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The interdependent effects of the pathogen *Didymella pinodes* and the symbionts rhizobia and mycorrhiza on *Pisum sativum*

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1 General introduction

1.1 Field pea cultivation and research

1.1.1 Field pea: agronomical relevance

Amongst all flowering plants, the legume family is the third largest [1] and in agriculture, after *Poaceae*, legumes are globally the second most produced crop accounting for 27% [2]. Their success is due to several properties, of which the most important is supposable the association with nitrogen fixing rhizobacteria. The symbiosis enables them to metabolize nitrogenous compounds deriving from the bacteria. By this, the biological nitrogen fixation not only contributes to the growth of legumes but, moreover, legumes gain an important role in the agricultural ecosystem. Thus, crop rotation and winter greening with legumes is common practise even beyond organic farming. Consequently, there are additional effects on reducing greenhouse gas emission as fertilizer production accounts for 1.2% of the total energy consumption [3].

Besides their success story based on symbiosis it is the nutritional value of legume seeds that makes them important for a balanced human diet but also for feeding of life stock [4]. For instance, seeds of *Pisum sativum* are rich in proteins, slowly digestible starch, soluble sugars (5%), minerals, vitamins and fibre [5]. Hence, in efforts to emphasize this agricultural and nutritional importance, the year 2016 was declared as the international year of pulses (http://www.fao.org/pulses-2016/).

Pisum sativum was possible the legume species receiving most attention in the past centuries because of the Austrian monk Gregor Mendel who discovered the laws of inheritance. Also, among all crops, pea was one of the first being domesticated, even preceding cereals [6, 7]. Its agronomical and economical importance originates in the Mediterranean, mainly in the Middle East [7]. The long farming history of field pea lead to enormous diversity. At present, the biggest germplasms worldwide are found in Russia (6 790 accessions; NI. Vavilov Research Institute of Plant Industry), the USA (6 106 accessions; Plant Germplasm Introduction and Testing Research Station, Pullman), Australia (6 567 accessions; Australian Temperate Field Crop Collection, Horsham) and Syria (6 105 accessions; International Center for Agricultural Research in the Dry Areas, Aleppo). A summary of the worldwide greatest germplasms in Smýkal,

Aubert [8] illustrates an overall diversity of more than 57 000 accession. Despite this extensive gene pool which is present *in vivo*, the majority of available accessions remains to be genotyped and phenotyped in order to select traits for breeding to adapt cultivars to environmental changes [9]. The diversity manifests phenotypically in great variation of pod number, seeds per pod, leaf area, or the number and distribution of nodules along the root. Although, the effectivity of the symbiosis is crucial for crop performance, there is just little information about nodulation patterns and interaction compatibility of rhizobial strains with modern cultivars that were mostly bred to increase yield [10]. This gap of knowledge of the belowground phenotype is caused by a lack of available methods. For instance, approaches exist to standardize and automate the mapping of nodulation patterns, however, procedures are still labour intense [11]. Still, reports depicting relationships of traits such as between shoot length and nodulation intensity could allow for more efficient breeding [12]. With respect to epidemiology, the leaf type (e.g. leafless, semi-leafless) and an erect habitus mostly influence microclimate and thus, fungal spore germination and disease progress. Useful genetic traits might not exclusively be hold by the cultivar species, but in other close relatives such as *Pisum fulvum*. However, this genetic diversity has so far been poorly exploited [13].

In the period from 2000 to 2010, a total of 94 countries largely placed in temperate zones, cultivated pea. While since 1990 field pea production declined in Europe, it increased in America, Canada and Russia (http://www.fao.org/) mostly due to the global market situation (e.g. low price level of soy bean). Plant derived proteins in Europe are to 75% imported and mostly consist of soy meal. Such circumstances affect global nitrogen distribution as places of input are far distant from leakage sites (e.g. spreading of life stock manure) what disturbs local ecosystems and is highly energy consuming compared to closed loop systems [14]. The re-establishment of low input systems (e.g. organic farming) involves the integration of pulses in agricultural practise. Out of the legume diversity, the *Pisum sativum* species is one of the most suited in the European moderate climate and its protein rich seeds meet the high demands for fodder. The rising food demand of a progressively growing population and climate change urge to breed for increased tolerance against abiotic and biotic stress in plants [15]. Some regions expect more frequent precipitation and humidity generating conditions that are favourable for fungal sporulation [16].

Today, fungal diseases are the most limiting factors of pea yield and the exploitation of current legume diversity is a key strategy to ensure efficient and sustainable agriculture. In the following chapters the role of belowground microsymbionts and the fungal disease complex of ascochyta blight will be introduced. An overview of plants general response to pathogenic fungal attacks is given to subsequently introduce the symbionts potential to decrease susceptibility.

1.2 Microbial - plant symbiotic interactions

In the past decade the exploration of the human gut microbiome let us revise our understanding of human health [17]. As a consequence, research interests expanded from the gut microbiota to the whole human microbiome (http://hmpdacc.org/). In ecology, it is long accepted that microbial communities tremendously affect ecosystem functioning by playing a crucial role in nutrient cycling consequently influencing plant diversity [18]. Vice versa, aboveground show a strong linkage to belowground biota [19]. And moreover, like for the human microbiome, reports describing newly discovered interactions with plant endophytes accumulate [20]. Yet, the plethora of microbes being in endophytic interaction is poorly described and we just scratch the tip of the iceberg. It must also be elucidated whether newly discovered endophytes have growth promoting capacities and if they are culturable [21]. Like for the human microbiome, new findings of the plant microbiome are promising to be ground breaking and our view of sustainable agricultural practice will be changed during the forthcoming decades.

Before the great plant microbiome diversity was suspected, other endophytes such as mycorrhiza and rhizobia were well described. Presumably it was their influence on nutrient availability what gave their hidden life an aboveground appearance as they affect the plants phenotype. The association with nitrogen fixing bacteria is not limited to members of the *Fabaceae* family and other examples are readily found for instance in the tropical rainforest where bacteria colonise the phyllosphere of higher plants [22]. However, scarcely any plant species other than legumes provide such optimised conditions for nitrogen fixation by forming specialised organs, where rhizobia are accommodated under oxygen low environment. In *Pisum*

ssp., nodules are indeterminate (maintaining an active apical meristem), which is decisive for their cylindrical shape [23]. Moreover, shape and nodule growth vary between cultivars. In any way, the nitrogenase complex is highly energy demanding (8 ATP per NH_3) and therefore bacteroids dependent on plant assimilates. Also nitrogenase activity varies between strains and cultivars as well as over time with maximum fixation rates at different stages of development [24]. The transport form of fixed nitrogen varies between legume species and *P. sativum* mainly obtains nitrate, amides, amino acids, and ureides [25].

Reports suggest that the association of land plants with mycorrhizal fungi was crucial for the evolution of vascular plants around 700 million years ago [26]. Most higher plants are able to associate with mycorrhizal fungi in different ways [27]. The fungi either colonises the root cortex extracellular space forming the so called Hartig net (ectomycorrhiza), or hyphae penetrate the cell wall, invaginate the cell membrane and form arbuscules (endomycorrhiza) [28]. These arbuscular mycorrhizal fungi (AMF) are exclusively formed by the division of *Glomeromycota*. Due to the fine structure of hyphae the surface is enlarged and uptake of mineral nutrients (e.g. phosphate) via infiltration of the soil is facilitated [28]. The translocation of phosphate in the mycelium is sped up by formation of polyphosphates, which is just prior transfer to the plant again converted to inorganic phosphate [29]. Nitrogen is transported in form of the amino acid arginine that is released in the intraradical mycelium as NH_4^+ [30]. Equally to the rhizobial symbiosis the plant in turn provides carbohydrates to the fungi accounting for up to 20% of photo-assimilates [31]. It has been suggested that parts of the freshly obtained C might be rapidly released to soil microbes [32]. This affirms the notion of the mycorrhiza symbiosis being a key player in soil ecosystems.

Although arbuscular mycorrhiza fungi and rhizobial bacteria are very different organisms, they share a common signalling cascade and course of morphological modulation whilst their initial interaction with plant root. The exudation of strigolactones by plant roots induces spore germination and branching of hyphae. AMF release Myc factors that in turn induce calcium oscillation in root epidermal cells [33] what activates genes required for symbiosis [34]. Similarly, in the rhizobial symbiosis plants release flavonoids in the rhizosphere that are perceived by the rhizobia which in turn produce nod (nodulation) factors activating plant signalling pathways leading to calcium oscillation [35]. While rhizobia enter by being trapped

in a curling root hair guiding their way through an infection thread into the root tissue, mycorrhiza first form a hyphopodia, like the known appressoria from pathogenic fungi, and then enter via a plant cell produced prepenetration apparatus that resembles the rhizobia infection thread [36]. The infection thread formed in rhizobial symbiosis, as well as the prepanatration apparatus in the mycorrhizal symbiosis have in common that they are lead and relocated by a plant nucleus which path is predicted by the alignment of the endoplasmatic reticulum and the cytoskeleton [36]. During the rhizobial infection process the meristematic active nodule primordia develops into a nodule in which bacteria are released into the symbiosome, surrounded by the peribacteroid membrane. There, bacteria continue to divide, enlarge soon after and differentiate into bacteroids which main purpose is the fixation of nitrogen. AMF on the other hand form the symbiotic arbuscular structures where nutrients are exchanged at the fungal plasma membrane and the plant derived periarbuscular membrane [37].

Both, mycorrhiza and rhizobia symbiosis are under strict control of the host plant which process is referred to as autoregulation of mycorrhization/nodulation. In legumes, the nodulation events on first originating roots inhibit later nodulation on younger roots to manage the balance between shoot and root development [38] and to control the energy investment for the obtained nitrogen which is reported to be 12-17 grams carbon for 1 gram of nitrogen [39]. Studies with split root systems showed that the regulation of nodulation involves a systemic feedback response including a leucine rich receptor kinase (pea: PsSym29) in the shoot [40]. Downstream signalling of this receptor kinase showed activation of jasmonic acid synthesis genes [41].

Likewise, novel root colonization by AMF is reported to inhibit establishment of subsequent mycorrhiza arbuscules [42] and fungal penetration of the root is regulated via the accumulation of flavonoids (formononetin, medicarpin) depending on the plant´s phosphor condition [43]. The repression AMF and rhizobia also correlates with higher levels of endogenous SA [44]. AMF is suggested to modulate the host´s immune system via excreting small cysteine rich apoplastic proteins resembling pathogen effectors [45].

Many reports showed increased nodulation in soybean roots colonised by AMF suggesting that the increased P supply enhances nodule formation [46-50]. However, others showed that nodulation is suppressed when co-inoculation with AMF was performed [51]. Vice versa, some studies found significantly increased AMF colonization [47, 52, 53] while others showed suppression of AMF by nodulation [54]. Recent reports indicated a common pathway for autoregulation of symbiont settlement what opens space towards interpretation of mutual suppression of nodulation and mycorrhization via a systemic signal [55].

1.2.1 Ascochyta blight and its causal agent *Didymella pinodes*

Viruses (pea common mosaic, pea seed borne mosaic, top yellow), bacteria (*Pseudomonas*) and fungi (e.g. *Fusarium*, ascochyta blight, powdery mildew, downy mildew) cause field pea diseases, leading to severe yield losses. Fusarium wilt (*F. oxyyporum* sp. *pisi*) was the first disease where resistance was discovered in a single dominant gene [56]. Next, higher resistance against *Ascochyta pisi* was discovered in the cultivar Austrian winter pea which was later used in combination with other lines to select for resistance against all physiological races [57, 58]. To date, in regions where field pea is regularly cultivated, ascochyta blight is the most harming foliar disease [59]. The disease is a fungal complex comprising the species *Ascochyta pinodes* (teleomorph: *Didymella pinodes*; Berk. and Blox.), *Phoma medicaginis* var. *pinodella* (L.K. Jones), *Ascochyta pisi* (teleomorph: *Didymella pisi*) [60, 61], *Phoma koolunga* [62] and others such as *Phoma herbarum* and *Boremia exigua* var. *exigua* [63, 64]. In a field experiment where plots were artificially infested with isolated species of the fungi complex, *D. pinodes* caused the greatest yield reduction [65]. Now, *D. pinodes* is commonly agreed to be the most damaging agent in this disease complex. It is most prevalent in Mediterranean and temperate regions, including main producing countries such as Canada, Australia, France, and China [60, 66, 67]. Yield losses due to ascochyta disease are mainly determined by environmental conditions (e.g. frequent rainfalls, high humidity, and strong winds) and the geographic location [68, 69]. The extent of damage, reflected in yield, ranges from 10%, that are annually consistent in Australia [60], to more than 50% in Canada [70] and even more severe penalties of over 75% in France, Australia, and Canada [59, 71]. In Western Australia the average yield loss is reported to exceed

50% [72]. Harvest penalties may directly correlate to reduced photosynthetic area caused by increased disease severity. Certainly, infection of pods reduces individual seed weight [73], but also, yield losses are connected to decreased plant growth caused by disease on stipules, internodes or stems [74]. Thus, already 48 days after sowing yield losses can be predicted with remarkable precision [68].

Epidemiology

Fungi survival over the wintertime was observed on plant debris, in the soil (sclerotiom generation), and on seeds, which is probably the most appearing form. Hence, *Ascochyta spp.* are typically considered seedborne what contributes to long distance dispersal such as between nations [75]. In the early season, ascospores rapidly develop as primary inoculum. On newly infected plants flecks with necrotic lesions evolve where pycnidia emerge, disseminating a secondary infection wave of pycnidiospores that generally infest plants in close vicinity. Again, pycnidia develop and pycnidiospores will re-infect newly grown tissue on upper parts of the plant. The reported latent period is 3-4 days and the process of re-infection is likely to repeat several times during a season [76]. Inoculation of first leaves by *D. pinodes* was reported not to decrease severity of later infections [77]. Secondary infections promote tissue senescence, which is indispensable for pseudothecia formation. Therefore, *D. pinodes* aggressiveness correlate with decreasing phytoalexin concentrations on older tissues and pseudothecia are predominantly formed at the basis of pea plants [71, 78]. Hence, alongside pycnidia also pseudothecia are formed frequently releasing ascospores that constitute an important source of secondary inoculum during the entire season [69]. It was hypothesized that different population cause ascochyta blight on winter or spring pea because the onset of disease appears at different timepoints although the growing season overlaps for four month. However, it was found that *D. pinodes* isolates of winter pea and spring pea are polymorphic but not different what indicates strong plasticity in one *D. pinodes* population [79].

Disease management

Given that host resistance levels towards to *D. pinodes* are low, disease management became important for successful field pea cultivation. The main approaches are minimisation of inoculum carry-over by decreasing the survival of inoculum (e.g. on crop residues, soil), and avoidance of initial infection of the

crop from aerial inoculum [80]. While burying of infected residues has been reported to decline pathogen survival [81, 82], tillage regimes and crop rotation did not lessen the disease magnitude [83]. Although, removal or burning of crop residue limits pathogenic expansion, it provokes soil erosion and is disadvantageous for carbon sink strategies. Delaying the sowing date is the main strategy to avoid initial ascosporal showers especially in Australia [84, 85]. However, this option is only suitable in regions with long growing seasons but not applicable in Europe or North America.

