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1 **NOVEL INSIGHTS INTO MANNITOL METABOLISM IN THE FUNGAL PLANT**
2 **PATHOGEN *BOTRYTIS CINEREA***

3
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21 Short title : Mannitol pathway in *Botrytis cinerea*

22
23 **SYNOPSIS**

24
25 In order to redefine the mannitol pathway in the necrotrophic plant pathogen *Botrytis cinerea*,
26 we used a targeted deletion strategy of genes encoding two proteins of mannitol metabolism, a
27 mannitol dehydrogenase (BcMTDH), and a mannitol-1-phosphate dehydrogenase (BcMPD).
28 Mobilization of mannitol and quantification of *Bcmpd* and *Bcmtdh* gene transcripts during
29 development and osmotic stress confirmed a role for mannitol as temporary and disposable
30 carbon storage compound. In order to study metabolic fluxes, we followed conversion of
31 labelled hexoses by wild type and *Bcmpd* and *Bcmtdh* mutant strains by *in vivo* NMR
32 spectroscopy. Our data revealed that glucose and fructose were metabolized via the BcMPD
33 and BcMTDH pathways, respectively. Existence of a novel mannitol phosphorylation
34 pathway was suggested by NMR investigations. This last finding definitively challenged the
35 existence of the originally postulated mannitol cycle in favor of two simultaneously expressed
36 pathways. Finally, physiological and biochemical studies conducted on double deletion
37 mutants (*Bcmpd/Bcmtdh*) showed that mannitol was still produced despite a complete
38 alteration of both mannitol biosynthesis pathways. This strongly suggests that one or several
39 additional undescribed pathways could participate to mannitol metabolism in *B. cinerea*.

40
41 Key words:

42 *Botrytis cinerea*, *in vivo* NMR spectroscopy, mannitol, carbon metabolism, osmotic stress,
43 trehalose.

44 Abbreviations used: BcMPD, *Botrytis cinerea* MPD; BcMTDH, *Botrytis cinerea* MTDH;
45 MPD, mannitol-1-phosphate dehydrogenase; MTDH, mannitol dehydrogenase; PCA,
46 perchloric acid; TLC, thin layer chromatography.

47
48
49 **INTRODUCTION**

51 Mannitol is one of the most abundant polyols occurring in nature. It is usually the most
52 abundant soluble carbohydrate within the mycelium [1]. In fungi, several physiological
53 functions have been ascribed to D-mannitol, such as reservoir of reducing power [2], carbon
54 storage compound accumulated in *Agaricus bisporus* basidiospores [3] or necessary for the
55 formation of fruit bodies in *Stagonospora nodorum* [4]. Mannitol contributes to stress
56 tolerance in fungi. In *Aspergillus niger*, mannitol appears to be essential for the protection of
57 spores against cell damage under a variety of stress conditions including cold, drought and
58 oxidative stress [5]. In mycorrhizal fungi, mannitol plays the role of carbon translocation
59 compound that enables fungi to assimilate carbohydrates from plant origin [6, 7].
60 Furthermore, mannitol has a role in fungal-plant interactions. During the biotrophic
61 interaction *Uromyces fabae/Vicia faba*, levels of mannitol markedly increase in apoplastic
62 fluids of infected leaves and in spores. Mannitol might have a dual function, sequestration of
63 plant hexoses and protection against reactive oxygen species [8]. A role for mannitol in
64 pathogenicity of *Alternaria alternata* on its host, tobacco, was demonstrated [9, 10]. Mannitol
65 was secreted by *A. alternata* in response to tobacco extracts. In addition, tobacco expressed an
66 endogenous mannitol dehydrogenase during infection [9, 11]. Mannitol, secreted by
67 *A. alternata* could play an antioxidant role and quench reactive oxygen species. Mannitol
68 dehydrogenase, induced in plant by fungal colonization, degrades pathogen-produced
69 mannitol, allowing for ROS-mediated plant defense to be effective against the fungus.

70 The metabolic pathway for mannitol biosynthesis and catabolism (Figure. 1), well
71 described in filamentous fungi, takes place through the mannitol cycle, involving two
72 pathways [12]. The direct reduction of fructose-6-phosphate into mannitol-1-phosphate
73 involves a mannitol-1-phosphate dehydrogenase (MPD, EC 1.1.1.17). Mannitol-1-phosphate
74 is then dephosphorylated into mannitol via a mannitol-1-phosphate phosphatase. This last
75 reaction was described as irreversible, consequently, mannitol degradation is supposed to
76 occur through oxidation of mannitol to fructose via a reversible mannitol dehydrogenase
77 (MTDH, EC 1.1.1.138). Both pathways exist in ascomycetes [2].

78 However, recent reports have challenged the existence of a mannitol cycle. In *S. nodorum*,
79 MPD is necessary for mannitol catabolism, whereas MTDH is not [4, 13]. A mannitol
80 phosphorylation pathway allowing conversion of mannitol into mannitol-1-phosphate might
81 exist. Mannitol pathway explorations conducted in *S. nodorum* and *A. alternata* revealed that
82 mannitol synthesis occurred mainly through MPD [13, 14]. *mpd* deletion mutant strains
83 revealed an intracellular mannitol concentration decreased by more than 80%, whereas *mtdh*
84 strains were almost completely phenotypically wild type. While mannitol metabolism through
85 mannitol-1-phosphate appears to be the dominant route, the physiological role of MTDH
86 branch remains unclear.

87 In a previous report, we showed that mannitol was accumulated during infection of
88 sunflower by the necrotrophic plant pathogen *B. cinerea* [15]. During pathogenesis, plant
89 hexoses were converted into mannitol, which was the major soluble carbon compound
90 detected in infected tissues. In previous experiments, we were unable to detect mannitol in
91 fungal growth media [15]. This polyol could be used as translocation compound to sequester
92 plant hexoses. Moreover, mannitol accumulated mainly during the final steps of fungal
93 development *in planta*, being stored when conidiophores emerged. Mannitol could be
94 necessary for spore survival or constitute a source of energy for germination. By means of
95 reverse genetics and metabolic investigations, we have explored the physiological role and
96 functioning of the mannitol pathway in *B. cinerea*.

97 98 **EXPERIMENTAL** 99

100 Fungal strain and growth conditions

101

102 *Botrytis cinerea* B05.10 was maintained at 21°C on rich medium as described in [16].
103 *B. cinerea* developmental stages were obtained from mycelia grown for 2 or 6 days
104 (mycelium and sporulating mycelium) on cellophane sheets deposited on 2% glucose-rich
105 solid medium. Conidia were harvested from 12-day-old mycelium grown on 2% glucose-rich
106 solid medium. Germinating conidia were obtained by cultivating 12-days-old spores (10^7 ml⁻¹)
107 in 2% glucose-rich liquid medium (110 rpm). For osmotic stress experiments, mycelia were
108 grown for 2 days on cellophane on solid Gamborg medium (pH 5.0) supplemented with 2%
109 glucose [16]. Mycelia were then transferred for 30 min, 1 h, 4 h, 9 h, and 24 h on fresh solid
110 medium supplemented with 2% glucose and 1M NaCl. For osmotic stress response analysis
111 by *in vitro* NMR spectroscopy, mycelia were first transferred for 4 h on labelled fructose
112 plates (1% fructose containing 10% ¹³C-fructose) and transferred for 1 and 4 h on fresh solid
113 medium supplemented with 2% glucose and 1M NaCl. Mycelia, conidia and germinating
114 conidia were harvested, frozen in liquid nitrogen then stored at -80°C. *In vivo* NMR analyses
115 were performed in a perfusion medium containing 1 g l⁻¹ KNO₃, 0.05 g l⁻¹ KCl, 0.1 g l⁻¹
116 MgSO₄, 0.1 g l⁻¹ CaSO₄, and ¹³C-labelled glucose or fructose as indicated in text. *B. cinerea*
117 transformant strains were selected on rich media supplemented with 70 µg ml⁻¹ hygromycin
118 (Invivogen, Toulouse, France) or with 100 µg ml⁻¹ nourseothricin (Werner BioAgents, Jena,
119 Germany).

