previously that some proteases are transcriptionally regulated (Poussereau *et al*., 2001; Rolland *et al*., 2009), the pH regulation of genes encoding secreted proteases was also investigated with mycelia growing under controlled culture conditions (Fig. S1, see Supporting Information).

During the colonization process, RT-qPCR analysis revealed that three protease genes (among 10 genes) showed a significant level of expression for *S. sclerotiorum* (*Ssacp1*, *Ssser2* and *Ssap10*), whereas five genes (among 13 genes) exhibited significant expression for *B. cinerea* (*Bcacp1*, *Bcser1*, *Bcser2*, *Bcap8* and *Bccp4*) (Table 2). Except for *Ssap10*, the expression level of the *B. cinerea* genes was always higher than that of the *S. sclerotiorum* genes. The relative expression of *Ssacp1* and *Bcacp1*, encoding the pepstatin-insensitive aspartic protease, increased as colonization proceeded, and the expression level of *Bcacp1* was 2.2-fold higher than that of *Ssacp1*. During the second stage of colonization, pH values increased to reach a neutral value with *B. cinerea* and the *Bcacp1* gene was fully repressed, as shown previously by Rolland *et al*. (2009). *Ssap10* and *Bcap8* genes, encoding two aspartic proteases, were expressed during the entire colonization process. The expression level of *Ssap10* increased during the first stage of colonization and then levelled off. A five-fold increase in the expression level of the *Bcap8* gene was noticed during the first stage of colonization as pH values decreased. However, this gene was repressed at the end of the colonization process as pH values became neutral. Consistent with the findings of ten Have *et al*. (2010), the *Bcap8* gene showed the highest expression level relative to all other examined protease genes. Regardless of the

Table 1 Primers used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of genes encoding secreted proteases.

Gene	Locus ID	Forward primer (5′–3′)	Reverse primer (5′–3′)
BcactA	AJ000335	CCGTGCTCCAGAAGCTTTGT	GTGGATACCACCGCTCTCAAG
Bcacp1	BC1G_14153	TGACGGAGACCTTTGCGAGTA	TCGCCCTCCTCGAAATCTTCA
Bcap5	BC1G_01794	TCAAAATCCAAGCCGCGTAT	AGTCGAAGCGCCGGAAA
Bcap7	BC1G_00545	ACTCTGTGCCATGCAATGCTA	ACGACACGCCGCCAAT
Bcap8	BC1G_03070	AAGCACCACCTACACCCTTACC	GAATTCGGAGTACTGGGCAGTT
Bcap9	BC1G_03579	GCAAGGTTCAAGGTGCTGTCA	TGGCAGCGCAAGGGAAT
Bcap10	BC1G_07521	CAAAATCGGCCCAACTGACT	CGACTGCGAAATTCATCAAAGA
Bcser1	BC1G_04708	ACCTCGCCCGCTTCCA	TCGGGCGTCGTTTGAATC
Bcser2	BC1G_06836	GTGAGGGAGTCGATGCTTATGTT	TGCACGACCTTCGAAATCAA
Bcser8	BC1G_12776	AACGGTGTCGGAGCAGATG	GGCGAGGAAAGTAGTGCTGTTG
Bccp4	BC1G_03711	TCTGCAACTGGCTCGGAAAC	TCTTGCCAGGCCATTCAAG
Bccp6	BC1G_08831	ACGACGCAGGTCACAGTATCC	CCGGGCAAAAACTTGGAAA
Bctpp2	BC1G_01026	CGCCGCTCCCGTAATTG	TTCCTGCTTTGAATCGTGCAT
Bcmep	BC1G_01418	TGTCGGAGAGATTAGGTGCTTTG	CAGCCGCTGGTGCA ACT
SsactA	XM_001589919	CATTGCACCACCCGAGAGA	GACAAAGATGCCAAAATAGAACCA
Ssacp1	SS1G_07836	CGGAGAATCCGTCGGTGTTA	GGTGAGGACGGAGTTTTGTTG
Ssap9	SS1G_03629	CGCCATGCCAGTTTTCACT	TTTCCGAATTCGTAGGATCCA
Ssap10	SS1G_03181	TCATCCTCATGAGCGATGCTA	GAGTAGGAAGCACCCTTGACTTG
Ssser1	SS1G_12605	TTGTAGCTGCCGGAAACGA	TGGGAGTAGAAGCAGGTGAAGTG
Sser2	SS1G_03282	CCTGCTGCTGCCGAGAA	TAGGCACGGGAATCATCGA
Sser8	SS1G_13922	CCTGATATCGCGGCTCAAG	TGAACCAATGTGCCATTCCA
Sscp4	SS1G_12500	TGTTCTTCTTTGACCGGTCTCTT	TTTGCCGTTCTTGTCGATTG
Sscp6	SS1G_11382	CCTCCGTAGCATCCCTGAAA	TTTCCGGCAATGATCATATCC
Sstpp2	SS1G_09225	CGGGCGCTAAGATTCCAA	CGCATTCCACGTCGCATA
Ssmep	SS1G_06094	GACTTGCACCGGCTCGAT	GTAAGAACGAAGACGATGGAGTGA

