

1 Hemibiotrophic fungal pathogen induces systemic susceptibility and systemic  
2 shifts in wheat metabolome and microbiome composition

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18

19 **Abstract**

20 Yield losses caused by fungal pathogens represent a major threat to global food production. One of  
21 the most devastating fungal wheat pathogens is *Zymoseptoria tritici*. Despite the importance of this  
22 fungus and wheat as main staple food crop the underlying mechanisms of plant-pathogen  
23 interactions are poorly understood. Here we present a conceptual framework based on coinfection  
24 assays, comparative metabolomics, and microbiome profiling to study the interaction of *Z. tritici* in  
25 susceptible and resistant wheat. We demonstrate that *Z. tritici* suppresses the production of  
26 immune-related metabolites in a susceptible cultivar. Remarkably, this fungus-induced immune  
27 suppression spreads within the leaf and even to other leaves, a previously undescribed phenomenon  
28 that we term “systemic induced susceptibility”. Using a comparative metabolomics approach, we  
29 identified defense-related biosynthetic pathways that are suppressed and induced in susceptible and  
30 resistant cultivars, respectively. We show that these fungus-induced changes also dramatically affect  
31 the wheat leaf microbiome. Our findings emphasize that immune suppression by this hemibiotrophic  
32 pathogen impacts specialized plant metabolism, alters its associated microbial communities, and  
33 renders wheat vulnerable to further infections.

34 Plant pathogens can be classified according to their lifestyle<sup>1</sup>. Biotrophic pathogens colonize and feed  
35 on living host tissue to complete their lifecycle, whereas necrotrophic pathogens can induce plant cell  
36 death and feed on nutrients released from dying host cells. Hemibiotrophic pathogens initially colonize  
37 hosts via biotrophic invasion and later switch to necrotrophic growth. Plant immune responses against  
38 biotrophic and necrotrophic pathogens differ considerably<sup>2</sup>. Immune defenses induced by biotrophic  
39 pathogens involve the accumulation of antimicrobial metabolites and local cell death conferred by a  
40 hypersensitive response<sup>2,3</sup>. Plant pathogens produce effector molecules to avoid or suppress immune  
41 responses<sup>3</sup>. While some effectors have evolved to avoid immune recognition by the plant, others  
42 protect the fungus from plant-derived apoplastic defense mechanisms or reprogram intracellular plant  
43 responses<sup>4-7</sup>. Plant defenses are often not specific to single pathogens, but generally target a broad  
44 range of microbes. As a consequence, different pathogens have evolved effectors targeting the same  
45 defense responses independently from one another<sup>8</sup>. Conversely, suppression of defense-related host  
46 responses by one pathogen may enable additional infections by other pathogens<sup>9</sup>.

47 *Zymoseptoria tritici* is a global hemibiotrophic plant pathogen that infects wheat, causing up to 50%  
48 yield loss<sup>10</sup>. Resistance breeding in wheat can confer complete resistance to particular isolates of the  
49 fungus. To date, 21 resistance genes against *Z. tritici* have been described<sup>11</sup>. Still, farmers mainly rely  
50 on chemical control to prevent and manage the disease. In fact, 70% of fungicides in the European  
51 Union are used to control *Z. tritici*. However, fungicide resistance is an increasing problem<sup>10,12</sup>. One  
52 challenge in decreasing fungicide usage in the control of *Z. tritici* is a long biotrophic infection phase  
53 before the switch to visible necrotrophy. The infectious process is poorly understood, and few *Z. tritici*  
54 effector proteins have been identified and functionally characterized to date<sup>5,13-16</sup>.

55 In this study, we looked at the interaction of *Z. tritici* with its host during the biotrophic stage of  
56 infection. We first addressed whether biotrophic fungal colonization of wheat involves active  
57 suppression of immune responses or if the pathogen only avoids host recognition. We also investigated  
58 the extent to which *Z. tritici* infection influences colonization of the plant by other microbes. If the  
59 fungus actively suppresses immune responses in susceptible wheat cultivars, this could influence the  
60 ability of other nonadapted microorganisms to colonize the plant. To test our hypotheses, we  
61 conducted coinfection experiments of *Z. tritici* with adapted and nonadapted *Pseudomonas syringae*  
62 bacteria. Here, we provide evidence for active immune suppression plant tissues both local and distant  
63 to the infection, a new effect that we termed “systemic induced susceptibility”. We applied a  
64 comparative Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) metabolomics  
65 approach to describe the observed differences on the wheat metabolome. In addition, we analyzed  
66 the bacterial microbiome to generalize the observations made during the coinfections.

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68

## 69 **Results**

### 70 **Resistance to *Z. tritici* is cultivar-dependent**

71 In compatible infections, *Z. tritici* propagates in the leaf mesophyll and later shifts to necrotrophic  
72 growth and pycnidia production<sup>17</sup>. In contrast, the fungus is not able to propagate in the mesophyll of  
73 incompatible host plants. In order to understand the underlying traits that define compatible versus  
74 incompatible interactions between different cultivars of wheat and the fungal pathogen *Z. tritici*, we  
75 conducted infection experiments with the susceptible wheat cultivar Obelisk and the resistant cultivar  
76 Chinese Spring<sup>18</sup>. The *Z. tritici* isolate IPO323 was used in all infection experiments. *Z. tritici* is able to  
77 infect and reproduce in leaves of Obelisk, but infection is aborted in leaves of the resistant cultivar  
78 Chinese Spring (Figs. 1a, 1b, S1).

79 There are no immune markers established for hexaploid wheat, and we lack good predictors of wheat  
80 response to fungal infection in different cultivars. We therefore developed a new assay based on  
81 bacterial infection to test our hypothesis that *Z. tritici* suppresses immune responses actively in  
82 compatible interactions. We predicted that bacterial growth would benefit from *Z. tritici*-mediated  
83 immune suppression and therefore could serve as a read-out for the extent of plant immune response  
84 following fungal infection. First, we tested the susceptibility of the cultivars Obelisk and Chinese Spring  
85 to the *P. syringae* pathovars *oryzae* (*Por*36), *tomato* (*Pst* DC3000 and its T3SS mutant *hrcC*-), and  
86 *maculicola* (*Psm* ES4326) (Fig. S2). While Obelisk and Chinese Spring differ in their susceptibility to *Z.*  
87 *tritici*, the 2 cultivars show the same extent of susceptibility and resistance to the *P. syringae* pathovars  
88 (Fig. 1c).

89

### 90 **Fungal infection of susceptible wheat promotes bacterial coinfection**

91 To assess the spatial and temporal impact of *Z. tritici* on the wheat immune response during biotrophic  
92 growth, we then coinfecting the *P. syringae* pathovars *oryzae* (*Por*) and *tomato* (*Pst*) on distinct leaf  
93 areas of Obelisk and Chinese Spring: 1) in the same area as the fungal spores (local), 2) adjacent to the  
94 fungal infection on the same leaf, and 3) on a leaf other than the one infected by *Z. tritici* (a systemic  
95 leaf) (Fig. S3a). Bacteria were inoculated on leaves 4 days after inoculation with fungal spores (4 dpi-  
96 f).

97 Local coinfection of Obelisk with *Z. tritici* and *Por* increases bacterial growth compared to a mock-  
98 treated leaf (Fig. 2a). The increase in bacterial growth on *Z. tritici*-infected leaves ceased at later stages  
99 of coinfection (Fig. S4). We predicted that *Z. tritici* efficiently suppresses the immune response in  
100 Obelisk, thereby promoting the growth of *P. syringae*. In support of this hypothesis, we observed  
101 reduced growth of the bacteria in leaves of Chinese Spring in which immune responses are induced by  
102 *Z. tritici* (Fig. 2a). Local coinfection cannot exclude a direct effect of fungal-bacterial interaction.  
103 Therefore, our experiment also included measures of bacterial growth in adjacent leaf areas. We

104 observed that fungal infection in Obelisk facilitated bacterial growth in adjacent leaf areas, while  
105 coinfecting Chinese Spring became more resistant to bacterial infection in adjacent leaf areas (Fig. 2b).  
106 This finding supports a direct effect of *Z. tritici* on the plant immune system. We further tested heat-  
107 killed fungal spores to confirm that only viable *Z. tritici* cells actively suppress plant immune responses.  
108 We confirmed the induction of susceptibility for the nonadapted *Pst* pathovar, which, in contrast to  
109 *Por*, hardly grows on Obelisk in the absence of *Z. tritici* but strongly benefits from fungal coinfection  
110 (Fig. 2c). Coinfection with *Z. tritici* also enabled growth of the T3SS-defective *Pst* hrcC- in the adjacent  
111 tissue (Fig. S5). A beneficial effect on bacterial growth was detected both distal (towards the leaf tip)  
112 and proximal (towards the leaf base), but the effect was more distinct in proximal tissue (Fig. S6). We  
113 then studied the reach of immune suppression by *Z. tritici* in infected plants. When the third leaf was  
114 inoculated with *Por*, bacterial growth was increased in the susceptible cultivar and reduced in the  
115 resistant cultivar, providing further support for systemic induced susceptibility (SIS) in Obelisk and  
116 systemic acquired resistance (SAR) in Chinese Spring (Fig. 2d).

