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Microbial symbionts affect *Pisum sativum* proteome and metabolome under *Didymella pinodes* infection

G. Desalegn [a,1], R. Turetschek [b,1], H.-P. Kaul [a], S. Wienkoop [b,*]

[a] University of Natural Resources and Life Sciences, Department of Crop Sciences, Austria
[b] University of Vienna, Department of Ecogenomics and Systems Biology, Austria

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ABSTRACT

The long cultivation of field pea led to an enormous diversity which, however, seems to hold just little resistance against the ascochyta blight disease complex. The potential of below ground microbial symbiosis to prime the immune system of *Pisum* for an upcoming pathogen attack has hitherto received little attention. This study investigates the effect of beneficial microbes on the leaf proteome and metabolome as well as phenotype characteristics of plants in various symbiont interactions (mycorrhiza, rhizobia, co-inoculation, non-symbiotic) after infestation by *Didymella pinodes*. In healthy plants, mycorrhiza and rhizobia induced changes in RNA metabolism and protein synthesis. Furthermore, metal handling and ROS dampening was affected in all mycorrhiza treatments. The co-inoculation caused the synthesis of stress related proteins with concomitant adjustment of proteins involved in lipid biosynthesis. The plant's disease infection response included hormonal adjustment, ROS scavenging as well as synthesis of proteins related to secondary metabolism. The regulation of the TCA, amino acid and secondary metabolism including the pisatin pathway, was most pronounced in rhizobia associated plants which had the lowest infection rate and the slowest disease progression.

Biological significance: A most comprehensive study of the *Pisum sativum* proteome and metabolome infection response to *Didymella pinodes* is provided. Several distinct patterns of microbial symbioses on the plant metabolism are presented for the first time. Upon *D. pinodes* infection, rhizobial symbiosis revealed induced systemic resistance e.g. by an enhanced level of proteins involved in pisatin biosynthesis.

1. Introduction

Legume crops such as field peas (*Pisum sativum* L.) are important components of the human and animal diet due to their content of protein, starch and other nutrients as well as their health benefit potentials. However, various aspects of field pea growth, development, productivity and expansion are threatened by abiotic and multiple biotic (pathogens or insects) stresses, of which Ascochyta blight is the most important necrotrophic foliar disease in most pea growing regions [1,2]. Particularly, *Didymella pinodes* (synonym: *Mycosphaerella pinodes*) which attacks seedlings and all the above ground parts of pea plants [3] is the most damaging one [4]. The two major damaging effects of this disease on crop growth distinguished by Shtienberg [5] were decreases in leaf area and photosynthetic efficiency of the remaining green leaf area. It has been reported that biotic stress globally downregulates photosynthetic genes [6]. *D. pinodes* alters carbohydrate metabolism, protein remobilization and free amino acid translocation from diseased leaves, what is likely to reduce photosynthesis [7] and causes significant yield losses. As reviewed by McDonald and Peck [8], between 30% and 75% losses have been measured in Australia, France and Canada. Although extensive breeding studies have been carried out, pea cultivars with durable resistance to *D. pinodes* are not yet available [1,9]. To reduce disease severity, minimise yield losses and improve the crop's contribution to food security, the suggested control measures are fungicide use and agronomic practices (burial or burning of infected crop residues, use of a suitable crop rotation and shifting of sowing dates). However, these control measures imply environmental threats (e.g. toxicity) or are often not suitable to many farm situations (e.g. sowing date). Therefore, alternative sustainable practices for pea production need to be developed and expanded.

Previously, the positive contribution of beneficial microbes for improving plant health, growth, development and productivity has been extensively reported, especially arbuscular mycorrhiza fungi (AMF) and rhizobia associations in the rhizosphere [9–11]. Plant growth-promoting rhizobacteria can induce systemic resistance in plants and minimise disease severity in both roots and leaves [12]. Likewise, phytohormones released from microorganisms activate plant immunity [13].

Recently, Kosova et al. and Perez-Alfocea et al. [14,15] reported that plant acclimation to stress is associated with profound changes in composition of the plant transcriptome, proteome, and metabolome.

* Corresponding author.
E-mail address: stefanie.wienkoop@univie.ac.at (S. Wienkoop).
[1] Authors contributed equally.