Sclerotinia sclerotiorum and *Botrytis cinerea* locus IDs were obtained from their respective genome annotation databases at http://www.broad.mit.edu. The accession numbers of actin-encoding genes *SsactA* and *BcactA* were obtained from GenBank and used as normalization controls.

Table 2 Relative expression of genes encoding secreted proteases during the colonization process on sunflower cotyledons.

	Percentage of colonized sunflower cotyledon area				
Strain	5%	10%	40%	80%	100%
S5					
Genes	pH 5.2	pH 4.3	pH 3.7	pH 4.7	pH 4.8
Ssacp1	0.20 ± 0.01	0.36 ± 0.05	0.59 ± 0.04	1.61 ± 0.15	1.81 ± 0.17
Ssap8	0.00	0.00	0.00	0.00	0.00
Ssap10	1.03 ± 0.07	1.62 ± 0.04	2.30 ± 0.33	2.40 ± 0.50	2.24 ± 0.20
Ssser1	0.00	0.00	0.00	0.00	0.00
Ssser2	1.07 ± 0.11	0.44 ± 0.12	0.00	0.24 ± 0.10	0.55 ± 0.13
Ssser8	0.22 ± 0.04	0.00	0.00	0.00	0.00
Sscp4	0.33 ± 0.19	0.00	0.00	0.00	0.00
B05.10					
Genes	pH 5.6	pH 5.4	pH 4.8	pH 6.6	pH 7.0
Bcacp1	0.35 ± 0.05	0.80 ± 0.13	4.01 ± 0.27	4.00 ± 0.28	0.00
Bcap8	1.73 ± 0.30	3.70 ± 0.09	8.76 ± 0.85	8.52 ± 0.98	0.28 ± 0.04
Bcap10	0.00	0.00	0.00	0.00	0.00
Bcser1	0.00	0.00	0.00	0.90 ± 0.07	6.42 ± 0.62
Bcser2	0.89 ± 0.08	0.34 ± 0.08	0.22 ± 0.08	0.46 ± 0.12	1.41 ± 0.45
Bcser8	0.00	0.00	0.31 ± 0.03	0.35 ± 0.03	0.00
Bccp4	0.42 ± 0.09	0.22 ± 0.09	0.21 ± 0.01	0.20 ± 0.02	1.32 ± 0.05

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed with total RNA extracted from sunflower cotyledons colonized by *Sclerotinia sclerotiorum* (strain S5) or *Botrytis cinerea* (strain B05.10). Relative quantification was based on the $-2^{\Delta Ct}$ method using, as reference, the *SsactA* actin gene with *S. sclerotiorum* and the *BcactA* actin gene with *B. cinerea*. Values given are the mean ± standard deviation of three independent biological replications.

colonization stage, the *Ssser1* gene, encoding a serine protease, was not expressed. In contrast, the *Bcser1* gene revealed a high expression level at the final stage of colonization as pH values became neutral. *Ssser2* and *Bcser2* genes, encoding serine proteases, showed similar levels of expression, which decreased during the first stage of colonization and increased during the second stage. Finally, the *Bccp4* gene, encoding a serine carboxypeptidase, showed a similar expression profile to *Bcser2*.

As it has been shown previously that some proteases are transcriptionally regulated (Poussereau *et al*., 2001; Rolland *et al*., 2009), the pH regulation of genes encoding secreted proteases was investigated under controlled culture conditions with both fungi (Fig. S1). This *in vitro* analysis confirmed that the expression of genes encoding ACP1 protease was fully repressed at pH 7.0, as described previously (Rolland *et al*., 2009). It was also revealed that the expression of *Ssap10* and *Bcap8*, encoding two aspartic proteases, was subject to the same pH regulation. *Ssser2* and *Bcser2* genes were expressed at pH 4.0 and pH 7.0, but the expression level of *Ssser2* was higher at pH 7.0, suggesting an induction at neutral pH. Surprisingly, the *Bcser1* gene, which exhibited the highest expression level by the end of sunflower cotyledon colonization, was not expressed at either pH 7.0 or pH 4.0 under our controlled culture conditions. Across medium pH values, the *Bccp4* gene, encoding a serine carboxypeptidase, showed a similar expression level, whereas that of *Sscp4* was higher at pH 7.0.