117

#### 118 ***Z. tritici* infection induces systemic changes in the wheat metabolome**

119 We next asked which physiological responses of wheat confer *Z. tritici* susceptibility in Obelisk and  
120 resistance in Chinese Spring. We conducted a suspected targeted metabolome analysis using FT-ICR-  
121 MS. We used infected and mock-infected leaves and included leaf areas both local and adjacent to the  
122 infection (Fig. S3b). Samples were taken on the same days post infection as in the measures of bacterial  
123 growth in the coinfection experiments (4 and 8 dpi-f). Overall, we measure 37,664 different  
124 metabolites, of which 296 were annotated in a list of plant secondary metabolites (Table S1). We  
125 focused on the annotated metabolites, especially those known to be immune-related metabolites and  
126 pathways. We compared the complete metabolome dataset of Chinese Spring and Obelisk and  
127 generated a principal component analysis (PCA) plot to visualize the overall differences in the  
128 metabolomes of the 2 plant genotypes and in infected versus uninfected samples. The wheat cultivar  
129 and the leaf position with respect to the site of fungal infection explained the main differences within  
130 the dataset (Fig. 3a). By comparing mock- and fungal-infected leaf areas (local and adjacent to  
131 infection), we found that fungal infection did not cause a global shift in the metabolomes of Chinese  
132 Spring or Obelisk suggesting that only particular metabolites are targeted by the fungus (Fig. S7).

133 We then set out to identify specific metabolites that accumulate differently in local and adjacent  
134 tissues to fungal infection of the 2 cultivars. We assessed the fold change of metabolite accumulation  
135 in 3 different comparisons. 1) In the cultivar comparison, an Obelisk sample was compared to a Chinese  
136 Spring sample (same treatment, same leaf position). 2) In the position comparison, we compared local  
137 samples to those from adjacent tissue (same treatment, same cultivar). 3) In the treatment  
138 comparison, samples infected with *Z. tritici* were compared to mock-treated samples (same cultivar,

139 same leaf position) (Fig. S8a,b). We identified 116 annotated metabolites with a significant difference  
140 in at least 1 of the comparisons (Table S2). We refer to these metabolites as differentially accumulating  
141 metabolites (DAMs). We applied a Fisher's exact test to assess the significance of the observed  
142 differences in the 3 comparisons with respect to the complete dataset (Table S3). Chinese Spring had  
143 significantly more DAMs than Obelisk (5191 of 296,031 metabolite incidences in Chinese Spring  
144 compared with 3820 of 297,482 metabolite incidences in Obelisk,  $P < .0001$ ). This significant difference  
145 was present in Chinese Spring tissues local and adjacent to infection compared to Obelisk (local: 4465  
146 of 29,906 in Chinese Spring compared with 2466 of 27,914 in Obelisk,  $P < .0001$ ; adjacent: 3390 of  
147 27,374 in Chinese Spring compared with 2474 of 30,945 in Obelisk,  $P < .0001$ ). In addition, significant  
148 differences between Chinese Spring and Obelisk were observed for metabolites that were both  
149 upregulated (839 of 30,063 in Chinese Spring compared with 397 of 28,248 in Obelisk,  $P < .0001$ ) and  
150 downregulated after fungal infection (584 of 24,672 in Chinese Spring compared with 584 of 29,060 in  
151 Obelisk,  $P = .0058$ ).

152

### 153 ***Z. tritici* targets immune-related biosynthetic pathways**

154 We studied *Z. tritici*-induced changes in entire biosynthetic pathways involving immune-related  
155 metabolites. We focused on 2 biosynthetic pathways that have been studied in other plant systems<sup>19–</sup>  
156 <sup>27</sup>: benzoxazinoids (BXs) and phenylpropanoids.

157 The BXs are a group of grass-specific antimicrobial phytoanticipines<sup>28,29</sup> (Fig. 3b). BXs are preformed  
158 and stored as inactive glycosides ready to be released as free BXs when needed<sup>30</sup>. Chinese Spring  
159 accumulates free BXs locally at 4 and 8 dpi-f (Fig. 3c, Fig. S9), and inactive BX glycosides accumulate in  
160 adjacent tissue without direct contact with the fungal pathogen (Fig. 3d, Fig. S9). In contrast, Obelisk  
161 does not accumulate free BXs or the storage forms. We suspect that the absence of BXs in Obelisk is  
162 partly responsible for the increased susceptibility of this cultivar.

163 Phenylpropanoids are a large class of phenolic secondary plant metabolites. Many stress-inducible  
164 phenylpropanoids and phenylpropanoid-derived compounds are categorized as phytoalexins against  
165 pathogens<sup>27,31–33</sup>. A comparative metabolome analysis revealed that *Z. tritici* has a strong effect on the  
166 biosynthesis of phenylpropanoids and compounds deriving from this pathway, such as  
167 hydroxycinnamic acid amides (HCAAs) and flavonoids (Fig. 4). We found high levels of  
168 (hydroxyl)cinnamyl alcohols and glucosides of HCAs in Chinese Spring at 4 and 8 dpi-f in local and  
169 adjacent tissues of infected leaves. On the other hand, there was a delay in the accumulation of  
170 phenylpropanoid compounds and majority of flavonoids in Obelisk. Here, single phenylpropanoid and  
171 flavonoid compounds accumulated only at 8 dpi-f. Chinese Spring also accumulated HCAAs locally after  
172 fungal infection at 4 and 8 dpi-f. We found a significant increase in HCAA levels in Obelisk only in

173 adjacent tissue at 4 dpi-f, and we detected no HCAs at 8 dpi-f. These results indicate that fungal  
174 infection disturbs the biosynthesis of phenylpropanoids and related compounds in Obelisk.  
175 Overall, we show that the regulation of immune-related pathways differs dramatically in response to  
176 *Z. tritici* infection in the Chinese Spring and Obelisk wheat cultivars. Immune-related DAMs are  
177 generally upregulated in the resistant cultivar Chinese Spring at the site of fungal infection and in  
178 adjacent tissues. We demonstrated that biosynthetic pathways and the accumulation of compounds  
179 involved in plant defense responses are manipulated by *Z. tritici* in Obelisk, a susceptible wheat  
180 cultivar.

181

### 182 **Infection with *Z. tritici* leads to systemic shifts in microbiome community structure**

183 Above we show that *Z. tritici* manipulates immune-related biosynthetic pathways in susceptible wheat  
184 to enhance fungal propagation. Moreover, the interaction of *Z. tritici* with the immune system of  
185 wheat in both the resistant and susceptible wheat has an effect on the growth of *P. syringae* locally as  
186 well as systemically. Based on these findings we next hypothesized that infection by *Z. tritici* could  
187 have a more general effect to alter the composition and structure of plant-associated microbiota in  
188 local and adjacent leaf tissue. To address this question, we profiled the bacterial communities in  
189 Obelisk and Chinese Spring leaves after *Z. tritici* infection at the same timepoints for which we  
190 monitored *P. syringae* growth, but also including 0 dpi-f (Fig. S3c).