1.2.2 Pathogen infection process and evasion strategy

The spore germ tube typically forms an appressorium and penetrates the cuticle [76, 86]. Host colonisation is characterised by subcuticular development followed by proliferation of the intercellular space in the palisade mesophyll 24 h after inoculation what results in host cell death [87-89]. Next, the fungal mycelium aggressively proliferates host tissue and releases phytotoxins and enzymes to suppress the host immune response. At the beginning, studies on *D. pinodes* largely focused on pathogen elimination, restriction of reproduction [90, 91] and on factors influencing the survival, such as the control via seed treatment [92-94] or fungal growth inhibition on leaves with chemicals [78, 87]. Research on the infection process and the pathogenic factors in *D. pinodes* responsible for triggering host susceptibility in *Pisum sativum* began in the 70´s by the discovery of cell wall degrading enzymes released by the pathogen [95]. To date, it was Ichinose, Oku, Shiraishi, and Toyoda who mainly investigated the mechanisms of action taken place during the infection process. They stated that during infection there must be a mechanisms that supresses pisatin synthesis, because leaf penetration is completely inhibited already by low concentrations (50 ppm) of this phytoalexin [96]. However, others showed that *D. pinodes* possesses pisatin degrading activity achieved through a cytochrome P450 complex that demethylates the phytoalexin with low K_m [97, 98]. Despite this pisatin degrading ability, *D. pinodes* suppresses pisatin synthesis within the first 24 h after infection [99]. In a germination fluid, conidia of *D. pinodes* release an elicitor and two suppressors [100, 101] that regulate pisatin synthesis in a competitive manner [102, 103]. The elicitor was reported to be a high molecular weight glycoprotein: A trisaccharide (Man-Man-Glu) that is O-glycosidically attached to a serine [104, 105]. The suppressors (named suprescin A&B) are an (A) α-N-acetylgalactosamin linked

tripeptide (SerSerGly) and a (B) β-Gal,1-4,O linked to an α-N-acetylgalactosamin-SerSerGlyAspGluThr [106]. The application of the elicitor results in mRNA accumulation of phenylalanine ammonium lyase (PAL) and chalcone synthesis (CHS) what is followed by an increase of these enzymes´ activity as well as pisatin accumulation [107]. The simultaneous application of elicitor and suppressors, however, resulted in 3 h delay of PAL and CHS mRNA transcription, and 6 h delay of their activity. Suppressor application also induces susceptibility to non-pea pathogens such as *M. ligulicola* and *M. melonis* [108]. In pea epicotyl pieces, pisatin synthesis is suppressed up to 9 hr (1 hr without suppressor) [107] and ATPase activity (H^+ pumping) in the plasma membrane is influenced at a concentration of 50 µg ml^{-1} of suppressor. In correlation to the decreased activity of plasma membrane ATPase is the suppressed, rather than delayed, activation of chitinase and β-1,3-glucanase what is exclusively in pea but not soy bean or kidney bean [109, 110]. However, later reports also identify alternative hosts for *D. pinodes* (e.g. soy, and cow pea) *[111, 112]*. After plasma membrane exposure to the elicitor, there is an immediate increase of phosphatidylinositol-4.5-bisphosphate (PIP2) and inositol-1,4,5-triphosphate (IP3) levels along with increased plasma membrane ATPase activity [113]. Upon simultaneous treatment with the suppressor, the release of PIP2 and IP3 as well as the plasma membrane ATPase activity are inhibited. ATPase activity can only partially be recovered upon addition of PIP2 [114]. It is probably the acidic amino acid domains of suprescin B (Asp-Glu-Thr and Gly-Asp-Glu) that inhibit plasma membrane phosphatase activity directly [115]. Additionally to the plasma membrane ATPase, a cell wall fraction ATPase, hydrolysing NTP, was found to be suppressor inhibited [116]. This ATPase contains five ACR domains (ADAM - transmembrane - cysteine rich) that are conserved among apyrases (calcium activated plasma membrane bound enzymes that catalyse the hydrolysis of ATP) [117]. The biotinylated elicitor and the suppressor bind to this ecto-apyrase (NTPase) that shows peroxidase activity and generates superoxide [117]. More precisely, the generation of superoxide appears to be dependent on the ATP hydrolysing activity releasing P_i, because when inorganic phosphate is induced artificially in pea tissues, superoxide is produced [118]. Equally, apoplastic P_i concentrations increase by about 30% upon elicitor treatment. Such increase of Pi also induces increase of peroxidase mRNA but not mRNA of PAL [118]. Consequently, penetration by *D. pinodes* is inhibited by P_i not during

the first 3 h, but at 6-12 h after infection, when synthesis of peroxidase is executed [118]. Later [119], it was shown that a copper amine oxidase with ammonia and hydrogen peroxide generating activity is forming a complex with the cell wall associated ecto-apyrase (NTPase). Activity is regulated by the elicitor, and complex formation is impaired by the suppressor as application causes appearance of apyrase monomers rather than complexes [119]. Similarly, the inhibition of ATPase (ecto-apyrase) activity by vanadate hinders the usual apoplastic efflux of Na^+ and K^+ ions upon elicitor treatment [120] what indicates impaired signalling as superoxide and induction of mRNA are also suppressed. Hence, ecto-apyrase constitutes a switch point in defense signalling against *D. pinodes* and is therefore targeted by the *D. pinodes* effectors/suppressors. The suppressor treatment alone causes the expression of 12-oxophytodieonate reductase [121, 122]. After establishing a functional pathosystem of *D. pinodes* and *Medicago truncatula* almost all members of the jasmonate (JA) synthesis pathway were shown to be upregulated upon infection and the salicylic acid (SA) induced PR10 protein was suppressed [123]. Hence, silencing of JA genes in pea results in remarkable reduced disease development [123]. Although, others argue for a positive role of JA in reducing disease severity [124], it is more conclusive that the suppressed synthesis of PR10 protein is the result of impaired SA signalling. To this, SA is thought to suppress JA synthesis [125], which in turn would promote resistance during the biotrophic phase of *D. pinodes* rather than susceptibility. Supporting this is suggestion of ecto-apyrase to positively regulate SA induction upon elicitor binding, and defense related genes are downregulated when ecto-apyrase is silenced, but, JA induced genes are upregulated when the suppressor interferes with the ecto-apyrase [126].

1.3 Plant pathogen interactions and systemic priming

1.3.1 Response to fungal pathogens

Abiotic stress such as fungal infestation affect the plant´s metabolism simultaneously at several levels. The process mostly onsets in the apoplast, where conserved pathogen associated molecular patterns (PAMP´s) are recognized by the host´s pattern recognition receptors that induce a signalling cascade which

eventually leads to PAMP triggered immunity (PTI). A PTI response includes accumulation of reactive oxygen species (ROS), activation of ion channels, activation of mitogen-activated kinase cascades (MAPK´s), and transcriptional reprogramming that results in callose deposition, H2O2 generation in the apoplast, phytoalexin synthesis, and synthesis of pathogenesis related (PR) proteins (e.g. chitinases). Successful colonisation is also achieved by biotrophic pathogen fungi through secretion of effectors. The task of such effectors is to silence the immune response in order to invade the plant. This is achieved by shielding MAMP´s or DAMP´s (damage associated molecular patterns) to avoid host detection, or by directly targeting host proteins to interfere with signal transduction. However, plants may develop mechanisms to sense pathogen effectors (e.g. guard proteins) that activate effector triggered immunity (ETI) what ultimately leads to a hypersensitive response cell death [127]. Pathogenic fungi with a biotrophic lifestyle commonly aim to avoid detection in order to invade the apoplast and thrive on host derived sugars [128]. Necrotrophs, however, might provoke a host hypersensitive response cell death to feed on content released from dead cells [129]. The infection mode of *D. pinodes* is mostly defined as hemibiotrophic because the fungi colonises living host cells (~ first 48 h) and subsequently produces toxic compounds [123, 130]. Nevertheless, some consider *D. pinodes* necrotrophic because of the short phase of apoplast invasion [79, 131]. As such, the penetration course mostly resembles the one of the necrotrophic fungi *Botrytis cinerea* infecting *Arabidopsis* [132]. In the following chapter, the plant response and adaptation to the infection of hemibiotrophic/necrotrophic fungi will be discussed in general with occasional links to examples in *D. pinodes*.

The cell wall

Typically, necrotrophs deploy phytotoxins and a battery of cell wall-degrading enzymes that ultimately induce cell death. They are known to induce synthesis of host proteins such as cellulases and expansins that contribute to cell wall loosening [133]. Also, *D. pinodes* was reported to show cell wall degrading activity by cellulase and pectinase [95]. Hence, the cell wall is an important physical barrier consisting of cellulose, hemicellulose, glycoproteins, and pectins. Pectin is found in the middle lamella where it binds cells together, or is also located in the primary cell wall. Some free adjacent carboxyl groups are linked by Ca^{2+} or Mg^{2+} ions and other carboxyl groups are esterified by methyl groups. Pectin methyl-

esterases facilitate cell wall modification and decomposition. Thus, the plant status of pectin methylesterification is essential for plant resistance towards *B. cinerea* and constitutive expression of pectin methylesterase inhibitors possibly decrease the cell wall degrading ability of the fungus [134]. Hence, during infection the cell wall undergoes several modifications such as lignification, deposition of callose, cell wall protein cross linking and accumulation of antimicrobial compounds [135]. The required enzymatic arsenal for this response includes for instance peroxidases and pectin-methyl-esterase inhibitors (Raiola 2011). Peroxidases and laccases catalyse oxidation of monolignols to radicals that consequently polymerise to form lignin.

The enzymatic activity of peroxidases depends on H_2O_2 supply. Plasma membrane located NADPH oxidases transfer electrons from cytosolic NADH or NADPH to apoplastic oxygen, resulting in superoxide (O_2^-) that is converted by superoxide dismutase to H_2O_2 [136]. These NADPH oxidases are typically referred to as respiratory burst oxidase homologs (RBOH) and are also involved in the immune response of mammal. Its EF-hand domain indicates the importance of calcium for its regulation. Indirectly Ca^{2+} also regulates phosphorylation of RBOH via a calcium dependent protein kinase (CPK). Likewise, two amino acid residues that are highly conserved among different plants RBOHs are phosphorylated by the *Botrytis* induced kinase (BIK1) upon PAMP perception [137, 138]. The compulsive regulation of RBOH by both, BIK and Ca^{2+} indicates a two-step activation [139]. And, CPK28 indirectly regulates ROS and Ca^{2+} burst by controlling BIK1 protein turnover [140]. The apoplastic ROS production itself is thought to induce Ca^{2+} influx in neighbouring cells where RBOH are activated generating a ROS wave which leads to a defense response in distal leaves [141].

Sensing of PAMPs and DAMPs by pattern recognition receptors (PRR) leads to downstream signal conversion by 3 kinases known as the MAPK cascade [142]. Amongst other PRR, the chitin elicitor receptor kinase 1 (CERK1) triggers the first line of MAPKs in *Arabidopsis [143, 144]*. In the third line the kinases MPK3 and MPK6 are also activated by oligo-saccharides and the fungal elicitor chitin [143, 145, 146]. Both kinases (MPK3, MPK6) regulate the expression of 1-aminocyclopropane-1-carboxylate synthase 2/6

(ACS) as well as its phosphorylation which leads to protein stabilisation and increase in cellular ACS activity and ethylene production [147, 148]. Vice versa, MPK3 and MPK6 mRNA is accumulating after treatment with benzothiadiazole, a functional analogue of SA [149]. In turn, MPK4 (third line) mutants accumulate SA but do not induce JA defense genes [150]. MPK6 together with its upstream kinase MKK3 was found as negative expression regulator of the transcription factor MYC2 that mediates JA sensitivity [151]. Inactivation of MPK4 and MPK6 by overexpression of phosphatases (AP2C1) comprises resistance of *Arabidopsis* against the necrotrophic fungi *B. cinerea* [152]. Direct impact on phytoalexin accumulation in Arabidopsis was achieved by constitutive expression of active MKK9 [153, 154]. Additionally, MAPKs responsive to pathogens actively promote the generation of ROS in chloroplasts [155].

Hormones

After recognition of PAMPs and activation of PTI or ETI, plant hormones trigger immune signalling [156]. Mainly the hormones salicylic acid (SA) and jasmonic acid (JA) coordinate the defense response [125]. SA, a phenolic compound synthesised from chorismate via PAL and isochorismate synthase [157], operates as transcriptional co-activator for several defense genes [158] effective against microbial biotrophic pathogens [159]. Downstream signalling of SA is largely regulated by the Non-expressor of PR-Genes 1 (NPR1), that resides in the cytosol as oligomer and is translocated as monomer to the nucleus via a nuclear pore protein [160]. In the nucleus NPR1 interacts with a transcription factor that binds to the promotor region of SA induced genes such as PR1 [161]. Activation of SA signalling entails a similar response in distal parts of the plant.

JA biosynthesis starts with the release of α-lineolic acid from the plasma membrane lipids followed by the oxylipin pathway [162]. After synthesis, JA can readily be methylated [163] or conjugated to amino acids such as Ile forming the highly active jasmonoyl-Ile [164, 165]. In inactive cells, the transcription repressor jasmonate-zim-domain protein (JAZ) binds to transcriptional regulators of JA signalling. The binding of JA-Ile to its receptor COI1 leads to ubiquitylation and degradation of JAZ resulting in activation of JA-responsive genes [166]. JA signalling is channelled in two directions: The MYC branch, being con-

trolled by the MYC-type transcription factors contributing to defense against insects, or the ethylene response factor (ERF) branch that requires JA and ethylene [167] what includes expression of the JA responsive marker gene plant defensin 1.2 (PDF1.2) which enhances defense against necrotrophic pathogens [168]. ET promotes ERF branch gene expression but antagonizes the MYC branch [169]. Moreover, genes of the JA biosynthesis pathway were found to be induced by JA [170].

Plant hormones are known to interfere in its signalling or synthesis. E.g. SA and its acetylated form potentially suppress the JA wound response [171]. However, low concentrations of SA and JA were shown to result in synergistically increased expression of PR1 and PDF1.2, but the effect was antagonistic at higher concentrations [172]. To this, NPR1 mutants were found not just impaired in SA signalling, but also failed to suppress JA signalling [173]. Despite the importance of nuclear localisation for SA responsive gene expression, NPR1 does not seem to play a role in JA depression [174]. The TGA transcription factors - important regulators of SA induced gene expression - bind to the SA-responsive core motif of PR-gene promotors [175] and also to the PDF1.2 promoter, what suggests a direct inhibition of JA-responsive promoter activity [176]. In the MAPK cascade, MPK4 was identified as negative regulator of SA [150]. MPK4 mutants showed elevated levels of SA but were unable to induce JA response.