Organic acids and ammonia production under controlled culture conditions

During the colonization process on sunflower cotyledons, *S. sclerotiorum* and *B. cinerea* differed significantly with regard to organic acid production. In order to investigate these differences, an *in vitro* kinetic analysis was performed under controlled conditions. In this analysis, *S. sclerotiorum* and *B. cinerea* mycelia were transferred into Gamborg's medium containing glucose or amino acids as the only source of carbohydrates. The pH value of the culture medium, organic acids, ammonia and residual glucose were followed during the kinetic analysis.

In all culture media tested, *S. sclerotiorum* culture pH values decreased. By contrast, with *B. cinerea*, two different pH profiles were obtained depending on the culture medium. In culture medium supplemented with glucose, pH values decreased from pH 6.0 to pH 4.0, up to 24 hpi, and then increased to reach pH 5.2. In medium supplemented with amino acids, pH values increased from pH 6.0 to pH 7.0 (Fig. 5a). Regardless of the culture medium, both fungi produced oxalic acid. With *S. sclerotiorum*, significant production of oxalic acid was measured in medium supplemented with glucose (Fig. 5b), but the level was 24-fold lower in culture medium supplemented with amino acids (Fig. 5d). With *B. cinerea*, the level of oxalic acid was insignificant in all culture media (Fig. 5c,e). Furthermore, a correlation between glucose consumption and oxalic acid production was observed with *S. sclerotiorum*, but not with *B. cinerea* (Fig. S2a,b, see Supporting Information). Succinic acid was produced first and its accumulation was similar for both fungi. Its level decreased as the culture time increased and as oxalic acid was produced. However, succinic acid production was three-fold lower than that of oxalic acid with *S. sclerotiorum* (Fig. 5b), and showed the opposite trend with *B. cinerea* (Fig. 5c). Surprisingly, citric acid was not quantifiable in any culture condition for either fungus.

Regardless of the culture medium, both fungi produced significant amounts of ammonia. It was noted that, in medium supplemented with glucose, ammonia production increased during the first 16 hpi, as pH values decreased, and then dropped. In medium supplemented with amino acids, the ammonia production increased, as succinic acid decreased, and its level was two-fold higher with *B. cinerea* than with *S. sclerotiorum* (Fig. 5d,e).

DISCUSSION

Comparative pathogenicity assays on sunflower cotyledons with *S. sclerotiorum* (strain S5) and *B. cinerea* (strain B05.10) show striking resemblances in symptom development, although *S. sclerotiorum* exhibits more rapid colonization. *Sclerotinia sclerotiorum* and *B. cinerea* produce organic acids, mainly oxalic acid (Cessna *et al*., 2000; Maxwell and Lumdsen, 1970; Verhoeff *et al*., 1988). During the colonization process on sunflower cotyledons, we have shown that both organic acids and ammonia are produced. With both *S. sclerotiorum* and *B. cinerea*, levels of organic acids and ammonia fluctuate during the colonization process and these variations may be responsible for the observed pH modulations. On the basis of the pH dynamics, the colonization process can be divided into two distinct stages. The first stage is associated with an ambient pH decrease, until 40% of the cotyledon area is colonized, and the second is correlated with a pH value increase, when greater than 40% of the cotyledon area is colonized.

During the first stage of colonization, the pH decrease may be attributed to the production of organic acids. Oxalic acid does not appear to be responsible for this initial pH decrease as the concentration of oxalic acid remains insignificant during the first stage of colonization. In contrast, citric and succinic acids are produced first and both accumulate to significant levels (Table 3). For both fungi, glucose, fructose and sucrose show a strong decrease during the early stages of sunflower cotyledon colonization (Dulermo *et al*., 2009; Jobic *et al*., 2007). As organic acids are directly linked to glycolysis, we can assume that glucose plays a major role in the highlighted pH fluctuation. In controlled culture medium, we have confirmed for both fungi that the glucose consumption and pH decrease are interdependent, as suggested previously for *Sclerotinia* (Rollins and Dickman, 2001), and that pH values increase as glucose is fully consumed (Fig. S2c,d). Moreover, we have validated that oxalic acid is produced later than succinic acid (Fig. 5b,c) and

