Proteomic analysis reveals how pairing of a Mycorrhizal fungus with plant growth-promoting bacteria modulates growth and defense in wheat

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Running head: AMF and PGPB modulate plant growth and defense

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Abstract

Plants rely on their microbiota for improving the nutritional status and environmental stress tolerance. Previous studies mainly focused on bipartite interactions (a plant challenged by a single microbe), while plant responses to multiple microbes have received limited attention. Here, we investigated local and systemic changes induced in wheat by two plant growth-promoting bacteria (PGPB), *Azospirillum brasilense* and *Paraburkholderia graminis*, either alone or together with an arbuscular mycorrhizal fungus (AMF). We conducted phenotypic, proteomic, and biochemical analyses to investigate bipartite (wheat–PGPB) and tripartite (wheat–PGPB–AMF) interactions, also upon a leaf pathogen infection. Results revealed that only AMF and *A. brasilense* promoted plant growth by activating photosynthesis and N assimilation which led to increased glucose and amino acid content. The

bioprotective effect of the PGPB–AMF interactions on infected wheat plants depended on the PGPB-AMF combinations, which caused specific phenotypic and proteomic responses (elicitation of defense related proteins, immune response, and jasmonic acid biosynthesis). In the whole, wheat responses strongly depended on the inoculum composition (single *vs.* multiple microbes) and the investigated organs (roots *vs.* leaf). Our findings showed that AMF is the best-performing microbe, suggesting its presence as the crucial one for synthetic microbial community development.

Keywords

Funneliformis mosseae, *Azospirillum brasilense*, *Paraburkholderia graminis*, *Xanthomonas translucens,* proteome, pathogens, bi- and tripartite interaction, wheat, growth and defense response.

Acknowledgments

V.F., D.G.S., P.B., L.M., and C.V designed the study; V.F., D.G.S., M.N., and D.G. carried out the majority of experiments; G.D., M.M., and C.V performed the bioinformatic analysis of proteomic data; C.V., V.F., P.B. L.M., F.W.-D., and M.B. interpreted the data and wrote the manuscript.

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INTRODUCTION

Like humans, animals, and fungi, plants live among a variety of microbial species, which together comprise the plant microbiota (Schlaeppi & Bulgarelli, 2015). Plant root-associated microbes have received increasing attention, starting from their taxonomic description (Bulgarelli et al., 2012; Lundberg et al., 2012) to their role in plant health (Müller, Vogel, Bai, & Vorholt, 2016). Data generated by multiple omics approaches demonstrate that the plant microbiota does not represent a random assembly of microbes living in the soil. On the contrary, plant microbiota composition is determined by an active host plant-driven selection process, which depends on the plant genotype, environmental conditions, and microbial interactions (Durán et al., 2018; Hacquard et al., 2015; Saad, Eida, & Hirt, 2020; Thiergart et al., 2019; Uroz, Courty, & Oger, 2019). The complexity of the microbial community structure parallels the many beneficial functions currently assigned to the plant

111 microbiota: stimulation of plant growth through phytohormone production,
112 improvement of the plant nutrient status through the increased uptake of
113 nutrients such as inorganic phosphate (Pi) and nitrogen (N) and increased
114 availability of nutrients such as iron, greater tolerance to abiotic stress (e.g.,
115 drought) and biotic stress, and increased activation of plant innate immunity
116 (Hacquard, Spaepen, Garrido-Oter, & Schulze-Lefert, 2017). Many of these
117 benefits have been traditionally associated with the so-called plant growth-
118 promoting bacteria (PGPB), as well as with the root symbionts, such as
119 arbuscular mycorrhizal (AM) fungi and N-fixing bacteria (Lugtenberg,
120 Caradus, & Johnson, 2016). Comparison of the data generated by culture-
121 independent approaches with the knowledge obtained from the investigation of
122 controlled binary interactions of bacterial and fungal isolates with their host
123 plant has led to the creation of the so-called synthetic communities (SynComs)
124 (Herrera Paredes et al., 2018; Tsolakidou et al., 2019). Simultaneous
125 inoculation of the host plant with several different beneficial microbes allows
126 the investigation of plant responses under controlled and reproducible
127 conditions. These new tools therefore form the basis of the so-called microbial
128 revolution, defined as the microbe-driven increase in crop productivity, leading
129 to higher sustainability (Baez-Rogelio, Morales-García, Quintero-Hernández,
130 & Muñoz-Rojas, 2017).

131 Together with rice and corn, wheat is one of the most important crops
132 worldwide (Fernie & Yan, 2019). Recently, many studies have been conducted

on wheat-associated microbes, providing a detailed list of bacteria and fungi associated with wheat plants under natural conditions (Kuźniar et al., 2020; Mahoney, Yin, & Hulbert, 2017; Naylor, DeGraaf, Purdom, & Coleman-Derr, 2017; Pagé, Tremblay, Masson, & Greer, 2019) or describing the core microbiome of wheat (Simonin et al., 2020), thus revealing the ecological rules that regulate microbial assembly (Hassani, Özkurt, Seybold, Dagan, & Stukenbrock, 2019). Ecological studies suggest that higher soil microbial diversity results in a greater resilience of the plant population (van der Heijden et al., 1998). However, the assumption that a mixture of beneficial microbes automatically provides greater plant protection is an oversimplification (Rosier, Bishnoi, Lakshmanan, Sherrier, & Bais, 2016). In this context, the responses of wheat to its microbiota are still unknown.

In this study, to disentangle the inherent complexity of plant–microbiota interactions (Vorholt, Vogel, Carlström, & Müller, 2017), we followed a reductionist approach, where we selected two PGPB species, *Azospirillum brasilense* and *Paraburkholderia graminis*, which are associated with wheat plants under natural conditions, as well as an arbuscular mycorrhizal fungus (AMF), *Funneliformis mosseae*. We hypothesized that targeted inoculation of wheat plants with the AMF and one of the two PGPB could provide an experimentally tractable system for evaluating the outcome of the interaction between beneficial microbes. Previously, we demonstrated that inoculation with *F. mosseae* improved plant growth and enhanced bioprotection in wheat

(Fiorilli et al., 2018). The current study aimed to investigate the long-term local and systemic effects of *P. graminis* or *A. brasilense* on the wheat proteome in non-mycorrhizal and mycorrhizal plants. We compared the proteomic changes in wheat triggered by co-inoculation of PGPB and *F. mosseae* with those elicited by single inoculations. We also investigated the bioprotective effects of bipartite (wheat–PGPB) and tripartite (wheat–PGPB–AMF) interactions on wheat plants against the leaf pathogen, *Xanthomonas translucens*. While *A. brasilense* drastically altered the bioprotective effect of the AMF, *P. graminis* did not affect AMF-induced pathogen resistance. Overall, proteomic changes revealed the molecular mechanisms underlying the tripartite interaction and showed that the beneficial effects of the AMF on plants are differentially modulated by the plant-associated PGPB.