Both, SA and JA influence the cellular glutathione buffer [177]. Whereas the amount and the ratio between reduced and oxidized GSH increases with SA, the GSH pool decreases with JA. Reversely, the overexpression of a glutaredoxin (GRX480) antagonizes the PDF1.2 transcription [178]. SA and JA pathways were also found to be changed by an enzyme (SSI2; suppressor of insensitivity) catalysing desaturation of stearic acid to oleic acid [179]. Mutants of SSI2 have reduced levels of oleic acid what signals upregulation of the SA and downregulation of the JA pathway [180]. The WRKY transcription factor genes potentially mediate the SSI2 induced SA-JA crosstalk. Mutants of WRKY50/51 reduced the SA levels mediated by lower SSI2 and restored JA responsive gene expression [181].

Auxin signalling is an additional factor that regulates SA levels [182] and some biotrophic pathogens exploit auxin mediated depression of SA signalling [183]. In return, along PTI, auxin receptors are targeted by mRNA and suppress auxin signalling, thereby preventing auxin from suppressing SA [184].

Hence, overexpression of this mRNA results in higher resistance to biotrophs, but susceptibility to necrotrophs, because of reduced camalexin synthesis [185]. Indirectly gibberellins were found to regulate susceptibility to necrotrophs. They regulate degradation of DELLA proteins that repress plant growth [186]. Degradation of DELLA proteins in turn was found to increase susceptibility to necrotrophs [187]. Similar influence was shown by cytokinins that activate the ARR2 transcription factor by binding to the SA-response transcription factor TGA3 what positively regulates PR-1 gene expression [188].

Primary metabolism

During infestation the primary metabolism underlies regulation directed by the plant immune response, but also changes that are induced by the pathogen (e.g. transcriptional reprogramming, feeding on host nutrients). Hence, approaches utilising elicitors tend to reflect the plant induced changes of primary metabolism, whereas studies with virulent pathogens show metabolic changes induced from both, the plant and the pathogen [189]. The role of primary metabolism during pathogen infection is diverse and besides energy provision for defense response, many primary metabolites themselves were found to induce defense signalling [190]. Foremost, primary metabolism drives plant growth and development. Thus, the onset of defense response signalling and expenses for development must be balanced. Such was demonstrated with reduced fertility in *Arabidopsis* plants that constitutively express defense genes, while mutants defective in defense signalling showed a taller phenotype [191] leading to the suggestion that the upregulation of defense related pathways is compensated by downregulation of photosynthesis and chlorophyll synthesis [192]. Although it is not fully clear how downregulation of photosynthesis happens, it was proposed that this is either triggered by pathogen effectors [193] or by a sugar signal feedback regulation [194]. The reason for downregulation of photosynthesis is possibly to lower the energy costs. This is coupled with upregulation of other pathways related with energy provision such as respiration, carbohydrate transport and cell wall invertase [195, 196]. In line with this, studies on *Arabidopsis* and tomato showed transcriptional upregulation of glycolysis, TCA cycle, pentose phosphate pathway, mitochondrial electron transport and ATP synthesis, whereas other pathways such as lipid metabolism, C1 metabolism and starch metabolism were downregulated [197]. Carbohydrate metabolism is of particular importance in defense signalling

since it was found that sugars (e.g. sucrose, glucose, and fructose) positively regulate defense-gene expression [198]. Glucose defense signalling was shown to rely on the hexokinase HXK1, which downregulation caused increased expression of defense transcripts, accumulation of H_2O_2 and increased occurrence of PCD [199]. Likewise, pyruvate carboxylase is an important player in defense regulation as overexpression leads to increased sugar levels, export of sucrose, callose deposition, expression of PR genes, and decreased pathogen expansion [200]. Subsequently, plants impaired in cell wall invertase functioning (cleaving sucrose to glucose and fructose) show reduced callose deposition, lower levels of H_2O_2, and susceptibility to pathogens [201]. Cell wall invertase enzymatic activity is being increased upon pathogen elicitor treatment [202].

One of the first events occurring after elicitor sensing is stomatal closure entailing increased photorespiration. Augmented formation of glycolate requires higher activity of glycolate oxidase. In hybrid *B. napus* and *Arabidopsis* plants that are resistant to the fungal pathogen *L. maculans*, this enzyme is synthesized abundantly [203] and silencing of this enzyme leads to delayed onset of a HR and susceptibility to non-host pathogens what is encompassed with reduction of ET and SA responsive defense-related genes [204]. The subsequent conversion of glyoxalate to glycine requires amino transferases which were found higher in expression in natural resistant melon cultivars against an oomycete pathogen [205]. However, it was suggested that amino transferases cause higher demand of glyoxylate which induces glycolate oxidase activity and H_2O_2 release. Hence, resistance would again be attributed to accumulated glycolate oxidase. Similarly important is the conversion of glycine to serine by the serine hydroxymethyltransferase (SHMT1) in the mitochondria. SHMT1 mutants of Arabidopsis showed constitutive expression of the SA-response genes PR1 and PR2 [206]. After infection with *P. syringae*, SHMT1 transcripts were reported to accumulate [206]. The expression of PDF1.2 increases in both wild type and *shmt1* mutants one day after infection with *Alternaria brassicola*. However, PDF1.2 expression decreased 4 days later in the mutants, while it was still visible in the wild type [206]. This suggests a mediating role of SHMT1 between SA and JA signalling, where it promotes the JA pathway. Besides amino acids involved in photorespiration, almost all other amino acids´ levels are affected upon infection. The accumulation varies depending on the applied pathogen [207].

Arabidopsis mutants of the lysine histidine transporter (LHT1) had lower levels of glutamine, alanine, and proline and showed enhanced resistance to bacterial, and fungal pathogens [208]. This was encompassed with SA accumulation, PR1 expression and callose deposition. Similar observations were made with the glutamine dumper 1 mutant, which has reduced glutamine levels along with increased callose deposition, H_2O_2 generation and development of spontaneous lesions [209]. Proline, which is known as stress responsive amino acid, increases after infection and was associated with a HR [210]. Thus *Arabidopsis* proline dehydrogenase mutants are rendered susceptible to avirulent pathogens [211] and proline application results in cell death independent of pathogen infection [212]. Ornithine delta aminotransferase, another enzyme involved in proline metabolism, participates in ROS accumulation resulting in non-host resistance [213]. Similarly, homoserine (precursor of threonine, isoleucine, and methionine) increased resistance against *H. arabidopsidis* independent of SA, JA and ET signalling which was shown with a homoserine kinase mutant that accumulated homoserine [214]. Increased resistance was also achieved with a mutation in the dihydrodipicolinate synthase 2 and the aspartate kinase 2 gene which was accompanied by accumulation of threonine [215].

1.3.2 Induced systemic resistance

Some soils were reported to accommodate more than 33000 different operational taxonomic units including bacteria and archaea [216] concluding that the type of soil essentially effects the microbial composition [217, 218]. Plants are known to shape the composition of the microbial community in the rhizosphere [219]. Several of these microbes located within the plant root tissue or in close vicinity might promote plant health, however, reports indicate a role of the whole microbiome for shaping plant vitality [220]. Since plants have evolved in the midst of microbial communities that essentially contribute to their life (e.g. growth, defense, vitality), the microbial genome might be considered as extension to the plant genome forming a pan-genome [20]. Considering the diversity of microbial organisms associated with plants, a definition of a core root and endophytic microbiome emerges [220-225] analogous to the human microbiome project [17]. Traits of this second genome, derived from microbes, are suggested to be major targets in breeding programs maybe leading to the next green revolution [226].

Plants take care of their relationships in the rhizosphere by sloughing root cells containing plant cell wall polymers (e.g. cellulose, pectin) that are decomposed releasing compounds such as methanol which is utilised as carbon source by microbes [227, 228]. Irrespective of the exact relationship to plants (symbiotic, non-symbiotic), some soil-borne microbes improve the defense capacity in above ground parts by inducing a state of enhanced resistance that protects non-exposed plant parts from a future pathogen attack [229]. To date, studies on induced systemic resistance (ISR) mainly include *Pseudomonas*, *Serratia*, *Bacillus*, *Trichoderma*, non-pathogenic *Fusarium oxysporum*, *Piriformospora indica*, and mycorrhiza species [226, 230]. In contrast to systemic acquired resistance (SAR), which is active in non-infected tissue of a plant upon pathogen attack, ISR neither depends on signalling by the hormone SA nor on the hormones transcriptional co-regulator NPR1 [231]. In the symbiotic interaction of legumes with rhizobia the SA-dependent infection response is suppressed by the bacteria´s released Nod-factors. Overexpression of NPR1 was shown to suppress root hair curling in *Medicago*, whereas NPR1 depletion accelerated root hair curling [232]. Nod-factor signalling is accompanied by increased levels of cytokinin and auxin in cortical cells suggesting hormonal crosstalk of these hormones and local suppression of SA [35, 233]. In this respect, rhizobia and mycorrhiza share a common signalling pathway [234, 235]. And similarly to the rhizobia interaction, SA accumulates transiently during early stages of mycorrhiza encounter, but, if persisting, the hormone negatively affects root colonisation, which was shown in the interaction of *Pisum* and the AMF *Glomus mossae* [44, 236]. In a typical ISR state, PR proteins are not directly accumulated, however, upon pathogen attack there is a significant stronger increase of PR proteins compared to non-primed plants [237, 238]. But, in ISR primed plants there is accumulation of the transcription factor genes AP2 and ERF2 which are involved in regulation of JA and ET dependent defenses [166]. Similar, *Arabidopsis* mutants of the JA regulated transcription factor MYC2 fail to generate an ISR priming induced by *Piriformospora indica* [239]. Rather, *P. indica* utilises the JA signalling pathway to suppress early onset of an SA mediated defense response [240]. Likewise to MYC2, the transcription factor MYB72 was shown to be required for early ISR signalling in Arabidopsis [241]. However, overexpression of MYB72 did not enhance resistance to the pathogen indicating MYB72 as signalling node [242]. It is to note that MYB72 is also induced under iron

limiting conditions and the iron-deficiency marker genes Fe^{3+} chelate reductase and a Fe^{2+} transporter are co-regulated with MYB72 suggesting a link between iron homeostasis and induction of ISR [243, 244]. Additionally to augmented levels of transcription factors, primed plants show accumulation of pattern recognition receptors (e.g. FLS2, CERK1) and the kinases MPK3 and MPK6 [245]. These are reported to regulate 1-aminocyclopropane-1-carboxylate synthase expression, and hence, regulation of ET synthesis. However, upon colonisation of *Arabidopsis* by the ISR inducing bacteria *P. fluorescens* JA or ET levels are not increased what lead to the conclusion that priming sensitises plants to these hormones [246, 247]. Sensitisation to JA and ET requires NPR1, which was shown with NPR1 and MYB72 mutants that fail to exhibit enhanced expression of JA/ET-responsive genes and associated callose deposition [241]. With respect to NPR1 dependency, SAR and ISR are alike, however, there is growing evidence that the cytosolic form of NPR1 functions in JA/ET signalling in ISR, whereas in SAR the monomerisation and translocation to the nucleus seems decisive [248-251]. As result of JA/ET sensitisation the JA and ET responsive genes PDF1.2 and HEL show potentiated expression after pathogen attack positioning them as marker genes for ISR [252]. The pivotal role of JA and ET during ISR were confirmed with signalling mutants of jar1, jin1 and coi1 (JA) as well as etr1, ein2, ein3 and eir1 (ET) that were defective in ISR [253-258]. In line with the increased sensitivity to JA and ET is the reported effectivity of ISR against JA and ET sensitive attackers such as necrotrophic pathogens or herbivores [259, 260]. For instance, the proliferation of the necrotrophic fungi *Alternaria solani* on tomato or *Bortrytis cinerea* on roses was hampered after priming [261-264]. Additionally to the JA and ET dependency of ISR it was shown that enhanced callose deposition was impaired in ABA related ibs3 mutants attributing ABA an important role in ISR execution [265, 266]. Augmented response to pathogen attack was also shown after mycorrhiza priming: Rhizoctonia infected potato plants showed amplified accumulation of phytoalexins [267]. Besides JA and ET as requirements for ISR, in the interaction of barley with *P. indica* it was shown that priming is associated with the activation of the GSH-ascorbate cycle without mediation of JA or ET [268].

The local accumulation of JA in roots colonised by mycorrhiza and *P. indica* are indicative for the restriction of colonisation via onset of a defense response [269-271]. Equally ET insensitive mutants render

Medicago truncatula defective in regulation of nodulation and roots are hyperinfected by *S. meliloti* [272]. In contrast, overexpression of ET responsive transcription factors lead to less *P. indica* colonisation but ET-mutants showed intensified colonisation [273]. The mechanism regulating the number of successful colonisations of microbes other than rhizobia or mycorrhiza are suggested to be systemic, which was demonstrated with split root experiments where colonisation of one half lead to defense and repressed colonisation in the other half [274, 275]. Such signal transduction resembles the autoregulation of nodulation in legumes in which numbers of infections are controlled in a systemic mechanism [276]. Additionally, the mere onset of ISR in above ground tissue was shown to shape the interactions in the rhizosphere by modifications in the quantity and composition of root exudates as shown in mycorrhiza experiments [277-279]. Such changes are induced by JA and SA proposing alteration of the rhizosphere community mediated by the plant [280]. The extent of hormonal alteration, however, depends on the associated AMF [271] and nitrogen availability strongly interferes with mycorrhiza derived resistance in tomato towards *Bortrytis cinerea* [281]. Although mycorrhiza induces changes of root exudates, phenolic compounds, ROS homeostasis, and defense related phytohormones, no antimicrobial compounds have yet been isolated from exudates of mycorrhizal roots [230, 271, 282, 283]. To the already mentioned effects of mycorrhiza on the root and its vicinity, it is to note that mycorrhiza changes the soil structure at the rhizosphere level [284] what additionally influences plant interactions with other microbes such as nitrogen fixing bacteria [285, 286] or with phosphate solubilising bacteria [287, 288]. Remarkably, above mediating changes in the rhizosphere, mycorrhiza was shown to induce pathogen resistance in neighbouring plants via its hyphal network [289].