120

121 *In vitro* germination and conidiation assays

122

123 Conidia were harvested in 0.5% Tween 80 from 12-days-old mycelium grown on solid rich
124 medium. For *in vitro* germination assays, 500 conidia of each strain were spread on solid rich
125 medium in 140 mm plates. Young germinations were counted after 4 days of incubation. For
126 conidiation assays, 1000 conidia were spread on solid rich medium in 24-well-plates. After 12
127 days of growth, conidia produced in each well were harvested and counted.

128

129 Pathogenicity tests

130

131 Phytopathogenicity assays were performed on sunflower cotyledons as hosts as previously
132 described [15]. Cotyledons from 1-week-old germlings were infected at the end of a dark
133 period by depositing a 5-mm-mycelium disk near the tip of the leaves. Necrosis was
134 detectable 24 hpi (hours post infection) by the appearance of a brown color surrounding the
135 starting point of infection. At 48 hpi, half of the cotyledons were macerated and necrosed. The
136 whole cotyledon was infected at 72 hpi. Conidiation began at 96 hpi and was achieved at 120
137 hpi.

138

139 Plasmid construction and transformation of *B. cinerea*

140

141 Transformation of *B. cinerea* B05.10 was performed using the *hph* gene as selectable marker.
142 To disrupt *Bcmpd* and *Bcmtdh*, constructs containing the hygromycin resistance cassette
143 (*OliC* promoter-*hph* gene coding sequence- *tubI* terminator) flanked by 5' and 3' *Bcmpd* and
144 *Bcmtdh* genomic DNA fragments were realized (Figure S1). The hygromycin cassette was
145 released from pLOB1 (provided by P. Tudzynski, Münster, Germany) after *EcoRI* and *EcoRV*
146 digestion, and then cloned in pBKSII. Flanking regions (5' *Bcmpd*, 3' *Bcmpd*, 5' *Bcmtdh* and
147 3' *Bcmtdh* fragments) amplified using P1/P2, P3/P4, P5/P6 and P7/P8 primers respectively
148 (Table S1 and Figure S1) were cloned on every side of the hygromycin cassette (Figure S1) to
149 form pTD5 and pTD6. To obtain pTD10 plasmid, *Bcmtdh* 5' and 3' flanking regions were

150 amplified using P9/P10 and P11/P12 respectively (Figure S1). Amplified fragments were
151 cloned in pCB04, on each side of the nourseothricin cassette, which contained the
152 *Streptomyces* nourseothricin N-acetyl-transferase gene (*nat1*) flanked by the *A. nidulans* *OliC*
153 promoter and the *B. cinerea* *tub1* terminator [17]. Preparation of protoplasts and
154 transformation were adapted from procedures described in [18]. Fungal cell wall was digested
155 using 50 mg ml⁻¹ Glucanex (Novozymes, Dittengen, Switzerland) for 2 h at 26°C. Wild type
156 strain (WT) was transformed with 10 µg of *NotI* linearized vector pTD5 or pTD6 to obtain
157 *Bcmtdh* and *Bcmpd* mutants respectively. The development of a double mutant was achieved
158 by transforming the previously generated Δ *Bcmpd* strain with 10 µg of *ScaI* linearized vector
159 pTD10. PCR analyses were performed to ensure replacement of gene of interest by the
160 selection cassette. gDNA from *Bcmpd* transformants was amplified using P13/P14 and
161 P15/P16 primers (Table S1), to check 5' and 3' insertions respectively. gDNA from *Bcmtdh*
162 transformants was amplified using P17/P14 primers and P18/P19 primers to verify 5' and 3'
163 insertions respectively (Table S1). *Bcmtdh* gene replacement by the nourseothricin cassette
164 was verified using primers P20/P21 and P22/P19 to check 5' and 3' insertions respectively
165 (Table S1). Southern analyses were performed to ensure single insertions. DNA (5 µg) was
166 digested with *EcoRI* for single mutants, and with *HindIII* for double mutants, separated
167 through an agarose gel (0.8%) and transferred to a nylon membrane. A ³²P-labelled probe,
168 (*hph* coding sequence for single mutants or the 5' *Bcmtdh* flanking region for double mutants),
169 was obtained from plasmids pLOB1 and pTD5 using Megaprime DNA Labelling system
170 (Amersham Biosciences). Hybridization was carried out as described in [19].

171 172 **RNA isolation and transcript quantification**

173
174 Biological materials were frozen in liquid nitrogen and kept at -80°C. Samples were crushed
175 in liquid nitrogen, and total RNAs were extracted by phenol/chloroform separation and
176 lithium chloride precipitation [20]. For Q-PCR experiments, 20 µg of total RNA of each
177 sample were treated with DNaseI (Ambion). Absence of genomic DNA was controlled by
178 PCR using the DNaseI-treated total RNA as template and Taq-DNA polymerase (MP
179 Biomedicals, Solon, USA). Quality of total RNA was verified using Agilent 2100
180 Bioanalyzer, Agilent RNA 6000 Nano reagents and RNA Chips. Total DNaseI-treated RNA
181 (5 µg) was treated with Thermoscript RT (Invitrogen, Carlsbad, USA) as described by
182 manufacturer. Q-PCR experiments were performed in ABI PRISM 7900HT (Applied
183 Biosystems, Foster City, USA) using the Power SYBR[®] Green PCR Master Mix (Applied
184 Biosystems) according to the instructions of the manufacturer. Relative quantification was
185 based on the 2^{ΔCT} method using *Bcact1* and *BctubA* as calibrator references. As gene
186 expression profiles were similar using both controls, only the results obtained using *BcactA*
187 transcripts are presented here. The amplification reaction was as follows: 95°C 10 min, 95°C
188 15 s, and 60°C 1 min (50 cycles), 95°C 15 s, 60°C 15 s, and 95°C 15 s. Three independent
189 replicates, prepared from independent biological samples, were analyzed. Primers used for Q-
190 PCR are shown in Table S1.

191 192 **NMR spectroscopy**

193
194 PCA (perchloric acid) extracts were prepared from 5 to 10 g *B. cinerea* mycelia according to
195 the method described in [21]. Values are given in mg g⁻¹ fresh weight (FW) of fungal
196 material. *In vitro* (tissue extracts) and *in vivo* (perfused tissues) spectra were recorded on a
197 Bruker NMR spectrometer (AMX 400, wide bore; Bruker, Billerica, MA) equipped either
198 with a 10-mm or a 25-mm multinuclear-probe tuned at 161.9 or 100.6 MHz for ³¹P- and ¹³C-
199 analyses. The deuterium resonance of ²H₂O was used as lock signal. For *in vitro*

200 measurements, ^{13}C -NMR and ^{31}P -NMR acquisitions were performed as described in [15].
201 Assignments and quantifications were made after running a series of spectra of extracts,
202 added with known amounts of authentic compounds, and at different pHs for the extracts
203 submitted to ^{31}P -NMR analysis, in particular to be certain that the peak of resonance
204 appearing at 4.77 ppm in samples incubated with mannitol corresponded well to mannitol-1-
205 phosphate. *In vivo* measurements were performed using 48 hpi sunflower cotyledons as
206 described in [15].

207

208 **Polyol and sugar detection by thin layer chromatography (TLC)**

209

210 Mannitol was extracted from conidia, germinating conidia, mycelia or conidiating mycelia
211 frozen in liquid nitrogen, crushed and then lyophilized. In order to obtain comparable data,
212 100 mg of desiccated fungal extracts were suspended in 900 μl of water and boiled for 10 min
213 for each sample. Supernatants were analyzed by thin layer chromatography (TLC). One μl of
214 each sample was deposited on TLC-plates SIL G-25 (Macherey-Nagel, France) and separated
215 in acetonitril:ethylacetate:propanol:water (85:20:20:15, by vol). Sugars and polyols were
216 revealed using 0,5% KMnO_4 , 1N NaOH and identified by using standard sugars or polyols (1
217 μl of 0.5% standard solution).