MATERIALS AND METHODS

Bacterial strains, mycorrhizal fungus and wheat genotype

Two plant growth promoting bacteria (PGPB), *Azospirillum brasilense* Sp245 (obtained from UMR Ecologie Microbienne, Villeurbanne) and *Paraburkolderia graminis* C4D1M (type strain of the species, LMG collection, Ghent, Belgium), one wheat pathogen, *Xanthomonas translucens* CFBP2054 (obtained from CFBP collection), and one mycorrhizal fungus, *Funneliformis mosseae* (BEG.12, MycAgro Lab, France) were used in our experiments.

In detail, *A. brasilense* strain Sp245 was isolated from wheat roots and was shown to stimulate root development and increase plant dry mass (Kapulnik, Okon, & Henis, 1985), while the strain C4D1M of *P. graminis* was isolated from senescent corn roots and found to positively interact with different species of wheat (L. Moulin, personal communication).

Gfp-tagged derivatives were also included for cytology analyses: *A. brasilense* Sp245 eGFP carrying the pMP2444 plasmid eGFP, GmR (Wisniewski-Dyé et al., 2011) and *P. graminis* C4D1M eGFP, constructed by triparental mating (using a Tn7 eGFP construct described in (Norris, Kang, Wilcox, & Hoang, 2010) with a single insertion of the Tn7 upstream of the *glmS* gene). *A. brasilense* was cultivated at 28°C on LBA medium (Luria Broth low salt, agar) and *P. graminis* in YMA medium (yeast extract, 3 g; mannitol, 10 g; KH_2PO_4, 0.5 g; $MgSO_4$, 0.2 g; NaCl, 0.1 g; agar, 18 g; distilled water, 1 L; pH 6.8) and grown overnight in the same broth medium for inoculation. Strains were stored at -80°c in 20% glycerol. *X. translucens* CFBP 2054 was grown at 28 °C on Peptone sucrose agar (PSA) medium, retrieved from Petri dish with sterile water to reach OD 0.5 for leaf clipping and infiltration assays.

The *Triticum aestivum* cv Chinese Spring was used for all experiments (seeds obtained from Valeria Terzi, CREA, Italy).

Plant material and plant inoculations

The methodologies have already been described in previous articles on the wheat response to *Xanthomonas* (Garcia-Seco et al., 2017a) and to mycorrhizal fungi (Fiorilli et al., 2018). Twelve combinations were studied: 1). Control plants (C), 2) *A. brasilense*-inoculated plants (Az), 3) *P. graminis*-inoculated plants (P), 4) *F. mosseae*-inoculated plants (M), 5) C+*Xanthomonas translucens* (X), 6) Az+X, 7) P+X, 8) M+X, 9) Az+M, 10) P+M, 11) Az+M+X, 12) P+M+X. An overview of the experiment is given in Figure S1.

Seeds were disinfected by immersing for 40 min in a sodium hypochlorite solution and washed with sodium thiosulfate (Hurek et al., 1994) and pregerminated. The seedlings were transferred to pots containing a mix of sterile quartz sand + either the *F. mosseae* carrier inoculum substrate (the substrate without the fungus) for control and PGPB conditions, or the *F. mosseae* inoculum (30% v/v) for mycorrhizal and mycorrhizal+PGPB conditions. PGPB were inoculated directly after seedling transfer to pots, with 1 ml per plant at OD 1 from an overnight broth culture washed once and diluted with water.

For each inoculated condition, 10 pots containing 1 plant were used for phenotyping of root and fresh weight at 50 dpi, 5 pots were used for proteomic, and 5 pots for leaf-clipping assays with *Xanthomonas translucens*.

All plants were maintained under glasshouse conditions under cycles of 12 h of light at 21 °C and 50% relative humidity (RH) and 12 h of dark at 21 °C and 50% RH, watered twice a week with water, and once with a modified Long-

219 Ashton solution containing a low phosphorous concentration (32 µM
220 Na_2HPO_4*$12H_2O$).
221 Spikes weight were measured separately in the mature plants at the end of their
222 natural cycle. The spikes were threshed and 1000-kernel weights were
223 determined.
224 For *Xanthomonas* infections, two types of inoculation were performed. A
225 phenotyping leaf-clipping assay with scissors soaked in a 0.5 OD *X.*
226 *translucens* culture was performed at 46 dpi on a first set of plants for
227 phenotyping the length of the symptoms at 4-day post-clipping (dpc; starting
228 point of the lesions) and 26 dpc. A second set of plants dedicated to proteomic
229 analyses was infiltrated at 49 dpi with a 0.5 OD *X. translucens* culture using a
230 microneedle, as described in Garcia-Seco et al. (Garcia-Seco et al., 2017b), and
231 sampled the following day.
232
233 **Evaluation of wheat roots microbial colonization**
234 The mycorrhizal and mycorrhizal+PGPB roots were stained with 0.1% cotton
235 blue and the level of mycorrhizal colonization was assessed as previously
236 described (Trouvelot, Kough, & Gianinazzi-Pearson, 1986).
237 For Colony Forming Unit (CFU) counting from plant roots, root fragments
238 were weighted then pulverized with a FastPrep™ in tubes containing a ceramic
239 bead in 500 µL of sterile water, centrifuged at 1000 rpm for 30 s and drops of
240 20 µL of serial dilutions were plated on bacterial media and counted 24 h later.

241

242 **Proteomic analysis and data processing**

243 Plant samples (root and leaves) were sampled at 50 dpi and pulverized with
244 liquid nitrogen. The used protocol for total protein extraction was based on
245 SDS and phenol extraction (Wu, Xiong, Wang, Scali, & Cresti, 2014). Then,
246 samples were digested and analysed by Liquid Chromatography-Mass
247 Spectrometry (LC-MS/MS) as described previously (Garcia-Seco et al.,
248 2017b). Mass spectrometer raw files were analysed by MaxQuant (version
249 1.6.2.3, default parameters) against UniProt *T. aestivum* (Version 2017-1,
250 150,716 entries), Uniprot *Rhizophagus irregularis* (Version 2015-10, 29,847
251 entries), Uniprot *P. graminis* (Version 2015-10, 6,732 entries) and Uniprot *A.*
252 *brasilense* (Version 2015-10, 7,636 entries). On January 2019, the UniProt *T.*
253 *aestivum* database has been updated. Therefore, we obtained the updated
254 protein IDs by BLAST search of our dataset against the Uniprot *T. aestivum*
255 2019 database (143,020 entries). Unknown proteins were annotated by BLAST
256 search against the Uniprot viridiplantae database (Version 2019-01, 6,913,939
257 entries), taking the first hit with a valid annotation.
258 All MS proteomic data have been deposited in the ProteomeXchange
259 Consortium via PRIDE partner repository with the Username:
260 reviewer04430@ebi.ac.uk and Password: 2vlyEEVZ.

MaxQuant output files were processed as described earlier (Vannini et al., 2019). Only proteins detected in at least two of the three biological replicates (75%) sharing the same treatment and tissue were considered.

To compare the differences among analytical groups we performed an ANOVA based multiple samples coupled with Tukey test using the *R* package LIMMA. Only proteins with false discovery rate (FDR) below 0.01 were considered Differentially Abundant Proteins (DAPs) within the various comparisons. In order to produce a reliable and robust dataset, all proteins which gave one nonzero and two zero outcomes (two-time imputation) in at least one of the samples in each comparison were considered unreliable and therefore eliminated.