1.4 Integrative systems biology: proteomics and metabolomics for a holistic view on the plant pathogen response

Cultivation of crop plants lead to enormous genomic diversity during the past millennia but breeding for enhanced tolerance against abiotic stress as well as for higher resistance towards rapidly adapting pathogens remains ongoing. Increasing world population and challenging environmental conditions in regions strongly affected by climate change require efficient breeding methods [290]. To date, genebanks

store enormous diversity being successively phenotyped to share quality traits with breeders internationally [291]. Technological advances in the last decades facilitated characterisation of genotypes on molecular level with high-throughput and immense amount of data is generated daily. Genetic information, nowadays mostly derived from next generation sequencing, forms the basis of our molecular analysis and the linkage with phenotypic characteristics is of utmost importance for breeders. The genome, however, is static and molecular stress response is often not directly related to gene expression due to post-transcriptional or post-translational modifications [292]. The proteome and the metabolome, on the other hand, are closer related to desired crop quality traits. To date, great developments in chromatography, mass spectrometry and increased computational power facilitate large scale analysis. The data obtained from such analysis strongly depends on the experimental design and on the sampling procedure. For instance, stress duration, tissue type, or cellular compartment are pivotal criteria shaping the outcome and accuracy of biological interpretations. To allow most comprehensive linkage between different -omic levels, proteins and metabolites are to be extracted from the same sample [293]. Such integrative obtained data can then be set in context with phenotypic plant traits (e.g. pathogen resistance, drought tolerance) to elucidate tolerance mechanisms and determine molecular markers for screening strategies. Hence, in the here presented studies I aimed to analyse the stress response of *P. sativum* towards *D. pinodes* in a large scale approach (shotgun LC-MS/MS proteomics, GC-MS metabolomics) to quantify a potentially high number of features.

1.4.1 Field Pea as non-model organism in MS-based proteomics

The developments in mass spectrometry from the 1960´s to 90´s form the basis of proteomics in systems biology [294, 295] and together with increasing computational power and sophisticated software, identification and quantification gained in confidence [296]. After extraction of proteins, workflows commonly include quantification of the total protein content followed by 2-D gel electrophoresis and in-gel digestion of interesting protein candidates, or in solution digestion of a complex sample mixture followed by direct application of peptides onto a reversed phase column prior tandem MS measurement [297]. In each case, peptides are subjected to fragmentation and the precursor mass together with a fragment ion series are required for a successful peptide sequence match to reasonably infer a protein identification. A

comprehensive database is essential in the analysis workflow as every spectrum match relies on the sequence information provided. In *P. sativum* genome sequencing is complicated due to 75-97% of repetitive DNA. Yet, full genome information is not available considerable hampering proteomic analysis [8, 298]. To circumvent this issue in shotgun proteomic experiments, interesting peptide candidates could be identified via mass accuracy precursor alignment with subsequent de novo sequencing of selected fragment spectra [299]. However, the advantages of a database are indispensable and alternatively experimenters choose a bigger not species-specific database to facilitate spectra matching. In case of *P. sativum* as non-model organism, sequence information from other legumes such as *Medicago* or *Glycine* (e.g. LegProt) can be merged into one database [300]. Due to a larger database this approach inevitably entails increased identification of false positives what requires stringent adjustment of matching criteria (e.g. lower FDR). Because the number of accurate identifications is connected to the database size and quality of contained sequences, it is evident to generate a protein sequence databases from all possible available DNA sequences of the organism at hand (e.g. RNA sequencing data, ESTs). For pea several datasets from preceding studies were available [301, 302], but merging of sequence information derived from several datasets results in redundancy and protein entries differing in single amino acids due to sequencing errors or genotypic variation. For this reason, other approaches align available DNA sequence to existing validated sequences of model organisms via BLAST algorithm to generate a comprehensive database minimized in redundancy [303]. The work with crop species routinely involves assessment of several genotypes with variation of protein sequences impairing shotgun proteomic results. The inclusion of genotypic sequence information in the database may be achieved via *de novo* sequencing of measured spectra and subsequent homology search in the before generated database derived from other sources [304]. This information might even be used to refine genomic sequencing in a proteogenomic approach [305]. Ideally, *de novo* sequencing is done with good quality spectra measured on a high-resolution tandem mass spectrometer [306]. In many proteomic experiments, MS2 scans are recorded in an LTQ to increase to number of acquired scans and identifications. However, LTQ obtained scans are more prone to cause *de novo* sequencing errors due to lower mass accuracy and a cut off in the lower mass range resulting in an incomplete fragment ion series. Despite recent

developments on the hardware and the software side [307, 308], *de novo* sequencing must be trusted with care and stringent exclusion criteria are needed. An extensive database including *de novo* derived sequence information for enhanced genotype specific protein identification is still missing functional annotation. Quick functional information is might be obtained with current software tools that link protein sequences to biological functions include software utilising algorithms such as BLAST [309].

1.4.2 Protein label free quantification: methods, chances and limitations

Proteomic label free quantification (LFQ) on a large scale requires the separation of the proteome prior identification. In the late 70s isoelectric focusing and gel electrophoresis were already applied successfully [310] and protein spots were analysed by Edman sequencing in the 80s [311]. To date, 2D electrophoresis is still popular and frequently applied when advantages such as detection of isoforms or PTMs are relevant. When it comes down to high through-put other methods have taken over mainly relying on 1D peptide separation prior measurement with different kind of mass spectrometers. Besides higher throughput, also quantification thereby gains in sensitivity and lower protein amounts are needed. On contrary, quantification in 1D-LC MS/MS experiments is most critical due to co-eluting peptides causing ion suppression in complex samples. Most notably peptides derived from RuBisCo, the most abundant protein in leaves, are detectable throughout entire chromatographic runs. This makes highest possible chromatographic resolution indispensable for accurate quantification as well as identification and column length of up to 50 cm are getting standard in unbiased proteomic approaches. Despite constant improvements in separating power of LC chromatography, still a high number of low abundant peptides co-elute making them inaccessible to data dependent shotgun MS analysis [312]. Today's mass spectrometers are increasingly sensitive combined with low acquisition times what improves the output of a proteomic analysis and its biological interpretation immensely. Still, the number of identifications is limited by speed, precursor ion isolation and sensitivity. In a data dependent proteomic analysis there always seems to be a compromise between high number of identification and accurate quantification determined by the maximum number of MS2 scans allowed per survey scan. For instance, a high number of MS2 scans allows potentially many

identifications, however, considering quantification with spectral counts, information is lost, because a peptide species´ chance being triggered is lower and similar quantification over precursor intensity would miss survey scans for adequate integration of eluted peptides. Hence, the number of maximum MS2 scans per survey scan is critical in a LFQ analysis and should be adapted carefully to the instrument's speed. Opinions differ in quantification using spectral counts, which is known for delivering a robust estimate of peptide abundances [313]. Alternatively precursor ions intensity and peak integration of the ion volume can be used in order to capture most quantitative information [314]. Both quantification methods have to struggle with the issue of varying intensity between different chromatographic runs caused by several sources (e.g. ionization, matrix effects). Such variation can be overcome with labelling experiments, in which for example cell cultures are cultivated with labelled amino acids [315], plants are irrigated with stable isotope labelled elements [316], or by isobaric tagging of peptides where reporter MS/MS ions are used for quantification [317]. If proteins of interest are present, other approaches such as selected reaction monitoring are preferred to increase sensitivity and dynamic range by making use of a triple quadrupole´s properties [318]. The advantages of a quadrupole instrument are also applied in the more recent method of sequential window acquisition of all theoretical mass spectra (SWATH) in which MS/MS spectra are obtained in data-independent acquisition mode with repeated cycling through 25 Da precursor isolation windows and subsequent fragmentation [319]. Peptide fragments are used for quantification even of high abundant proteins and identification is done via database match. To date, data-dependent shotgun proteomic experiments are common practice, however, they have to cope with several issues. For instance, the same peptide species is not triggered for fragmentation in every run, or protein inference results in variability of peptides aligned to a protein between runs what considerably influences protein quantification. To minimise such errors it is critical to choose an appropriate workflow for analysing raw data. In the past years many efforts have been made to develop user friendly software with confident identification and quantification algorithms and even implemented statistical tools [320-322]. In the here presented studies, MaxQuant was used for identification and quantification of samples measured on an LTQ-Orbitrap. This software´s features (e.g. retention time

alignment, mass recalibration, match between runs, LFQ ratio) were found to produce most confident output for further statistical evaluation [314]. Additionally, tools are available, to qualitatively assess MaxQuant LFQ output [323].

1.4.3 Metabolomics in integrative systems biology

As mentioned above, genomic information is static and as such, the molecular stress response cannot be predicted. Also the tempting assumption of a correlation between mRNA and protein expression holds only partially true due to alternative splicing, RNA silencing, different half-lives and post transcriptional settings [324]. The metabolome is regularly depicted as the final of all omic levels reflecting the actual outcome of a response. Hence, the integration of all levels (transcriptome, proteome, metabolome) provides promising insights with data allowing for metabolic modelling [325, 326].

Proteins are mostly located in a specific cellular compartment, and if present in various compartments they are frequently found as distinguishable isoforms. Metabolite extracts of complex samples, however, do not allow us to draw any conclusions of the subcellular origin. Moreover, unlike proteins in which peptide bonds link various amino acids that might be modified or hold a prosthetic group, metabolites exhibit an enormous structural diversity. The extraction and analysis of this diversity requires a combination of techniques if a large part of the metabolome shall be identified [327]. Consequently, a non-targeted profiling of the metabolome is complicated with regard to the plethora of chemical properties. In the past decades, GC-MS has proven as robust approach for identification and quantification of a few hundred metabolites essentially covering key elements of the primary metabolism [328]. Although the number of identifications would be increased in an LC-MS approach, the method still lacks reproducibility due to retention time variation, and ion suppression effects occurring during electron spray ionisation. Despite the reliability of the system, the application of standards yields additional confirmation of identification [329]. Hence, GC-MS is still regarded the gold standard in terms of reliable identification and accurate quantification.

2 Research objectives

The introductory chapters provided an overview of the present knowledge about plant pathogen interactions and cultivar influence with specific focus on the infection process of *D. pinodes* on *P. sativum*. It was recapitulated how microsymbionts such as rhizobia or mycorrhiza contribute to plant nutrition and how they render host immunity to permit successful invasion. I presented that below ground symbionts effect the host in a systemic way and that they enable enhanced defense responses in above ground tissues. Subsequently the benefits of an integrative proteomic and metabolomic approach were outlined. Hence, from the above introduced state of knowledge I deduce the following questions.

2.1 How to improve the quality of protein identification in a non-model species (i.g. *Pisum sativum*) by additionally accounting for cultivar typic sequence variation?

Many shotgun proteomic studies on non-model organisms face the problem of lacking a comprehensive database. Although *P. sativum* served as a model for Mendel to discover the laws of genetic inheritance, the research community is still awaiting the genome to be fully sequenced. Thus, I aimed to setup a simple workflow to gather and assemble all available sequence information in a database with subsequent inclusion of cultivar typic sequence variation. This should be accomplished by utilising data obtained in course of a data dependent analysis.

2.2 Does the host association with common microsymbionts such as mycorrhiza and rhizobia affect the resistance level against the hemi-biotrophic fungi *Didymella pinodes*? And does the metabolic stress response vary between cultivars?

In a randomised design the influence of varying symbiotic associations should be tested in a greenhouse experiment. First, the influence of the symbionts on the uninfected host should be determined (i.g.

morphology, proteome, metabolome) in order to derive the symbionts influence on the host immune response. The symbiotic effects should then be assessed in a higher resistant cultivar to elucidate stable effects of symbionts and, to determine metabolic resistance traits associated with the cultivar.

3 Publications

The following chapter provides an overview of all published as well as submitted manuscripts (chapters 3.1 - 3.3) during the period of this PhD.

3.1 A proteomic workflow using high throughput de novo sequencing towards complementation of genome information for improved comparative crop science.

Turetschek R, Lyon D, Desalegn G, Kaul H-P, Wienkoop S. In: Proteomics in Systems Biology: Methods and Protocols. Ed. Reinders J, New York, NY: Springer New York; 2016. p. 233-43.

High-throughput proteomic workflows established as standard procedure for many organisms. The basis of such studies is a comprehensive database. The first assembly of this constantly complemented database was accomplished with the programming language python. Newly available sequence information from other groups were included by the author of this thesis via use of the programming language R. Prior this publication, LC/MS methods were optimised (i.e. gradient steepness, automated gain control, fill time, signal threshold) to obtain highest possible quality spectra through data dependent acquisition on an LTQ-Orbitrap in order to gain certainty in the subsequent *de novo* analysis. The idea for this workflow was already presented in form of a poster at the conference of the International Plant Proteomic Organization in Hamburg (2014). The published book chapter comprises a detailed workflow description of confident identification of cultivar sequence variations that are obtained via *de novo* sequencing and subsequently included into a database for more accurate identification with peptide mass fingerprint. The book chapter additionally illustrates the improved identification (peptide spectrum matches) due to inclusion of sequence variations.

Author contributions

The presented work was published in the book series ‘Methods in Molecular Biology‘ with the book title ‘Proteomics in Systems Biology - Methods and Protocols’. The data utilised for this publication and the setup of the workflow was generated by myself. I performed the statistical analysis and wrote this work.

Published manuscript

Chapter 17

A Proteomic Workflow Using High-Throughput De Novo Sequencing Towards Complementation of Genome Information for Improved Comparative Crop Science

Reinhard Turetschek, David Lyon, Getinet Desalegn, Hans-Peter Kaul, and Stefanie Wienkoop

Abstract

The proteomic study of non-model organisms, such as many crop plants, is challenging due to the lack of comprehensive genome information. Changing environmental conditions require the study and selection of adapted cultivars. Mutations, inherent to cultivars, hamper protein identification and thus considerably complicate the qualitative and quantitative comparison in large-scale systems biology approaches. With this workflow, cultivar-specific mutations are detected from high-throughput comparative MS analyses, by extracting sequence polymorphisms with de novo sequencing. Stringent criteria are suggested to filter for confidential mutations. Subsequently, these polymorphisms complement the initially used database, which is ready to use with any preferred database search algorithm. In our example, we thereby identified 26 specific mutations in two cultivars of *Pisum sativum* and achieved an increased number (17 %) of peptide spectrum matches.

Key words Proteomics, De novo sequencing, Polymorphism, Crop science, Cultivars, Mass spectrometry, *Pisum sativum*

1 Introduction

In recent decades entire genome sequences of many organisms were acquired and the amount of sequence information is continuously expanding at an increasing rate. Advanced functional annotation of genomic data in model organisms facilitates interpretation of newly generated data. Despite the fact that specific sequence information is unavailable for non-model organisms, a growing number and a broad range of phylogenetic diverse species, reaching from snake venoms [1–6] to whole microbial communities [7–11], are being subjected to proteomic studies. A great evolutionary distance to well-characterized species considerably complicates the compilation of comprehensive databases (DBs),

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which is crucial for every bottom-up proteomic approach. Prevalently this hurdle can be overcome by combining a relatively large unspecific database (e.g., viridiplantae, NCBI) with a custom-built specific database consisting of translated RNA-sequences (e.g., 6-frame translation of nucleotide to amino acid sequences, BLAST homology searches for functional annotation) [12]. In most of the cases, such a composite database is sufficient to gain new insights into the protein level. However, conclusions are often hampered when it comes to comparison of cultivars within the same species. Among cultivar-specific sequence polymorphisms match to a greater or lesser extent with the aforementioned database and consequently result in distinct identifications, not related to functional differences. Thus, differentiation of cultivars in the proteomic domain may be composed of both sequence variation and molecular processes. However, the identification of molecular adaptations of cultivars upon environmental constraints is a major focus of crop science. With the use of shotgun proteomics, comparative crop science not only aims to identify homologues but moreover quantifies differences among cultivars, thus supporting the development of breeding strategies [13]. Yet, most common database search algorithms (e.g., SEQUEST) require a good match with in silico-generated spectra (PMF and fragment ion series) and fail to identify polymorphisms derived from cultivar-specific sequences. Hence, the amino acid sequence is required for detailed DB comparison.