218

219 **Preparation of cell extracts and enzyme assays**

220

221 Cell extracts were prepared as described in [5]. Lyophilized mycelia were ground,
222 resuspended in extraction buffer (50 mM phosphate buffer pH 7.0, 0.5 mM EDTA, 5mM β -
223 mercaptoethanol), then centrifuged for 30 min at 15000 rpm at 4°C. Supernatants were
224 collected and desalted on a Zeba Desalt Spin column (ThermoScientific, Cergy Pontoise,
225 France) before enzyme activity measurements. Enzyme assays were performed as described
226 in [22] using 50 μg of proteins in a final volume of 1 ml and by monitoring absorbance
227 changes of NAD(P)+/NAD(P)H at 340 nm. For MTDH activity, the reduction of fructose to
228 mannitol was assayed in a reaction mixture containing 0.6 M fructose, 0.2 mM NADPH, 10
229 mM HEPES pH 7.0. The oxidation of mannitol to fructose was conducted using 0.4 M
230 mannitol, 2mM NADP, 10 mM HEPES pH 9.0. For MPD activity, the reduction of fructose-
231 6-phosphate was assayed in a reaction mixture containing 5 mM fructose-6-phosphate, 0.3
232 mM NADH, 10 mM Hepes pH 7.0. For the reverse reaction, the oxidation of mannitol-1-
233 phosphate was measured using 5 mM mannitol-1-phosphate, 0.5 mM NAD, 10 mM HEPES
234 pH 9.0. Enzyme activity was expressed as nmoles of cofactor (NAD(P)H reduced or oxidized)
235 per minute and per μg of total cell extract protein.

236

237 **Western Blot analysis**

238

239 BcMPD and BcMTDH were produced as His-tag fusion proteins in *E.coli* M15 using the
240 QiaExpress System (Qiagen, Courtaboeuf, France). cDNAs were amplified by PCR with
241 forward primers containing *Nco*I restriction sites and reverse primers containing *Bgl*II sites
242 (Table S1). PCR products were cloned in pQE60. Optimal protein expression was achieved
243 using 2 mM IPTG after 4 h of induction at 37°C and 1 mM IPTG after 20 h of induction at
244 23°C, for BcMPD and BcMTDH respectively. BcMPD was detected in inclusion bodies while
245 BcMTDH remained in soluble fraction. Purification of tagged proteins was performed on Ni-
246 NTA columns, under denaturing and native conditions for BcMPD and BcMTDH
247 respectively, according to manufacturer's instructions. Anti-BcMPD and anti-MTDH sera
248 were obtained by immunization of rabbits with the purified proteins (Covalab, Villeurbanne,
249 France). Detection of BcMPD and BcMTDH in fungal strains was performed by Western blot

250 analyses. Total cell extract proteins were quantified in order to load 75 μ g of each extract in
251 each lane. Proteins were then separated by SDS-PAGE in a 10 % acrylamide gel and blotted
252 onto a nitrocellulose membrane. Nitrocellulose membranes were probed with rabbit
253 polyclonal BcMPD (1:2500) and BcMTDH (1:2500) antisera. Bands were visualized with
254 ECL (ElectroChemiluminescence) using HRP (horseradish peroxidase) conjugated with goat
255 anti-rabbit IgG (dilution 1:40000). The detection was performed as described in the
256 manufacturer's instructions for the ECL Western detection kit (Super Signal West Pico
257 Chemiluminescent Substrate, Perbio, France).

258

259

260

261

262 RESULTS AND DISCUSSION

263

264

265 Construction of *Bcmpd* and *Bcmtdh* mutant strains

266

267 To understand the role of mannitol in *B. cinerea* (Figure 1), we searched for mannitol
268 dehydrogenase and mannitol-1-phosphate dehydrogenase coding sequences, in order to
269 construct single and double deletion mutant strains. *Bcmtdh* and *Bcmpd* genes were identified
270 in the genome in sequence of *B. cinerea*
271 (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/). Low stringency Southern
272 blot indicated that only one copy of each gene was present in the *B. cinerea* genome (data not
273 shown). Each sequence (respectively 1314 bp and 862 bp for *Bcmpd* and *Bcmtdh*) had one
274 intron. Sequence analyses revealed that BcMPD and BcMTDH had the features of mannitol-
275 1-phosphate 5-dehydrogenases and mannitol 2-dehydrogenases and coded respectively 43 and
276 28 kDa isoforms. BcMDTH shares 71% and 78% identity with *S. nodorum* Mdh1 and
277 *A. alternata* MtDH, respectively. BcMPD shares 62% and 59% identity with *S. nodorum*
278 Mpd1 and *A. alternata* MpdH, respectively. Whereas BcMPD belongs to the long-chain
279 dehydrogenases/reductases superfamily (LDR), BcMTDH falls into the short-chain group of
280 dehydrogenase/reductase superfamily [23-24].

281

282 In the *Bcmpd* deletion construct, a region including 567 bp of the promoter, the open
283 reading frame (ORF) and 328 bp of the 3' of the gene was replaced by a hygromycin
284 resistance cassette. In the case of the *Bcmtdh* deletion vector, 60 bp of the promoter and
285 423 bp of the coding sequence were replaced by the hygromycin resistance cassette (Figure
286 S1A, B). Protoplasts of *B. cinerea* B05.10 strain were transformed with the linearized vectors
287 and hygromycin-resistant transformants were tested by PCR and Southern analyses (data not
288 shown, Figure S1D). In both cases, two transformants, $\Delta Bcmpd19$ and $\Delta Bcmpd24$, for *Bcmpd*,
289 and $\Delta Bcmtdh11$ and $\Delta Bcmtdh16$, for *Bcmtdh*, were found to have undergone gene
290 replacement as expected (Figure S1D). Gene deletions were further confirmed through
291 transcriptional analyses and western blot that revealed an absence of transcripts and proteins
292 in the mutants as compared to the wild type strain (Figure 2A, B). Interestingly, as compared
293 to the wild type strain, *Bcmtdh* expression was clearly increased in *Bcmpd* mutant, whereas
294 *Bcmpd* transcription level was not affected by *Bcmtdh* deletion. Western blot experiments
295 showed that BcMPD and BcMTDH proteins seemed to be more abundant in both deletion
296 mutants (Figure 2B). Enzymatic assays (Figure 2C) confirmed that *Bcmpd* deletion abolished
297 mannitol-1-phosphate dehydrogenase activity. However, in the *Bcmtdh* mutant, mannitol
298 dehydrogenase activity was strongly reduced but not completely eliminated (Figure 2C).
299 Indeed, a low mannitol dehydrogenase activity remained in *Bcmtdh* mutant. All experiments
presented in this work were conducted on *Bcmpd19* and *Bcmpd24* for BcMPD pathway and

300 on *Bcmtdh11* and *Bcmtdh16* for BcMTDH. In both cases, results were clearly similar, in all
301 experiments, for the two mutants analyzed. Consequently, in order to improve reading of the
302 manuscript, results presented here concern *Bcmpd19*, *Bcmtdh16* deletion mutant strains, only.
303