191 We sequenced leaf-associated bacterial communities (V5-V7 regions of the 16S rDNA) and identified  
192 3139 bacterial operational taxonomic units (100% OTUs). To assess the effect of *Z. tritici* infection on  
193 both local and adjacent leaf tissues, we computed 2 alpha diversity measures (Shannon Index and  
194 observed OTUs, Fig. 5a) for both wheat cultivars (Chinese Spring and Obelisk). Remarkably, we found  
195 that fungal infection significantly reduced community richness at 4 and 8 dpi-f in local leaf tissues and  
196 at 4 dpi-f in adjacent leaf tissues in Chinese Spring. In contrast, *Z. tritici* infection had no significant  
197 effect on the community composition of either local or adjacent leaf areas in the susceptible cultivar  
198 Obelisk (Fig. 5a). To further analyze how infection by *Z. tritici* altered the leaf bacterial community  
199 structure, we computed Bray-Curtis distances between samples and applied principal coordinates  
200 analysis (Fig. 5b, Fig. S10). As expected, *Z. tritici* treatment induced a shift in the community structure  
201 of both leaf tissues in Chinese Spring (Fig. 5b, Table S4) that is mainly explained by the depletion of  
202 several Actinobacteria and Proteobacteria OTUs at 4 dpi-f in local leaf tissue and the enrichment of  
203 diverse bacterial OTUs at 8 dpi-f (Fig. 5c). Although the infection had no significant effect on the  
204 bacterial community composition of Obelisk leaves (Fig. 5a), it induced a significant shift in the  
205 community structure of both local and adjacent leaf tissues of Obelisk at 4 dpi-f (Fig. S10, Table S4).  
206 These data indicate that leaf-associated bacteria are strongly altered by *Z. tritici* infection in a resistant

207 wheat cultivar at early time points. We speculate that this is an indirect effect of upregulated immune-  
208 related metabolites and reflect SAR in the plant.

209

### 210 **Systemic induced susceptibility may promote *Z. tritici* dissemination**

211 The increased growth of different *P. syringae* pathovars provides evidence for highly efficient immune  
212 suppression of *Z. tritici* during biotrophic colonization, an effect that spreads into systemic plant tissues  
213 (Fig. 2). We considered the biological relevance of this phenomenon, because bacterial proliferation  
214 also may imply competitors in the wheat tissues. One possible scenario is that *Z. tritici* induces SIS to  
215 facilitate systemic infection by new *Z. tritici* spores. *Z. tritici* is propagated both sexually and asexually  
216 during the growing season. The asexually produced pycnidiospores are splash-dispersed upwards from  
217 leaf to leaf<sup>34</sup>. We have previously shown that *Z. tritici* isolates can differ dramatically in the timing of  
218 disease development, not only between isolates but also between spores of the same cultivar<sup>17</sup>. This  
219 variability in spore germination and host penetration may be adaptive if early colonizers can facilitate  
220 the infection of late colonizers by suppressing immune responses in systemic leaf areas.

221 To test this hypothesis, we set up an experiment in which we coinfecting the systemic third leaves of  
222 Obelisk seedlings with *Z. tritici* spores 4 days after *Z. tritici* infection on the second leaf (Fig. S3d). In  
223 addition to increased bacterial growth, we expected to see the same effect on *Z. tritici* development  
224 on the third leaf. We quantified the fungal biomass in the systemic leaf over a time course of 16 days  
225 using a qPCR assay because fungal biomass correlates with fungal success. Additionally, we quantified  
226 the development of necrosis and pycnidia production in both the second and third leaves. Mock-  
227 infected second leaves served as the control for SIS. Visible symptom development on the third leaf  
228 started from 10 dpi onwards, but varied strongly between replicates (Fig. S11-13). The development  
229 of necrosis appeared to be independent of the treatment on the second leaf (Fig. 6a). Regarding  
230 pycnidia formation and fungal biomass on 16 dpi, fungal success on the systemic leaf was increased  
231 with infection on the second leaf at 16 dpi (Fig. 6b,c).

232

### 233 **Discussion**

234 SAR is known to be a central component of the plant immune system<sup>35-37</sup>. The spread of immune  
235 signals from the site of infection acts to prime systemic plant parts against further pathogen infection.  
236 In this study, we demonstrated for the first time that systemic immune signaling can confer increased  
237 susceptibility in systemic tissues of plants infected by virulent pathogens. We termed this new  
238 phenomenon systemic induced susceptibility, or SIS.

239 In our studies, we have shown increased growth of nonadapted *P. syringae* bacteria in the wheat  
240 cultivar Obelisk during infection with the fungal pathogen *Z. tritici* (Fig. 2a-d). Obelisk is susceptible to  
241 the *Z. tritici* isolate IPO323, and biotrophic phase invasion is followed by necrosis and asexual

242 sporulation<sup>17</sup>. We hypothesized that the increased bacterial growth in leaf tissues local and adjacent  
243 to fungal infection is conferred by the efficient suppression of immune responses by *Z. tritici*-produced  
244 effectors. Infection with heat-killed fungal spores provides further evidence for active immune  
245 suppression by fungal effectors in Obelisk, as the presence of denatured fungal elicitors from heat-  
246 killed spores was not sufficient to trigger SIS in this cultivar. *P. syringae* proliferate to the same extent  
247 in Obelisk when treated with heat-killed fungal spores as when mock-treated (Fig. 2b,c). In contrast,  
248 we found evidence for *Z. tritici*-induced SAR in the resistant wheat cultivar Chinese Spring; *P. syringae*  
249 bacteria grew less in leaf tissues local and adjacent to fungal infection when leaves were treated with  
250 *Z. tritici* spores. (Fig. 2b,c).

251 Our hypothesis that fungal effector proteins confer SIS in Obelisk was further supported by the  
252 observation that the bacterial T3SS mutant strain *Pst hrcC*- grows like the wild-type *Pst* strain when  
253 coinfecting with IPO323. The T3SS mutant is unable to secrete effectors and, in our experiment, relies  
254 on the immune suppression conferred by *Z. tritici* to grow (Fig. S5). The increased growth of *Pst hrcC*  
255 in *Z. tritici*-infected Obelisk suggests that the fungal effectors partially replace the function of the  
256 absent *P. syringae* effectors and promote bacterial colonization. We also show that the effect of SIS is  
257 limited to the biotrophic infection phase of *Z. tritici*, as effects are reduced during later infection stages  
258 (Fig. S4). Effector candidate genes of plant pathogens like *Z. tritici* show a dynamic expression pattern  
259 during the development of infection<sup>3,17,38</sup>. Therefore, the expression of effectors conferring SIS may be  
260 downregulated as the fungus develops into a necrotrophic lifestyle. In addition to fungal effectors, SIS  
261 could also be caused by yet to be identified fungal non-proteinogenic toxins with similar effects on  
262 plant immunity. Because we identified the induced susceptibility effect in adjacent and systemic  
263 tissues, we excluded a nutritional effect of the fungus on bacterial performance, which would be  
264 limited to the infection site.

265 We investigated the underlying metabolic components of SIS. We compared local and systemic  
266 responses in wheat cultivars resistant and susceptible to fungal infection. We applied a suspected  
267 targeted metabolomics approach using FT-ICR-MS. Due to the ultrahigh resolution and the high  
268 sensitivity of this method, we identified a wide range of immune-related plant compounds. For the  
269 first time, we have provided an overview of plant compounds and secondary metabolite pathways that  
270 are affected by *Z. tritici* infection in 2 wheat cultivars of differing susceptibility. We identified a variety  
271 of immune-related and antimicrobial plant compounds differentially produced in the resistant cultivar  
272 Chinese Spring, notably in local tissues after fungal infection. For example, we detected a salicylate  
273 conjugate at early stages after fungal infection specifically in the resistant cultivar (Fig. S8d). Salicylic  
274 acid regulates local defense responses against biotrophic pathogens and is a crucial component of SAR  
275 in both monocot and dicot plants<sup>35-37,39</sup>. The observed increase of salicylate conjugate levels confirms  
276 the local and systemic activation of defense responses against a biotrophic pathogen in Chinese Spring.



