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Identification and characterization of parasitism genes from the pinewood nematode *Bursaphelenchus xylophilus* reveals a multi-layered detoxification strategy

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1 **Identification and characterization of parasitism genes from the pinewood**
2 **nematode *Bursaphelenchus xylophilus* reveals a multi-layered detoxification**
3 **strategy.**

4

5 **Running title: Effectors of *B. xylophilus*.**

6

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9

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20

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22 metabolism, gland cells.

23

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25 **Summary**

26 The migratory endoparasitic nematode, *Bursaphelenchus xylophilus*, which is the
27 causal agent of pine wilt disease, has phytophagous and mycetophagous phases
28 during its life cycle. This highly unusual feature distinguishes it from other plant-
29 parasitic nematodes and requires profound changes in biology between modes. During
30 the phytophagous stage the nematode migrates within pine trees, feeding on the
31 contents of parenchymal cells. Like other plant pathogens, *B. xylophilus* secretes
32 effectors from pharyngeal gland cells into the host during infection. We provide the
33 first description of changes in the morphology of these gland cells between juvenile
34 and adult life stages. Using a comparative transcriptomic approach and an effector
35 identification pipeline we identify numerous novel parasitism genes which may be
36 important for mediating interactions of *B. xylophilus* with its host. In-depth
37 characterisation of all parasitism genes using *in situ* hybridisation reveals two major
38 categories of detoxification proteins, those specifically expressed in either the
39 pharyngeal gland cells or the digestive system. These data suggest that *B. xylophilus*
40 incorporates effectors in a multilayer detoxification strategy in order to protect itself
41 from host defence responses during phytophagy.

42

43 **Introduction**

44 The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is a migratory plant
45 endoparasitic nematode and is the causal agent of Pine Wilt Disease (PWD). The
46 PWD complex includes the pathogenic agent, its insect vector (cerambycid beetles of
47 the genus *Monochamus*) and the host, which can be one of several different *Pinus*
48 species. *Bursaphelenchus xylophilus* is native to North America and causes little
49 damage to indigenous tree species. However, it was introduced into China and Japan

50 at the start of the 20th Century and here it has caused significant damage under the
51 appropriate environmental conditions (Jones *et al.*, 2013). The nematode was found
52 in Europe for the first time in 1999 (Mota *et al.*, 1999) and has now been detected in
53 mainland Portugal, Madeira Island and Spain (Mota *et al.*, 1999; Robertson *et al.*,
54 2011; Fonseca *et al.*, 2012). Pine wood represents a major proportion of the forestry
55 industry and the rapid spread of this disease has become a major problem with the
56 potential to cause significant economic losses and damage to forests on an ecological
57 scale (Mota and Vieira, 2008; Vicente *et al.*, 2012a).

58

59 The PWN has two different life cycle stages – a phytophagous parasitic stage and a
60 mycetophagous stage. This highly unusual feature distinguishes it from other plant
61 parasitic nematodes (PPN) and enables it to reproduce and survive in the host at the
62 later stages of PWD when healthy plant tissues may be absent but fungi are abundant
63 (Vicente *et al.*, 2012a; Jones *et al.*, 2013). Like many other nematode species, *B.*
64 *xylophilus* has four juvenile stages prior to the mature adult and all life stages are
65 vermiform. Nematodes can feed on fungi in dead or dying trees and as nematode
66 numbers increase, and food becomes scarce, a survival and dispersal stage develops
67 (the *dauer* juvenile) that migrates to beetle pupal chambers. When the adult insect
68 emerges, the *dauer* stage of the nematode enters the tracheid and is transported to a
69 new host. The nematode may be transported to a dead or dying tree colonised with
70 fungi, in which case the mycetophagous cycle described above begins again.
71 Alternatively, the nematode can infect healthy host trees through maturation feeding
72 wounds made by the insect. Once inside the pine cortex the nematode migrates to the
73 xylem resin and ray canals and feeds on parenchyma cells leading to cell death
74 (Mamiya, 2012). The tree releases polyphenolic compounds (causing browning of

75 the tissues during infection), terpenoids, reactive oxygen species (ROS) and lipid
76 peroxides during the early stages of infection as part of a strong defence response
77 (Fukuda, 1997). Nematode numbers increase and water transport through the infected
78 tree is compromised leading to wilt and, consequently, to death of the tree (Jones *et*
79 *al.*, 2008; Futai, 2013).