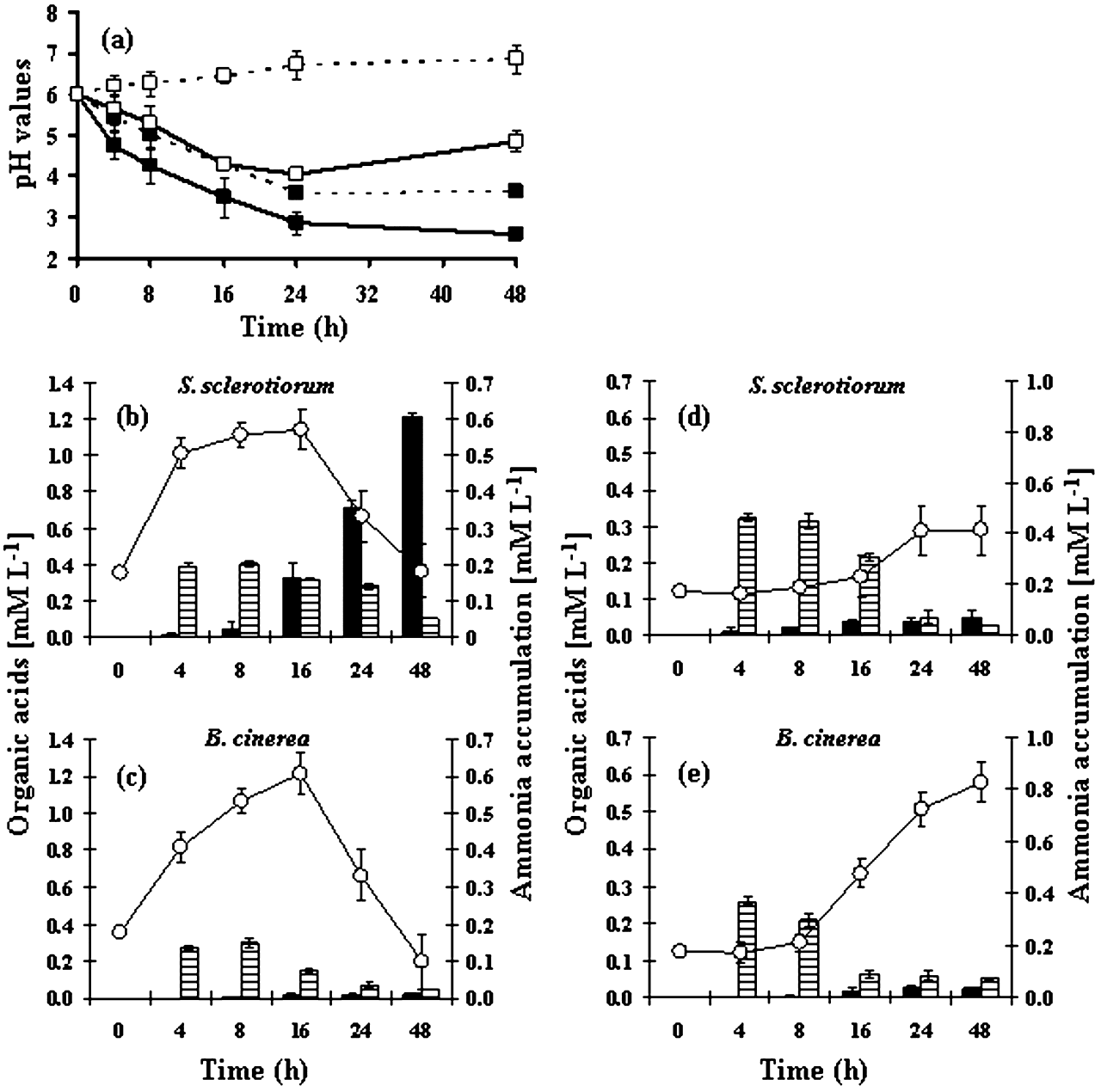

Fig. 5 Comparison of pH changes, organic acids and ammonia production in controlled culture medium. Significant biomasses of *Sclerotinia sclerotiorum* and *Botrytis cinerea* were transferred into Gamborg's medium adjusted to pH 6.0 and supplemented with 10 mM glucose or 2 mM amino acids. A kinetic analysis was performed and pH changes, organic acids and ammonia were measured in all culture filtrates. Three biological replications were conducted and the values are expressed as the average of at least triplicate assays for each replication. (a) pH changes with *S. sclerotiorum* (■) or *B. cinerea* (□) growing with amino acids (----) or glucose (——). Ammonia (○), succinic acid (▤) and oxalic acid (▬) production in media supplemented with glucose (b, c) and amino acids (d, e).