304 **Conversion of hexoses by BcMPD and BcMTDH pathways**

305
306 To investigate the respective role of BcMPD and BcMTDH branches in mannitol metabolism,
307 hexose assimilation and conversion through the mannitol pathway were performed in a plant
308 infection context, by *in vivo* NMR spectroscopy. Transfer rates of $^{13}\text{C}_1$ -glucose or $^{13}\text{C}_2$ -
309 fructose by $\Delta Bcmpd$ or $\Delta Bcmtdh$ strains were followed in real time using sunflower
310 cotyledons infected by *B. cinerea* (Table 1) and compared to the data previously obtained
311 with the wild type strain [15]. $^{13}\text{C}_1$ -glucose was converted by $\Delta Bcmpd$ and $\Delta Bcmtdh$ mutant
312 strains into $^{13}\text{C}_1$ -trehalose, glycogen and $^{13}\text{C}_{1/6}$ -mannitol as previously shown [15]. Whereas
313 transfer of $^{13}\text{C}_1$ from glucose to mannitol was strongly impaired in $\Delta Bcmpd$, amounts of
314 labelled trehalose and glycogen were increased. In $\Delta Bcmtdh$, labelling transfer rate of $^{13}\text{C}_1$
315 from glucose into $^{13}\text{C}_{1/6}$ mannitol was similar to the WT (wild type) strain, while the amounts
316 of labelled trehalose and glycogen were seriously lowered. $^{13}\text{C}_2$ -fructose was converted by all
317 the strains in a unique compound, mannitol. However, $\Delta Bcmtdh$ transferred $^{13}\text{C}_2$ from fructose
318 to mannitol with a lower efficiency, as compared to the WT strain (30% decrease). *In vivo*
319 NMR data showed trehalose (and glycogen) labelling during growth in the presence of
320 glucose but not in the presence of fructose. Our data clearly demonstrate that trehalose
321 accumulation is a consequence of MPD pathway deletion, preferentially used during glucose
322 assimilation by *B. cinerea*. Trehalose (and glycogen) synthesis is directly linked to glucose-6-
323 phosphate (Figure 1). Mannitol biosynthesis is also connected to this metabolite via fructose-
324 6-phosphate, used as substrate by BcMPD. Consequently, mannitol synthesis from glucose by
325 BcMPD pathway could deplete the pool of glucose-6-phosphate. *Bcmpd* mutants, affected in
326 mannitol biosynthesis, could produce trehalose and glycogen to prevent glucose-6-phosphate
327 accumulation.
328

329 **Mannitol is mobilized during *in vitro* development of *B. cinerea***

330
331
332 Metabolic profiling revealed that mannitol is found in large amounts in developing *B. cinerea*
333 mycelium [15]. To try to determine the role of mannitol in *B. cinerea* development, we
334 analyzed sporulation and germination rate. No obvious differences were observed between the
335 WT and single mutant strains (Figure S2). This lack of effect of mannitol gene deletion on
336 sporulation and/or spore germination, prompted us to analyze sugar and polyol content by
337 TLC (thin layer chromatography), after growth in the presence of glucose (Figure 3). For this
338 purpose, fungal extracts were analyzed during distinctive phases of development: growing
339 mycelium (2-day-old cultures), conidiation (6-day-old cultures), mature conidia (collected
340 from 12-day-old cultures) and germination of conidia (performed for 2, 4 and 6 hours).

341 Mannitol, and to a lesser extent trehalose, were accumulated during sporulation and in
342 mature conidia in WT strain. These compounds, rapidly degraded during germination, were
343 almost undetectable 2 hours after germination initiation (Figure 3A). While $\Delta Bcmtdh$
344 exhibited a similar profile to WT, sugar profile revealed by TLC was modified in $\Delta Bcmpd$.
345 Mannitol content was markedly reduced in mycelium, whereas trehalose store drastically
346 increased in mature conidia and mycelium (2-d and 6-d old). Surprisingly, TLC profiles
347 revealed an almost unaltered mannitol content in mutant strain conidia.

348 *Bcmpd* and *Bcmtdh* gene expression was also checked during development phases, in
349 the WT strain. The results presented in Figure 3B show that both genes exhibited similar

350 expression patterns during *in vitro* development of WT strain. Despite the dissimilar
351 expression levels, their transcription was particularly high during spore genesis (6-day-old
352 cultures) and in mature conidia. In contrast, during germination, transcript and protein levels
353 drastically decreased (Figure 3B, C). It should be noticed that BcMTDH detection was higher,
354 particularly two hours after germination initiation. This could be explained by a major role
355 played by this protein in mannitol degradation during germination. Nevertheless, both
356 mannitol metabolism branches could participate in mannitol accumulation in conidiating
357 mycelium and spores.

358 Given its high abundance in spores, the idea of mannitol acting as carbohydrate
359 reserve, as suggested in several reports, can be considered [25, 26]. Data on sporulation and
360 germination rate in fungal pathogens affected in mannitol metabolism by gene disruption
361 strategies are controversial. In *S. nodorum*, *mpd* and *mpd/mtdh* mutants, the production of
362 conidia was strongly affected [4, 13], whereas in *A. alternata*, *mpd/mtdh* mutant is affected
363 only [10]. As *S. nodorum* and *A. alternata*, *B. cinerea* accumulates mannitol in sporulating
364 mycelium [4, 10] but, despite a lower mannitol content, mutants were not affected for *in vitro*
365 or *in planta* conidiation (Figure S2). A reduced mannitol concentration could allow
366 sporulation. Moreover, trehalose could be stored or degraded instead of mannitol.

367 368 **Mannitol is involved in osmotic stress response in *B. cinerea***

369
370 To further dissect the mannitol pathway, it was necessary to identify physiological conditions
371 that could induce synthesis or degradation of this compound in *B. cinerea*. An osmotic shock
372 is known to induce remobilization of intracellular carbon pool in fungi [27]. Most reports
373 have so far excluded a role for mannitol in enabling fungi to cope with osmotic stress.
374 *Sclerotinia sclerotiorum*, *Saccharomyces cerevisiae* and *A. nidulans* store glycerol in response
375 to a hyperosmotic stress [28, 29, 30], whereas *Magnaporthe grisea*, *Cladosporium fulvum* and
376 *S. nodorum* accumulate arabitol [31, 32, 33]. In *B. cinerea*, osmotic stress response is
377 controlled through glycerol accumulation [34]. But mannitol, directly connected to central
378 carbon metabolism could also be implicated in osmotic stress response. To address this
379 question, we investigated the polyol content of *B. cinerea* WT, *Bcmpd* and *Bcmtdh* mutant
380 strains during growth under hyperosmotic conditions (Figure 4A).

381 In WT strain, mannitol was transiently degraded during stress response. Its
382 concentration decreased between 1 and 4 h of stress and then increased from 9 to 24 h after
383 transfer. In parallel, glycerol accumulated as mannitol was degraded (Figure 4A). Mannitol
384 degradation during osmotic stress response could suggest its contribution to osmo-adaptation.
385 Analysis of *Bcmpd* and *Bcmtdh* gene expression by Q-PCR, revealed that *Bcmpd* was
386 transiently downregulated, whereas *Bcmtdh* transcription increased as mannitol pool
387 decreased (Figure 4B). After one hour of stress, *Bcmtdh* gene expression reached its maximal
388 level. A 4.5 fold expression increase was detected. BcMPD and BcMTDH protein profiles
389 were analyzed by Western blot (Figure 4A). During osmotic stress, BcMPD was degraded
390 from 0 to 1 h whereas BcMTDH was accumulated. Hence, BcMTDH could be preferentially
391 dedicated to mannitol degradation during osmotic stress response.