In order to use bioinformatic tools available only for *A. thaliana,* a local BLAST of *T. aestivum* proteins against the TAIR10 database (version 2012-05-07) was performed.

The enrichment analysis was performed using the Gene Ontology Resource (http://geneontology.org), running PANTHER algorithm with *A. thaliana* as background and FDR < 0.05 or using the AgriGO Singular Enrichment Analysis (SEA) compare tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php?method=compare), with *A. thaliana* TAIR10_2017 protein database as background, default parameters and a FDR threshold of 0.05 (Du, Zhou, Ling, Zhang, & Su, 2010).

282

283 **Amino acid analysis**

284 For the amino acids (AAs) extraction, 0.1 g of lyophilized samples were re-
285 suspended in 10 mL of 0.1% (v/v) formic acid in water/methanol (50:50). 10
286 µL of 10 mM deuterated internal standards (L-Phenyl-d5-alanine and L-alanine
287 ^{15}N Met), were added. Free AAs were quantified by Liquid Chromatography
288 Tandem Mass Spectrometry (LC-MS/MS) as described previously (Fiorilli et
289 al., 2018).

290

291 **Total glucose and nitrogen content**

292 Soluble sugars were extracted as described by (Shi, Wang, Yang, Li, & Miao,
293 2016) with minor modifications. Briefly, 0.2 g of leaves material were boiled
294 (80°C) in ethanol 80% for 30 min. After centrifugation (13000g x 5 min) the
295 supernatant was recovered. The extraction was repeated twice and all
296 supernatants were collected. Sucrose in solution was hydrolysed with HCl (2%
297 of HCl concentrated V/V) for 5 min at 90°C. After acid neutralization by KOH
298 (5% of 5N KOH V/V) total glucose was estimated by the dinitrosalicylic (DNS)
299 method (Miller, 1959).

Wheat root N content was determined by CHNS elemental analyzer Thermo Fisher Scientific following the manufacturer's specifications. About 2–3 mg of sample for each replicate were weighed and placed in a tin capsule containing 9.5 to 10.5 mg of vanadium pentoxide. The N_2 product by sample combustion was quantitatively determined through a separation with a gas chromatograph (GC) followed by a quantification using a thermal conductivity detector. Three tests were prepared for each sample.

Statistics

Phenotyping data of plant weight, CFU and lesion length were analyzed, depending on normality of data, by ANOVA followed by Tukey post-hoc test or Kruskal-Wallis test followed by Mann–Whitney pairwise comparisons, in R Environment (rstatix) and figures produced by ggplot.

Data from quantification of amino acid, total glucose and nitrogen were subjected to statistical analysis by ANOVA and Tukey post-hoc test.

RESULTS

PGPB and AMF impact plant growth

Wheat plants were inoculated with *A. brasilense* Sp245, *P. graminis* C4D1M, *A. brasilense* Sp245 plus *F. mosseae*, or *P. graminis* C4D1M plus *F. mosseae* (hereafter referred to as Az, P, AzM, and PM plants, respectively) and grown under controlled conditions (Figure S1). The root and shoot biomass of these

322 plants was determined at 50 days post-inoculation (dpi) and compared with that
323 of mock-inoculated control (C) and *F. mosseae* only-inoculated (M) plants. In
324 isolation, *A. brasilense* Sp245 exerted a strong positive effect on the growth of
325 roots and shoots, whereas *P. graminis* C4D1M did not induce statistically
326 significant growth of these organs (Figure 1). Monitoring these plants until
327 seed production revealed that *P. graminis* significantly increased the seed
328 yield, doubling the spike weight (Figure S2).

329 To determine whether the positive impact of the two PGPB on plant
330 growth was associated with an efficient colonization process, bacteria on the
331 root surface were counted at different time points. *A. brasilense* exhibited the
332 greatest colonizing potential, with the bacterial count remaining constant
333 across different time points. Colonization by *P. graminis* decreased with time
334 to 1×10^2 colony forming units (CFU) at 21 dpi (Figure S3). The success of
335 AMF colonization was evaluated at 50 dpi by calculating the total length of
336 colonized roots (F%) and total number of arbuscules (A%) in plants inoculated
337 with AMF alone or AMF plus PGPB (*A. brasilense* or *P. graminis*).
338 Colonization by the AMF resulted in abundant arbuscules in all plants. No
339 differences were detected in F% and A% among plants inoculated with AMF
340 alone or together with PGPB, indicating that the presence or absence of PGPB
341 does not affect mycorrhizal colonization (Figure S3).

342 The shoot weight of AzM and PM plants was significantly higher than
343 that of Az and P plants (Figure 1B) but comparable with that of M plants,

indicating that PGPB did not lead to any additional yield benefit compared with the mycorrhizal condition.

AMF alone or in combination with a PGPB triggers different responses to *X. translucens* infection

Inoculated wheat plants were assessed for protective effect to *X. translucens* leaf infection by leaf-clipping plants with the pathogen at 46 dpi and recording leaf symptoms at 4 and 26 days post-leaf clipping (dpc). Pathogen-infected plants were identified as AzX, PX, MX, AzMX, and PMX and positive control plants as CX. Disease symptoms were evident in CX plants at 4 and 26 dpc (Figure 2). Lesion length in MX plants was significantly less than that in CX plants both at 4 and 26 dpc, consistent with our previous results (Fiorilli et al., 2018).

Lesion length appeared extended in AzX and CX plants at both time points. AzMX plants showed reduced symptoms in comparison with AzX plants at 4 dpc but showed extended lesions compared with CX and AzX plants at 26 dpc. This result indicates that the *F. mosseae*-induced bioprotection in wheat is abrogated between 4 and 26 dpc in the presence of *A. brasilense*.

At 26 dpc, a significant reduction in symptoms was observed only in MX and PMX plants when compared with CX plants, whereas lesions were significantly increased in AzX and AzMX plants compared with PX, MX, and PMX plants. These results indicate that inoculation with *F. mosseae* alone

366 (Fiorilli et al., 2018) or in combination with *P. graminis* increased protection
367 against *X. translucens*. Overall, this experiment showed that *A. brasilense*
368 inoculation alone did not protect wheat plants against the pathogen, and rather
369 undermined the positive effect exerted by *F. mosseae*.
370
371 **Quantitative overview of proteomics analysis**
372 We conducted proteomic analysis of the roots (R) of Az, P, M, AzM, and PM
373 plants (hereafter referred to as RAz, RP, RM, RAzM, and RPM samples,
374 respectively) as well as the leaves of these plants (hereafter referred to as LAz,
375 LP, LM, LAzM, and LPM samples, respectively). We also performed
376 proteomic analysis of the leaves of these plants following infection with *X.*
377 *translucens* (hereafter referred to as LAzX, LPX, LMX, LAzMX, and LPMX,
378 respectively). Each treatment was analyzed in triplicate.
379 A total of 3,846 and 3,883 wheat proteins were identified and quantified
380 in root and leaf samples, respectively. Samples were clustered by condition
381 according to their protein expression patterns (Figure S4). Replicates within
382 each analytical group clustered together, confirming the high reproducibility of
383 biological replicates. Protein abundance was compared between samples, and
384 differentially abundant proteins (DAPs) were identified using the following
385 thresholds: false discovery rate (FDR) < 0.01 and $\log_2$fold-change ($\log_2$FC) >
386 0.5 (Tables S1–S5 and S7–S15).