The sequence may be acquired de novo, by deriving the amino acid composition from fragment ions of peptides.

The idea of determining peptide primary structure via mass spectrometry without prior knowledge of the sequence was already developed in the 1970s by studying penicillinase [14]. In the 1980s, first tandem MS scans were manually sequenced with 2200 mass resolution [15]. Today, various automated de novo sequencing algorithms are available enabling high-throughput processing of MS/MS data [16–19]. Still, the reliability scoring, inherent to all de novo algorithms, remains an ongoing issue which highly influences accuracy and computation time [20]. Once confident sequence tags are obtained, these can be matched to a database with the help of various search engines [21–24] to retrieve homologue proteins.

After assigning homologues, comparing de novo tags with an adequate database is just one more step to extract sequence differences in order to determine mutations. This additional step, however, requires special care as de novo sequencing is prone to specific errors (e.g., the inability to distinguish between K and E in low mass accuracy measurement). Such errors are taken into account by a few programs, such as SPIDER [21], TagRecon [22], and OpenSea [23], that correct de novo tags and additionally allow inexact matches to DB sequences. By matching de novo tags

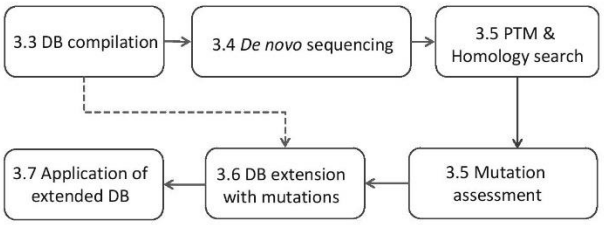

Fig. 1 Workflow with main steps from initial DB compilation and de novo sequence analysis to the application of the new organism aligned DB, explained in detail as follows (cf. Subheadings 3.2–3.6)

inexactly to the DB these algorithms show the capacity to more or less accurately—depending on the spectra quality and the search algorithm—identify posttranslational modifications, homologies, and mutations. With the use of such an automated search for homologies and mutations, our workflow (Fig. 1) aims to amend the initial DB with newly identified sequences (*see* Subheading 3.4). In case of cultivars, mutated sequences are not replacing original entries in the database, but are added with a header corresponding to the cultivar. Identified homologies to DB entries from different organisms are as well amending the new DB, but additionally the name of the organism must remain in the header not to confuse a homology with a mutation. Using this (extended) DB with any conventional algorithm (e.g., SEQUEST, Mascot) facilitates high-throughput MS/MS data analysis (compared to de novo sequencing) and additionally increases the confidence- and probability-based peptide identification (e.g., Xcorr) as well as protein sequence coverage, which results in more accurate quantification of cultivar-specific proteins (*see* Subheading 3.6). However, the determination of mutations via de novo sequencing is delicate and requires a few criteria. Therefore, particular attention has to be paid to reliable identification of mutations by critically taking mismatches with PTMs into account (*see* Subheading 3.2) and setting further criteria for stringent consideration of mutations (*see* Subheading 3.3).

2 Materials

2.1 Plant Material

1. Seeds from *P. sativum* ssp. cultivar Messire were provided by the Institute for Sustainable Agriculture CISC (Department of Plant Breeding, Cordoba, Spain). Cultivar Protecta was obtained from Probstdorfer Saatzucht GmbH & Co KG (Probstdorf, Austria).

2.2 Protein Extraction: Materials

1. Lyophilized plant material.
2. TRIzol Reagent® RNA Isolation Reagent.
3. Precipitation solution: 0.5 % β-Mercaptoethanol in acetone.
4. Protein digestion: Endoproteinase LysC Sequencing grade, Poroszyme® Immobilized Trypsin Bulk Media.

2.3 LC-MS/MS Instrumentation

1. One-dimensional nano-flow LC (Dionex UltiMate 3000; Thermo Scientific, USA).
2. EASY-Spray column, 15 cm × 75 μm ID, PepMap C18, 3 μm (Thermo Scientific, USA).
3. LTQ Orbitrap Elite (Thermo Scientific, USA).

2.4 LC-MS/MS Analysis: Materials

1. Mobile-phase solvent A: 0.1 % Formic acid; solvent B: 90 % acetonitrile, 0.1 % formic acid.

2.5 Software

1. mEMBOSS 6.5 (European Molecular Biology Open Software Suite).
2. PEAKS 7.0 (Bioinformatics Solutions Incorporation, Canada).
3. SEQUEST (Proteome Discoverer 1.3; Thermo Scientific, USA).

3 Methods

3.1 Protein Extraction

Leaves of 4-week-old plants were sampled, immediately quenched, and ground in liquid N_2. Protein from lyophilized material was extracted in TRIzol® according to Lee et al. [25] with a few modifications: 3 ml of β-mercaptoethanol in acetone was used for precipitation overnight at −20 °C. The protein pellet was washed and digested with LysC and trypsin according to Staudinger et al. [26].

3.2 LC-MS/MS Analysis

Peptide digests (1 μg) were applied to a one-dimensional nano-flow LC. The peptides were separated using a 95-min nonlinear gradient from 98 % of solvent A to 45 % of solvent B at a flow rate of 300 nl/min. The nLC-ESI-MS/MS analysis was optimized for standard high-throughput analysis at a resolution of 120,000 (FTMS) with 20 MS/MS scans in the LTQ at the following settings: rapid scan mode, minimum signal threshold counts 1000, prediction of ion injection time, repeat count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 60 s, exclusion mass width 5 ppm relative to reference mass, early expiration enabled (count 1, S/N threshold 2), monoisotopic precursor selection enabled, rejected charge state: 1, normalized collision energy: 35, and activation time 30 ms.

3.3 Custom Database Design

A composite protein-fasta file was created by merging the following six databases:

Uniprot UniRef100 (all identical sequences and subfragments with 11 or more residues are placed into a single record—http://www.uniprot.org/help/uniref) sourced at 15-05-2013 from the following Taxa:

1. *Pisum sativum*
2. *Rhizobium leguminosarum*
3. *Glomus*
4. *Mycosphaerella*
5. Legume-specific protein database (LegProt) [27] including information from the following organisms: *Pisum sativum*, *Lotus japonicus*, *Medicago sativa*, *Glycine max*, *Lupinus albus*, *Phaseolus vulgaris.*
6. Processed dbEST NCBI sourced from http://www.coolseasonfoodlegume.org/

Pisum Sativum Unigene v1, P. Sativum Unigene wa1, Pisum Sativum Unigene v2;

Nucleotide sequences were six-frame-translated using mEMBOSS. For each accession number the longest continuous amino acid sequence (longest ORF) within a frame was chosen. If multiple sequences (of different frames) were of the same maximum length, all of them were kept (each with a different accession number, including the frame number).

The 6 fasta files described above were combined, producing a new fasta containing 135,754 entries. Protein sequences 100 % identical in sequence and length were combined by subsequently adding one header after the other, separating them by the following characters " __***__ " (no matter if the redundancies originated from one or multiple fasta files). All other entries were simply added to the end of the new file. The first accession number of the header was repeatedly written at the very beginning of the header line, separated by a " | " in order to consistently view and parse the accession numbers.

3.4 De Novo Sequencing and Homology Search

Several automated software solutions for de novo sequencing are available to date. The outcome very much depends on the quality of processed spectra and the selected algorithm [28]. Higher spectra quality can be achieved by adaptation of the fragmentation (*see* **Note 1**). Here, the de novo search was performed with PEAKS [18] employing settings according to the resolution and mass accuracy of the mass spectrometer used (*see* Subheading 2.3). By calculating the narrowest possible mass error tolerance most occurring PTMs cannot be mistaken for a mutation. Hence, a mass error

of 5 ppm from the monoisotopic precursor was allowed with fragment ion mass error of 0.5 Da (*see* **Note 2**). De novo tags with a minimum average local confidence (ALC) of ≥15 were subjected to PTM identification to determine the most frequent modifications in order to recalculate an adequate mass error tolerance (*see* **Note 2**). Accordingly, de novo tags were matched against the designed database (*see* Subheading 3.1) and searched for mutations with the implemented SPIDER tool. Maximum three of the previously searched PTMs were allowed. The peptide spectrum matching score (−10 lgP) was set to 20. A maximum of two missed cleavages per peptide and nonspecific cleavage at one end of the peptide were allowed—this apparently loose restriction facilitates the identification of mutated K or R residues.

3.5 Evaluation of Homology and Mutation

Various automated programs [21, 23, 24] crucially simplify the identification of sequence variance. However, these identifications must be critically filtered to obtain only confident sequence amendments to the original database. First, exclusively proteotypic peptides are added to the original database, because a variation in any other peptide cannot be specifically attributed to one protein. However, as a result of using a merged DB containing sequence information of several RNA data (*see* Subheading 3.1), variations are sometimes assigned to multiple protein entries with the same function but slightly different sequences. In such case, the protein entry with the most assigned peptides and highest coverage is chosen for further processing. If the number of assigned peptides and coverage is similar for several DB entries, all of them are chosen for further processing. Second, the mutated peptide must not have a non-mutated counterpart: if a mutated and a non-mutated peptide are attributed to the same sequence in the database, the identified mutation is likely to be a false positive. Third, the sequence of the mutation must be confirmed by at least two MS/MS spectra. Thereby, a de novo error—identifying a mutation—caused by a low-quality spectrum is largely avoided and mutations gain confidence. A typical quantitative shotgun proteomics experiment requires the measurement of several replicates, which usually acquire enough MS/MS to confirm mutations.

3.6 Database Extension with Mutations

Confidently identified mutated amino acid residues are added to the database by copying the original fasta entries with the mutated sequence. Original entries must be kept, because the located mutations may be characteristic for just one cultivar. The mutated sequences are found in new entries with modified accession and header (*see* example below).

Accession: ACU20233.1_**m1**

Header: unknown [glycine max] [**Me_IV25**]

The accession shows a suffix ("_m1") indicating a sequence alteration. If cultivars show different mutations at the same protein entry, the number of the suffix is ascending. The header is supplemented with squared brackets including the information about the cultivar and the polymorphisms in the sequence (in the current example in cv. Messire isoleucine substitutes valine at position 25). Here, an entry of the LegProt database (NCBI) [27] is shown which is not yet annotated. After inserting the mutation, the sequence can again be blasted to achieve improved annotation, albeit a change in only one amino acid will result in a similar BLAST result. Additionally, when working with non-model organisms, it is worth considering re-annotation of the genome by use of proteomic data in a proteogenomics approach [29].

3.7 Application of Extended Database

The database, amended with mutated sequences, potentially enhances the identification of any preferred DB search algorithm and enables high-throughput processing of MS/MS data. By increasing peptide scores (e.g., Xcorr) and protein sequence coverage, proteins are more confidently identified. Moreover, inclusion of exclusively proteotypic peptides (*see* Subheading 3.5) expands the list of candidates for other proteomic approaches (e.g., SRM, MRM).

3.8 Iterated Search with DB Search Algorithm

The new extended DB was used for a standard DB search using the SEQUEST algorithm with the following settings: 5 ppm precursor mass tolerance, 0.5 Da fragment mass tolerance, acetylation of the N-terminus, and oxidation of methionine as dynamic modifications. Minimum peptide confidence was set to medium, and minimum Xcorr to 2. A minimum of two peptides per protein were required for identification.

In the present study of *P. sativum* with the cultivars Messire and Protecta we identified 48 variations to original DB entries, of which 26 are mutations showing high cultivar specificity. Both cultivars have five mutations in common. Messire showed 12 and Protecta 9 characteristic mutations. Furthermore, 22 homologues were identified from entries of different species (e.g., *G. max* from the LegProt DB). The ratio of replaced and substituting amino acids (Fig. 2) shows that most frequently valine and alanine are both replacing and substituting other amino acids in our experiment.

The number of peptide spectral matches (PSMs) shows how many of fragmented ions match to the applied DB. Thus, a rather complete DB will result in a higher number of PSMs compared to an imperfect DB. Here Fig. 3 shows that the number of PSMs increased significantly (17 %) for the two studied cultivars after amending the initial DB with sequence variations. Besides improving protein identification, the elevated number of PSMs crucially contributes to more accurate and confident quantification.

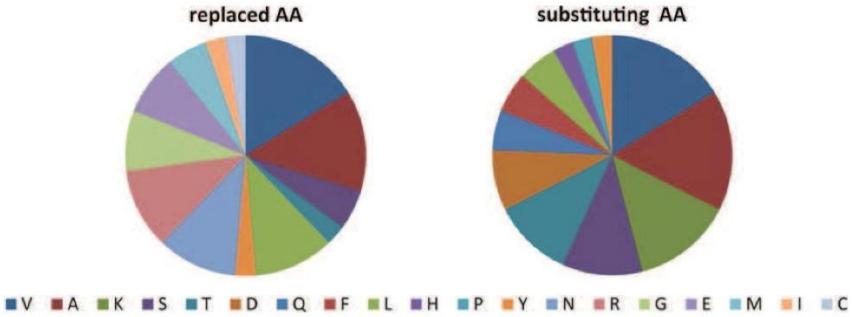

Fig. 2 Ratio of replaced (from the initial DB) and substituting amino acids

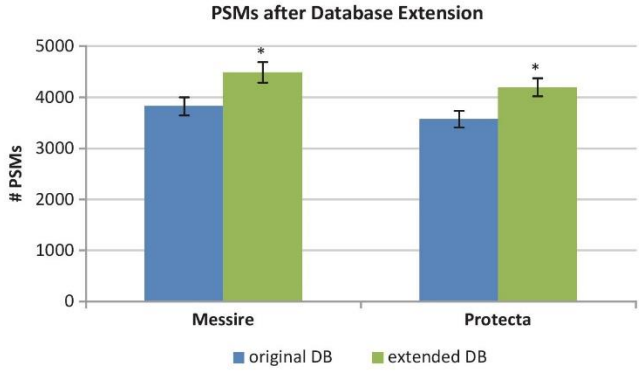

Fig. 3 The total number of PSMs is significantly increased (student's *t*-test, $p < 0.05$) in both cultivars when the extended DB is applied; $n = 24$, error bars indicate standard error at 95 % confidence intervals

4 Notes

1. Optimizing prerequisites for de novo sequencing
   De novo sequencing essentially depends on a complete series of fragment ions which are generated according to the applied fragmentation technique. Thus, the quality of de novo sequencing can be enhanced crucially by applying different fragmentations (CID/HCD/ETD) to the same precursor and subsequently merging the spectra in order to achieve a great number of fragment ions [30]. The choice of instrumentation often points to high speed and sensitivity (e.g., LTQ using CID) with the drawbacks of reduced mass resolution and accuracy, resulting in an impossibility to resolve

fragment ions' charge state. This deficit causes considerable difficulties to de novo sequencing because of additional ambiguity. Through performance of MS/MS scans at high resolution (i.e., FT) the precision of de novo sequencing benefits especially for precursors of higher charge states (≥3+). However, high resolution requires higher AGC values, which increases acquisition time remarkably. The trade-off between spectra-quality (FT) and speed (LTQ) can be accounted for by a data-dependent decision tree [31], where the site of the fragment scan depends on the charge state and the m/z of the peptide. Consequently MS/MS scans from ≥3+ charged precursors crucially improve in quality.