392 In $\Delta Bcmtdh$ mutant, TLC profile was similar to that of the WT strain (Figure 4A).
393 $\Delta Bcmpd$ showed a constant and low mannitol content, whereas trehalose, produced by this
394 mutant strain was degraded concomitantly to glycerol accumulation. While in the WT and in
395 the *Bcmtdh* mutant, mannitol pool was partially restored from 9 to 24 h of stress, trehalose
396 pool was not restored in $\Delta Bcmpd$ mutant. Expression profiles of *Bcmpd* or *Bcmtdh* genes in
397 WT and both mutant strains were similar (Figure 4B). However, western blot experiments
398 showed that, in $\Delta Bcmtdh$ strain, BcMPD was not degraded during osmotic stress (from 0 to
399 4 h). Therefore, while transcriptional control of *Bcmpd* gene was the same, post-translational

control differed in WT and $\Delta Bcmtdh$ mutant. Mannitol content decreased during osmotic stress response in $\Delta Bcmtdh$. This could be due to the low MTDH activity still detected in $\Delta Bcmtdh$ cell extracts. On the other hand, BcMPD could participate in mannitol degradation. Consequently, this could suggest, contrary to the proposed cycle [12], that the mannitol-1-phosphate dephosphorylation reaction could be reversible to allow mannitol degradation through MPD pathway (Figure 1).

Our data showed that mannitol degradation in WT and *Bcmtdh* strains paralleled glycerol accumulation during osmotic stress response, suggesting that mannitol could constitute a carbon store in which *B. cinerea* could draw, in order to quickly synthesize glycerol for osmoprotection. For this purpose, mannitol pool of *B. cinerea* wild type strain was labelled using $^{13}\text{C}_2$ -fructose (fructose is almost solely converted in mannitol in WT strain). An osmotic shock (1 M NaCl), in the presence of unlabelled glucose, was applied to mycelium and allocation of labelled carbon during osmotic response was then followed by *in vitro* NMR spectroscopy (Figure S3). Before osmotic stress, labelled carbon was found exclusively in mannitol molecules (C_2 and C_5). Between 1 and 4h of stress, mannitol was degraded and 50% of labelled carbon originating from mannitol was allocated for glycerol synthesis and detected in $^{13}\text{C}_2$ glycerol. This result demonstrates that mannitol is not directly implicated in osmotic stress response but could contribute indirectly to osmoprotection via carbon relocation to glycerol molecules. This direct connection between mannitol and glycerol for stress answer underlines the central role of mannitol in *B. cinerea* carbon metabolism. In WT and single mutants, mannitol and trehalose disappearance correlated to glycerol accumulation (Figure 4). In order to participate to this transient response, trehalose could also be mobilized into glycerol.

***Bcmpd* deletion mutant reveals a mannitol phosphorylation pathway**

Radial growth experiments revealed that *Bcmtdh* mutants were able to grow as well as the WT strain on mannitol, used as sole carbon source (Figure S4). Moreover, mannitol degradation occurred in $\Delta Bcmtdh$ during osmotic stress response and during germination of spores. This degradation could be performed by the remaining MTDH activity detected in $\Delta Bcmtdh$ or reveal that another pathway to degrade mannitol, via mannitol-1-phosphate probably, may exist. To confirm this hypothesis, WT, $\Delta Bcmpd$ and $\Delta Bcmtdh$ were grown for two days in the presence of 2% mannitol. Metabolic spectra of mycelial PCA extracts were then analyzed by *in vitro* ^{31}P -NMR spectroscopy. Mannitol-1-phosphate peaks were detected at 4.77 ppm for each strain. ^{31}P spectra of $\Delta Bcmpd$ are presented in Figure 5. Mannitol-1-phosphate was detected after growth on mannitol, only. MPD is the only known enzyme in fungi able to produce mannitol-1-phosphate from fructose-6-phosphate [1]. Consequently, biosynthesis of mannitol-1-phosphate by $\Delta Bcmpd$ requires a pathway which could phosphorylate mannitol into mannitol-1-phosphate. Moreover, *Bcmpd* and *Bcmtdh* gene expression analysis, performed after growth of WT strain on synthetic medium containing 2% mannitol as sole carbon source confirmed this hypothesis. In this case, *Bcmpd* expression level was 35 fold higher than *Bcmtdh* (Figure S5). This suggests that mannitol catabolism could occur through phosphorylation of mannitol and subsequent conversion in fructose-6-phosphate via MPD pathway.

Mannitol metabolism in fungi was proposed to occur as a cycle [12]. In this cycle, mannitol is synthesized through MPD pathway and degraded via MTDH pathway. However, several recent reports do not support the metabolism of mannitol operating as a cycle [4, 14]. The ability of the $\Delta Bcmpd$ strains to use mannitol as sole carbon source clearly indicates that mannitol can be metabolized through other routes, most probably through mannitol-1-

450 phosphate. The remaining objection was the absence of mannitol kinase activity in fungi [2,
451 25]. In the present work, detection of mannitol-1-phosphate when $\Delta Bcmpd$ mutants were
452 grown on mannitol reveals for the first time the occurrence of a mannitol phosphorylation
453 pathway in fungi. As a matter of fact, this phosphorylation of mannitol seems specific to fungi
454 (at least to *B. cinerea*), since it has not been detected in any vascular plant cells or tissues that
455 were preincubated in the presence of mannitol (Bligny, unpublished data). Accumulation of
456 mannitol-1-phosphate could result from a one step reaction involving a mannitol kinase and
457 ATP, or from a series of several enzymatic reactions that remain to be discovered.

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461 **Deletion of BcMPD and BcMTDH pathways did not abolish mannitol metabolism in** 462 ***B. cinerea* and reveal a new metabolic route**

463

464 Development and pathogenicity were not affected in $\Delta Bcmpd$ and $\Delta Bcmtdh$ mutant strains
465 (Figure S2). *In vitro* and *in planta* conidiation and *in vitro* spore germination tests showed that
466 mutant strains were not affected in those developmental steps. In that case, deletion of whole
467 pathway by creating a *Bcmpd/Bcmtdh* double mutant was indispensable to assign a
468 physiological role to mannitol.

469 To construct such a double deletion strain, *Bcmpd* mutant strain was transformed with
470 a *Bcmtdh* disruption cassette containing a nourseothricin resistance marker (Figure S1A, B,
471 C). To check deletion and homologous recombination, nourseothricin-resistant strains were
472 screened by PCR (data not shown) and Southern blot (Figure S1D). Two strains, *db32.3* and
473 *db35.2* were selected as double gene replacement mutants. Transcriptional analyses and
474 western blotting confirmed deletion of both genes in the double mutants (Figure 2A, B).
475 However, as found in the $\Delta Bcmtdh$ single mutant, mannitol dehydrogenase activity was still
476 detected in the double mutant strains, with a reduction of 70%, as compared to the wild type
477 strain (Figure 2C).

478 Radial growth experiments revealed that the double mutants were able to grow as well
479 as the WT strain on mannitol as sole carbon source (Figure S4). Furthermore, germination and
480 sporulation and infection process were not altered in $\Delta Bcmpd\Delta Bcmtdh$ as compared to the
481 WT strain (Figure S2). TLC profiles of intracellular sugars and polyols performed during
482 development of $\Delta Bcmpd\Delta Bcmtdh$ revealed that mannitol was still produced in double mutants
483 (Figure 6). While mannitol store was severely lowered in mycelium it was not affected in
484 conidia (Figure 6). Quantification of intracellular metabolites by *in vitro* NMR spectroscopy
485 in 2-day-old $\Delta Bcmpd\Delta Bcmtdh$ mycelium revealed that mannitol was synthesized from glucose
486 and fructose (Table 2). As compared to the WT strain, mannitol content in $\Delta Bcmpd\Delta Bcmtdh$
487 mycelium after growth on glucose or fructose was decreased by 70% and 31%, respectively.
488 The low mannitol dehydrogenase activity still detected in double mutant strains could explain
489 why these mutants are still able to produce mannitol, principally from fructose. Moreover, this
490 compound was still degraded during double mutant spore germination (Figure 6). This may
491 suggest that the remaining mannitol dehydrogenase pathway could compensate mannitol
492 production in double mutant conidia rather than in mycelium and be implicated in mannitol
493 degradation during germination. These last findings strongly suggest that mannitol could be
494 synthesized and degraded through potentially other metabolic routes.