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Presumably, such changes could be affected by belowground microbial symbionts (e.g. AMF and/or rhizobia). The research on crop plants progressively enters the field of proteomics [16], and methods were proposed to analyse the proteome of non-model organisms [17,18]. In recent years, the main goals of proteomics [19] and its advancement in techniques and protocols for high-throughput proteomics are extensively presented [20]. In the investigation of symbiotic and plant–pathogen interactions, proteomics especially proved to be a successful approach to understand molecular mechanisms [21,22]. Similarly, metabolomics has a promising role in agriculture to unravel responses of stressed plants [23] or to assign plant molecular modifications associated with symbionts [24]. Besides the biosynthesis of secondary metabolites, the regulation of primary metabolites is known to crucially contribute to the plants' defence response [25]. The integration of proteomic and metabolomic data was prioritized frequently [26,27] to obtain a systemic view of the plants' molecular adjustment.

In the past, most reports claimed for synergistic effects of co-inoculation of AMF and rhizobia on legume growth and development [28–33], because of enhanced N uptake or N_2-fixation [34,35]. However, other studies showed a negative effect of mycorrhiza on nodule development and legume plant growth [36–38]. In general, the productivity of a legume crop cultivar depends on the effectiveness and compatibility of the AMF [39] and the *Rhizobium* bacteria in the rhizosphere [29]. So far, information on the priming effects of AMF and rhizobia, particularly on the proteome and metabolome of field pea under biotic and abiotic stresses, is scarce.

Hence, in this study, we investigated:

1) Whether the bipartite- (peas–mycorrhiza or peas–rhizobia) or tripartite-interactions (peas–mycorrhiza–rhizobia) affect the pea leaf proteome and metabolome compared to non-symbiotic plants.
2) Whether these interactions interfere with the plants phenotypic and molecular response to *D. pinodes* infection.

Hence, with this study we aim to provide new insights into potential benefits of microbial symbionts to develop induced systemic resistance in pea plants against *D. pinodes*. This knowledge contributes to breeding strategies in order to improve field pea productivity, yield security and expansion in cropping systems globally.

2. Materials and methods

2.1. Materials and plant growth conditions

2.1.1. Experimental design and soil conditions

In an effort to investigate whether a single or dual inoculation of arbuscular mycorrhiza fungi (AMF) and rhizobia affect dry matter, photosynthetic components (e.g. green area), as well as the proteome and the metabolome of pea plants, a factorial experimental design with four treatments and two biotic conditions was carried out in a randomized complete block design. The four treatments were: AMF, *Glomus mosseae* (M), *Rhizobium leguminosarum* bv. *viceae* (R), dual microbial symbionts of AMF and *Rhizobium* (MR) and a control with dual synthetic NP mineral fertilizer but without symbionts (NS). Four or three biological replicates were sampled for phenotypic characterisation as well as for proteomic and metabolomic studies, respectively.

The soil used in this experiment was collected from the 0–20 cm horizon of arable fields in Tulln, Austria. Table S1 shows its chemical and physical characteristics. It was air-dried, sieved to pass a 2 mm sieve, mixed with expanded clay and silica sand (1:1:1 w/w/w), and sterilised at 121 °C for 20 min. Prior to planting, plastic pots (3 L) were disinfected with 12% sodium hypochlorite, cleaned with deionised water, filled with 2 kg growing substrate (described below) and moistened with 400 mL sterilised deionised water. To maintain the moisture content at optimum levels, pots were irrigated with sterilised deionised water every second day until a drop came out from their bottom. As can be seen from Table S1, the soil was low in both plant available nitrogen and phosphorus. In low N soils, a starter dose as little as 5–10 kg N ha^{-1} can stimulate seedling growth and early nodulation such that both N_2 fixation and eventual yield are enhanced [40]. The same is true for phosphorus and AM-symbiosis.