2. Definition of mass error tolerance for accurate de novo sequencing

   The calculation of the mass error tolerance comprises the comparison of an AA mass with a PTM to another AA. This gap is the mass difference which needs to be resolved to distinguish a PTM from a mutation. Since a database search with more than 200 possible PTMs [32] requires infeasible computing capacity, the calculation refers to the seven most observed PTMs in this experiment. The most frequently occurring PTMs were determined by peaks with 5 ppm precursor mass error tolerance and 0.6 Da fragment mass error tolerance: oxidation of methionine (+15.99), sodium adduct (+21.98, on D-, E-, and C-term), carbamylation (+43.01 on N-term), methyl ester (+14.02 on D-, E-, and C-term), deamidation (+0.98 on N and Q), acetylation (+42.01 on N-term), and replacement of two protons by calcium (+37.95 on D-, E-, and C-term). The mass error tolerance can be calculated as follows: The mass of a possible modification is added to the AA's mass. When modifications affect the C- or N-term, the PTM's mass is added to each AA's mass. These values are subtracted from the masses of each amino acid. Thus, a mass error window to differentiate between an AA and a modified AA is calculated. For MS/MS scans in an ion trap a mass accuracy of 100–200 ppm must be additionally subtracted from this mass difference. Accordingly, a bulk of false positives (modified peptides identified as mutations) can already be excluded by setting the mass error tolerance to 0.5 Da in an MS/MS scan, that is, e.g., the identification of valine as asparagine which mass differs in 0.94 Da from a peptide with methyl ester at the C-terminus. Considering a mass accuracy of 200 ppm in an ion trap (0.4 Da at 2000 m/z) the mass difference narrows to 0.54 Da. For an FT MS/MS scan the mass error tolerance is preferably set a lot lower (e.g., 0.05 Da). Additionally, setting the precursor mass tolerance to ≤5 ppm critically minimizes false positives.

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

Desalegn G., Turetschek R., Kaul H.P. & Wienkoop S. (2016). *J Proteomics*, Vol 143, Food and Crop Proteomics, p. 173-187.

Microorganisms are frequently reported to contribute to plant health and enhanced immune response. This effect named 'induced systemic resistance' was described in several plants and on various levels including genomics and transcriptomic studies. In this publication we described the effects of microsymbionts on the plant metabolic infection response in a high-throughput proteome/metabolome study. The previously published book chapter was the groundwork for the proteomic analysis in this publication. The original database was completed with up to date sequence information and database entries were annotated with BLAST (UniRef100) and the mercator software [309]. Several studies before stated the effect of enhanced resistance triggered by beneficial microbes. As mentioned above, this enhanced resistance depends much on the beneficial microbes, but moreover on the type of pathogen. Due to hormonal sensitivity, resistance against necrotrophic pathogens is suggested to be increased. Thus, the unbiased analysis of the symbionts effect on the infection response against this hemi-biotrophic fungi was one of the major motivations for this study. While we found remarkable effects of varying symbiotic association on uninfected plants, it was essentially the combination with rhizobia that provoked an enhanced infection response in the host.

Author contributions

The presented work was published in the Journal of Proteomics in the special issue 'Food and Crop Proteomics'. The first authorship is credited equally to Getinet Desalegn and myself. I aided in assessment of phenotypic characteristics (i.e. symbionts effectiveness, dry matter, leaf area). Additionally, I performed experimental parts, laboratory labour, statistical analysis and writing of paragraphs related to proteomics and metabolomics.

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

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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.

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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, $p < 0.05$).

				i/h		
Accession	Description	Peptides	Max. peptide Score	*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	***

plants pot^{-1} for morphological characterisation and for proteomic/metabolomic studies, respectively. Pots were arranged on benches at 20 °C day and 12 °C night temperature. Relative humidity was at 60 ± 5% with 14 h photoperiod and a light intensity of 300 μmol m^{-2} s^{-1}. 1. Pots were rotated every ten days.

A pathogen inoculum of pea plants (i.e. *D. pinodes* isolate) was also obtained from the Rubiales Lab, Cordoba (Spain), and multiplied in Petri dishes containing PDA media (23 °C, 12 h photoperiod). Approximately thirty days after planting (at the 8th to 10th leaf stage), plants were inoculated by spraying a conidia suspension containing 5 × 10^6 conidia mL^{-1} and 120 μL of Tween-20 in 100 mL as a wetting agent. After inoculation, plants were covered with a polyethylene sheet. High humidity was ensured by adjustable and automatic humidifiers operating for 15 min every 2 h in the controlled greenhouse.

2.2. Assessment of phenotypic characteristics

2.2.1. Plant disease severity

After the initial 36 h the polyethylene cover was removed and plants were maintained in the controlled greenhouse throughout disease development and assessment and any other measurements. For assessment disease severity (DS), two scorings (i.e., on day three and nine after *D. pinodes* inoculation) were performed as described by Roger and Tivoli [45]. Visual estimation of the percentage of the plant surface covered by symptoms, such as on stipules and leaflets at first, fourth and eighth nodes and the plants' mean were taken using a 0–5 scale (0, no lesion; 1, a few scattered flecks; 2, numerous flecks; 3, 10–15% leaf area necrotic and appearance of flecks; 4, 50% of leaf area covered by lesions; 5, 75–100% of leaf area dehydrated or necrotic).

2.2.2. Microbial symbionts' effectiveness and plant growth components

At full flowering stage (BBCH 65) plants were cut at ground level, the shoot fresh weight was taken, subsequently all the dead leaves or not green ones and flowers were removed, and the green area was measured using a scanning planimeter (LI-COR 3100 area meter, Lincoln, NE, USA). Finally, for dry matter (DM) determination, all shoot parts were dried at 40 °C for five days.

Mycorrhizal root colonisation and *Rhizobium* root nodulation were assessed after rinsing off the remaining substrate on the roots with running tap water on a fine (1 mm) sieve bench in the root washing facility. For mycorrhizal colonisation assessment, roots were cleared and stained according to Vierheilig et al. [46] by boiling them in 10% KOH for 10 min and in a 5% ink (Schaeffer black ink) plus household vinegar (equal to 5% acetic acid) solution for 5 min. The percentage of root length colonised by AMF was estimated according to Newman [47]. For *Rhizobium* nodulation assessment, nodules were removed from the roots, to record their number as well as fresh weight and dried after five days at 40 °C.

2.3. Proteome and metabolome studies

2.3.1. Plant sampling and preparation

For proteomic and metabolomic studies, plants were sampled at 36 h (late morning hours) after infection in order to obtain the least circadian effect on the proteome/metabolome. It was reported that *D. pinodes* penetrates the leaf cuticle after 24 h and small lesions become visible [43,48] and infection is in general most effective in the evening. Three pots were sampled each representing one biological replicate. Leaves from 3 out of 4 plants per pot were pooled to eliminate effects of a varying infection success. The fourth plant was used for extraction of the plasma membrane (Section 2.3.3). After immediate quenching in liquid nitrogen, leaves were ground to a fine powder in liquid nitrogen and then samples were stored at −80 °C for further processing.

2.3.2. Integrative extraction of metabolites and proteins

About 25 mg fresh weight of the ground samples were used for extraction with 1 mL of freshly prepared and pre-cooled extraction buffer (MeOH:$CHCl_3$:H_2O, 2.5:1:0.5). Samples were kept on ice for 8 min with regular agitation before centrifugation (4 min, 14,000 *g*, 4 °C). The supernatant was transferred to a new tube containing 500 μL ultrapure water and shaken thoroughly. After centrifugation (4 min, 14,000 *g*, 4 °C) the upper phase, containing polar metabolites, was split into two aliquots and dried in a vacuum concentrator. The remaining plant material was kept for further extraction of proteins (Section 2.3.2.2).

2.3.2.1. Derivatisation and analysis with GC–MS. Vacuum dried metabolites were dissolved in 20 μL of a 40 mg mL^{-1} solution methoxyamine hydrochloride in pyridine through shaking at 30 °C for 90 min *N*-methyl-*N*-trimethylsilyltrifluoroacetamid (40 μL) spiked with 60 μL/mL of an even-numbered alkane mix (C10–C40) was added followed by incubation for 30 min at 37 °C under continuous shaking and centrifugation at 14,000 *g*. The supernatant was transferred into a glass vial for measurement. The sample (1 μL) was injected into a GC coupled triple quadrupole (Thermo Scientific TSQ Quantum GC™, Bremen, Germany). GC and MS adjustments were set as described by Staudinger et al. [49] with minor variations. To enable the quantification of sugars, the samples were measured in split less and split mode with a ratio of 10. Before and after a set of 10 randomly queued samples a set of 5 different concentrations of external standard mix was measured. Identification was based on the matching of MS-spectra and retention time index (calculated through the spiked alkane mix) against an in-house library (extended gmd database) in AMDIS [43]. Peak areas were integrated with the software LCquan (version 2.5, Thermo Xcalibur). Absolute metabolite quantities were calculated by normalization to the slope of the external standard and to the fresh weight. Metabolites not found in the external standard were normalized to the slope of a similar substance with nearby retention time (e.g. nicotinate was normalized to the slope of citric acid).

2.3.2.2. Integrative protein extraction. Proteins were extracted from the plant material pellet (2.4.1) using trizol (TRI Reagent®, Sigma-Aldrich) according to Carrillo et al. [43], with minor modifications. The plant material left over from metabolite extraction was sonicated in 800 μL trizol reagent. For phase separation of phenolic and water soluble compounds 160 μL of chloroform was added prior centrifugation (15 min, 12,000 *g*, 4 °C). The upper aqueous phase was discarded and 240 μL EtOH were added to facilitate pelleting of the insoluble leaf material by centrifugation (5 min, 7000 *g*, 4 °C). The supernatant was transferred and proteins were precipitated overnight with five times of the initial volume in −20 °C cold acetone containing 0.5% β-mercaptoethanol. The precipitate was pelleted (10 min, 4000 *g*, 4 °C), subsequently the supernatant removed and the protein pellet dissolved in 700 μL urea buffer (8 M urea, 50 mM HEPES, pH 7.8).

2.3.3. Plasma membrane preparation

The fresh leaves of one plant (2–4 g) were homogenized in breaking buffer (0.1 M HEPES, 1 mM EDTA, 0.33 M sucrose, 5 mM DTT, 1.5% PVPP, 1 mM PMSF, pH 7.5). The homogenate was centrifuged for 30 min at 30,000 *g* to pelletise the microsomal fraction, consisting of membranes derived from different vesicles. The supernatant (fraction with the cytosol) was precipitated and dissolved in urea buffer as described for the integrative protein extraction. Plasma membranes in the microsomal fraction were purified by use of an aqueous two phase partitioning optimized for *Pisum* leaves according to Luthje et al. [50].

2.3.4. Protein digestion

Protein concentration was determined via Bradford assay [49] and a BSA standard calibration line. A volume corresponding to 100 μg (integrative extraction and cytosolic fraction) and 20 μg (plasma

membrane) proteins was transferred and LysC (1:100 vol/vol, 5 h, 30 °C, Roche, Mannheim, Germany) was added for initial digestion. Samples were diluted with trypsin buffer (10% ACN, 100 mM AmBic, 1 mM $CaCl_2$, 5 mM DTT) to a final concentration of 2 M urea and incubated over night at 37 °C with Poroszyme immobilized trypsin beads (1:10, vol/vol; Applied Biosystems, Darmstadt, Germany). The digest was applied on C18-SPEC 96-well plates (Varian, Darmstadt, Germany) and washed twice with 250 μL water. The sample flow-through and the first wash step were kept for second desalting step with graphite. Peptides were eluted from C18-spec-plates with 500 μL of 100% MeOH. Proteins from the flow through and the first wash step were desalted with 10 mg graphite in spin columns (MobiSpin Column F, MoBiTec) according to the manufacturer's instructions (Thermo scientific, Pierce® graphite spin columns). Eluates from graphite and C18 desalting were subsequently merged, split into two technical aliquots, and dried in a vacuum concentrator.

2.3.5. Nano ESI LC–MS/MS

Peptides were dissolved in 100 μL 2% ACN, 0.1% FA and 1 μg of each sample were applied randomly on a reverse phase C18 column. Integrative extracted peptides (3 biological and 2 technical replicates) were loaded on a 15 cm × 50 μm column (PepMap®RSLC, Thermo scientific, 2 μm particle size) and separated during a 90 min gradient with a flow rate of 300 nL min^{-1}. MS measurement was performed on an LTQ-Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) with the following settings: Full scan range 350–1800 *m/z*, max. 20 MS2 scans (activation type CID), repeat count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 60 s, charge state screening enabled with rejection of unassigned and +1 charge states, minimum signal threshold 10,000.

Cytosolic peptides (3 biological and 2 technical replicates) and plasma membrane peptides (3 biological replicates) were applied on a 15 cm × 100 μm column (Supelco Ascentis® Express Peptide ES-C18, 2.7 μm particle size) for separation during a 90 min gradient with a flow rate of 400 nL min^{-1}. MS measurement was performed on an LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) with the following settings: Full scan range 350–1600 *m/z*, max. 9 MS2 scans (activation type CID), repeat count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 60 s, charge state screening enabled with rejection of unassigned and +1 charge states, minimum signal threshold 10,000.

2.3.6. Protein identification and label free quantification

P. sativum remains to be sparsely sequenced because of repetitive DNA that complicates a shotgun proteomic approach. Thus a database (db) was assembled according to [51] with minor modifications: UniProt UniRef100 db (22 January 2015) were sourced from the following taxa: *P. sativum*, *R. leguminosarum*, *Glomus*, and *Mycosphaerella*. Because of little entries for *P. sativum* at UniProt, we included organism specific sequences from NCBI. Additionally, assembled ESTs of *P. sativum* were sourced from www.coolseasonfoodlegume.org (unigene v2, unigene) [49] and subsequently six-frame-translated using mEMBOSS v6.5. The longest continuous amino acid sequence (longest open reading frame) within forward and reverse translation was chosen. Protein annotations were derived via BLAST against the complete UniRef100 db. Specific blast information was provided in brackets in the protein description (UniRef100 accession, identical coverage, query coverage, e-value). The identical sequences and subfragments were placed into a single record to avoid redundancy. The db was finally completed with cultivar specific mutations according to [51] and comprised 140,560 entries.