80

81 Although a genome sequence has been reported for *B. xylophilus* (Kikuchi *et al.*,
82 2011) the details of the mechanisms underlying the interaction between this nematode
83 and its host remain unclear. Although peptides and plant hormones have been
84 suggested to play important roles in the interactions between plants and nematodes,
85 some of the most important nematode-derived factors that manipulate the host are
86 ~~These interactions are mediated by~~ effector proteins, many of which are produced in
87 the pharyngeal gland cells and secreted into the host through the stylet. In
88 aphelenchids (Ord. Rhabditida), which include *B. xylophilus*, these glands are
89 composed of two subventral and one dorsal gland cell. Despite the morphological
90 similarity of *B. xylophilus* to other PPNs, it is taxonomically unrelated (van Megen *et*
91 *al.*, 2009) and has a uniquely complex mode of parasitism.

92

93 Effectors have been identified from PPNs, including effectors that induce changes in
94 the host cells, facilitate migration and modulate host defences (reviewed by
95 Haegeman *et al.*, 2012; Mitchum *et al.*, 2013). However, the vast majority of these
96 studies have focused on cyst and root-knot nematodes. Previous studies on PWN
97 have often relied on attempting to identify orthologues of cyst nematode or root-knot
98 nematode effectors from Expressed Sequence Tag (EST) and genomic datasets
99 (Kikuchi *et al.*, 2011; Yan *et al.*, 2012). This has allowed identification of a range of

100 cell wall degrading enzymes that disrupt the plant and fungal cell wall, such as GH45
101 cellulases, several pectate lyases, expansins and beta-1,3-endoglucanases (Kikuchi *et*
102 *al.*, 2004; Kikuchi *et al.*, 2005, Kikuchi *et al.*, 2006; Kikuchi *et al.*, 2009). However,
103 PWN has an entirely different parasitic strategy from cyst nematodes and root knot
104 nematodes, which does not require the nematode to keep host tissues alive for a
105 prolonged period of biotrophy, and is taxonomically unrelated to these nematodes. It
106 is therefore important to consider alternative approaches which do not make *a priori*
107 assumptions about the nature of effector molecules. For example, one study has used
108 proteomic analysis of secreted proteins collected from nematodes stimulated with
109 pine extracts and identified cell wall degrading enzymes, detoxification enzymes and
110 peptidases amongst the secreted proteins (Shinya *et al.*, 2013). In an alternative
111 approach, microarray analysis has been used to identify secreted proteins upregulated
112 during infection (Qiu *et al.*, 2013).

113

114 Here we describe a differential expression based approach for identification of
115 effectors from PWN. We use RNAseq and bioinformatic analyses to identify a panel
116 of potentially secreted proteins upregulated after infection. Importantly, and in
117 contrast to other studies of this type, we use *in situ* hybridisation to examine spatial
118 expression profiles of candidate effectors and confirm that some are expressed in the
119 pharyngeal gland cells. We show that detoxification proteins are deployed in a two-
120 layer strategy, most likely in order to counter defence responses of the host. In
121 addition, we examine morphological changes in the PWN pharyngeal gland cells
122 across the life cycle and compare this with the development of these structures in cyst
123 and root-knot nematodes.

124

125 **Results**

126 *Characterisation of the pharyngeal gland cells of PWN*

127

128 Previous studies on effectors of PWN have not attempted to identify the specific
129 gland cells in which different putative effectors are expressed. This is frequently
130 justified on the basis that the pharyngeal gland cells are difficult to distinguish as they
131 are dorsally overlapping and all connect to similar positions in the large median
132 oesophageal bulb (Nickle *et al.*, 1981). To rectify this, and to allow the precise site of
133 expression of effectors to be determined, we first undertook a detailed morphological
134 analysis of the structure of the pharyngeal gland cells in juveniles and adults of *B.*
135 *xylophilus*. The dorsal and subventral gland cells were readily distinguished in both
136 juveniles and adults (Figure 1). Measurements of the gland cells showed that although
137 there was no significant difference in the size of the subventral gland cells between
138 juveniles and adults, the dorsal gland is significantly larger ($p < 0.05$) in the adult
139 stage than in the juvenile stages (Figure 1; Table 1).

140

141 *Differential gene expression in mycetophagous and phytophagous stages of B.*
142 *xylophilus and identification of candidate effectors*

143

144 Differential gene expression analysis showed extensive variation between replicates
145 of some life conditions, in particular the fungal feeding (FF) and 15 days post
146 infection (DPI) samples which failed to cluster in a heat map analysis (~~Supplementary~~
147 ~~Figure 1~~). This meant that only twenty-nine transcripts were identified as being
148 differentially expressed between the mycetophagous and phytophagous life stages
149 (Supplementary Figure 1). These genes represent a much lower proportion of the *B.*

150 *xylophilus* genes than expected, given the very different environments that these life
151 stages represent. In spite of this, genes that may have a role in the host-parasite
152 interaction were included in the sequences identified as differentially expressed after
153 infection, including glutathione S-transferase (GST), GHF45 cellulases, peptidases
154 and GH16 endoglucanases (Supplementary Table 1).