Functional characterization of DAPs was conducted with Gene Ontology (GO) enrichment analysis to determine the main biological processes stimulated by microbial inoculations.

In root samples, approximately 7%, 0.6%, and 0.6% of all identified proteins were assigned to AMF, *A. brasilense,* and *P. graminis* proteomes, respectively, confirming the presence of all three root-associated microbes at harvest.

Wheat response to single inoculation: an overview

A large number of significant DAPs ($P < 0.01$) were identified; 639 DAPs (386 in leaves and 253 in roots) in the C vs. Az comparison, and 1,085 DAPs (424 in leaves and 661 in roots) in the C vs. P comparison (Tables S1–S4). In leaves, approximately 50% of the DAPs were common between the C vs. Az and C vs. P comparisons (Figure S5A). By contrast, in roots, proteomic expression was highly specific, mirroring the stronger impact on the colonized niche; only 12% of the DAPs were common between the C vs. Az and C vs. P comparisons (Figure S5B).

To decipher the molecular mechanisms involved in *A. brasilense*-induced plant growth promotion, we performed GO enrichment analysis of LAz vs LC, LP vs LC, Raz vs RC and RP vs RC (Figure 3). In leaf samples, "photosynthesis light harvesting" and "photosynthetic electron transport chain" were the two most enriched GO terms. These data were consistent with

409 the higher total glucose content of LAz samples compared with LC, LP, and
410 LM samples (Figure 4A). Our experiments, therefore, confirmed that *A.*
411 *brasilense* exhibits a greater ability to drive an increase in the glucose content
412 of shoots than other beneficial microorganisms (*F. mosseae* and *P. graminis*).
413 Moreover, the LAz sample showed a higher abundance of sucrose transporter
414 2D (SUT2D) than the LC sample; SUT2D shows high similarity to rice SUT2
415 (OsSUT2), which is involved in sucrose mobilization to sink cells (Eom et al.,
416 2011).

417 The "amino acid metabolism" GO term was highly enriched in enzymes
418 involved in the synthesis of aspartate, proline, and branched-chain amino acids.
419 The increased content of amino acids both in root and leaf samples validated
420 the proteomics data (Table S6). The presence of *A. brasilense* on wheat roots
421 also increased the abundance of the phosphate transporter Pht1-10*,* which is
422 induced by the AMF (Fiorilli et al., 2018) and enhances Pi uptake.
423 Interestingly, the abundance of Pht1-10 was lower in the RP sample vs RC
424 (Figure S6). Moreover, Pht1-10 was among the four proteins whose abundance
425 showed opposite trends between RAz and RP samples (Table S5). Plants
426 assimilate and metabolize ammonium (NH_4^+) provided by diazotrophs,
427 including *Azospirillum* spp. (Carvalho, Balsemão-Pires, Saraiva, Ferreira, &
428 Hemerly, 2014). In LAz samples, we observed an increase in the abundance of
429 ferredoxin-glutamate dehydrogenase, 2-oxoglutarate (2-OxG)/malate
430 translocator, and isocitrate dehydrogenase (ICDH), and a decrease in the

abundance of ferredoxin-nitrite reductase; both these trends are indicative of a higher NH_4^+ assimilation rate in the leaves of Az plants. The increase in photosynthesis could contribute to enhanced tolerance to NH_4^+ toxicity by increasing NH_4^+ assimilation (Setién et al., 2013). Higher concentrations of free amino acids and N in RAz samples support these proteomic results (Table S6, Figure 4B).

In roots and leaves, inoculation with *A. brasilense* stimulated the mitochondrial electron transport for ATP synthesis, even if the proteins involved were different (Tables S1 and S2).

Proteomic data showed that *P. graminis* inoculation also had a substantial impact on primary metabolism (glycolysis, tricarboxylic acid cycle, and aerobic respiration) in roots and leaves (Figures 3, Figure S6, Tables S3–S5). In the LP sample, DAPs related to photosynthesis did not showed a clear pattern (Table S4).

Similar to Az plants, *P. graminis*-inoculated plants showed significantly higher concentrations of N and almost all amino acids than C plants, particularly in the roots (Figure 4B, Table S6). In P plants, the more efficient N uptake could be due to the increased abundance of the high-affinity nitrate transporter NAR2 and its activator NRT2. The abundance of the wheat ortholog of rice ammonium-inducible transporter 1-2 (OsAMT1-2) was also increased in RP sample (Figure S6, Table S5).

Overall, most of these proteomic changes affecting respiration, photosynthesis, N assimilation, and mineral nutrition mirror the differential growth response of wheat upon PGPB inoculation, as illustrated in Figure 1, with a significant systemic effect of only *A. brasilense* on plant growth.

AMF plays a dominant role in plant roots upon binary association with PGPB

We previously showed that *F. mosseae* elicits a significant proteomic change in wheat roots and leaves during colonization (Fiorilli et al., 2018). Consistent with this observation, AzM and PM samples showed high numbers of DAPs; RAzM vs. RC and RP vs. RC comparisons revealed 709 and 1055 DAPs, respectively (Tables S7 and S8), whereas LAzM vs. LC and LPM vs. LC comparisons revealed 504 and 808 DAPs, respectively (Tables S9 and S10, Figure S7).

Venn diagrams showed a specific contribution by *F. mosseae* in the roots of co-inoculated plants, mainly AzM plants (Figure S7B and S7D). These data are consistent with our previous proteomic data showing that *F. mosseae* has a stronger local but a weaker systemic impact on wheat (Fiorilli et al., 2018). In roots, inoculation with *F. mosseae* alone or together with *A. brasilense* or *P. graminis* led to an increased abundance of 196 proteins, several of which have been previously shown to increase in abundance during mycorrhization (Fiorilli et al., 2018). In particular, we found increased levels of key enzymes

474 involved in glycolysis and the pentose phosphate pathway (glyceraldehyde-3-
475 phosphate dehydrogenase, pyruvate kinase, and 6-phosphogluconate
476 dehydrogenase), fatty acid biosynthesis and metabolism (plastid acetyl-CoA
477 carboxylase and 3-oxoacyl-[acyl-carrier-protein] reductase, GDSL
478 esterase/lipase), and mineral nutrition (OsAMT3;1 homolog). We also detected
479 significant up-regulation of some proteins involved in plant defense, including
480 one acidic endochitinase protein, cysteine-rich receptor-like protein kinase 25
481 (CRK25), and some Germin-like proteins (GLPs).

482 Venn diagrams also showed that most DAPs were exclusively expressed
483 in AzM and PM plants. This trend was mainly detected in leaves (Figure S7A
484 and S7C). In fact, GO enrichment analysis showed that oxidative
485 phosphorylation, response to oxidative stress, photosynthesis, and response to
486 abiotic stimulus were among the up-regulated biological processes in the
487 LAzM vs. LPM comparison. Ribosome biogenesis, translation, and gene
488 expression were down-regulated processes (Table S11, Figure S8).