495 Our data are in agreement with those reported in the case of *A. alternata* *mtdh/mpd*
496 double mutant that was still able to grow on mannitol as sole carbon source [14]. In that case,
497 the authors suggested that the fungus contain other enzymes that allow utilization of mannitol
498 as a substrate. The ability of *B. cinerea* double mutant to metabolize mannitol, together with
499 the presence of a mannitol dehydrogenase activity, suggest that additional unrelated mannitol

500 dehydrogenase encoding gene(s) should be present in *B. cinerea* genome. Recently, a new
501 mannitol dehydrogenase showing no similarity with any known fungal MTDH has been
502 described in *Tuber borchii* [35]. The phylogenetic analysis showed TbMDH to be the first
503 example of a fungal mannitol dehydrogenase belonging to the medium-chain
504 dehydrogenases/reductases (MDRs). Consequently, the *T. borchii* enzyme identified a new
505 group of proteins forming a distinct subfamily of polyol dehydrogenase [35]. A BlastP search
506 (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/Home.html) for possible
507 homologies with *T. borchii* *Tbmdh* mannitol dehydrogenase [35] was thus performed.
508 Surprisingly, our BlastP results clearly pointed out a *B. cinerea* sequence (BC1G_15343),
509 annotated as an alcohol dehydrogenase, sharing 84% identity (89% positive) with TbMDH,
510 while it shared only 11% identity with BcMTDH. In *T. borchii*, TbMDH produces mannitol
511 from fructose. Such an enzyme, with a high preference for fructose and mannitol is a good
512 candidate to perform mannitol synthesis from fructose and degrade mannitol during
513 development in double mutant *B. cinerea* strains. However, further experiments are necessary
514 to clearly implicate this new gene in *B. cinerea* mannitol metabolism.

515 In conclusion, a physiological role has been assigned to MTDH pathway that could be
516 dedicated to a favourable conversion of fructose to mannitol and to mannitol degradation
517 during osmotic stress response. On the other hand, we have shown that MPD pathway could
518 also be implicated in mannitol catabolism, which abrogates the existence of a mannitol cycle.
519 And finally, analyses of double mutant revealed the existence of a new mannitol pathway
520 which could parallel mannitol dehydrogenase pathway functions. During plant infection,
521 mannitol metabolism could help pathogen to efficiently sequester plant hexoses. Gene
522 expression studies revealed a regulated developmental control for mannitol pathway.
523 Interconnections of mannitol metabolism with the central carbohydrate pathway suggested
524 that it could regulate carbon metabolic fluxes. Mannitol constitutes then an easily mobilisable
525 carbon store, for growth and dissemination but also to cope with stresses and to maintain
526 hyphal turgor pressure.

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Tables :

Allocation of labelled carbon	Labelling transfer rate $\mu\text{moles g}^{-1} \text{FW h}^{-1}$					
	3mM Glucose			3mM Fructose		
	WT	$\Delta Bcmpd$	$\Delta Bcmtdh$	WT	$\Delta Bcmpd$	$\Delta Bcmtdh$
Glycogen	0.9	1.4	0.3	n.d.	n.d.	n.d.
Mannitol C1/6	0.55	0.09	0.5	n.d.	n.d.	n.d.
Mannitol C2/5	n.d.	n.d.	n.d.	1.4	1.37	1
Trehalose C6	0.39	0.73	0.08	n.d.	n.d.	n.d.

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Table 1 Transfer rates of labelled hexoses through carbon mannitol metabolism in *B. cinerea* WT strain and single mutants.

Transfer rates were obtained from proton-decoupled ^{13}C -NMR *in vivo* spectra of sunflower cotyledons infected by *B. cinerea* (48 hpi). At time zero, 3 mM $[1-^{13}\text{C}]$ glucose or 3 mM $[2-^{13}\text{C}]$ fructose were added to perfusion medium. Sugar conversion was analysed during 3.5 h. Transfer rates were determined at the end of the analysis period. Spectral conditions were as given in experimental procedures. Two independent experiments were done from separate infection series and cultures. A representative result is presented. n.d., not detected.

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	2% Glucose				2% Fructose			
	WT	$\Delta Bcmpd$	$\Delta Bcmtdh$	$\frac{\Delta Bcmtdh}{\Delta Bcmpd}$	WT	$\Delta Bcmpd$	$\Delta Bcmtdh$	$\frac{\Delta Bcmtdh}{\Delta Bcmpd}$
Glucose	0.8	0.7	0.9	1.0	n.d.	n.d.	n.d.	n.d.
Fructose	n.d.	n.d.	n.d.	n.d.	1.0	0.8	1.4	0.4
Mannitol	10.9	1.3	4.9	2.8	18.2	10.2	5.8	11.0
Tréhalose	1.0	6.6	n.d.	6.4	0.3	n.d.	n.d.	n.d.
Arabitol	0.3	0.6	0.7	0.5	1.0	1.5	1.3	1.3
Glycérol	7.7	6.7	3.7	5.9	4.1	1.2	0.5	0.8

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Table 2 Impact of carbon sources on polyol and sugar content of WT, $\Delta Bcmpd$, $\Delta Bcmtdh$, $\Delta Bcmpd\Delta Bcmtdh$

$\Delta Bcmpd\Delta Bcmtdh$ strains were grown 2-d on 2% glucose or 2% fructose. Sugars and polyols were extracted and analysed by NMR in vitro spectroscopy, as described in experimental procedures. Two independent experiments were done from separate cultures. A representative result is presented. n.d., not detected

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Figure Legends

Figure 1 Model showing mannitol metabolism and main carbohydrate conversion pathways in fungi

Dotted arrows indicate series of enzymatic reactions.

Figure 2 Effect of mannitol pathway alteration on *Bcmpd* and *Bcmtdh* transcript levels, proteins and enzymatic activities

Mycelia were cultivated 2-d, on synthetic medium supplemented with 2% glucose. (A) *Bcmpd* and *Bcmtdh* gene expression. *Bcmpd* and *Bcmtdh* gene expression was measured by quantitative PCR using gene-specific primers and calibrated to *BcactA* transcripts. Amplifications were done in triplicate, from 3 independent biological replicates. Bars represent SD. (B) BcMPD and BcMTDH detection by western blot analysis. Gels were loaded with 75 µg of proteins in each lane. Western blots were repeated twice, representative results are presented here. (C) BcMPD and BcMTDH enzymatic activities. Total mannitol-1-phosphate dehydrogenase and mannitol dehydrogenase activities were measured from 50 µg of proteins by monitoring absorbance changes of NAD(P)⁺/NAD(P)H at 340 nm. Analyses were performed in duplicate from two independent biological replicates. A representative result is presented here.

Figure 3 Sugars content and *Bcmpd* and *Bcmtdh* transcript levels during *in vitro* development

(A) Sugars and polyols were extracted from 12-d-old fresh spores (conidia), germinating conidia (2, 4, and 6 h after activation of spore germination in rich liquid medium), young mycelium (2-d-old) and sporulating mycelium (6-d-old), after growth of WT strain, $\Delta Bcmpd$ and $\Delta Bcmtdh$ in the presence of glucose. Fungal extracts were then analysed by TLC as described in experimental procedures. Sugars and polyols were extracted from at least three independent biological replicates. A representative chromatography is presented here. (B) Total RNA was extracted from WT strain. *Bcmpd* and *Bcmtdh* gene expression was measured by quantitative PCR using gene-specific primers and calibrated to *BcactA* transcripts. RNA was extracted from at least three independent biological replicates. Bars represent SD. (C) Detection of BcMPD and BcMTDH in WT conidia and germinating conidia by Western blot analysis. Gels were loaded with 75 µg of proteins in each lane. Proteins were extracted from at least three independent biological replicates. A representative western blot is presented here.