155

156 An alternative differential expression approach was used in parallel. The top 200
157 sequences upregulated in the parasitic life stage of the nematode were identified.
158 These sequences included numerous known effectors from this species (*e.g.* cell wall
159 degrading enzymes). The most highly represented Gene Ontology (GO) terms in this
160 set of 200 genes in the molecular function category were hydrolase, oxidoreductase
161 and lyase activity (Supplementary Figure 2). Seventy three of these 200 genes were
162 predicted to have a signal peptide and to lack transmembrane domains. This
163 represents a significant enrichment of potentially secreted proteins compared to the
164 proportion in the whole predicted gene set for this nematode (36.5% versus 12.7%; p
165 = <0.0001; chi-square test analysis). Fewer than half (33) of these 73 potentially
166 secreted proteins gave matches in BLAST searches against the non-redundant (NR)
167 database while the other 40 sequences encoded proteins that gave no matches and
168 were therefore considered pioneers. A subset of 46 putatively secreted proteins were
169 subsequently selected for further analysis ([Table 2](#)); these were the most highly
170 upregulated during infection and/or had matches in the database which suggested a
171 potential role in parasitism. These sequences include transcripts encoding several
172 classes of proteases, fatty acid transport proteins, putative V5/TPx1 allergen-like
173 proteins (VAPs), a lysozyme, several enzymes involved in the detoxification of

174 xenobiotic compounds and the most highly expressed pioneer genes (Table 2). The
175 pipeline used to generate this list of candidate effectors is summarised in Figure 2.

176

177 *Localisation and validation of effectors*

178

179 *In situ* hybridisation was used to investigate the spatial expression patterns of the 46
180 putatively secreted proteins in mixed life stage-nematodes. The majority of the genes
181 that gave a signal (18 sequences) were expressed in the intestine (Figure 3) while one
182 gene was expressed in the glandular tissues surrounding the anterior sense organs
183 (Figure 3A) and seventeen genes gave no signal in *in situ* hybridisation reactions (not
184 shown). Ten genes were expressed in the gland cells; four in the dorsal gland cell and
185 six in the subventral gland cells (Figure 4). The gland cell genes were similar in
186 sequence to a putative fatty acid and retinoid binding protein (*BUX.s00422.201*)
187 (Figure 4a), two pioneer genes (*BUX.s00083.48*, *BUX.s01109.178*) (Figure 4b, d), one
188 cytochrome P450 (*BUX.s00116.698*) (Figure 4c), a lysozyme protein (*BUX.s01066.2*)
189 (Figure 4e) and a predicted VAP protein (*BUX.s00116.606*) (Figure 4f) expressed in
190 the subventral gland cells. Genes similar in sequence to two putative GSTs
191 (*BUX.s01254.333*, *BUX.s00647.112*) (Figure 4h, j), one pioneer gene
192 (*BUX.s01144.122*) (Figure 4i) and a peptidase C1A (*BUX.01147.177*) (Figure 4k)
193 were expressed in the dorsal gland cell. No signal was detected using sense probes
194 (*e.g.* Figure 4l, n). The ten gland cell localised sequences represent novel effectors
195 that could be delivered into the host through the stylet during infection.

196

197 The expression levels of the ten putative effectors identified as being expressed in the
198 gland cells were validated by semi-quantitative RT-PCR and compared with the

199 results from the normalized expression values obtained by RNAseq ([Supplementary](#)
200 [Figure 3](#)). The RT-PCR ~~showed~~confirmed that all the ten putative effector genes were
201 more highly expressed in nematodes after infection of the host. All of them, with the
202 exception of the putative lysozyme (*BUX.s01066.2*) and cytochrome P450
203 (*BUX.s00116.698*), were also expressed in the fungal feeder condition. These latter
204 two genes were only expressed at 15dpi and 6dpi, respectively. ~~compared to~~
205 nematodes feeding on fungi (Supplementary Figure 3). Most of the genes showed
206 highest expression levels at 6 dpi. However, one gene (*BUX.s00422.201*) was equally
207 highly expressed at both 6 and 15 dpi while three of the genes (*BUX.01144.122*;
208 *BUX.s01147.177* and *BUX.s01066.2*) showed highest expression at 15 dpi.