489 Altogether, our results revealed that organ-specific proteomic changes in
490 AMF-inoculated wheat plants (Fiorilli et al., 2018) are mostly maintained upon
491 binary inoculation. This suggests that the AMF plays a dominant role in the
492 root protein profile.

493

494 **Defense proteins are induced locally and systemically during bipartite and**
495 **tripartite interactions**

Some rhizosphere-associated beneficial bacteria trigger a plant immunization phenomenon, called induced systemic resistance (ISR), thus priming the plant immune system (C. M. Pieterse et al., 2014). To verify whether *A. brasilense* and *P. graminis* possess the tools to elicit immune system priming in wheat, we first examined the enriched GO terms involved in plant–microbe interactions. Whereas the "response to wounding" was one of the up-regulated GO enriched categories in RAz samples, the DAPs enriched in the category "response to bacterium" showed a decrease, in LAz samples (Figure 3). In particular, we found reduced abundance of two chitinases, two pathogenesis-related (PR) proteins, and one protein (A0A3B6BXY2) highly similar to the Arabidopsis heat stable 1 (HS1) protein, which exhibits antibacterial activity (Park et al., 2007). This decrease in the abundance of defense-related proteins in leaves could at least partly explain the susceptibility of Az plants to *X. translucens* infection in leaf-clipping tests (Figure 2). The sucrose transporter SUT2D specifically induced by *Azospirillum* (Table S2) in wheat leaves could also play a role in the susceptibility of Az plants to pathogen infection. *Xanthomonas* TAL effectors usually target SWEET family sugar transporters to sustain pathogen growth (Verdier et al., 2012).

In RP samples harvested at 50 dpi, one of the enriched GO terms was "defense response/incompatible interaction" with the induction of several defense proteins (Figure 3, Table S5). Among them, the A0A3B6DGK2 protein, which is similar to RPM1-interacting protein 4 (RIN4), a major

518 regulator of plant defense that plays important roles in both pathogen-
519 associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-
520 triggered immunity (ETI) (Ray, Macoy, Kim, Lee, & Kim, 2019). Additionally,
521 lipoxygenase 1 (LOX1), 12-oxophytodienoate reductase 1 (OPR1), OPR3, and
522 allene oxide cyclase 3 (AOC3) point to up-regulation of the biosynthesis of
523 jasmonic acid (JA), a plant hormone that plays a key role in the biotic stress
524 response and overall plant immunity (C. M. J. Pieterse et al., 1998). Moreover,
525 RP samples showed an increased abundance of a protein (A0A3B6KT24) that
526 is similar to the respiratory burst oxidase homolog protein D (RbohD) and is
527 involved in the generation of reactive oxygen species (ROS) during
528 incompatible plant–pathogen interactions (Torres, Dangl, & Jones, 2002), a
529 CERK homolog (A0A3B6RF20), and proteins required for lignin biosynthesis,
530 which are activated in tomato plants associated with native microbiota (M.
531 Chialva et al., 2018). Lastly, *P. graminis*-inoculated plants showed an
532 increased abundance of some proteins involved in isoprene metabolism,
533 suggesting the up-regulation of pathways involved in plant defense. These data
534 support the hypothesis that similar to other PGPB (C. M. J. Pieterse et al., 1998)
535 and unlike *A. brasilense*, *P. graminis* elicits an immune response in the roots
536 but does not elicit strong ISR at 50 dpi in the leaves against *X. translucens*
537 infection (Figure 2).

538 To describe the systemic bioprotective effect of AMF and PGPB co-
539 inoculation in wheat, we performed LAzM vs. LAz and LPM vs. LP

comparisons (Tables S12 and S13). Our analysis showed an increase in several DAPs, putatively involved in the biotic stress response, in both LAzM and LPM samples (Figure 5A). Among these DAPs, we found proteins either involved in JA biosynthesis, such as a phospholipase D, three LOXs, and an AOC, or induced by JA, such as a dirigent protein, PR4 (wheat protein) (Desmond et al., 2005), and OsMPK1 (Singh & Jwa, 2013). Moreover, we observed an increase in the abundance of other proteins involved in signaling of the plant immune response, such as the homolog of brassinosteroid (BR)-signaling kinase 1 (BSK1) (Shi et al., 2013) and a calcium-transporting ATPase, whose homolog (ACA8) is required for limiting the growth of virulent bacteria in Arabidopsis (Frei dit Frey et al., 2012). Another protein, whose expression was highly induced in LPM and LAzM samples, was manganese superoxide dismutase 1 (Mn-SOD1), which belongs to the polyphyletic family of enzymes and protect cells from reactive superoxide radical-induced damage, thus conferring increased stress tolerance (~~S.~~ Wang et al., 2017).

Overall, this analysis revealed some unexpected features: *A. brasilense* alone down-regulates plant defense (which is consistent with the observed disease susceptibility phenotype shown in Figure 2), while single inoculations of *P. graminis* and *F. mosseae* trigger a similar number of proteins involved in the plant immune response in an organ-dependent way. Co-inoculation of wheat plants with the AMF and *A. brasilense* or *P. graminis* elicits the plant immune response not only in LPM but also in LAzM samples, at least in the

562 short term, suggesting that both microbial pairs (AMF–*A. brasilense* and
563 AMF–*P. graminis*) induce a priming response at least at the proteome level
564 (Figure 5A).
565
566 **Microbial pairs modulate wheat response to *X. translucens* by inducing**
567 **different proteomic changes**
568 Leaf inoculation of M, Az, P, and C plants with *X. translucens* revealed that
569 pathogen susceptibility detected in the leaves of LAzX plants was alleviated by
570 the presence of mycorrhizal colonization at 4 dpc (based on the LAzMX vs.
571 LAzX comparison) (Figure 2A). To decipher the main proteins responsible for
572 the reduction of symptoms at 4 dpc, we analyzed the DAPs identified in the
573 LAzMX vs. LAzX and LAzMX vs. LMX comparisons (Tables S14 and S15).
574 The up-regulated proteins included those involved in JA biosynthesis and
575 response. Several LOXs and two lipases including phospholipase A1-II and
576 phospholipase D, which generate fatty acid substrates for JA biosynthesis
577 (Browse, 2009), were highly induced in the LAzMX vs. LAzX and LAzMX
578 vs. LMX comparisons (Ishiguro, Kawai-Oda, Ueda, Nishida, & Okada, 2001;
579 Lee & Park, 2019; C. Wang et al., 2000; Wasternack & Hause, 2013) (Figure
580 5A, Tables S14 and S15). In addition, two allene oxide synthase (AOS)
581 enzymes, which catalyze the first step in the JA biosynthesis pathway, and two
582 AOCs, which are committed for the second step in this pathway (Schaller &
583 Stintzi, 2009), were highly induced in LAzMX samples (Figure 5A) compared

584 with LAzX and LMX samples. We also found that two JA-induced dirigent-
585 like proteins, which act downstream of the JA biosynthesis pathway, were
586 induced in LAzMX samples. This increase of proteins involved in JA
587 biosynthesis was also observed in LPMX samples (Figure 5A).