277 The phenylpropanoid pathway acts as a central hub for the biosynthesis of many immune-related  
278 compounds. In Chinese Spring, we found increased levels of several lignin precursors, such as alcohols  
279 of (hydroxy)cinnamic acids and diferulic acid (Fig. 4b, Fig. S8d). These phenolic compounds are well  
280 known for their antimicrobial and antioxidant properties and support cell wall reinforcement after  
281 pathogen infection<sup>40–43</sup>. Similarly, flavonoid compounds as gallicocatechin and kaempferol rhamnoside  
282 accumulate mainly in the resistant cultivar and are known to act as antimicrobials and  
283 antioxidants<sup>23,33,44,45</sup>. Accumulation of HCAAs is associated with resistance to various filamentous plant  
284 pathogens<sup>21,22,25,46</sup>. Interestingly, these compounds accumulated in leaf tissues adjacent to infection in  
285 the susceptible cultivar Obelisk at early stages of infection, whereas HCAAs were mainly increased  
286 locally in the Chinese Spring cultivar (Fig. 4b). Accumulation of HCAAs in adjacent tissues of Obelisk but  
287 not Chinese Spring might point to an overreaction of the HCAA pathway in the susceptible cultivar due  
288 to local *Z. tritici*-mediated inhibition of this pathway.

289 A second important pathway activated during fungal infection in many *Poaceae* species is BX  
290 biosynthesis<sup>30</sup>. BXs are released from constitutively stored precursors following microbial invasion  
291 attempts. The antimicrobial effect of BXs, such as DIMBOA, has been described previously in maize,  
292 wheat, and other grasses<sup>20,29,47</sup>. In contrast to free BXs, BX glycosides represent inactive storage forms.  
293 Accumulation of BX glycosides in Chinese Spring tissues adjacent to infection (ie, not directly  
294 challenged by *Z. tritici*) (Fig. 3d) may reflect priming of the immune system in the resistant cultivar for  
295 further fungal infection<sup>48,49</sup>. Interestingly, BX biosynthesis is blocked at an early stage in Obelisk leaf  
296 tissues both local and adjacent to infection (Fig. 3b-d), which might contribute to the virulence of *Z.*  
297 *tritici* in this cultivar. We speculate that microbial manipulation of plant biosynthetic pathways and  
298 antimicrobial plant compounds contributes largely to fungal success in leaf tissues. In maize kernels,  
299 the virulence gene FUG1 of *Fusarium verticillioides* has an impact on DIMBOA biosynthesis<sup>50</sup>, and many  
300 effector candidate genes are upregulated during early stages of *Z. tritici* infection<sup>17,38</sup>. Using our  
301 metabolomics approach, we may be able to predict steps in metabolic pathways that are potentially  
302 manipulated by such fungal effectors. This also suggests a large diversity in the effector repertoire and  
303 potential redundancy in immune manipulation.

304 Plants utilize an extensive spectrum of secondary metabolites to defend against attacking pathogens.  
305 Depending on the individual properties of such compounds, plants may be indirectly protected against  
306 other pathogens. However, these compounds may also influence the composition of other microbes  
307 systemically. For example, antimicrobial DIMBOA can impact the composition of the maize rhizosphere  
308 microbiome<sup>51</sup>. DIMBOA attracts *Pseudomonas putida* (which is beneficial for the plant) to the maize  
309 rhizosphere, which shows increased tolerance towards the antimicrobial compound. In our study, we  
310 observed a strong shift in the microbial communities in the Chinese Spring cultivar (Fig. 5a,b). Because  
311 growth of *P. syringae* bacteria was reduced in Chinese Spring during *Z. tritici* infection, it is likely that

312 other bacteria are impacted by SAR; most OTUs have reduced abundance in Chinese Spring during  
313 early *Z. tritici* infection (Fig. 5c). At later times, this effect is reversed. We assume that the persistent  
314 microbial taxa can tolerate the accumulating immune-related compounds in Chinese Spring as shown  
315 for *P. putida* in the maize rhizosphere<sup>51</sup>.

316 Systemic changes in rhizosphere community structure due to activation of plant immune responses  
317 are not a new phenomenon<sup>52,53</sup>. Plants are thought to respond to an infection, such as through the  
318 recruitment of beneficial microbes from the soil to increase resistance<sup>54,55</sup>. Our unique approach of  
319 combining coinfection studies with microbiome analysis, points to an active role of pathogens in  
320 manipulating the microbiome in a susceptible host. Experimental validation of future studies (eg, using  
321 fungal effector mutant strains) will confirm the direct or indirect role of *Z. tritici* on microbial  
322 community structure.

323 Finally, we considered the biological relevance of the observed SIS. Increased proliferation of other  
324 microbes could eventually be harmful to *Z. tritici* by increased competition in the phyllosphere  
325 environment. However, induced susceptibility in systemic leaf tissues could be a mechanism to  
326 promote infection by new *Z. tritici* spores. To test this hypothesis, we designed an experiment based  
327 on coinfection of asexual spores on different leaves of wheat seedlings (Fig. 6). While we did not  
328 observe a major effect, our results indicated that infection of the second leaf increases the efficiency  
329 of subsequent fungal infection of the third leaf. We selected timepoints for analysis that corresponded  
330 to those used for bacterial coinfection. However, it is possible that these timepoints reflect minor  
331 effects of fungal coinfection. *Z. tritici* infection is a continuous rather than synchronous process. *Z.*  
332 *tritici* can persist as spore and hyphae on the leaf surface for up to 10 days before penetrating through  
333 stomata<sup>56</sup>. In addition, *Z. tritici* isolates display high variability in disease development<sup>17</sup>. Because  
334 infection in the field typically involves multiple *Z. tritici* strains,<sup>57,58</sup> strains infecting later could mediate  
335 SIS in favor of already released pycnidiospores of fast colonizers. This would benefit the subsequent  
336 generation of fast-maturing pycnidiospores during the development of individual plants<sup>34</sup>. In addition  
337 to the production of asexual pycnidiospores, SIS may also promote sexual mating. *Z. tritici* has a  
338 heterothallic mating system, but both mating types (Mat1-1 and Mat1-2) can act as either male or  
339 female in sexual crosses. Previous studies demonstrated that avirulent *Z. tritici* isolates can mate with  
340 virulent strains but the avirulent strain will exclusively act as the male partner<sup>59</sup>. SIS may promote the  
341 coexistence of avirulent and virulent mating-compatible strains on the same leaf and thereby play a  
342 role in the outcrossing efficiency of *Z. tritici*.

343 Our findings emphasize the relevance of systemic dynamics that take place at various levels during  
344 plant infection. These dynamics include effects on the plant metabolome, but also organismic  
345 interactions at the microbial community level. Thus, further knowledge is needed to understand the  
346 interaction of the plant metabolome with the plant microbiome. Progress in this field is crucial for the

347 development of future crop protection strategies based on plant probiotics and plant health-  
348 promoting microbes.

349

## 350 **Material and Methods**

### 351 **Wheat infection assays**

352 *Triticum aestivum* cultivars Obelisk (obtained from Wiersum Plant Breeding BV, the Netherlands) and  
353 Chinese Spring (kindly provided by Bruce McDonald, ETH Zurich, Switzerland) were used for all  
354 infection experiments. Plants were grown in phytochambers on peat under constant conditions  
355 (16/8 hr light (~200  $\mu\text{mol}/\text{m}^2/\text{s}$ )/dark cycle, 20°C, 90% relative humidity).

356 The *Z. tritici* isolate IPO323 (kindly provided by Gert Kema, Wageningen University, the Netherlands)  
357 was used in all fungal infection experiments. Plants were infected with a spore concentration of  $1 \times$   
358  $10^7$  cells/mL as previously described<sup>17</sup>. For the systemic fungal coinfection experiment, local infection  
359 with *Z. tritici* on the second leaf was followed by coinfection with the same strain on the third leaf 4  
360 days after initial infection of the second leaf. The second and third leaves were harvested at the  
361 indicated timepoints. For further information on quantification of necrosis, pycnidia formation, and  
362 fungal biomass and detection of hydrogen peroxide, please see the Supplemental Methods.