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210

211 Discussion

212 A range of morphobiometric, ecological and population genetic studies have been
213 carried out on *B. xylophilus* (Moens and Perry, 2009). Other studies have identified
214 host physiological changes that occur upon the infection of the nematode (Fukuda,
215 1997; Hirao *et al.*, 2012; Mamiya, 2012). However, compared to cyst and root-knot
216 nematodes, little information is available on the nature of effectors secreted by PWN
217 or the details of the molecular basis by which it parasitizes plants. *Bursaphelenchus*
218 *xylophilus* has a unique feeding behaviour, a complex life cycle and infests a narrow
219 host range of pine tree species. These features, coupled with the economic damage
220 that it causes, make further studies on effector biology of *B. xylophilus* a priority.

221

222 The pharyngeal gland cells are the source of the majority of nematode effectors (*e.g.*
223 Haegeman *et al.*, 2012). Like most tylenchid nematodes (including root-knot and cyst

224 nematodes) and other nematode groups, *B. xylophilus* has two subventral gland cells
225 and one dorsal gland cell (Gheysen and Jones, 2006; Maule and Curtis, 2011;
226 Haegeman *et al.*, 2012). In *B. xylophilus*, the three pharyngeal gland cells dorsally
227 overlap the intestine and are connected to similar positions in the large median bulb,
228 which can make them difficult to distinguish (Nickle *et al.*, 1981). Despite this, we
229 were able to show that the dorsal gland cell in *B. xylophilus* is larger in the adult
230 stages than in juveniles, as is seen in the sedentary stages of root-knot and cyst
231 nematodes such as *Meloidogyne incognita* and *Heterodera glycines* (Endo, 1987;
232 Hussey and Mims, 1990; Endo 1993). In sedentary nematodes the subventral gland
233 cells decrease in size after the formation of the feeding structure (Maule and Curtis,
234 2011). By contrast, the subventral gland cells of *B. xylophilus* remain similar in size in
235 juvenile and adult stages, suggesting a prolonged role in parasitism. Consistent with
236 this, the majority of putative effectors identified here were subventral gland
237 expressed. Together our findings align well with a recent study on *B. mucronatus*, a
238 species closely related to *B. xylophilus*, which showed that a larger number of
239 secretory granules are present in the subventral glands during the juvenile stages and
240 in the dorsal gland during the adult stages (Carletti *et al.*, 2013).

241

242 We generated transcriptomic datasets from mycophagous (pre-invasive of the host)
243 and phytophagous (post-invasion of the host) stages of the nematode. Our first
244 analysis unexpectedly showed extensive variation between replicates of the nematode
245 samples, particularly at the later stages of infection. A similar independent study (T.
246 Kikuchi pers. comm.) has shown that the environmental conditions (*e.g* time of year)
247 experienced by the host have a profound effect on gene expression in parasitic *B.*
248 *xylophilus* and it is likely that the variability seen here reflects a similar process. In

249 order to collect the relatively large numbers of nematodes required for analysis,
250 samples were collected from many different trees that may have been exposed to
251 different environmental conditions. In spite of these issues we were able to identify a
252 panel of genes that were significantly upregulated after infection and secreted proteins
253 were enriched in these sequences. Subsequent *in situ* hybridisation experiments
254 identified ten putative effector proteins expressed in the gland cells, validating the
255 approach. A comparison of these secreted proteins with the PWN secretome dataset
256 obtained in a previous study using a proteomic approach (Shinya *et al.*, 2013),
257 showed that five of the effectors identified here were also identified in secreted
258 proteins collected from *B. xylophilus* (data not shown). Although there are clearly
259 differences in the results obtained using the two approaches, it is reassuring to see
260 some measure of cross validation between the two studies.

261

262 A significant proportion of the sequences upregulated during the transition to
263 parasitism, including some of the identified effectors, are likely to have roles in
264 protecting the nematode from host defence responses. Pine trees respond to nematode
265 infection by releasing a range of defence compounds in the areas surrounding the
266 entry wound including ethylene, terpenoids (alpha and beta-pinene), ROS and lipid
267 peroxides (Fukuda, 1997). Our study revealed that one secreted cytochrome P450 and
268 two secreted GSTs upregulated at the early stages of infection (6dpi) are expressed in
269 the subventral and dorsal gland cells respectively (Figure 5). These two enzymes are
270 major components of the pathway leading to metabolism of xenobiotic compounds in
271 the free-living nematode, *Caenorhabditis elegans* (Lindblom and Dodd, 2006). A
272 secreted GST has also been identified that plays an important role in parasitism of
273 plants by root-knot nematodes, and that most likely protects the nematode against host

274 defences (Lindblom and Dodd, 2006; Dubreuil *et al.*, 2007). Our results suggest that
275 GST plays a similar role in *B. xylophilus* parasitism.