588 Our results showed that *A. brasilense*–AMF and *P. graminis*–AMF
589 interactions amplified JA signaling during pathogen attack. In addition,
590 proteins involved in biotic stress, which were induced during *A. brasilense*–
591 AMF and *P. graminis*–AMF interactions in LAzM and LPM samples,
592 respectively (Figure 5A), were also recruited during *X. translucens* infection,
593 as testified by their higher abundance in LAzMX and LPMX samples,
594 respectively.

595 These proteomic data correlate with the reduced lesion length observed
596 at the early time point (4 dpc) in AzMX plants with respect to AzX plants.
597 However, at 26 dpc, a significant reduction in lesion size resulted only in MX
598 and PMX plants compared with CX plants (Figure 2B). These data suggest that
599 when co-inoculated with *A. brasilense*, the bioprotective effect exerted of *F.*
600 *mosseae* is transient and probably related to prompt induction of the JA
601 response.

602 Further analysis is needed to clarify the molecular changes during the
603 later stage of pathogen attack under different conditions. However, the
604 proteomic profile of LAzMX samples was very different from that of LPMX
605 samples (799 DAPs; Table S16). Among the most abundant proteins identified

606 in the LAzMX vs. LPMX comparison, we found proteins involved in the
607 response to abiotic stimulus and oxidative stress, while those implicated in
608 translation, ribosome biogenesis, gene expression were down-regulated. A
609 similar pattern was already observed in the LAzM vs. LPM comparison (Figure
610 S8).

611 Overall, these data highlight the intricate network of processes that
612 regulate wheat–PGPB–AMF–pathogen interactions (as observed in LAzMX
613 and LPMX samples). However, elicitation of defense priming in the proteome
614 of LAzM and LPM samples does not necessarily lead to better performance
615 once the plant is under pathogen attack.

616

617 **DISCUSSION**

618 Wheat, one of the earliest food crops to be domesticated, is currently the second
619 most widely cultivated crop in the world and one of the most important grain
620 sources for humans. Given the increasing relevance of plant microbiota, many
621 researches have described wheat-associated microbiota by considering the
622 effects in different organs as well as in grain production. The results of this
623 study illustrate how proteomic changes in wheat plants depend on the inoculum
624 composition (single or multiple microbes) and the organ under study, and lead
625 to differential growth effects and pathogen resistance. All analyses revealed
626 that the AMF was the crucial driver of plant growth and defense priming under
627 our growth conditions (low P). However, the overall changes induced by the

AMF–PGPB consortium can interfere with the final mycorrhizal-induced resistance (MIR) outcome (Figure 6).

Effect of beneficial microbes on wheat growth is organ- and microbial identity-dependent

In addition to their N-fixing ability, *Azospirillum* spp. exhibit a remarkable capacity to benefit a wide range of plant species by activating multiple mechanisms (Fukami, Cerezini, & Hungria, 2018); however, the available omics data are limited to the effects of *A. brasilense* inoculation on roots (Drogue et al., 2014; Spaepen, Bossuyt, Engelen, Marchal, & Vanderleyden, 2014). In this study, we showed that the higher root and shoot biomass of plants colonized by *A. brasilense* is supported by the sustained activation of the main metabolic processes (respiration, photosynthesis, and N assimilation), while the roots act as a strong sink for nutrients, such as hexoses and amino acids. These results are consistent with the findings of Zeffa and colleagues, who showed that *A. brasilense* promotes plant growth in maize by enhancing the plant photosynthetic potential or by increasing the N use efficiency (Zeffa et al., 2019) On the other hand, *P. graminis* did not efficiently increase the root and shoot biomass of wheat plants but increased the spike biomass.

Wheat actively responds to *P. graminis* inoculation by eliciting many metabolic processes, which involve a higher number of DAPs compared with those induced by *A. brasilense*. Some of these processes were, however,

650 common to the two bacterial species (e.g., processes involved in ROS
651 scavenging) as well described for many other PGPB (Fukami et al., 2018).
652 Wheat responds well to AMF, particularly *F. mosseae* (Fiorilli et al.,
653 2018). The wheat–PGPB–AMF tripartite interaction led to intensive proteomic
654 changes where nutrient transporters and many enzymes involved in primary
655 and secondary metabolism, protein biosynthesis, and ROS homeostasis were
656 elicited.
657 Overall, plant growth experiments, nutrient quantification, and
658 proteomic analyses demonstrated that the AMF plays a leading role in tripartite
659 interactions, particularly in the root, while PGBP (at least *Azospirillum*) affects
660 systemic growth, as evident from the leaf proteome.
661
662 **The bioprotective effect of the AMF is modulated by the nature of the co-**
663 **inoculated PGPB**
664 PGPB are considered essential components of the plant microbiota because of
665 their ability to improve plant growth via multiple mechanisms, including plant
666 health protection (Berendsen, Pieterse, & Bakker, 2012; Lugtenberg et al.,
667 2016). *Azospirillum* is not a typical biocontrol agent, despite studies showing
668 its ability to increase pathogen resistance in plants (Bashan & de-Bashan, 2002;
669 Kusajima et al., 2018; Tortora, Díaz-Ricci, & Pedraza, 2012; Yasuda, Isawa,
670 Shinozaki, Minamisawa, & Nakashita, 2009). On the other hand, some
671 *Paraburkholderia* taxa, such as *P. phytofirmans,* induce resistance against a

672 broad range of plant pathogens by inducing plant-mediated responses in aerial
673 organs (Miotto-Vilanova et al., 2016). Proteomic analysis of wheat plants
674 inoculated with a single microbe showed that proteins involved in plant defense
675 were down-regulated in LAz samples. Moreover, according to the "pathogen
676 starvation" model, which links plant resistance with soluble sugars (Bezrutczyk
677 et al., 2018), the high sugar and amino acid contents of LAz leaves coupled
678 with an enhanced abundance of sugar transporters could guarantee a nutrient-
679 rich niche for the pathogen. Under these conditions, the plant could not activate
680 any defense mechanisms, notwithstanding a light improvement in the presence
681 of the AMF at 4 dpc.

682 *P. graminis* induced diverse proteomic changes in roots characterized by
683 an increase in the abundance of proteins involved in microbe-associated
684 molecular pattern (MAMP) perception, PTI and ETI regulation (RIN4), ROS
685 production and detoxification, lignin biosynthesis, and isoprene metabolism.
686 These findings suggest that *P. graminis* elicits an immunomodulatory
687 response; however, this does not lead to ISR.

688 The protein profiles clearly indicate the capacity of mycorrhizal plants,
689 associated with PGPB, to increase the number of defense-related proteins in
690 leaves in the absence of the pathogen, and an augmented capacity to express
691 these proteins upon pathogen infection (Figure 5A). The up-regulation of JA
692 biosynthesis proteins was a key finding because this hormone is considered the
693 first regulator of the plant immune response (Hickman et al., 2017; C. M. J.

694 Pieterse et al., 1998). Several studies reported that AM symbiosis protects
695 plants against pathogens, suggesting that JA defense mechanisms play a key
696 role in MIR (Jung, Martinez-Medina, Lopez-Raez, & Pozo, 2012).