Figure 4 Intracellular sugar content and *Bcmpd* and *Bcmtdh* transcript levels in WT, $\Delta Bcmpd$, and $\Delta Bcmtdh$ during an osmotic stress response

Sugar, polyols and total RNA were extracted from young mycelium cultivated for 2 d on synthetic medium supplemented with 2% glucose and then transferred 0, 0.5, 1, 4, 6, 9 or 24 h on the same medium supplemented with 1M NaCl. (A) Analysis of mannitol, trehalose and glycerol content in WT and mannitol mutant strains was performed by TLC experiments. Culture transfers and TLC experiments were repeated three times from independent biological replicates. Representative chromatographies are presented here. (B) *Bcmpd* and *Bcmtdh* gene expression was quantified, in WT and mutant strains, by PCR using gene-specific primers and

728 was calibrated to *BcactA* transcripts. Amplifications were done in triplicate from 3
729 independent biological replicates. Bars represent SD. (C) Western blot detection of BcMPD
730 and BcMTDH in WT, $\Delta Bcmpd$ and $\Delta Bcmtdh$ strains. Gels were loaded with 75 μ g of proteins
731 in each lane. Proteins were extracted from at least three independent biological replicates.
732 Representative results are presented here.

733

734 **Figure 5** *Bcmpd* deletion mutant reveals a mannitol phosphorylation pathway in
735 *B. cinerea*

736 Proton-decoupled ^{31}P -NMR spectra of PCA extracts of $\Delta Bcmpd$ mycelium during growth in
737 the presence of mannitol as sole carbon source. Extracts were prepared from 5 to 10 g of
738 mycelium grown on 2% glucose (A) or on 2% mannitol (B). Peaks assignments are as
739 follows: Mnt-1-P, mannitol-1-phosphate; Glcn-6-P, 6-phosphogluconate; α/β -Glc-6-P, α/β -
740 glucose-6-phosphate; Tre-6-P, trehalose-6-phosphate; Gly-3-P, glycerol-3-phosphate; PGA,
741 Phosphoglyceric acid; n.d., not determined. This experiment was done twice. Representative
742 spectra are presented here.

743

744 **Figure 6** Sugar and polyol content during *in vitro* development of $\Delta Bcmpd\Delta Bcmtdh$

745 Sugars and polyols were extracted from 2-day-old double mutant mycelium after growth in
746 the presence of 2% glucose and analysed by TLC as described in experimental procedures.
747 This experiment was done in triplicate using independent biological replicates. A
748 representative chromatograph is presented here.

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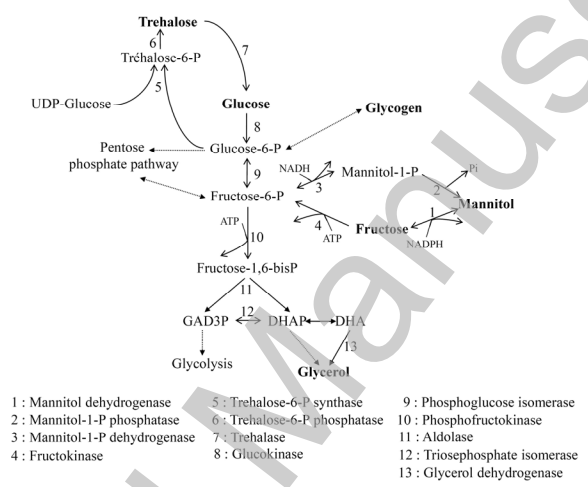


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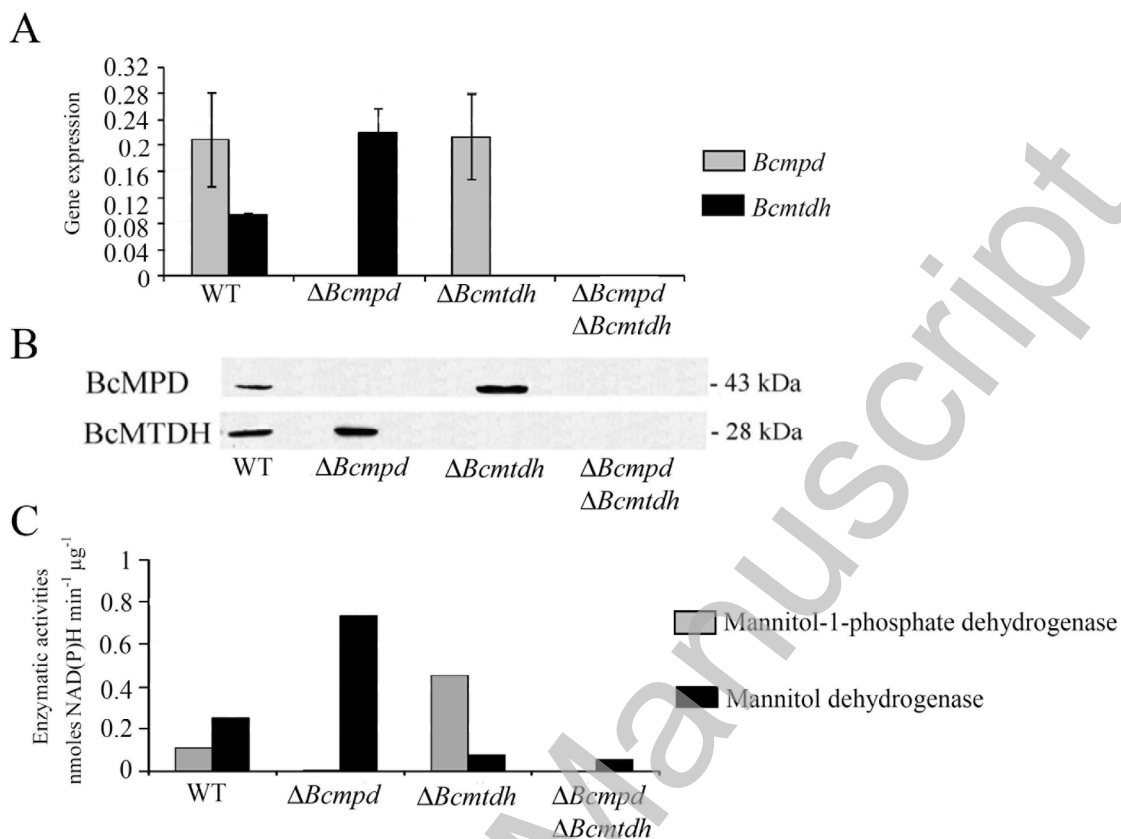