276

277 Our analysis showed that a range of transcripts encoding other enzymes potentially
278 involved in the detoxification of xenobiotic compounds (including epoxide hydrolase,
279 multicopper oxidase, flavin monooxygenase, UGT and cytochrome P450) are
280 upregulated after infection but are expressed in the intestine (Figure 3). A recent study
281 in *C. elegans* showed that the intestine is the first line of defence against xenobiotic
282 compounds to oxidative-stress and emphasized the importance of phase 2
283 detoxification enzymes in this process (Crook-McMahon *et al.*, 2014). Our data
284 suggest that *B. xylophilus* uses a two-layered approach to protect itself against host-
285 derived xenobiotic compounds. Some enzymes involved in detoxification pathways
286 are secreted into the host representing the first layer, while others are upregulated in
287 the digestive system, which will be exposed to ingested host materials, and represent
288 the second.

289

290 The other identified effectors have a range of potential roles in the host-parasite
291 interaction. One effector was similar to secreted venom allergen like proteins (VAPs)
292 from other nematodes and was highly expressed 6 dpi. Three secreted VAPs have
293 previously been characterized from PWN (Lin *et al.*, 2011). It has been suggested that
294 one of these (*Bx-vap-1*) is involved in migration of PWN inside the host (Kang *et al.*,
295 2012). More recently, a study of the potato cyst nematode *Globodera rostochiensis*
296 has shown that VAPs from this species are required for suppression of host immunity,
297 possibly through a proteinase inhibition activity (Lozano-Torres *et al.*, 2014). VAPs
298 are conserved throughout nematodes and are frequently upregulated in parasitic

299 nematodes upon infection. It is therefore possible that VAPs are widely deployed
300 against host defence responses that require the activity of host proteinases.

301

302 The *B. xylophilus* genome encodes hundreds of proteinases (Kikuchi *et al.*, 2011).
303 Our RNAseq analysis showed that several, including cysteine, metallo, aspartic and
304 serine catalytic classes, are upregulated after infection. The majority of these were
305 expressed in the intestine (Figure 3), consistent with a role in digestion. However, we
306 identified a cysteine proteinase C1A that is expressed in the gland cells and
307 upregulated at the later stage of infection (15dpi). This enzyme could have a role in
308 digesting host tissues during migration or may also target host proteins involved in
309 defence responses, as has been shown in animal parasitic nematodes (Sajid and
310 McKerrow, 2002; Malagón *et al.*, 2013). Consistent with this, plants are known to
311 deploy proteinase inhibitors against pathogens (Xia, 2004).

312

313 A secreted fatty acid and retinol binding protein (FAR) was identified that is
314 expressed in the subventral gland cells during the infection of the host. Most
315 nematode lipid binding proteins are thought to be important for internal transport of
316 lipids. However, FAR proteins have been identified both cyst (*Globodera pallida*)
317 and root-knot nematodes that bind precursors of lipid-based plant defence signalling
318 compounds important in the jasmonate signalling pathway (Prior *et al.*, 2001;
319 Iberkleid *et al.*, 2013). The role of these pathways in terms of the interaction between
320 *B. xylophilus* and its host remains to be determined.

321

322 One effector sequence was similar to lysozymes from a range of nematode species.

323 Nematode lysozymes may have a role in digestion of host proteins and may also be

324 important in protection of nematodes against other pathogens. Several lysozymes with
325 antibacterial activity have been described from *C. elegans* (Boehnisch *et al.*, 2011)
326 that are thought to play an important role in defence against pathogenic bacteria. It is
327 known that *B. xylophilus* is associated with a range of bacterial species that may form
328 an important component of the infection process (Vicente *et al.*, 2012b). The
329 deployment of lysozyme by *B. xylophilus* may restrict bacterial growth in the regions
330 infected by the nematode, reducing competition for food resources.

331

332 Our analysis also identified three pioneer genes expressed in the subventral and dorsal
333 gland cells that are highly upregulated at 6 and 15 dpi. Given the absence of these
334 proteins from other nematodes, they are likely to play key roles in the biology of *B.*
335 *xylophilus*. Effectors from other nematodes are frequently novel proteins (*e.g.* Gao *et*
336 *al.*, 2003). Characterising the function of such sequences in detail is likely to be
337 challenging.