Thermo raw files were identified and quantified in MaxQuant [52]. Identification parameters were adapted to the respective instrument (Orbitrap Elite/Orbitrap XL): first search peptide tolerance 20 ppm/20 ppm, main search tolerance 4.5 ppm/6 ppm, ITMS MS/MS match tolerance 0.8 Da/0.8 Da, intensity threshold 500/500. All files were searched with maximum 5 of the following variable modifications: oxidation of methionine and acetylation of the N-term. Maximum two missed cleavages were allowed. A retention time window of 20 min was used to search for the best alignment function and identifications were matched between runs in a window of 0.7 min. A revert decoy db was used to set a cut-off at a FDR of 0.01 (at PSM and protein level). A minimum of 6 amino acids was required for identification of a peptide and at least two peptides necessary for protein identification. Label free quantification (LFQ) was done when at least one MS2 scan was present. LFQ minimum ratio was set to 2. Stabilisation of large LFQ ratios was active.

2.4. Statistical analysis

A two-way ANOVA was used to examine the main effects and interaction of biotic stress (with/without *D. pinodes* infection), and NP nutrient sources (microbial symbionts or mineral fertilizer) on shoot green area, shoot dry matter yield, root AMF colonisation and rhizobial nodulation. All assumptions required by ANOVA were verified. Differences between treatments were compared with Tukey's multiple range test, and statistical significance was defined at $P < 0.05$. These analyses were performed using SAS v. 9.4.

In proteomic and metabolomic studies, all statistical computation was done in R [53]. Outliers (1.5 times the interquartile range) of protein and metabolite intensities) were removed. Only proteins/metabolites present in more than half of the observations of a group were considered for statistical analysis. If less than half of the observations in a group were missing, the missing values were estimated via k-nearest neighbour algorithm. Remaining missing values were filled with half the minimum value of the respective protein/metabolite. Significant differences between groups were determined with an ANOVA followed by a Post-hoc test (Tukey HSD, $P < 0.05$). Significant proteins additionally required a minimum fold change of ≥2.

The average intensity of statistically significant proteins among healthy treatments was scaled (*z*-transformation). Proteins were hierarchically clustered with Euclidian distance and complete linkage method and plotted with heatmap.2 function [54] and 'YlOrRd' colour palette from the 'RColorBrewer' package [55]. The proteins were functionally classified with Mercator [56], filtered to the most representative functions (only proteins with assigned function; minimum count of 4 proteins per function) and visualised in a stacked bar plot (Fig. 7B). Proteins/metabolites significantly responding to disease were determined via a comparison (ANOVA, Tukey HSD) of the diseased versus the healthy plants (e.g. Md/Mh) and a comparison (Student's *t*-test) of all diseased versus healthy plants (d/h). Corrected *p*-values (Benjamini Hochberg) are additionally provided.

The scaled protein averages were clustered and plotted in a heatmap. Stress related categories were visualised as well as responsive categories with >4 proteins per functional category. Averages of metabolite intensities were used to calculate the ratios between diseased and healthy treatments. The ratios are visualised with the 'RdBu' colour palette (RColorBrewer package). Pathway visualisation was adopted from Schweiger et al. [24].

3. Results

3.1. Phenotypic characterisation

For the determination of symbiont root colonisation and its effect on pathogen disease severity as well as on plant growth, several analyses where carried out at the stipules, leaflets and roots with the following results:

3.1.1. Disease severity

We noted significant difference in disease development between two scoring dates of disease severity (DS) on both stipules and leaflets, but not among symbiotic treatments. After nine days of pathogen

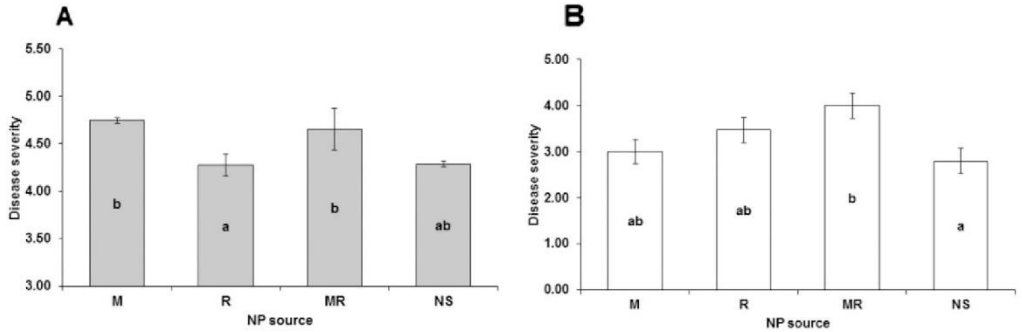

Fig. 1. Effects of absent, dual or tripartite symbiosis with arbuscular mycorrhizal fungi (AMF) and/or *Rhizobium leguminosarum* bv. *viceae* on pathogenic disease severity of *P. sativum*: leaflets (A) and stipules (B) nine days after infection of *D. pinodes*. Abbreviations are: M (AMF); R (*Rhizobium* bacteria); MR (dual M and R); NS (non-symbiotic synthetic dual nitrogen and phosphorus fertilizers). Values are means ($n = 4$) and error bars indicate standard error. Bars labelled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

infection, however, the mean DS on leaflets of plants inoculated with single R was significantly lower than on plants with AMF treatment (M and MR). This effect of Rhizobia on the disease severity was not found on stipules (Fig. 1A, B). The overall DS ranged from 2.3 to 4.5 on the leaflets and from 0.4 to 3.2 on the stipules between the two scoring dates. The DS range was much smaller on leaflets than on stipules, however, DS was higher. Possibly leaflets were most accessible to the spray of spores that led to rapid disease progress and higher initial DS score. A general downward (not upward) disease progress from leaflets to petioles, stipules and to the base of stems was noticed.

3.1.2. Mycorrhizal root colonisation

To assess the efficiency of dual and tripartite AMF associations with host plants under healthy conditions and *D. pinodes* attack, mycorrhizal root colonisation was measured. Healthy plants inoculated with single mycorrhiza (M) showed the maximum fungal root colonisation of 62% (Fig. 2). The biotic stress significantly reduced root mycorrhizal colonisation by 30% compared to healthy plants. Furthermore, antagonistic effects of dual MR inoculation were noted under both healthy and diseased plant growth conditions with a 13% to 15% reduction compared to single AMF inoculation, respectively. For control, random samples from plants without AMF inoculation never showed any AMF colonised roots.

3.1.3. Efficiency of root nodulation by rhizobia

To evaluate inoculation with *Rhizobium*, we determined nodule dry weight (DW) as well as number. Plants inoculated with single R had approximately 30% significantly higher nodule DW than co-inoculated (MR) plants (Fig. 3). The interaction between pathogen and *Rhizobium* inoculation showed significant effect on nodule DW both for treatments R and MR (Fig.3). Pathogen infection reduced nodule DW by 75%. Overall, nodule distribution and size were bigger and confined around the crown root, particularly in the single R system. As control, random samples from plants without *Rhizobium* inoculation never showed any nodule-like structures on the roots.

3.1.4. Green area production

A two-way ANOVA indicated that there were no statistically significant interaction effects between pathogen infection and symbiotic treatments on green area (GA) production (Fig. 4). However, about 80% GA reduction was found due to *D. pinodes* infection as compared to healthy pea plants. Independent of the treatment, in our experimental setup GA was only affected by plant health (e.g. Fig. S1).

3.1.5. Shoot dry matter production

To reveal the effects of biotic (pathogenic) stress and microbial symbionts' interactions on photosynthetic performance of plants, the dry weight (DM) of shoots was determined. Overall, the mean DM

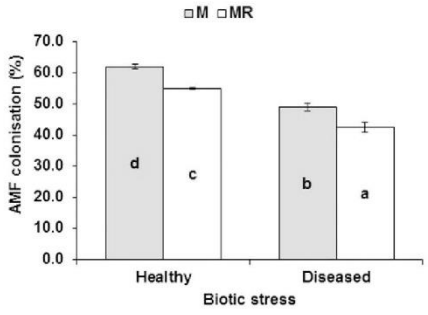

Fig. 2. Efficiency inoculation of field pea (*Pisum sativum* L.) with single AMF or combined with *Rhizobium* bacteria (MR) on mycorrhizal root colonisation under healthy and biotic (*D. pinodes*) stress plant growth conditions. Error bars indicate standard error ($n = 4$). Bars labelled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

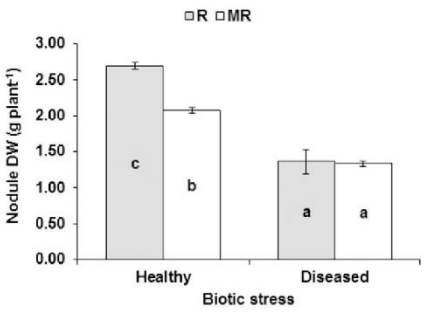

Fig. 3. Efficiency of inoculation of field pea (*Pisum sativum* L.) with single *Rhizobium* bacteria (R) or combined with AM fungal (MR) on root nodule dry weight (DW) under healthy and biotic (*D. pinodes*) stress plant growth conditions. Error bars indicate standard error ($n = 4$). Bars labelled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

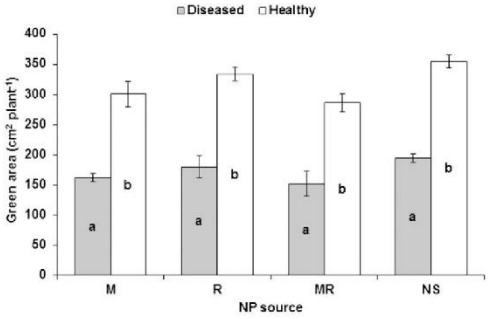

Fig. 4. Effects of NP sources from microbial symbionts and synthetic fertilizers on green area production of field pea (*Pisum sativum* L.) under healthy and diseased plant growth conditions. Abbreviations are: M (AMF); R (*Rhizobium* bacteria); MR (dual AMF and R); NS (non-symbiotic synthetic dual nitrogen and phosphorus fertilizers). Values are means ($n = 4$) and error bars indicate standard error. Bars labelled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

production was significantly lower in diseased plants than in healthy ones by 70% (Fig. 5). DM was also found to be partly treatment specific. The R treatment showed significantly higher mean shoot DM of healthy and diseased plants (2.4 g pot^{-1}) compared to M with lowest mean DM production of 1.8 g pot^{-1}. Again, statistically significant interaction effects between pathogen infection and treatments were found.

3.2. Integrative molecular and subcellular analyses of the proteome and metabolome

For the investigation of the molecular effects of the different symbiotic treatments and their possible influence on the plants leaflet response to the pathogen attack, an integrative proteome and metabolome extraction as well as an additional subcellular proteome extraction of the cytosol and the plasma membrane were performed. For best data presentation, we decided to combine quantitative proteomics data of the integrative and cytosolic extractions separately from plasma membrane proteins and metabolites.

3.2.1. Functional and local proteome characterisation

Altogether, 1564 proteins were used for quantitative analysis. Because of the largest complexity the integrative leaf extracts accounted for the majority of identifications (1383; Fig. 6) including especially

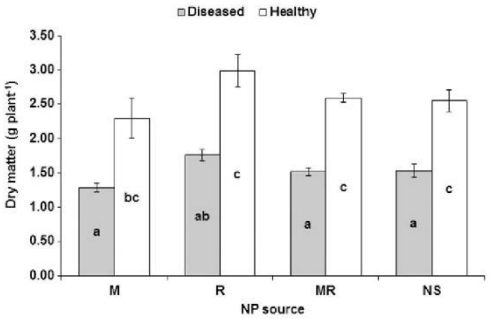

Fig. 5. Effects of NP sources from microbial symbionts and synthetic fertilizers on dry matter production of field pea (*Pisum sativum* L.) under healthy and diseased plant growth conditions. Abbreviations are: M (AMF); R (*Rhizobium* bacteria); MR (dual AM fungi and R); NS (non-symbiotic synthetic dual nitrogen and phosphorus mineral fertilizers). Values are means ($n = 4$) and error bars indicate standard error. Bars labelled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

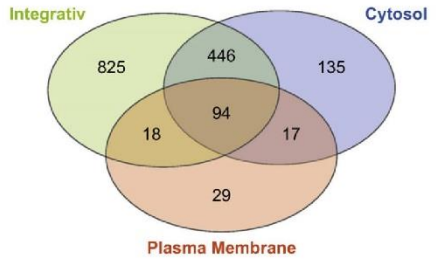

Fig. 6. Number of quantified leaf proteins from total integrative extraction, cytosolic fraction and plasma membrane enrichment.

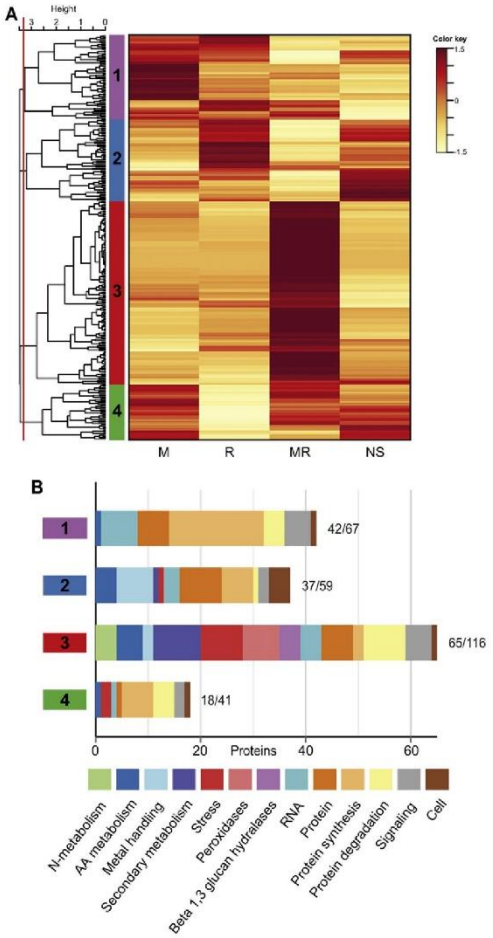

Fig. 7. (A) Heatmap of 293 protein abundances from the integrative and cytosolic extraction which showed a significant difference among symbiont treatments of healthy plants. Each cell represents the scaled average protein intensities ($n = 3$). Euclidean distance and complete linkage method were used for cluster analysis. Clusters (1–4) were grouped at a height of 3.3; (B) clusters from (A) were functionally categorized by adopting functional bins from the MapMan Mercator tool. Categories were plotted when containing a minimum of 4 proteins in at least one cluster. Values at the bars' right side represent the number of categorized proteins per number of proteins in the corresponding cluster (1–4) derived from (A).