697 AMF are a crucial component of the plant microbiota (Bonfante, Venice,
698 & Lanfranco, 2019) and the first inducers of plant immunity. A previous study
699 showed that co-inoculation of wheat with an AMF and *Pseudomonas* spp.
700 (PGPB) leads to synergistic effects, priming the host immunity through
701 chitosan-induced callose deposition (Pérez-de-Luque et al., 2017). A
702 comparable result has been described in tomato plants grown in native soil
703 containing multiple bacteria and AMF; MAMPs released by various microbes
704 enhance the plant immunity, thus activating PTI markers. When challenged by
705 pathogenic *Fusarium* spp., the tomato plants were strongly protected because
706 of the activation of specific antifungal proteins (Chialva, Zhou, Spadaro, &
707 Bonfante, 2018).

708 According to a previously proposed hypothesis (Cameron, Neal, van
709 Wees, & Ton, 2013), JA could act as a long distance signaling molecule that in
710 mycorrhizal wheat, and also in the presence of both PGPB species, activates
711 the systemic priming of plant defense. However, in our system, lesion length
712 was reduced only at the early time point (4 dpc) in AzMX plants in comparison
713 with AzX plants. At 26 dpc, a significant reduction in lesion length was
714 observed only in MX and PMX plants in comparison with CX plants (Figure
715 2B). We speculate that additional determinants induced by the AMF–PGPB

716 interactions interfere with cellular processes, leading to MIR. Proteomic data
717 showed that in LAzMX samples, the abundance of proteins involved in
718 ribosome biogenesis and gene expression decreased compared with their
719 abundance in LMX and LBMX samples (Figure 5B). Ribosomal genes are
720 highly responsive to stress and signaling molecules, indicating that the encoded
721 proteins play roles in stress amelioration, besides house-keeping. The
722 instantaneous up-regulation of ribosomal genes in response to stress might
723 function as an prompt defense response (Moin et al., 2016). In addition, a
724 reduction in HMGA subfamily transcription factor and H2B and H1 histones
725 could affect the transcription of defense-related genes (Isaac, Hartney, Druffel,
726 & Hadwiger, 2009). Finally, a reduction in the abundance of two proteins
727 involved in stomata regulation, H1.3 and ubiquitin-specific protease 24
728 (Rutowicz et al., 2015; Zhao et al., 2016), and some proteins involved in
729 cuticular wax production, could promote leaf pathogen invasion in AzMX
730 plants.
731
732 **CONCLUSION**
733 Plant-associated microbiota hold great promise for the development of
734 sustainable crop systems, and this can be guaranteed by the use of SynComs
735 (Kong, Hart, & Liu, 2018). However, results obtained from on-field microbiota
736 census and those obtained using reductionist approaches, mostly through
737 laboratory-based experiments, have not yet been fully integrated (Fitzpatrick

et al., 2020). Our results suggest that beneficial microbes have different impacts on plants, at least in wheat, and the plant growth-promoting effects of beneficial microbes are not always accompanied by enhanced pathogen resistance, as shown by *A. brasilense* inoculation (Figure 6). On the other hand, a bacterium that does not show strong growth-promoting effect, such as *P. graminis*, may be more effective against pathogen attack, if associated with an AMF (Figure 6). Our data highlight the crucial role of AM fungi, which are often absent in SynComs, as well as the potential contrasting effects of different AMF–PGPB consortia on plant defense. In a wider context, these findings suggest that SynCom efficiency should be validated by checking the outcome of the interaction under different conditions (microbe-microbe interactions; nutritional status, plant life cycle and biotic stress) before their exploitation for crop growth.

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AUTHOR CONTRIBUTIONS

V.F., D.G.S., P.B., L.M., and C.V designed the study; V.F., D.G.S., M.N., and D.G. carried out the majority of experiments; G.D., M.M., and C.V performed the bioinformatic analysis of proteomic data; C.V., V.F., P.B. L.M., F.W.-D., and M.B. interpreted the data and wrote the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPORTING INFORMATION

Table S1. Differentially abundant proteins (DAPs) identified by comparative proteomic analysis of the roots (R) of *Azospirillum brasilense*-inoculated (Az) and mock-inoculated (control; C) wheat plants (RAz vs. RC comparison).

Table S2. DAPs identified by comparative proteomic analysis of the leaves (L) of Az and C plants (LAz vs. LC comparison).

Table S3. DAPs identified by comparing the leaf proteome of *Paraburkholderia graminis*-inoculated (P) and C plants (RP vs. RC comparison).

Table S4. DAPs identified by comparing the leaf proteome of P and C plants (LP vs. LC comparison).

781 Table S5. Root-specific DAPs identified by comparing the root proteome of
782 Az or P plants with that of C plants (RAz vs. RC and RP vs. RC comparisons,
783 respectively).
784 Table S6. Amino acid content (ratio).
785 Table S7. DAPs identified by comparing the root proteome of wheat plants co-
786 inoculated with the arbuscular mycorrhizal fungus (AMF), *Funneliformis*
787 *mosseae*, and *A. brasilense* (AzM) with that of C plants (RAzM vs. RC
788 comparison).
789 Table S8. DAPs identified by comparing the root proteome of wheat plants co-
790 inoculated with the AMF and *P. graminis* (PM) with that of C plants (RPM vs.
791 RC comparison).
792 Table S9. DAPs identified by comparing the leaf proteomes of AzM and C
793 plants (LAzM vs. LC comparison).
794 Table S10. DAPs identified by comparing the leaf proteomes of PM and C
795 plants (LPM vs. LC comparison).
796 Table S11. DAPs identified by comparing the leaf proteomes of AzM and PM
797 plants (LAzM vs. LPM comparison).
798 Table S12. DAPs identified by comparing the leaf proteomes of Az plants
799 treated with or without the AMF (LAzM vs. LAz comparison).
800 Table S13. DAPs identified by comparing the leaf proteomes of P plants treated
801 with or without the AMF (LPM vs. LP comparison).

802 Table S14. DAPs identified by comparing the leaf proteomes of Az plants
803 infected with or without the leaf pathogen, *Xanthomonas translucens* (LAzMX
804 vs. LAzX comparison).
805 Table S15. DAPs identified by comparing the leaf proteomes of AzM plants
806 and AMF only-inoculated (M) plants infected with *X. translucens* (LAzMX
807 vs. LMX comparison).
808 Table S16. DAPs identified by comparing the leaf proteomes of AzMX plants
809 and PM plants infected with *X. translucens* (LAzMX vs. LPMX comparison).
810 Figure S1. Overview of the experimental set up.
811 Figure S2. Spike fresh weight.
812 Figure S3. Evaluation of microbial root colonization.
813 Figure S4. Hierarchical clustering analysis of protein intensities.
814 Figure S5. Overlap of differentially abundant proteins (DAPs) between Az vs.
815 C and P vs. C comparisons.
816 Figure S6. Heat map of DAPs belonging to "carbohydrate metabolism",
817 "photosynthesis", "ion transport", and "defense" GO terms in LAz vs. LC, LP
818 vs. LC, RAz vs. RC, and RP vs. RC comparisons.
819 Figure S7. Venn diagrams of DAPs identified by comparing the proteome of
820 mock-inoculated control (C) wheat plants with that of plants inoculated with
821 only plant-growth promoting bacteria (PGPB; single inoculation) or co-
822 inoculated with PGPB and arbuscular mycorrhizal fungus (AMF) (double
823 inoculation).