363 The rifampicin-resistant *P. syringae* strains *Por36\_1rif* (kindly provided by Henk-Jan Schoonbeek, John  
364 Innes Centre, UK), *Pst DC3000*, *Pst DC3000 hrcC-*, and *Pma ES4326* (kindly provided by Tiziana Guerra,  
365 Leibniz-Institut für Gemüse- und Zierpflanzenbau, Germany) were used for bacterial (co)infection  
366 experiments. Analysis of *in planta* growth of *P. syringae* in wheat was adapted from Schoonbeek et  
367 al<sup>60</sup>. For wheat inoculation, bacterial suspension ( $\text{OD}_{600 \text{ nm}} = 0.02$ ) or control treatment was applied to  
368 the second leaf of 15-day-old wheat plants. The plants were sealed in a plastic bag and incubated for  
369 4 days in phytochambers. Bacterial growth was assessed by counting bacteria after extraction and  
370 serial dilution. A detailed description of bacterial infection and quantification of bacterial growth can  
371 be found in the Supplemental Methods. For local bacterial coinfection experiments, the area for  
372 bacterial infection was the same as the area previously infected with IPO323. For adjacent bacterial  
373 coinfection experiments, the labeled area for bacterial infection was separated from the fungal  
374 infection area by a 1-cm buffer zone (Fig. S14). Systemic bacterial coinfection took place on the third  
375 leaf while fungal spores were applied to the second leaf. If not stated otherwise, fungal infection was  
376 done 4 days prior to the bacterial infection.

377

### 378 **Metabolomics profiling**

379 Leaf material (6-cm leaf area) was harvested at 4 and 8 dpi-f, weighed, deep-frozen, and then extracted  
380 with methanol/water (80/20, v/v). For cell distribution, a Precellys Tissue Homogenizer was used. For

381 extraction and suspensions, ultrapure LC-MS solvents and water were used. After extraction, all  
382 samples were stored at -80°C until measurement.

383 An FT-ICR-MS (7 Tesla, SolarixR, Bruker, Bremen, Germany) was used in the flow-injection mode (a  
384 HPLC 1260 Infinity from Agilent (Waldbronn, Germany) was used). Water/methanol (50/50, v/v) with  
385 0.1% acetic acid was used as transport eluent. The samples were ionized with an electrospray  
386 ionization source (both modes) and with 2 methods, so the detection range was from 65 to 950 Da.  
387 The average resolution at 400 m/z was 600,000. The main instrument parameters were dry gas  
388 temperature (nitrogen) of 200°C at 4 L/min; nebulizer 1 bar; time-of-flight time section 0.35 ms and  
389 quadrupole mass 150 m/z with an RF frequency 2 MHz; and detector sweep excitation power of 18%.  
390 Data evaluation was conducted with DataAnalysis 5.0 and MetaboScape 4.0, both from Bruker  
391 (Bremen, Germany). Sum formulas were calculated based on the mass error and isotopic fine  
392 structure. To reduce false-positive results, the 7 golden rules of Kind and Fiehn were used<sup>61</sup>. In addition,  
393 annotation was conducted with customized databases by suspected targeted methods. The database  
394 was created based on 15 plant-related pathways from KEGG<sup>62-64</sup>, such as general secondary plant  
395 metabolism or phenylpropanoid biosynthesis (Table S5).

396 All m/z values with assigned sum formulas were exported and further processed with the statistical  
397 software R (version 3.4.2). For each mode (SM/UM, positive/negative), measurements of m/z values  
398 assigned to the same sum formula were added, and metabolites with measurements in fewer than 3  
399 samples were removed. To create a single metabolomics dataset, metabolite data of the 4 modes were  
400 merged. For metabolites that were available in several modes, the one with the smallest number of  
401 missing values, or if ambiguous the largest median, was kept. Metabolite measurements were  $\log_2$   
402 transformed, and missing values were replaced by the limit of detection ( $= \log_2(10^6)$ ).

403 Metabolite measurements were compared separately between breeds, positions, and treatments, and  
404 each comparison was stratified by the 3 remaining factors (including day). A linear regression model  
405 was fit for each metabolite separately, and *P* values were corrected for multiple testing using the  
406 Benjamini-Hochberg procedure<sup>65</sup>.

407

#### 408 **Microbiota profiling**

409 Seeds of Chinese Spring and Obelisk were washed 3 times and germinated on filter paper prior to  
410 sowing in pots containing peat inoculated with soil slurry (for details, see Supplemental Methods).  
411 Plant growth and fungal infection were carried as described above. Leaf material (6-cm leaf area for  
412 both local and adjacent tissue) was harvested at 0, 4, and 8 dpi-f. Each leaf sample was washed 3 times  
413 and stored at -80°C for downstream processing. For DNA extractions, samples were homogenized and  
414 pretreated with lysozyme and proteinase K for 5 min at room temperature. DNA was extracted

415 according to the manufacturer's protocol and stored at -20°C for MiSeq library preparation. Detailed  
416 information on sample preparation and DNA extraction can be found in the Supplemental Methods.  
417 The 16S rRNA gene DNA library for Illumina sequencing was prepared through a 2-step PCR-  
418 amplification protocol. The 16S rDNA regions were PCR-amplified in triplicate using the primer set  
419 forward 799F (AACMGGATTAGATACCKG) and reverse 1193R (ACGTCATCCCCACCTCC)<sup>63</sup>. To block plant  
420 mitochondrial DNA, 10 times the volume 799F or 1193R of the blocking primer  
421 (GGCAAGTGTCTTCGGA/3SpC3/) was added to each reaction. Technical replicates were pooled, and  
422 leftover primers were enzymatically digested. Amplicons from the first reaction were PCR-barcoded  
423 over 10 cycles using reverse Illumina compatible primers (B1 to B120). The PCR replicates from the  
424 second reaction were pooled, cleaned, and extracted from agarose gel plugs. PCR products were  
425 quantified and pooled. The library was cleaned twice with the AgencourtAMPure XP Kit (Beckman  
426 Coulter, Germany) and submitted for DNA sequencing using the MiSeq Reagent kit v3 with the 2× 300  
427 bp paired-end sequencing protocol (Illumina Inc., USA). A detailed description of the library  
428 preparation procedure is included in the Supplemental Methods.  
429 The forward and reverse sequencing reads were joined and demultiplexed using Qiime2 pipeline  
430 (2018.2.0)<sup>66</sup>. PhiX and chimeric sequences were filtered using Qiime2-DADA2<sup>67</sup>. All scripts used for read  
431 preprocessing are available at [https://github.com/hmamine/raw\\_data\\_preprocessing](https://github.com/hmamine/raw_data_preprocessing). For alpha  
432 diversity analyses, count reads were rarefied to an even sequencing depth based on the smallest  
433 sample size of 601 reads using the R package "phyloseq"<sup>68</sup>. For community structure analyses, count  
434 reads were normalized by cumulative sum scaling normalization factors<sup>69</sup> prior to computing Bray-  
435 Curtis distances between all samples. To test for significantly enriched OTUs, the data were fitted to a  
436 zero-inflated Gaussian mixture model<sup>69</sup>, and OTUs with Benjamini-Hochberg<sup>65</sup> adjusted  $P < .05$  were  
437 displayed. R scripts used for the described analyses are accessible at  
438 [https://github.com/hmamine/ZIHJE/community\\_analysis](https://github.com/hmamine/ZIHJE/community_analysis), and raw data are deposited in  
439 <http://www.ncbi.nlm.nih.gov/bioproject/549447>.