338

339 In summary, we describe a transcriptomic approach that has allowed identification of
340 ten novel effectors and eighteen proteins from the digestive system of *B. xylophilus*.
341 We also demonstrate that the gland cells of this species, like those of other plant-
342 parasitic nematodes change in structure during the life cycle. Our data suggest that *B.*
343 *xylophilus* uses a multi-layered system of enzymatic detoxification to metabolise host
344 derived xenobiotics within the host and in the digestive system.

345

346 **Experimental procedures**

347 *Biological material*

348 The Portuguese isolate of *B. xylophilus*, BxPt75OH, used in this study originated from
349 a symptomatic pine tree in Oliveira do Hospital district, in the central region of
350 mainland Portugal. The nematode was identified to species level (Nickle *et al.*, 1981)
351 and cultures were maintained in Erlenmeyer flasks containing *Botrytis cinerea* on
352 barley seeds at 25°C (Evans, 1970). Nematodes were extracted using the Baermann
353 funnel technique (Southey, 1986) for 24 hours followed by sieving (38µm).

354

355 *Morphometric studies of the pharyngeal gland cells*

356 Mixed life-stage nematodes were killed by heat (water bath for approximately 15
357 minutes until the temperature reaches 60°C) and fixed in 4% formaldehyde and
358 prepared for mounting according to Siddiqi (1964). The nematodes were transferred
359 into lactophenol and incubated for 24 hours at 40°C. Nematodes were then transferred
360 to a solution of 75% glycerine: 25% lactophenol for approximately 24 hours at 40°C,
361 until the lactophenol had evaporated and the nematodes were in pure glycerine. The
362 nematodes were then mounted in glycerine surrounded by a ring of paraffin on a glass
363 slide. A coverslip was placed on the top of the paraffin ring and the preparation was
364 heated until the paraffin had melted. The slides were observed under a laser scanning
365 microscope (Zeiss LSM 710) using the DIC (Differential Interference Contrast)
366 method.

367 Measurements of the dorsal and subventral glands cells were performed from ten
368 individuals for each of the life stages (juveniles and adults) , mounted using an agar
369 pad technique as described by Eisenback (2012). Statistical significance was tested
370 using Mann-Whitney U test analysis (STATISTICA v12.0) (Mann and Whitney,
371 1947). Images (measurements) were recorded using an Olympus BX50 light
372 microscope and Cell Software (Olympus).

373

374 *PWN inoculation trials*

375 Two-month old maritime pine trees (*Pinus pinaster*) obtained from a Portuguese
376 nursery were used for inoculation of the PWN isolate. Approximately 2000 mixed
377 life-stage nematodes were cultured on fungi as described above and inoculated into a
378 small wound (5mm) made on the pine stem using a sterilized scalpel. Infections were
379 conducted under controlled conditions (average temperature 23°C, 50% humidity). A
380 subset of the nematodes prepared for each biological replicate were frozen in liquid
381 nitrogen and stored at -80°C for RNA extraction as the mycetophagous controls. The
382 inoculated nematodes were collected from the trees, six and fifteen days post
383 infection. For this, the pine stems were cut and nematodes were collected by the
384 Baermann funnel technique for approximately 2hrs. Nematodes were centrifuged by
385 sucrose flotation (50%), washed three times in 1X Phosphate Buffered Saline (PBS)
386 and frozen in liquid nitrogen.

387

388 *RNA Extraction and Sequencing*

389 Nematode RNA was extracted from samples corresponding to three different
390 conditions: fungal feeding (pre-inoculation), 6 days post infection (dpi) and 15 dpi.
391 RNA extraction was performed using the GeneJET RNA Purification Kit (Fermentas-
392 ThermoScientific) following the manufacturer's instructions. RNA integrity number
393 was assessed using a Bioanalyser (Agilent Technologies). The samples (two
394 biological replicates for fungal feeding condition and three biological replicates for
395 the other two conditions), each with a RNA Integrity Number - RIN over the value of
396 7, were used for paired end sequencing at The Genome Analysis Centre (TGAC, UK),

397 on the Illumina HiSeq platform. [RNAseq data described in this manuscript are](#)
398 [available through ENA under accession number PRJEB9165](#)

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400 *Differential gene expression analysis*