Therefore, each pot received starter N and P with synthetic fertilizers at the same rate of 20 mg kg^{-1} soil after planting. Furthermore, the non-symbiotic treatment received N and P forms as described by Hoffmann et al. [41] for beans (i.e., 80 mg N and 28 mg P kg^{-1} soil). Similar to this dual NP mineral fertilizer group, pots with single AM fungi or single *Rhizobium* bacteria treatment also received nitrogen or phosphorus, respectively. Additionally, a modified NP free nutrient solution prepared according to Broughton and Dilworth [42] ($CaCl_2$ 147 ppm, Fe-citrate 3.35 ppm, $MgSO_4 \cdot 7H_2O$ 61.6 ppm, K_2SO_4 43.5 ppm, $MnSO_4$ 0.17 ppm, H_3BO_3 0.123 ppm, $ZnSO_4$ 0.144 ppm, $CuSO_4$ 0.05 ppm, $CoSO_4$ 0.028 ppm, $NaMoO_2$ 0.024 ppm; pH 6.7) was applied at a rate of 10 mL pot^{-1} once a week.

2.1.2. Biological materials

Commercial inoculants (Vaminoc) containing a *Glomus* species and a *R. leguminosarum* bv. *viceae* were obtained from former Becker Underwood Ltd. UK. The inoculants were applied as prescribed by the company. The *P. sativum* seeds obtained from Rubiales Lab, Cordoba (Spain) was cultivar Messire, which is reported to be susceptible to *D. pinodes* [1,43,44]. From the bulk seeds, we selected uniform and healthy ones for surface sterilisation (70% ethanol for 30 s, 12% sodium hypochlorite for 5 min). Subsequently, the seeds were rinsed six times with sterilised deionised water, and pre-germinated in previously autoclaved (20 min at 121 °C) perlite. From three days old pre-germinated seeds, five healthy-looking plantlets pot^{-1} were chosen. To avoid potential cross-contamination of microbial inoculants between treatments, planting and covering of the germinated seeds were started with NS pots. Ten days after planting, seedlings were thinned down to three and four

Table 1
Plasma membrane proteins significantly responding to infection (Student's *t*-test, $p < 0.05$).

Accession	Description	Peptides	Max. peptide Score	i/h		
				p-Val (*p*-adjust)	Ratio	Significance
gi|118933	Disease resistance response protein Pi49 (PR10)	13	252.12	0.000 (0.007)	7.8	***
gi|1708427	2′-Hydroxyisoflavone reductase (NADPH: isoflavone oxidoreductase)	22	255.39	0.004 (0.062)	5.3	**
gi|257632899	Unnamed protein product [*Pisum sativum*]	16	179.65	0.000 (0.001)	6.7	***
frv2_47806	Plastocyanin-like domain protein (UniRef100_A0A072TWJ3 icov:100% qcovs: 73.98% e-val: 6e-78)	4	174.94	0.000 (0.007)	8.9	***
frv2_110760	12-Oxophytodienoate reductase-like protein(UniRef100_G7K3S2 icov: 96% qcovs: 91.11% e-val: 0)	9	165.89	0.002 (0.042)	3.9	**
frv2_111907	Archaeal/vacuolar-type H^+-ATPase subunit B (UniRef100_A0A072VSL4 icov: 98% qcovs: 99.18% e-val: 0)	17	206.74	0.013 (0.178)	3.5	*
frv2_53662	Protein disulfide-isomerase (UniRef100_B7FM01 icov: 98% qcovs: 84.41% e-val: 0)	17	202.4	0.018 (0.213)	2.3	*
frv2_75243	Translational elongation factor 1 subunit Bbeta (UniRef100_Q6SZ89 icov: 90% qcovs: 95.67% e-val: 3e-133)	5	187.78	0.003 (0.051)	2.1	**
frv2_83550	PfkB family carbohydrate kinase (UniRef100_G7IAA1 icov: 91% qcovs: 92.08% e-val: 0)	10	314.42	0.000 (0.007)	2.5	***
frv2_86187	Glucan endo-1,3-beta-d-glucosidase (UniRef100_Q9ZP12 icov: 98% qcovs: 87.01% e-val:0)	11	307.43	0.000 (0)	2.4	***
frv2_86875	Proteasome subunit beta type (UniRef100_B7FGZ8 icov: 85% qcovs: 96.98% e-val: 4e-164)	4	192.4	0.001 (0.015)	2.4	***

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