440

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447

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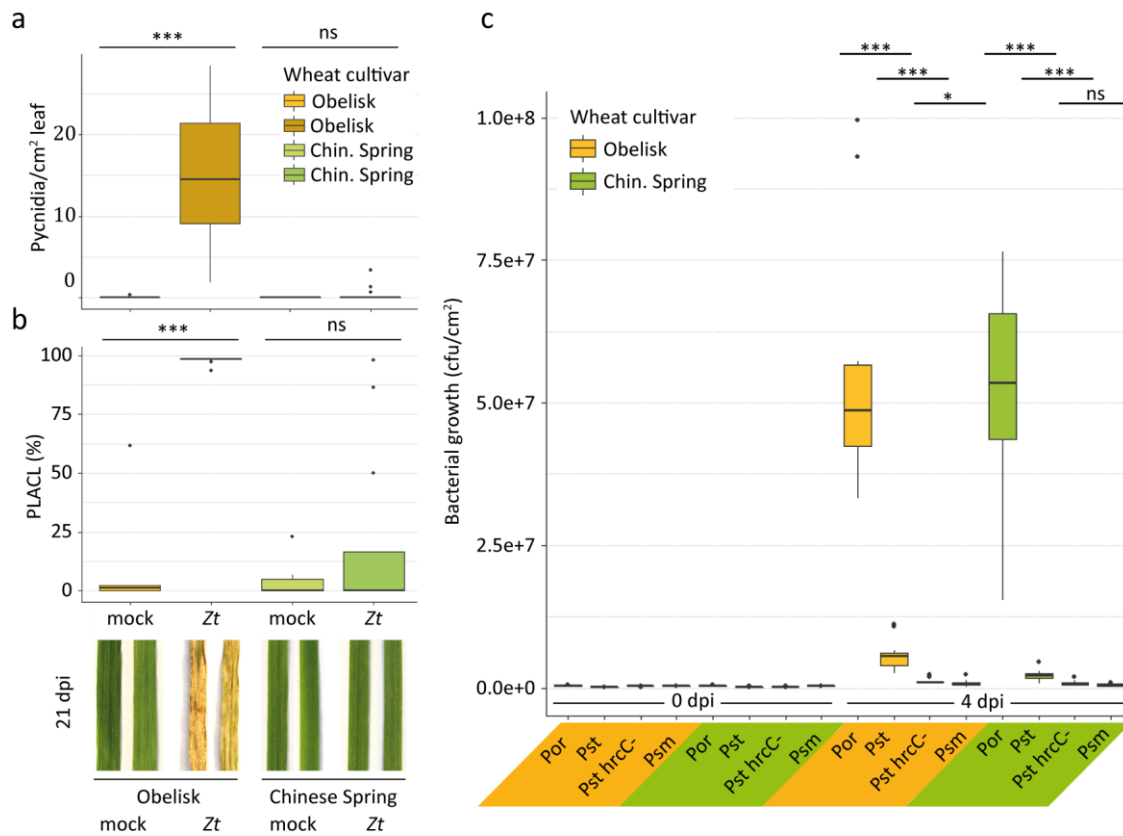
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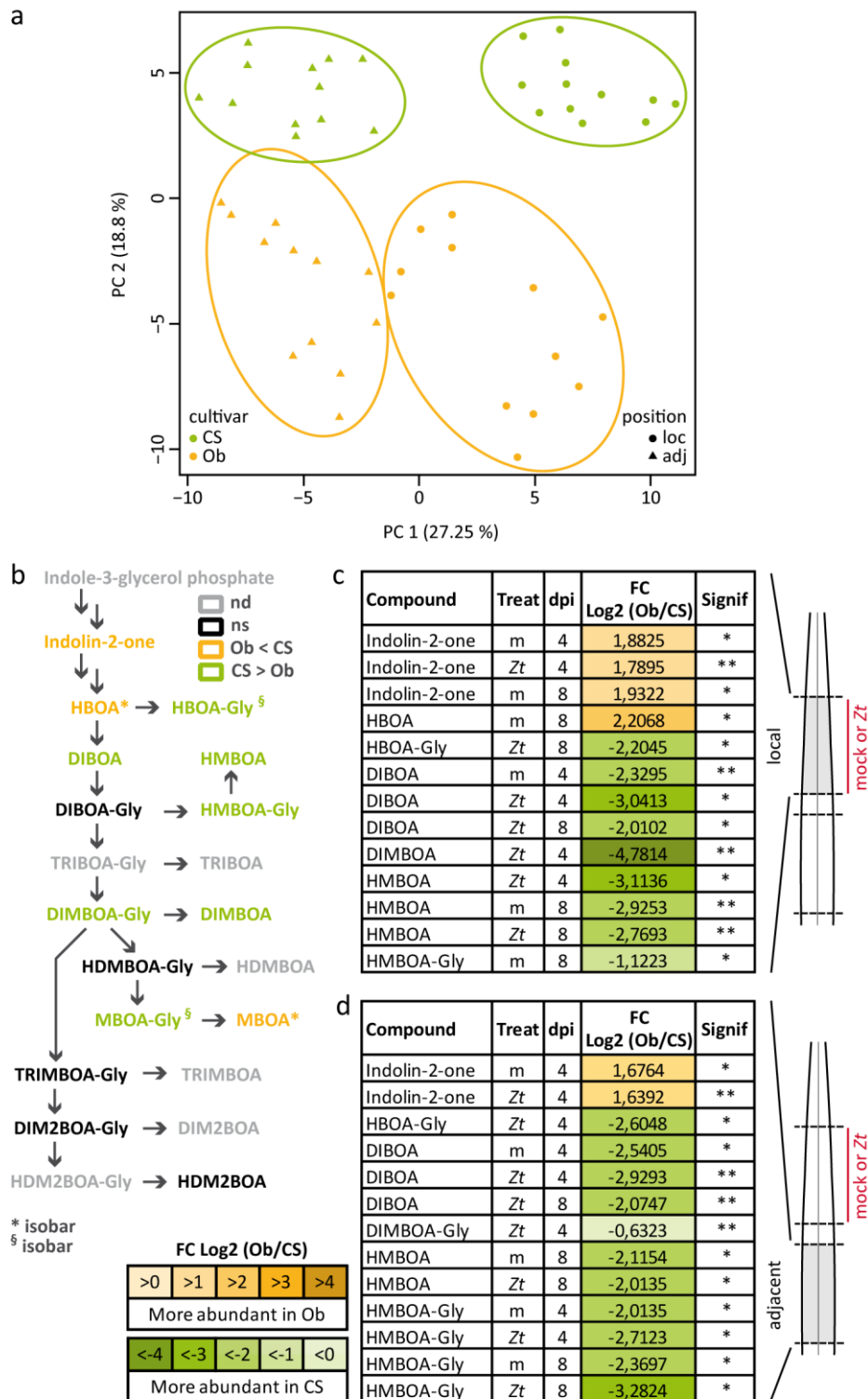
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614 **Fig. 1 | Contrasting resistance phenotype of wheat against *Z. tritici* but not *P. syringae***

615 **a.** Number of *Z. tritici* (Zt) IPO323 pycnidia per cm<sup>2</sup> leaf of wheat cultivar Obelisk (orange/brown) and  
 616 cultivar Chinese Spring (green) at 21 dpi-f. **b.** Percentage of leaf area covered by lesions (PLACL) as in  
 617 **a** and representative leaf phenotypes at 21 dpi-f. **c.** Bacterial growth of *P. syringae* pv. *oryzae* (Por)/  
 618 pv. *tomato* (Pst)/ pv. *maculicola* (Psm) at 0 days post bacterial infection (dpi-b) and 4 dpi-b in wheat  
 619 cultivars as in **a**. Statistical analysis was performed using a Shapiro-Wilk test of normality followed by  
 620 a Wilcoxon rank-sum test of null hypothesis. \**P*<.05; \*\*\**P*<.001. Number of biologically independent  
 621 replicates: **a-b**, Ob/CS mock (n=6), Ob Zt (n=14), CS Zt (n=16); **c**, 0 dpi-b (n=3), 4 dpi-b (n=9).





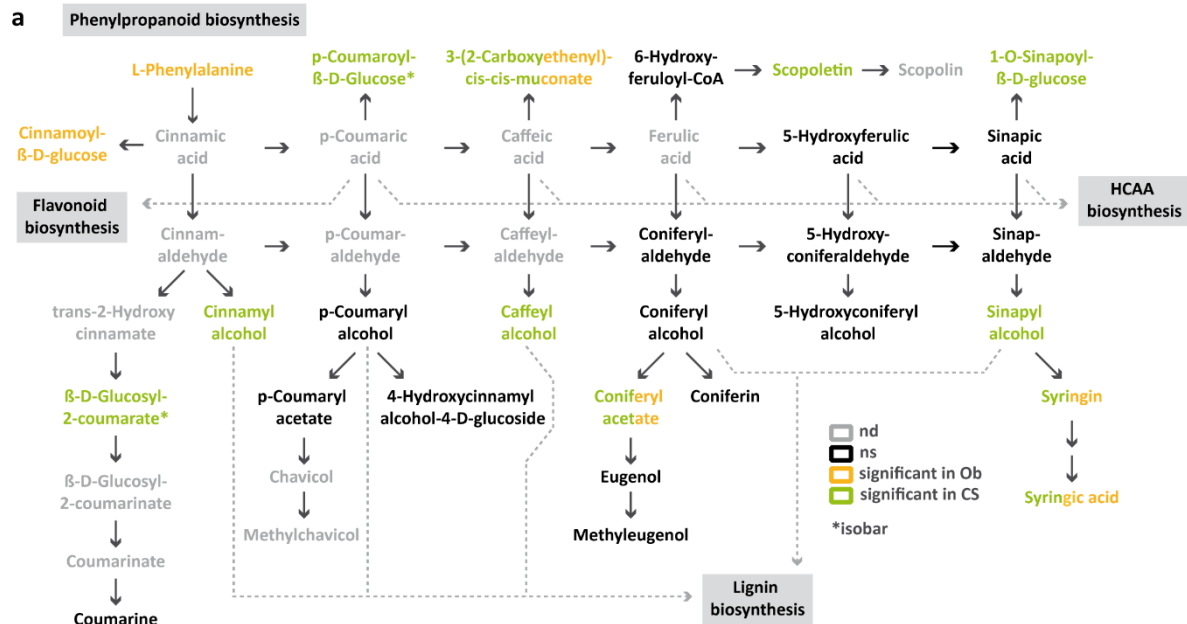
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641 **Fig. 3 | Biosynthesis of benzoxazinoids is altered locally and in adjacent tissues upon infection with**  
 642 ***Z. tritici***