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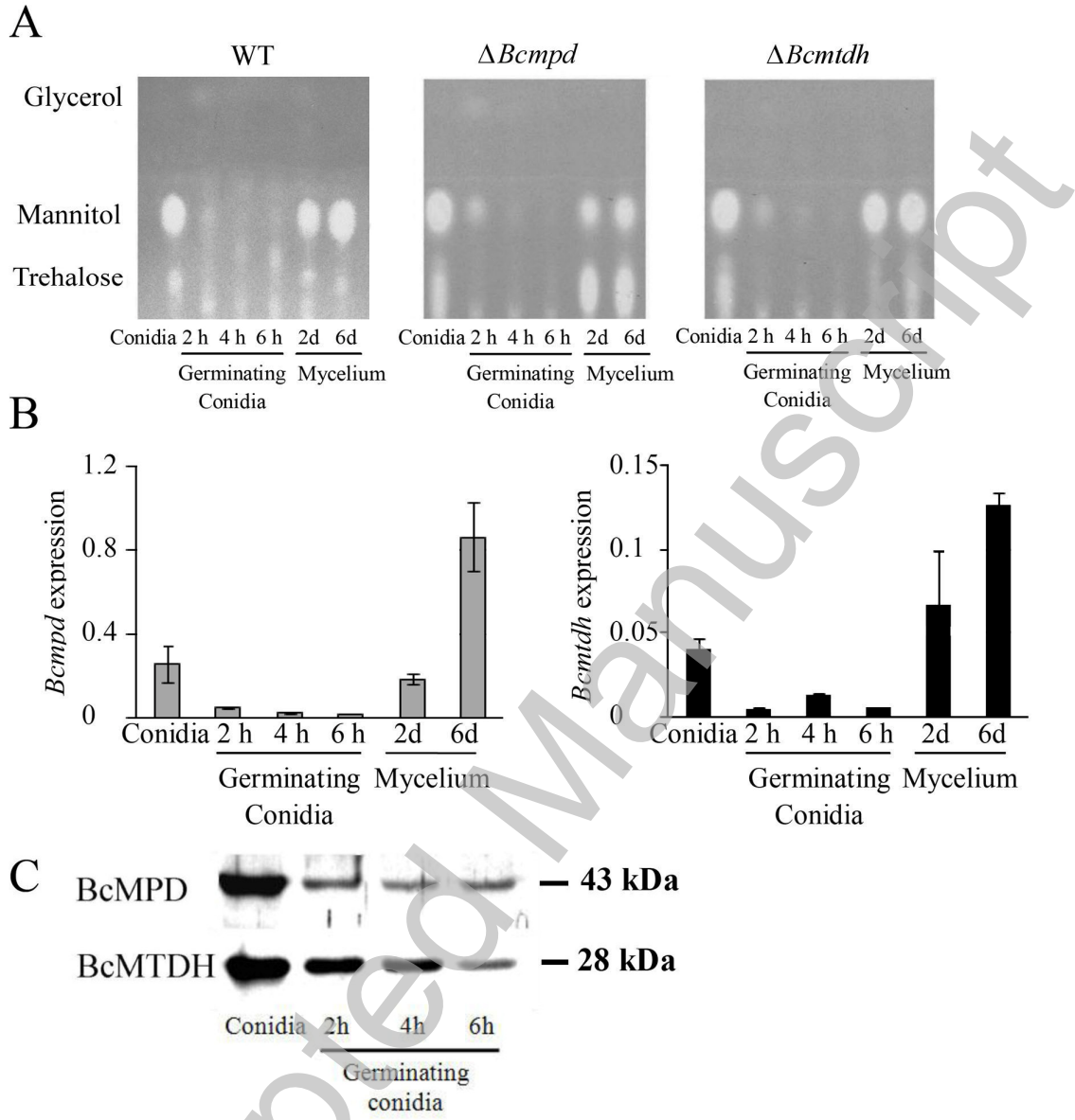


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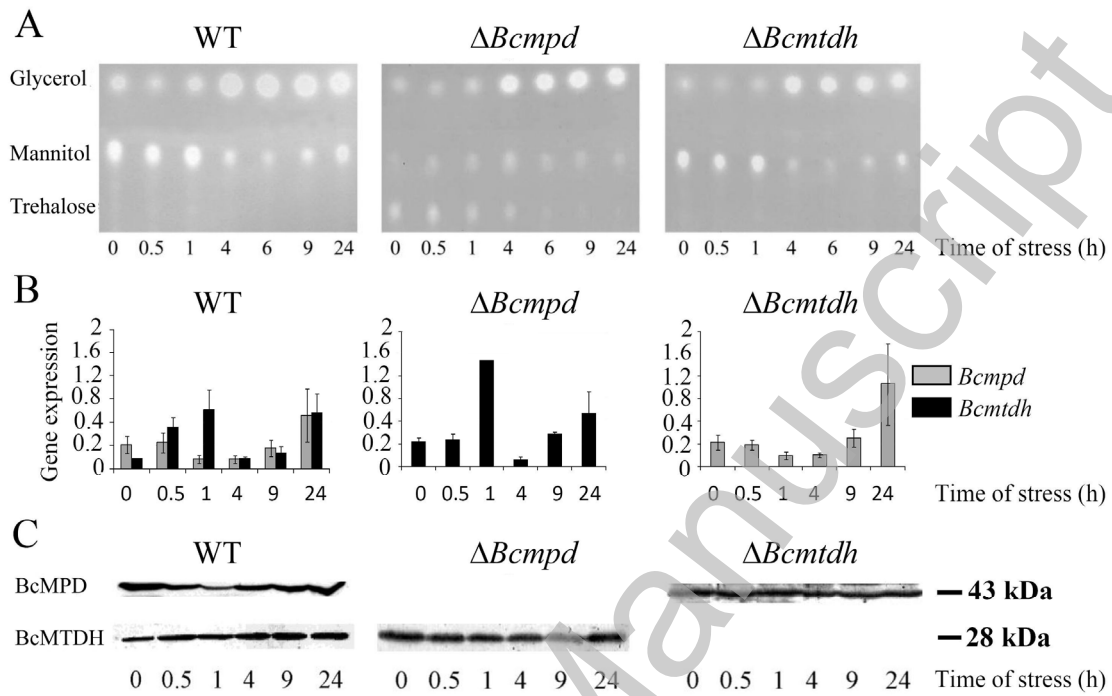


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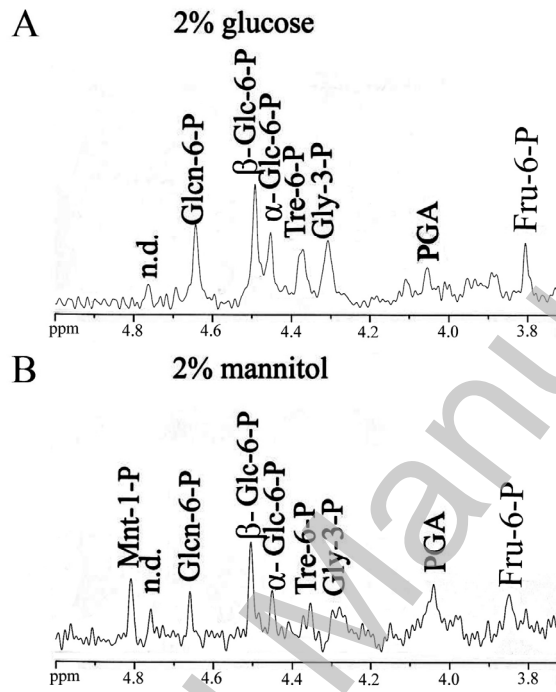


Figure5.tif

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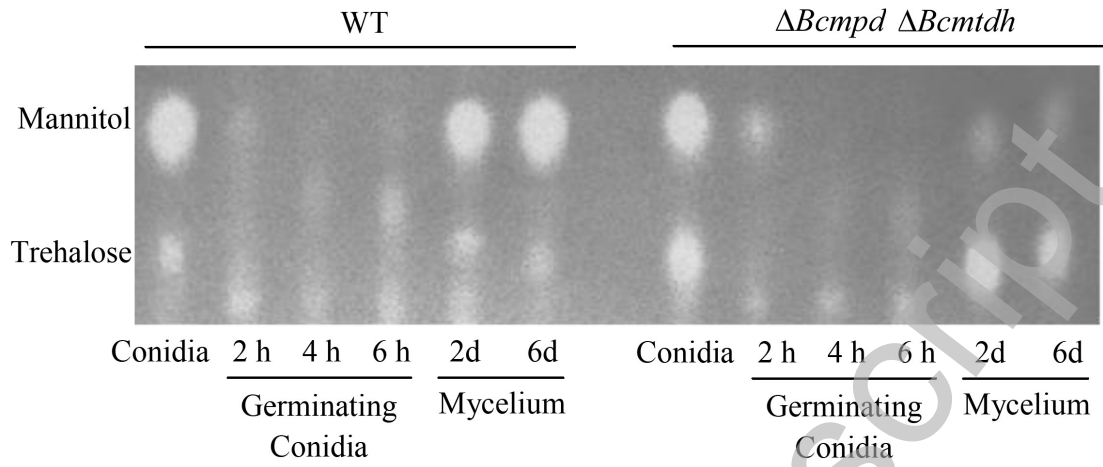


Figure6.tif

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