that, in contrast with *B. cinerea*, the production of oxalic acid appears to be closely linked to glucose consumption with *S. sclerotiorum* (Fig. S2a,b). These phenomena can explain the pH decrease measured during the first stage of colonization on sunflower cotyledons. Moreover, they can also explain, for both fungi, the ongoing pH decrease during the colonization on carrot root or apple fruit because of the high glucose concentration of these tissues (Bourquin *et al*., 1992; Jobic *et al*., 2007). However, as organic acids are directly linked to glycolysis via the tricarboxylic acid (TCA) cycle, this work suggests that *S. sclerotiorum* and *B. cinerea* differ significantly in glycolysis or in TCA cycle regulation, as *S. sclerotiorum* has neither the same level of end products from

Table 3 Comparative overview on organic acid and ammonia production during sunflower cotyledon colonization by *Sclerotinia sclerotiorum* and *Botrytis cinerea*.

	Control	*S. sclerotiorum*			*B. cinerea*		
Colonized area	0%	5%	40%	100%	5%	40%	100%
Citric acid accumulation	474	689	0	0	1621	1136	0
Succinic acid accumulation	455	811	13	5	1543	688	4
Malic acid accumulation	91	71	0	0	214	3	0
Oxalic acid accumulation	1.1	3.0	23	289	1.2	19	34
Ammonia accumulation	106	106	97	259	106	33	400
Resulting pH values	6.5	5.2	3.7	4.8	5.6	4.8	7.0

Values given in the 'Control' column are those measured in uninfected sunflower cotyledons and are used as reference. Columns '5%', '40%' and '100%' show the results when 5%, 40% and 100%, respectively, of the area was colonized. All values are expressed as μM/g of dry matter.

glucose metabolism nor the same rate of glucose consumption as *B. cinerea*. Nevertheless, in controlled culture medium, we have verified that both fungi produce succinic acid, but citric acid is not detected. As citric acid, a known antioxidant, is produced in high concentrations in the earliest stages of the infection process, we suggest that citric acid production may be a result of the host plant reaction in response to oxidative stress. Not only *B. cinerea* does not suffer from H_2O_2-induced oxidative stress, but it is also able to generate reactive oxygen species (Rolke *et al*., 2004; Temme and Tudzynski, 2009), and the level of citric acid is 2.5-fold higher with *B. cinerea* than with *S. sclerotiorum*. Ascorbic acid, synthesized throughout the life of the plant, is a substrate for oxalic acid synthesis, and a 30-fold decrease in ascorbic acid was noted in plant leaves colonized by *B. cinerea* (Loewus, 1999; Muckenschnabel *et al*., 2002). Therefore, during the first stage of the colonization process, the resulting pH of colonized tissues depends not only on the accumulation of citric and succinic acids, but also on the decrease in ascorbic acid.

During the second stage of the colonization process on sunflower cotyledons, the pH of colonized tissues increases with both fungi. Ammonia is an end product of amino acid catabolism (Voet and Voet, 2005) and we have shown that amino acids, an important source of carbon, nitrogen and sulphur, decrease during the second stage of colonization. Therefore, the decrease in amino acids appears to be concomitant with ammonia production and, consequently, with the pH increase. Other phenomena must be considered during the second stage of colonization: citric and succinic acids disappear and oxalic acid increases significantly. However, with *S. sclerotiorum,* oxalic acid increases 12.6-fold and the accumulation of ammonia is similar to that of oxalic acid. With *B. cinerea*, oxalic acid increases weakly (1.8-fold) and its level remains 11.8-fold lower than that of ammonia. Consequently, and consistent with this low production of oxalic acid and the strong production of ammonia, the resulting pH of necrotic tissues reaches a neutral value with *B. cinerea* (Table 3). These findings reveal that *B. cinerea* is able to adapt from an acidic to a neutral environment. With *S. sclerotiorum*, as oxalic acid and ammonia are produced at similar levels, the resulting ambient pH of necrotic tissues increases weakly and then stabilizes. Therefore, *S. sclerotiorum* remains in an acidic environment during the entire colonization process on sunflower cotyledons.