401 Raw RNA reads were trimmed of adapter sequences and low quality bases (phred
402 score < 22) using Trimmomatic v0.32 (Bolger *et al.*, 2014) (Supplementary Table 2).
403 Remaining high quality reads (79%) for each library were mapped back to the
404 reference genome (<http://www.genedb.org/Homepage/Bxylophilus>) (Kikuchi *et al.*,
405 2011) using Tophat2 (Kim *et al.*, 2013). Read counts for each gene were determined
406 using bedtools v2.16.2 and normalised (TMM) using Trinity wrapper scripts (Haas *et*
407 *al.*, 2013) for EdgeR (Robinson *et al.*, 2010). Two differential expression analyses
408 were carried out on normalised read counts: 1) Transcripts with a minimum fold
409 change of 4 ($p < 0.001$) between conditions were identified using Trinity wrapper
410 scripts for EdgeR, and clustered based on 20% tree height. 2) All genes were ranked
411 by the ratio of their average normalized expression during all *in planta* stages (6 dpi +
412 15dpi) compared to fungal feeding. The top two hundred most differentially regulated
413 genes were selected for further analyses. Potentially secreted protein sequences were
414 identified using a workflow within a local installation of Galaxy on the basis of the
415 presence of an N-terminal signal peptide (predicted by SignalP 3.0; Bendtsen *et al.*,
416 2004) and the absence of a transmembrane domain (predicted by TMHMM 2.0;
417 Krogh *et al.*, 2001) (Cock and Pritchard, 2014). A BLASTp search ([using Galaxy tool](#)
418 [version 0.1.01](#)) was performed against the non-redundant (NR) database ([cutoff value](#)
419 [of 1e-03](#)), for all candidates, in order to predict their functions based on sequence
420 similarity. [Putative protein domain description is based on the annotation of the B.](#)

421 [xylophilus genome \(version 1.2\) available on Gene DB](http://www.genedb.org/Homepage/Bxylophilus)
422 [\(http://www.genedb.org/Homepage/Bxylophilus\).](http://www.genedb.org/Homepage/Bxylophilus)

423

424 *In situ hybridisation*

425 *In situ* hybridisation using digoxigenin labelled probes was performed in order to
426 determine the spatial expression patterns of candidate effectors based on the protocol
427 described by de Boer *et al.*, 1998. For each candidate gene a fragment of
428 approximately 200 base pairs was amplified from the coding region and used as
429 template for synthesis of both sense and antisense probes. The primers used for these
430 reactions are shown in Supplementary Table 3.

431

432 *Validation of the expression profiles of candidate effectors*

433 The expression profiles of the genes identified as expressed in the gland cells were
434 validated by semi-quantitative PCR as described in Chen *et al.*, 2005. Actin was used
435 as a control for all reactions (Supplementary Table 3). Expression levels of each gene
436 relative to the actin control were determined in the three different conditions (FF, 6
437 and 15 dpi), using cDNA synthesised from total RNA as a template [and after 30-35](#)
438 [cycles. The results were analysed by electrophoresis in agarose gels. The qualitative](#)
439 [results were compared to the predicted expression values obtained by RNAseq data.](#)

440

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452

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629 ~~RNAseq data described in this manuscript are available through ENA under accession~~

630 ~~numbers X-Y.~~

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632 **Supporting information legends**

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634 **Supplementary Figure 1** - Differential expression analysis of the transcripts. The
635 heatmap resulting from the RNAseq analysis, using 8 samples in three different
636 conditions – pre-invasive/mycetophagous (Fungal Feeding) and post
637 invasive/Phytophagous (6 and 15 DPI).

638

639 **Supplementary Figure 2** - Analysis of the most represented molecular function
640 (level 3) in the Top 200 set of up regulated genes obtained by a bioinformatics
641 pipeline.

642

643 **Supplementary Figure 3** - Validation of the expression of the secreted effectors by
644 semi-quantitative-PCR [using the actin as housekeeping and the primers described in](#)
645 [Supplementary Table 3. The results were analysed by gel electrophoresis and for each](#)
646 [candidate the results of both actin and the candidate gene were presented. On the](#)
647 [right, the bar chart represents the ~~and~~ normalized expression values \(FPKM\)](#)
648 [predicted by RNAseqTMM ~~for each candidate gene.~~](#)

649

650 **Supplementary Table 1** – List of the twenty-nine differential expressed transcripts
651 between mycetophagous and phytophagous stages. ~~–~~Detailed description of the
652 twenty-nine transcripts includes [the presence or absence of putative signal peptide,](#)
653 [their putative protein domain \(according to Gene DB annotation of the version 1.2. of](#)
654 [the genome; available at <http://www.genedb.org/Homepage/Bxylophilus>\), the top](#)
655 [match and e-value of the BLASTp analysis against nr \(non redundant\) database](#)
656 [\(cutoff value of 1e-03\) ~~and their orthologous protein sequences in other organisms.~~](#)

657 and also the normalised expression profile in the three different conditions (fungal
658 feeding nematodes [FF], nematodes 6 and 15 days post infection [dpi]). The
659 normalized expression values are in FPKM (Fragments Per Kilobase of exon per
660 Million mapped fragments).