Figure S8**.** Schematic representation of AgriGO SEA COMPARE function of up/down-regulated in LAzM vs. LPM and LAzMX vs. LPMX comparisons.

FIGURE LEGENDS

Figure 1. Effect of arbuscular mycorrhizal (AM) symbiosis on the biomass of different organs of wheat plants. (A) Fresh weight of roots (RFW; grams). (B) Fresh weight of shoots (SFW; grams). Plants were either mock-inoculated (control; C) or inoculated with different microbial combinations: *Azospirillum brasilense* only (Az), *Paraburkholderia graminis* only (P), *Funneliformis mosseae* only (M), *A. brasilense* plus *F. mosseae* (AzM), and *P. graminis* plus *F. mosseae* (PM). Wheat plants were harvested at 50 days post-inoculation (dpi). Data represented as mean ± standard deviation (SD; $n \geq 6$) were subjected to a one-way analysis of variance (ANOVA). Asterisks indicate significant differences ($P < 0.05$; Tukey's test). Different lowercase letters indicate significant differences.

Figure 2. Phenotypic evaluation of disease symptoms caused by the bacterial pathogen *Xanthomonas translucens.* Lesion length (mm) was assessed on leaves of C, Az, P, M, AzM, and PM plants at 4 (A) and 26 (B) days post-leaf clipping (dpc). Data at 4 dpc (not normally distributed) were analysed using the Kruskal-Wallis test. Asterisks indicate significant differences at the 5%

845 level using Mann–Whitney pairwise comparisons. Data at 26 dpc were
846 subjected to one-way analysis of variance (ANOVA). Asterisks indicate
847 significant differences ($P < 0.05$; Tukey's test). Different lowercase letters
848 indicate significant differences.
849
850 **Figure 3.** Gene Ontology (GO) enrichment analysis of DAPs identified by
851 comparing Az or P vs. C roots (RAz vs. RC and RP vs. PC) and leaves (LAz
852 vs. LC and LP vs. LC). Enriched GO terms were selected using the following
853 thresholds: false discovery rate (FDR) ≤ 0.05 and fold enrichment > 3.5. Red
854 and blue indicate the enrichment of GO biological process terms for up-and
855 down- regulated DAPs, respectively.
856
857 **Figure 4.** Total glucose and nitrogen (N) contents of wheat leaves and roots,
858 respectively. (A, B) Total glucose content of leaves (A) and N content of roots
859 (B) of C, Az, P, M, AzM, and PM wheat plants harvested at 50 dpi. Data
860 represented as mean ± SD ($n \geq 3$) were subjected to a one-way ANOVA.
861 Different lowercase letters indicate significant differences ($P < 0.05$; Tukey's
862 test).
863
864 **Figure 5.** Heat map of the main DAPs involved in plant defense (A) and in cell
865 wall production, epigenetic regulation, translation (B) found in wheat leaves
866 inoculated with PGPB and/or AM fungus (AMF) and treated with or without

867 *X. translucens*. Log_2fold-change (Log_2FC) values indicate the changes in
868 protein abundance with respect to the control. Red and blue indicate maximum
869 and minimum values, respectively. Asterisks indicate significant differences
870 (ANOVA FDR < 0.01; Tukey's test).
871
872 **Figure 6.** (A) Scheme showing the molecular and phenotypic responses of non-
873 mycorrhizal wheat colonized by *A. brasilence* (left side) and *P. graminis* (right
874 side). (B) Scheme showing the molecular and phenotypic responses of
875 mycorrhizal (*F. mosseae*) wheat alone (center) or colonized by *A. brasilence*
876 (left side) and *P. graminis* (right side). The green boxes include the effects on
877 the leaves while the brown ones include the effects on the roots.
878
879 **Figure S1**. Overview of the experimental set up. Twelve treatments of two
880 different plant growth-promoting bacteria (PGPB) and one arbuscular
881 mycorrhizal fungus (AMF) were tested. Surface-sterilized wheat seeds were
882 pre-germinated for 4 days, transplanted into pots (see Experimental
883 procedures) at t0, and inoculated with PGPR 1 day after. The AMF was present
884 in the substrate at t0 (M treatment). The leaf-clipping assay was performed by
885 infecting plants with *Xanthomonas translucens* on day 46, and the length of
886 lesions was monitored at 4 and 26 days post-clipping (dpc; corresponding to
887 day 50 and 72, respectively). To perform proteomics analysis, leaves were

infiltrated with a micro needle on day 49, and the infiltrated zone was sampled on day 50 and frozen in liquid nitrogen.

Figure S2. Spike fresh weight of control, Az and P plants evaluated at the end of wheat natural life cycle. Data (means ± SD, $n \geq 6$) were subjected to one-way analysis of variance (ANOVA). The asterisks indicated significant differences at the 5% level using Tukey's test.

Figure S3.

Evaluation of microbial colonization of wheat plants. (A) Quantification of bacterial population (colony forming units [CFU]) per gram of wheat roots at 7, 14, and 21 dpi. (B, C) Frequency of mycorrhizal hyphae (F%) (B) and arbuscule abundance (A%) (C) in plant samples stained with trypan blue. One hundred, 1-cm root fragments were analyzed for each sample. Data represent mean ± standard error (SE) of two biological replicates per treatment. Az, *Azospirillum brasilense* Sp245; P, *Paraburkholderia graminis* C4D1M; M, *Funelliformis mosseae*; AzM, *A. brasilense* Sp245 plus *F. mosseae*; PM, *P. graminis* C4D1M plus *F. mosseae*.

Figure S4. Hierarchical clustering analysis of protein intensities in different organs of wheat plants. (A) Leaf dataset; (B) root dataset.

Figure S5. Overlap of differentially abundant proteins (DAPs) between Az vs. C and P vs. C comparisons. (A, B) Venn Diagrams of DAPs in leaves (A) and roots (B). Red and blue values indicate up- and down-regulated proteins, respectively. (C, D) Heat map of DAPs regulated in response to both plant growth-promoting bacteria (PGPB) in leaves (C) and roots (D). Red and blue indicate maximum and minimum values, respectively.

Figure S6. Heat map of DAPs belonging to "carbohydrate metabolism", "photosynthesis", "ion transport", and "defense" GO terms in LAz vs. LC, LP vs. LC, RAz vs. RC, and RP vs. RC comparisons. Log_2fold-change (Log_2FC) values are shown. Red and blue indicate maximum and minimum values, respectively.

Figure S7. Venn diagrams of differentially abundant proteins (DAPs) found in all single and double inoculations vs. control (C) plants. DAPs exclusively and commonly regulated by *A. brasilense* and AMF upon single and double inoculations in leaves (A) and roots (B). DAPs exclusively and commonly regulated by *P. graminis* and AMF upon single and double inoculations in leaves (C) and roots (D).

Figure S8. Schematic representation of AgriGO SEA COMPARE function of up/down-regulated in LAzM vs LPM and LAzMX vs LPMX comparisons. The

colored blocks represent the level of regulation of each term, where the degree of color saturation (yellow-to-red) of the corresponding box, was determined by the adjusted P-value of the GO term (red = more significant).

DATA AVAILABILITY STATEMENT

Data available on request from the authors

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