To penetrate and extensively colonize the host plant, *S. sclerotiorum* and *B. cinerea* secrete numerous cell wall-degrading enzymes (Choquer *et al*., 2007; van Kan, 2006; van Kan *et al*., 1997). Several proteolytic activities have been reported from *S. sclerotiorum* (Billon-Grand *et al*., 2002; Poussereau *et al*., 2001) and *B. cinerea* (Gamboa-Melendez *et al*., 2009; Manteau *et al*., 2003; ten Have *et al*., 2010). Proteolysis of plant proteins can provide essential nutriments when they are no longer easily assimilated from other sources, and ammonia accumulation can result directly from protease activities (Voet and Voet, 2005). Here, we report that genes encoding secreted proteases are predominantly activated during the second stage of colonization as ammonia accumulates (Table 2). Genes encoding secreted proteases are known to be transcriptionally regulated by ambient pH (Kim *et al*., 2007; Poussereau *et al*., 2001; Rolland *et al*., 2009) and, as described previously, *Bcacp1* and *Ssacp1* genes are only expressed under acidic conditions. *Bcap8* and *Ssap10* are expressed over a wider pH range, but we have validated that they are fully repressed at pH 7.0 (Fig. S1). In contrast, the expression level of *Bcser2* and *Ssser2*, genes encoding serine proteases, increases as the pH value increases, and the *Bcser1* gene is only expressed as necrotic tissues reach a neutral pH value. Therefore, during the colonization process, both fungi are able to secrete acidic as well as neutral proteases, depending on the prevailing ambient pH of necrotic tissues. However, the expression level of *B. cinerea* secreted protease genes is two- to four-fold higher than that of *S. sclerotiorum* (Table 2), and proteases are potentially a more significant factor during the colonization process of *B. cinerea* relative to *S. sclerotiorum*.

Despite the observed differences during colonization (i.e. protease gene expression, organic acid production and ammonia accumulation), these two pathogens share an ability to modulate the host pH environment. An understanding of ambient pH-regulated gene expression and its role in regulating organic acid biosynthesis may provide key insights into the differing colonization strategies employed by these two fungi. A random mutagenesis nonpathogenic mutant (A336), negatively affected in

bcacp1 expression and oxalic acid production, has been described in *B. cinerea* (Kunz *et al*., 2006). This loss of both oxalic acid accumulation and *bcacp1* expression further strengthens the ties of these two factors to pathogenicity. Knockout and constitutively active mutants of Pac1 in *S. sclerotiorum* are also negatively affected in pH-responsive gene expression of *Ssacp1*, oxalic acid production and virulence (Kim *et al*., 2007; Rollins, 2003). A functional characterization of the *B. cinerea pac1* orthologue would provide valuable insight into its role in protease gene regulation, oxalic acid biosynthesis and virulence in different hosts. To understand the role of oxalic acid in infection and colonization, specific gene deletion mutants of the oxaloacetate acetyl hydrolase encoding gene in *B. cinerea* (*Bcoah*) have been created (Han *et al*., 2007). This mutant does not produce oxalic acid and is reported to have no effect on virulence on tomato leaves and fruits, cucumber fruits, apples and carrots. On *Arabidopsis thaliana* and cucumber plants, the expansion of primary lesions was strongly reduced (Stefanato *et al*., 2008). In contrast, *S. sclerotiorum* oxalate minus mutants, created by random mutagenesis (Godoy *et al*., 1990) and by target gene deletion of the *Ssoah1* gene (J. A. Rollins, unpublished), exhibit defects in pathogenicity or virulence on all tested hosts. These reports are consistent with the observations of the current study, in which acidic pH environments were not created in all hosts with *B. cinerea*, but were consistently associated with host colonization with *S. sclerotiorum*. A systematic comparison of these *B. cinerea* and *S. sclerotiorum* mutants is warranted to understand how these similar necrotrophic fungi may use dissimilar strategies in the colonization process of the same hosts.

In conclusion, this work suggests that the overt similarities observed in symptom development during the colonization processes of *S. sclerotiorum* and *B. cinerea* mask our recognition of the dynamic and potentially different metabolic pathways employed for host colonization by these two necrotrophic fungi.

EXPERIMENTAL PROCEDURES

Fungal strains and phytopathogenicity assays

Sclerotinia sclerotiorum, strain S5, provided by Bayer SAS, Lyon, France, and *Botrytis cinerea*, strain BO5.10, were maintained on potato dextrose agar (PDA; Difco, Paris, France) at 21 °C. Phytopathogenicity assays were performed on sunflower cotyledons (sunflower seeds were provided by Limagrain, Chappes, France). Sunflowers were grown at 21 °C in a glasshouse. Cotyledons from 1-week-old germlings were infected on the adaxial surface with a 4-mm agar disc from 48-h-old mycelial PDA cultures. Infected cotyledons were covered with transparent plastic boxes in 100% relative humidity air. They were placed under alternating near-UV light (10 h) and daylight (14 h) at 21 °C. At different stages of symptom development, cotyledons were harvested and immediately frozen at –80 °C. Healthy cotyledons were collected at the same time, frozen at –80 °C and used as controls.