661 **Supplementary Table 2** - Summary of RNAseq data.

662

663 **Supplementary Table 3** - List of pair of primers used for amplification of probes for
664 *in situ* hybridisation. Gene model are according to Kikuchi *et al.*, 2011 and sequences
665 available at <http://www.genedb.org/Homepage/Bxylophilus>. bp: base pair.

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Tables

	Juveniles	Adults	690
Dorsal gland cell	9030.9±4.439 (8024-38.296)	13566.9±6.48111 (11953.5-73.8151)	
			694
Subventral gland cells	10557.5±8.6293 (41.993-72122)	41.5106±2.2686 (39.294-45.1146)	695
			696

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Table 1 - Measurements of the dorsal and subventral pharyngeal gland cells of *B. xylophilus*, BxPt75OH isolate [in μm and in form: mean \pm SD (range)]. calculated from ten individuals for each life stage.

702

Predicted function	Putative protein domain (GeneDB annotation)
PROTEASES (10)	Aspartic protease A1 (5) Cysteine proteases C1A (1); C46 (1) Serine-type protease (2) Metallo-type protéase M13 (1)
FATTY ACID METABOLISM (2)	Fatty acid retinoid binding proteins
DETOXIFICATION OF XENOBIOTIC COMPOUNDS (12)	FMO (flavin monooxygenase) (2) UDP-glucuronosyl transferase (2) Multicopper putative acid oxidase (1) Glutathione S-transferase (2) Cytochrome P450 (3) Acid phosphatase (1) Epoxide hydrolase (1)
UNKNOWN PROTEINS DOMAIN (PIONEERS) (16)	None
PROTEIN WITH TOXIN DOMAIN (2)	Metridin-like Sht toxin domain
ALLERGENS (1)	Putative allergen V5/TPx1
GLYCOSYL HYDROLASE CLASSES (2)	GH29 (alpha-L-fuco domain) GH30- GH2
LYSOZYME ACTIVITY (1)	Lysozyme 7,8

703

704 **Table 2** – List of candidate effector genes categorized by predicted function.

705

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706 **Figure legends**

707

708 **Figure 1-** Positions of pharyngeal gland cells in ~~juvenile-adult~~ (A) and ~~adult-juvenile~~
709 (B) *B. xylophilus*. M: Median bulb; DG: Dorsal glands; SVG: Subventral glands; S:
710 Stylet. Subventral glands (white) and dorsal gland (orange) are outlined in the
711 duplicate figures below the main panels. (Scale bar = 20µm)

712

713 **Figure 2-** Bioinformatic pipeline for the identification of candidate effectors from *B.*
714 *xylophilus*. FF: Fungal feeder; DPI: days post infection.

715

716 **Figure 3-** Localisation of the candidate proteases and detoxification enzymes
717 encoding genes expression in the intestine by *in situ* hybridization, with the exception
718 of putative epoxide hydrolase (a) (*BUX.s00298.34*) that was expressed in the
719 glandular tissues surrounding the anterior sense organs. b, putative multicopper
720 oxidase (*BUX.s01281.17*); c, putative flavin monooxygenase (*BUX.s01337.7*); d,
721 putative peptidase C46 (*BUX.s01109.245*); e, putative UDP-glucuronosyl transferase
722 (UGT) (*BUX.s00422.680*); f, putative CYP33 C-related (*BUX.s01144.121*); g,
723 putative peptidase M13 (*BUX.s01661.67*); h, putative peptidase A1 (*BUX.s00532.10*);
724 i, putative peptidase S28 (*BUX.s01144.130*).

725

726 **Figure 4 -** Localisation of the candidate effectors expression in the pharyngeal gland
727 cells by *in situ* hybridization. a, *BUX.s00422.201*, b, *BUX.s00083.48*, c,
728 *BUX.s00116.698*, d, *BUX.s01109.178*, e, *BUX.s01066.2*, f, *BUX.s00116.606*, h,
729 *BUX.s01254.333*, i, *BUX.s01144.122*, j, *BUX.s00647.112*, k, *BUX.s01147.177*, l and n

730 are control Forward probe. M/MB: Median bulb; G: Dorsal gland cell; SVG:
731 Subventral glands.