643 **a.** Principle component analysis (PCA) based on the complete metabolomics dataset. Samples are  
 644 colored according to cultivar, and shapes refer to the position in the leaf. **b.** Biosynthesis pathway of  
 645 benzoxazinoids. Compounds with significant differences in the comparison between cultivars are  
 646 highlighted in orange (Obelisk) or green (Chinese Spring). nd = not detected, ns = not significant. **c.**

647 Compounds of the biosynthesis pathway of benzoxazinoids with significant differences at local site of  
648 inoculation with *Z. tritici* (Zt) IPO323 or mock (m) at 4 and 8 dpi-f between wheat cultivars Obelisk and  
649 Chinese Spring. **d.** Significantly different compounds in the benzoxazinoid biosynthesis pathway at an  
650 adjacent site to the inoculation in **c.** Number of biologically independent replicates: n=3. For details on  
651 the statistical analysis of the metabolomics dataset, please see the methods section. \* $P < .05$ ; \*\* $P < .01$ ;  
652 \*\*\* $P < .001$ .



**b**

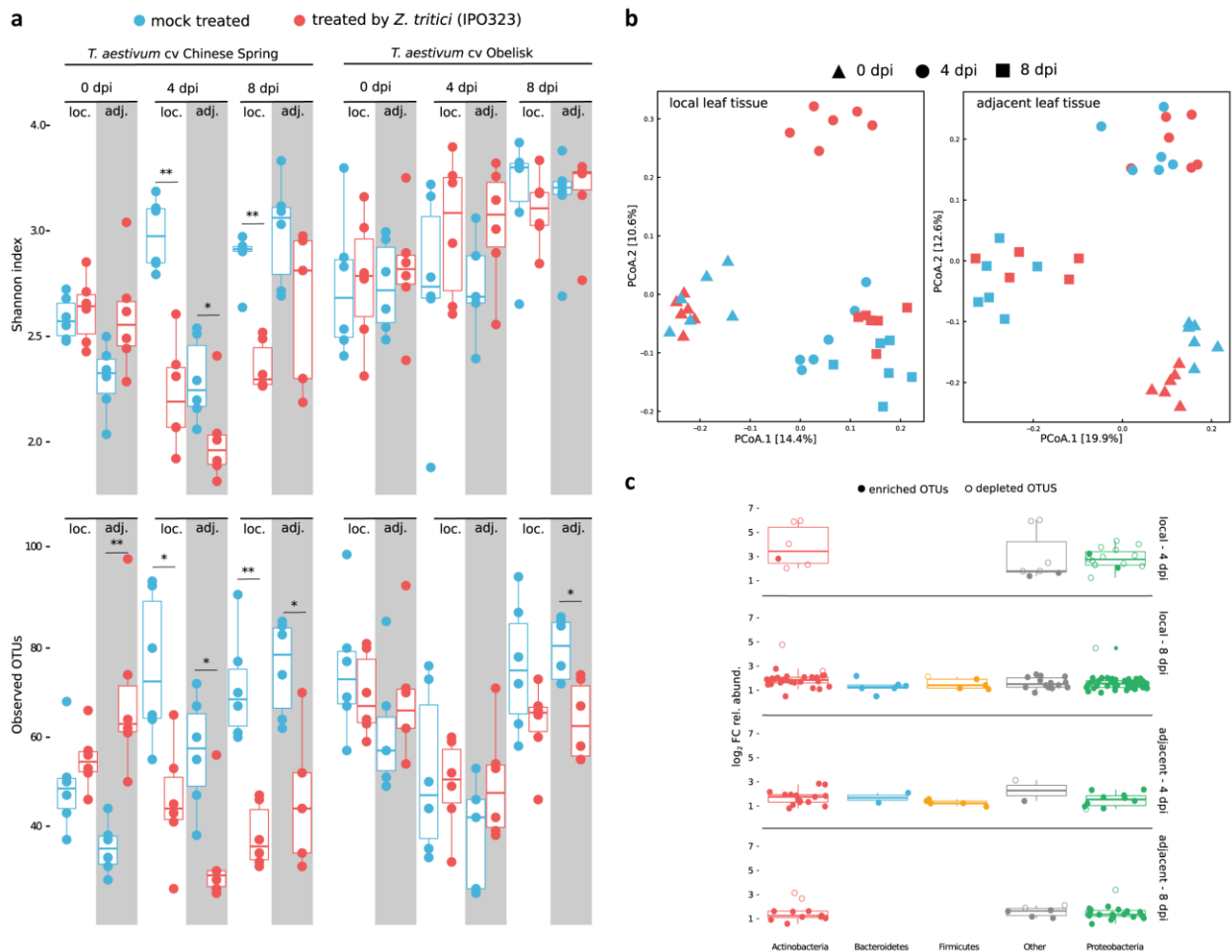
Phenylpropanoid biosynthesis	4dpi		8dpi	
	local	adjacent	local	adjacent
L-Phenylalanine	ns	ns	ns	adj_Ob-m***
Cinnamoyl-β-D-glucose	ns	ns	loc_Ob-Zt*	ns
1-O-Sinapoyl-β-D-glucose	ns	m_CS-adj*, CS_m-adj** adj_CS-m**	ns	ns
β-D-Glucosyl-2-coumarate	CS_m-loc*	CS_m-adj*, CS_Zt-adj*	CS_m-loc*	CS_Zt-adj*
Cinnamyl alcohol	CS_m-loc***, CS_Zt-loc***	CS_m-adj***, CS_Zt-adj***	CS_m-loc***, CS_Zt-loc***	CS_m-adj***, CS_Zt-adj*** adj_CS-m*
Caffeyl alcohol	nd	nd	ns	CS_Zt-adj*
Sinapyl alcohol	ns	ns	ns	CS_Zt-adj*
3-(2-Carboxyethenyl)-cis-cis-muconate	Ob_Zt-loc***	adj_CS-Zt***	ns	ns
Coniferyl acetate	ns	ns	CS_m-loc**, CS_Zt-loc**	adj_Ob-m**
Scopoletin	ns	ns	ns	adj_CS-m*
Syringin	ns	ns	loc_CS-m* loc_CS-Zt**	loc_Ob-m** loc_Ob-Zt**
Syringic acid	ns	ns	loc_CS-Zt*	CS_m-adj** Zt_Ob-adj**
<b>Flavonoid biosynthesis</b>	<b>local</b>	<b>adjacent</b>	<b>local</b>	<b>adjacent</b>
Flavonol	ns	ns	CS_m-loc**, CS_Zt-loc**	adj_Ob-m**
(+)-Gallicocatechin	CS_m-loc***, CS_Zt-loc**	CS_m-adj***, CS_Zt-adj***	CS_m-loc***, CS_Zt-loc**	CS_m-adj***, CS_Zt-adj**
4'-O-Methylneobavaisoflavone 7-O-(2''-p-coumaroyl)glucoside	ns	adj_CS-Zt***	nd	nd
5-Hydroxy-6,7,3',4',5'-pentamethoxy- flavanone 5-O-rhamnoside	nd	nd	ns	adj_CS-m** adj_Ob-m**
Kaempferol 3-rhamnoside- (1->2)-rhamnoside	ns	ns	loc_CS-Zt* CS_m-loc*	loc_Ob-m*
Kaempferol 3-glucoside- 3-rhamnoside	loc_CS-Zt* loc_Ob-Zt**	ns	loc_CS-m*, loc_CS-Zt**	ns
Kaempferol 7-rhamnoside	loc_CS-Zt*	ns	ns	ns
Neocarlinoside	Ob_Zt-loc***, Ob_m-loc*	Ob_m-adj*	Ob_m-loc*	Ob_Zt-adj*, Ob_m-adj*
4,2',3',4'-Tetrahydrochalcone 4'-O-(2''-O-p-coumaroyl) glucoside	nd	nd	ns	Ob_Zt-adj**
Aureusidin 6-O-glucoside	Ob_Zt-loc*	ns	ns	ns
<b>HCAA biosynthesis</b>	<b>local</b>	<b>adjacent</b>	<b>local</b>	<b>adjacent</b>
Coumaroylhydroxyagmatine	loc_CS-Zt*	Ob_Zt-adj*	loc_CS-Zt* Zt_Ob-loc*	ns
N-Coumaroylserotonin	ns	ns	Ob_m-loc*	ns
N-Caffeoylputrescine	ns	ns	loc_CS-Zt**	ns
Feruloylagmatine	ns	Zt_Ob-adj*	nd	nd
Feruloylhydroxyagmatine	loc_CS-Zt*	Ob_Zt-adj*	loc_CS-Zt**	ns
Feruloylputrescine	ns	Ob_Zt-adj*	loc_CS-Zt*	ns
Sinapoylagmatine	loc_CS-Zt**	Ob_Zt-adj*, Zt_Ob-adj*	loc_CS-Zt*	ns
Hydroxy-hordatine A	loc_CS-m* m_CS-loc*	ns	nd	nd
Hordatine D + 2 hex	loc_CS-Zt**	ns	nd	nd