Pathogenicity assays were also performed on chicory leaves, carrot roots and apple fruits var. 'Golden', collected at full maturity from a local grocery. Carrot roots were divided into two pieces along their longitudinal axis, apple fruits were cut into 5-mm-thick slices and individual chicory leaves were isolated. All these tissues were infected with 4-mm discs of 48-h-old mycelial PDA cultures and placed in 100% relative humidity air under alternating near-UV light (10 h) and daylight (14 h) at 21 °C.

In vitro culture conditions

Malt medium (malt extract 2% w/v, glucose 0.5% w/v, yeast extract 0.1% w/v, tryptone 0.1% w/v, acid hydrolysate of casein 0.1% w/v, ribonucleic acid 0.02% w/v) (100 mL) was inoculated with agar mycelium plugs of 48-h-old cultures on PDA (Difco) for *S. sclerotiorum*, or with conidia (0.5×10^7 spores/mL) obtained from a 15-day-old culture on PDA for *B. cinerea*. Cultures were placed on a rotary shaker for 30 h at 110 rpm and 21 °C. Mycelia were collected by filtration on miracloth (Calbiochem, Lyon, France), washed twice with sterile water and blended for 3×30 s in 10 mL of sterile water. For kinetic analysis, 1 mL of the blended mycelia was added to 20 mL of Gamborg medium (Gamborg *et al*., 1968) supplemented with 2 mM amino acids (400 μM alanine, 400 μM arginine, 400 μM aspartic acid, 400 μM cysteine and 400 μM glutamine), used as sole nitrogen, sulphur and carbon sources, or with 10 mM glucose or 2 mM amino acids plus 10 mM glucose. All media were adjusted to pH 6.0 with 1 M NaOH. Cultures were incubated under constant light at 21 °C on a rotary shaker at 110 rpm for 4, 8, 16, 24 and 48 h. Culture filtrates were collected by filtration on ashless filter papers (Whatman, Versailles, France), sterilized over 0.2-μm filters (Schleicher and Schuell, Dassel, Germany) and pH measurements were immediately taken. Culture filtrates were stored at –20 °C and used for the quantification of glucose, oxalic, citric, malic and succinic acids and ammonia.

Effect of ambient pH and glucose on protease gene expression

To analyse the effect of ambient pH and glucose on protease gene expression, 2×30 mL of malt medium (containing 20 g/L malt extract and 100 mM NH_4Cl) were inoculated with agar mycelium plugs for each fungus. These cultures were incubated at 21 °C for 30 h, with shaking at 110 rpm. Mycelia were collected by filtration, washed twice with sterile distilled water and blended (3×30 s in 10 mL of sterile water). Aliquots of 100 μL were grown on solid malt medium on the surface of cellophane sheets. After 22 h of incubation at 21 °C, cellophane sheets were transferred onto the surface of 10 mL of Gamborg medium buffered at pH 4.0 (with 0.2 M citrate/phosphate buffer) or pH 7.0 [with 0.2 M piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (PIPES) buffer] and supplemented or not with 100 mM glucose. Three hours after transfer, mycelia were washed in sterile water, collected by scraping, freeze–dried and stored dry for RNA extraction.

pH measurement of plant tissues

Infected and uninfected sunflower cotyledons were detached from stems. Mycelial plugs were removed with a scalpel. Cotyledons were turned with the inoculated surface down and left on a glass plate. All pH values were

measured with a flat contact electrode (Bioblock Scientific, Illkirch, France) placed directly either on the lesion centre for infected sunflower cotyledons, chicory leaves, carrot roots and apple fruits, or on the middle of uninfected cotyledons. pH values were recorded after 2 min. For each stage of symptom development, 10–15 samples were used and four biological replications were carried out. Healthy tissues were used as comparative controls.

Quantification of organic acids and ammonia

Forty infected and uninfected sunflower cotyledons per time point were frozen in liquid nitrogen, ground to a fine powder and freeze–dried. For each infection time, 300 mg of ground tissues were rehydrated into 4 mL of freshly distilled water. After 10 min at 4 °C, 500 mg of activated charcoal was added to chelate the pigments. The mixture was shaken for 10 min and centrifuged twice at 10 000 ***g*** for 15 min at 4 °C. Clear supernatants were filtered through 0.2-μm filters (Schleicher and Schuell), neutralized with 1 M NaOH and stored at –20 °C.

As oxalic acid could be trapped as oxalate crystals, colonized tissues needed an additional treatment. For each infection time, 300 mg of ground tissues were rehydrated into 3 mL of freshly distilled water. Samples were adjusted to pH 3.0 with 1 M hydrochloric acid, heated at 100 °C for 15 min, and the final volume was adjusted to 4 mL with freshly distilled water. After 10 min at 4 °C, 500 mg of activated charcoal was added to chelate the pigments. The mixture was shaken for 10 min and centrifuged twice at 10 000 ***g*** for 15 min at 4 °C. Clear supernatants were filtered through 0.2-μm filters (Schleicher and Schuell), neutralized with 1 M NaOH and stored at –20 °C. Uninfected cotyledon extracts, used as controls, were subjected to the same experimental procedure.

For oxalic acid quantification, an enzymatic assay kit (Cat. No. 10 755 699 035) was provided by R-Biopharm (St Didierau Mt d'Or, France) and assays were performed according to the manufacturer's instructions. For citric, malic and succinic acids, enzymatic assay kits were provided by Megazyme (Bray, Ireland) and assays were performed according to the manufacturer's instructions. All values represent the means of triplicate assays. Uninfected cotyledon extracts and uncultured liquid media were used as controls.

The quantification of ammonia was performed according to Werber *et al*. (1997). Briefly, 100 μL of 0.1 M sodium and potassium tartrate and 100 μL of Nessler's reagent (Merck, Darmstadt, Germany) were added to 2 mL of plant extracts (*in planta* assays) or 2 mL of culture filtrates (*in vitro* assays). After 10 min at room temperature, the absorbance was measured at 420 nm. Controls were performed with 2 mL of plant extracts from uninfected cotyledons, or 2 mL of sterile liquid medium. Values represent the average of at least four assays.

Amino acid quantification in plant tissues

Forty infected and uninfected sunflower cotyledons per time point were frozen at –80 °C, freeze–dried and ground to a fine powder. To 5 mg of powder, 1 mL of 50 mM HCl/50% acetonitrile (v/v) was added. Final suspensions were incubated on ice for 10 min and centrifuged at 13 000 ***g*** for 10 min at 4 °C. Supernatants were used for the quantification of amino acids.

Amino acid separation and quantification were performed by reversed phase chromatography using high-performance liquid chromatography (HPLC) (Waters HPLC system coupled to an Uptisphere HP5HDO-25K column, St Quentin en Yvelines, France) after labelling with *ortho*-phthadialdehyde (50 mg *ortho*-phthadialdehyde, 1 mL absolute ethanol, 10 mL borate/0.5 M NaOH, pH 9.5, 200 μL β-mercaptoethanol) (Ravanel *et al*., 1995). Elution was carried out with the following buffers: 50 mM sodium acetate/NaOH, pH 4.5, and 3% tetrahydrofuran (buffer A) and 100% methanol supplemented with 50 mL tetrahydrofuran (buffer B). The *ortho*-phthadialdehyde adduct fluorescence was monitored at 455 nm on excitation at 340 nm using an SFM25 fluorimeter (UVK-Lab, Trappes, France). The identification of amino acids was assessed by the co-injection of standards, and quantification was performed by measuring the peak areas with 450-MT2 software (UVK-Lab).

Real-time RT-qPCR

Total RNA was extracted by phenol/chloroform separation and lithium chloride precipitation (Verwoerd *et al*., 1989). For RT-qPCR experiments, 20 μg of total RNA of each sample were treated with DNAse I (Ambion, Villebon sur Yvette, France) to remove genomic DNA. The absence of genomic DNA was controlled by PCR using DNAse I-treated total RNA as template and primers specific to the actin-encoding genes *BcactA* (accession number AJ000335) and *SsactA* (accession number XM_001589919). The quality of total RNA was verified using an Agilent 2100 Bioanalyser, Agilent RNA 6000 Nano reagents and RNA chips from Agilent (Garches, France). Total cDNA was produced by treating 5 μg of DNaseI-treated total RNA with Thermoscript RT (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer. qPCR experiments were performed on an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) with specific primers for genes encoding proteases (Table 1) and using the Power SYBR® Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. Following examination of the primer efficiencies, the amplification reactions were carried out as follows: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min (50 cycles), 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s. Relative quantification was based on the $2^{-\Delta Ct}$ method using *BcactA* (for *Botrytis* genes) and *SsactA* (for *Sclerotinia* genes) as references. Three independent biological replications were analysed.

Determination of residual glucose

An enzymatic assay kit (R-Biopharm, Darmstadt, Germany) was used for the determination of residual glucose in culture filtrates, according to the manufacturer's instructions. All values represent the mean of four assays. Uncultured liquid media were used as controls.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Ambient pH regulation of genes encoding secreted proteases.

Fig. S2 Effect of glucose concentration on ambient pH and oxalic acid production in controlled culture medium.

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