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655 **Fig. 4 | Phenylpropanoids and branching pathways are differentially regulated in *Z. tritici*-resistant**  
656 **and *Z. tritici*-susceptible wheat cultivars**

657 **a.** Biosynthesis pathway of phenylpropanoids and branching biosynthesis pathways (modified from  
658 KEGG map00940). Compounds with significant differences in the comparison between treatments,  
659 cultivars, or leaf positions are highlighted in orange (Obelisk), green (Chinese Spring), or green/orange  
660 (Chinese Spring and Obelisk), respectively. nd = not detected, ns = not significant, Ob = Obelisk, CS =  
661 Chinese Spring. **b.** Detailed information on metabolites with significant differences as highlighted in **a**  
662 and metabolites with significant differences from branching biosynthesis pathways. DAMs from the  
663 comparison “treatment” are named “m\_” or “Zt\_”, DAMs from the comparison “cultivar” are named  
664 “Ob\_” or “CS\_”, DAMs from the comparison “position” are named “loc\_” or “adj\_”. Details on the  
665 labelling can be found in Fig. S8c. Number of biologically independent replicates: n=3. For details on  
666 the statistical analysis of the metabolomics dataset, please see the methods section. \* $P < .05$ ; \*\* $P < .01$ ;  
667 \*\*\* $P < .001$ .

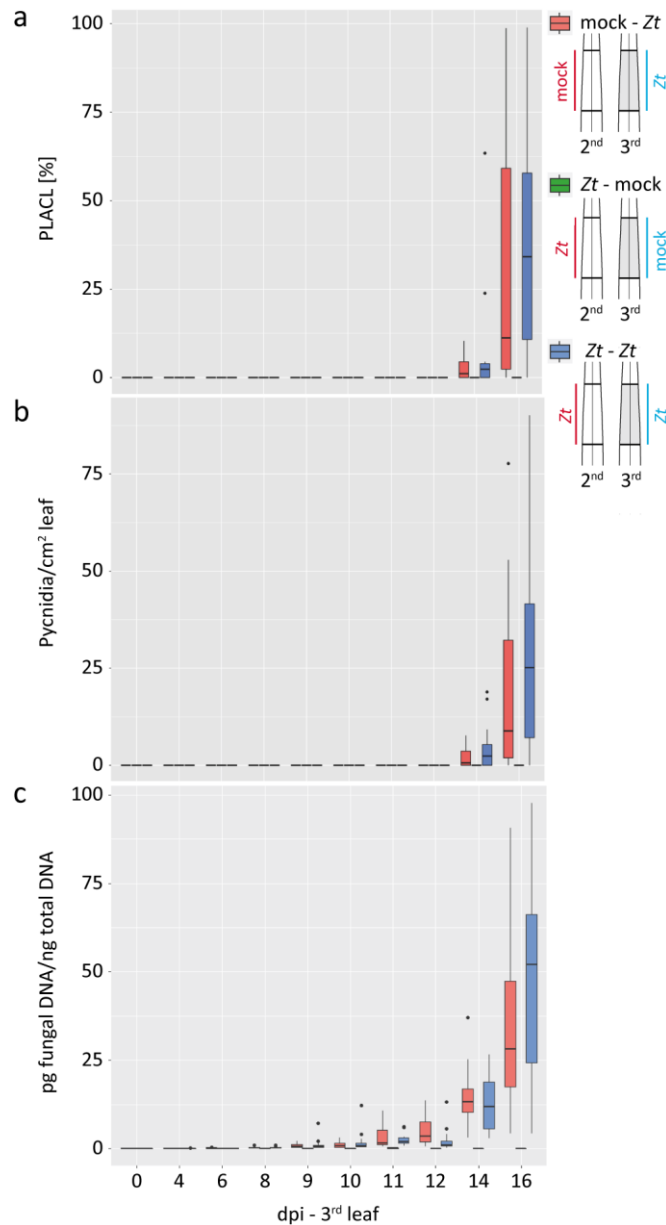


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670 **Fig. 5 | Infection by *Z. tritici* alters the wheat microbiota in local and adjacent leaf tissues**

671 **a.** Two measures of the community composition, Shannon index and observed OTUs, are depicted in  
672 upper and lower panels, respectively. Reads were rarefied to an even sequencing depth corresponding  
673 to the smallest simple size of 601 reads. Community composition in leaf samples treated by *Z. tritici*  
674 (red boxplots) were compared to mock treatment (blue boxplots) for each corresponding time point  
675 (ie, 0, 4, and 8 dpi-f) and leaf tissue types, local (loc.) and adjacent (adj.). Infection by *Z. tritici* leads to  
676 a significant drop in the computed Shannon indexes and observed OTUs in both types of leaf tissues  
677 (local and adjacent). Testing for significance was performed using a Kruskal-Wallis rank sum test.  
678 \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ . **b.** Principal coordinates analysis (PCoA) computed on Bray-Curtis  
679 distances of microbial communities associated with *T. aestivum* cultivar Chinese Spring. Each shape in  
680 the plot represents the community structure of one sample. Red- and blue-colored shapes designate  
681 mock- and *Z. tritici*-treated leaf samples as in **a**. PCoA plots in the left and right panels correspond to  
682 local (left) and adjacent (right) leaf tissues, respectively. **c.** Boxplot depicting log<sub>2</sub> fold changes in the  
683 relative abundance of OTUs. Colors in the graph depict bacterial phyla. OTUs with  $P > .05$  were filtered  
684 out. Filled and unfilled circles indicate OTUs that are significantly enriched and depleted, respectively,  
685 from *Z. tritici*-treated leaf samples compared to mock-treated samples.



686

687

688 **Fig. 6 | Local infection with *Z. tritici* promotes systemic dissemination of the fungus**

689 **a.** Percentage of leaf area covered by lesions (PLACL) throughout infection of cultivar Obelisk with *Z.*  
690 *tritici* (Zt) IPO323. **b.** Number of pycnidia of Zt per cm<sup>2</sup> leaf of cultivar Obelisk as in **a.** **c.** The picogram  
691 (pg) fungal DNA/nanogram (ng) total DNA measured by quantitative real-time PCR. Statistical analysis  
692 was performed using the Shapiro-Wilk test of normality followed by a Wilcoxon rank-sum test for null  
693 hypothesis. \**P*<.05. Number of biologically independent replicates: Zt-mock (n=3), mock-Zt and Zt-Zt:  
694 0-6 dpi (n=12), 8-12 (n=12), 14-16 (n=12).