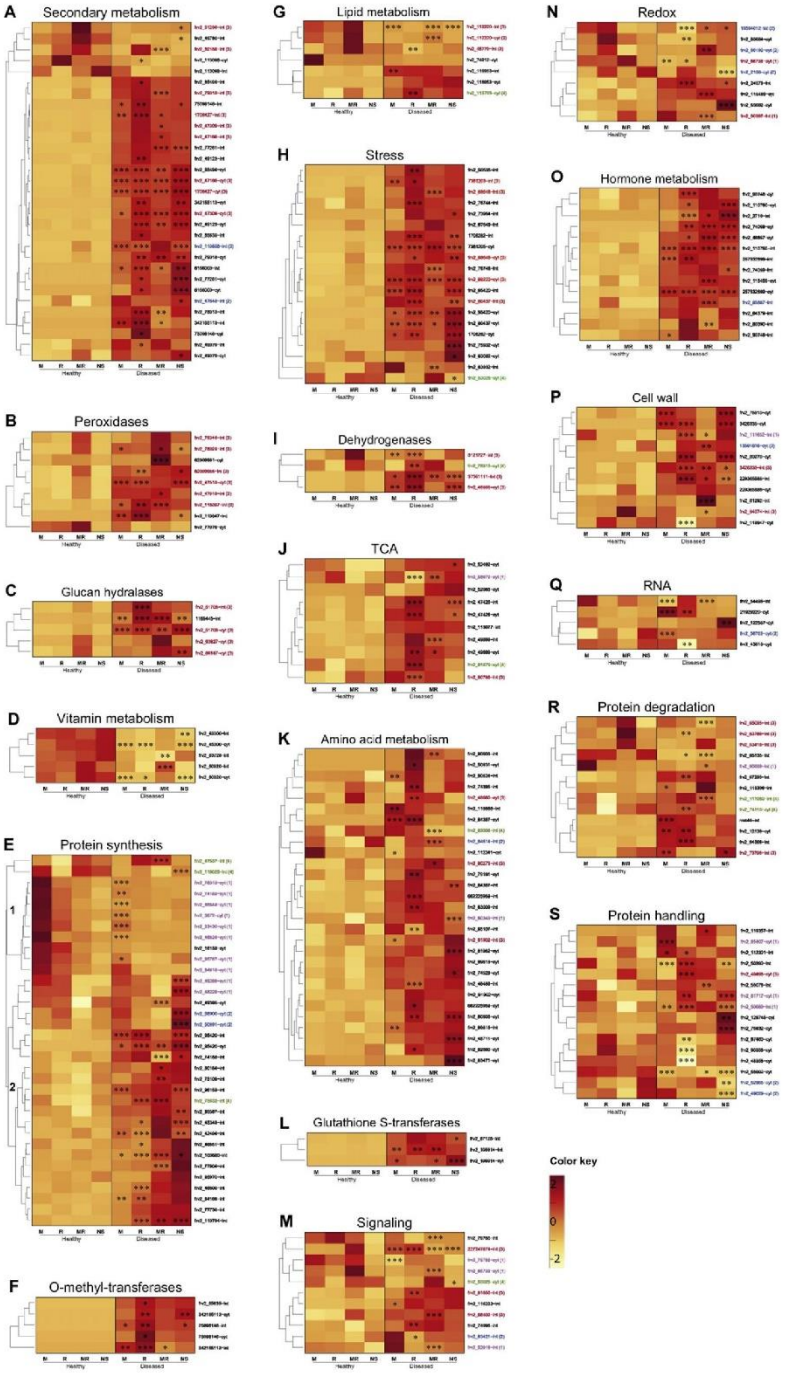

Fig. 8. (A–S) Heatmaps of functionally annotated proteins that significantly changed upon infection; exclusively proteins with known function and a minimum of 4 proteins per category were hierarchically clustered (Euclidean distance, complete linkage). Protein accessions also found significant among healthy plants (Fig. 7) are colour coded and marked with the respective cluster number. Asterisks indicate *p*-values of treatment response to infection: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the plastidic proteome with the most abundant proteins of the photosynthetic apparatus (e.g. Calvin Cycle). The additional subcellular enrichment of the cytosol (692) and the plasma membrane (158) fractions, lead to the quantification and localisation of additional 135 and 29 proteins, respectively (Fig. 6). Many of those proteins provided additional information especially to amino acid metabolism, protein synthesis and protein handling, which were also involved in pathogen response (Fig. 8N and C). Cytosolic proteins mostly overlap with integrative extracted proteins, whereas identifications of the plasma membrane overlap likewise with both integrative and cytosolic fractions.

By using the agriGO tool (v. 1.2) [57], the cellular compartment was predicted for all identifications of the plasma membrane purified fraction. Supplementary Fig. S2 indicates that a good portion (39/135) of these identifications was related to the plasma membrane but also to the cytoplasm. On one hand, this means that several plasma membrane proteins where also found in the integrative and cytosolic extractions and some proteins of unknown annotation may possibly be associated with the plasma membrane. On the other hand, several proteins might be derived from expected contaminations mainly of the cytosol due to incomplete purification [58]. In the following accessions of significantly responding proteins these are marked with -int (integrative extraction only) or -cyt (cytosolic extraction only) or -int-cyt (from both extractions), depending on where these have been found.

3.2.2. Symbiont treatment effect on proteome and metabolome of healthy plants

A heatmap was generated with all 293 proteins of the integrative and cytosolic extraction, significantly different in abundances between treatments of the healthy plants (Table S2; $p < 0.05$, fold change ≥ 2) (Fig. 7A). The intensity values for label free quantification were used for hierarchical cluster analysis. Four treatment specific clusters (cluster height 3.3) were further functionally grouped (Fig. 7B).

The first set (violet) represents proteins with elevated abundance in R and specifically in M treated plants (Fig. 7A). To a high degree these proteins are involved in RNA and protein metabolism (Table S2; protein targeting, post translational modifications, synthesis, and degradation). The second set (blue) with highest levels in R and NS treatments includes a large part of metal handling proteins (mostly ferritins: frv2_45286-int-cyt, frv2_45302-int-cyt, frv2_74959-int) as well as proteins involved in protein regulation. However, MR plants showed by far the largest set (116 proteins — red) which mainly consisted of proteins categorized in, or related to stress response (secondary metabolism, Fig. 8A; beta 1,3 peroxidases, Fig. 8B; glucan hydralases, Fig. 8C). The majority of those proteins involved in secondary metabolism belonged to flavonoid synthesis (e.g. chalcone–flavanone isomerase, isoflavone reductase). The functional category signalling noteworthy comprises more proteins in sets with high abundance in symbiotic treatments (violet, red), but not in the NS ones (blue, green).

Differences were observed in 15 metabolites among healthy plants that are explained by two main observations: (1) Elevated levels of glutamine and oxaloacetate and lower levels of leucine in NS plants; (2) high levels of galactinol, galactose, isoleucine, phenylalanine, valine, threonine, asparagine, benzoate, butanoic acid, nicotinic acid and butyro-1,4-lactam in MR plants (Table S2). In healthy plants, we observed significantly higher abundance of γ-aminobutyric acid (GABA) in M than in R.

3.2.3. Effect of pathogen infection on the plant proteome and primary metabolism

With this proteomic approach a quantity of 301 proteins were found to change abundance upon infection (Table S3).

Proteins from the plasma membrane extract were exclusively compared on the infection level as a consequence of too little observations caused by low protein yield in samples of specific treatments (MR, R). All 11 proteins that were found significantly responding to infection were accumulating (Table 1). About half of the proteins responding upon infection in the plasma membrane were also found to be induced in the integrative and cytosolic samples. Among them was the disease resistance response protein Pi49 (PR10 like), which showed the highest fold change in the plasma membrane fraction.

The following proteins were solely found significantly upregulated upon infection in the plasma membrane fraction but not in the cytosolic or the integrative extraction: V-type H^+-ATPase subunit β (frv2_111907), translational elongation factor 1 subunit β (frv2_75243), PfkB family carbohydrate kinase (frv2_83550), and proteasome subunit β (frv2_86875).

Abundances of proteins which showed response to the infection (including some proteins that contributed to significant differences among healthy treatments) were grouped according to their function and visualised in cluster heatmaps (Fig. 8; functional clusters A–S). All proteins with information about ratios and *p*-values can be found in Table S3.

Only proteins of the categories vitamin metabolism (Fig. 8D) and few proteins of the secondary metabolism (Fig. 8A) as well as from protein synthesis (Fig. 8E) were substantially depleted upon pathogen infection. In vitamin metabolism (Fig. 8D), phosphomethylpyrimidin synthase (frv2_80920-int-cyt), thiamine thiazole synthase (frv2_45000-int, frv2_45000-cyt) as well as its possible reaction centre (frv2_83728-int) were significantly reduced upon infection. This oxidative stress upon infection is as well reflected in the higher levels of SOD (frv2_85684-cyt; redox).

Overall, most functional categories reveal a substantial protein accumulation upon infection. We found an isoflavone-7-*O*-methyltransferase (frv2_85635-int) and two isoforms of the (+)-6a-hydroxymaackiain 3-*O*-methyltransferase 1/2 (342165113-int-cyt, 75098146-int-cyt) to be induced exclusively at disease response (Fig. 8F; *O*-methyl-transferases).

Noticeable, the single rhizobia inoculated plants showed the most significant response of all treatments in the pisatin pathway. Upstream the flavonoid synthesis, we also found that 2-hydroxyisoflavanone dehydratase (frv2_118953-int-cyt; Fig. 8G; lipid metabolism) was exclusively upregulated in diseased plants.

Stress related proteins were similarly induced independent of the symbiotic treatments, whereas certain subsets (Fig. 8H; accessions indicated red; PR proteins, endochitinases) showed high abundance also in healthy co-inoculated (MR) plants. This applies also for proteins related to secondary metabolism, peroxidases and dehydrogenases (Fig. 8A, B and I). However, such difference was not observed among infected treatments, where R and/or NS plants seemed to show the strongest response in abundance (Fig. 8F, J and K; *O*-methyl-transferases, TCA, amino acid).

Glutathione-*S*-transferases (Fig. 8L), which are catalysing the binding of glutathione to Xenobiotics, were exclusively upregulated in diseased plants. Glutathione synthesis requires the amino acid glycine, which was mainly downregulated (Fig. 9), while glutamate and cysteine showed no significant regulation. Proteins attributed to signalling function show diverse characteristics among healthy, as well as diseased plants (Fig. 8M).

Plants inoculated with rhizobia exhibited high levels of thioredoxin (15594012-int, frv2_56192-cyt) and superoxide dismutase (SOD; frv2_85684-cyt) which were downregulated upon disease, whereas other treatments responded contrary (Fig. 8N; redox). Despite this rhizobial dominated priming, we also noted a common upregulation of redox associated proteins (Fig. 8N) in disulphide isomerases (frv2_24576-int, frv2_53662-cyt), and 1-aminocyclopropane-1-carboxylate oxidase (frv2_115459-cyt). Upstream, methionine is processed to *S*-adenosyl-L-methionine via *S*-adenosylmethionine synthase to 1-aminocyclopropane-1-carboxylate. We observed that several isoforms of the *S*-adenosylmethionine synthase (Fig. 8K and O) involved in amino acid and hormone metabolism (frv2_46489-int, frv2_46489-cyt, frv2_74528-int, frv2_74528-cyt, frv2_99818-cyt, frv2_46489-int, frv2_74528-int) were significantly

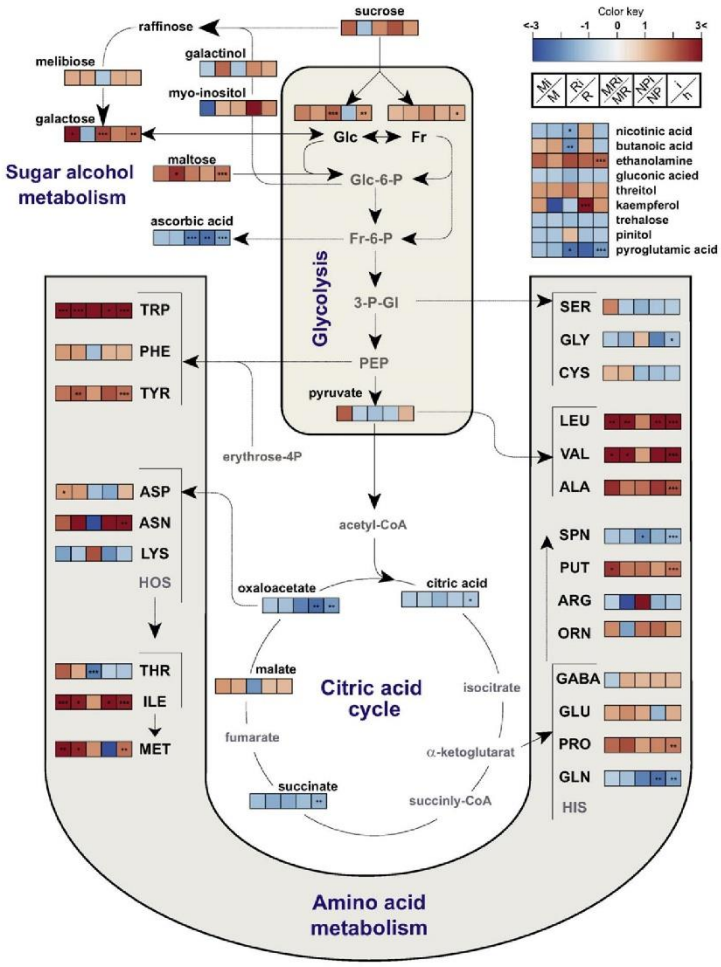

Fig. 9. Schematic overview of the primary metabolism: Metabolite levels of different symbiotic treatments that significantly changed upon infection (blue: decrease, red: increase); i = infected; h = healthy; Asterisks indicate *p*-values: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

upregulated upon infection. Furthermore, ascorbate and methionine pools showed notable changes in diseased plants: In M and R treatments with increased methionine, there was no significant decrease in ascorbate. In contrast, MR and NS treatments with no significant increase in methionine showed a significantly depleted ascorbate pool.

Fig. 8O indicates hormone metabolism, besides, ET and SA (acyl-[acyl-carrier-protein] desaturase, frv2_113703-cyt) responding pathway proteins, a set of two isoforms of 12-oxophytodienoate reductases (frv2_110760-int, frv2_110760-cyt, 257632899-int, 257632899-cyt), belonging to the jasmonate (JA) synthesis pathway showed increased abundance in all diseased plants.

Fig. 8P shows the cell wall metabolism that responded with increased levels of pectin esterases (3426335-int-cyt, frv2_80270-cyt) as well as UDP-D-glucuronate carboxy-lyase (13591616-int-cyt) and UDP-glucose 4-epimerase (229365688-int). Additionally, a leucine rich repeat showed response especially in diseased M and NS plants.

The three initial enzymes of the citric acid cycle (Fig. 8J; TCA), citrate synthase (frv2_113077-int), aconitase (frv2_52492-cyt/int), isocitrate dehydrogenase (frv2_81879-int-cyt), and also succinyltransferase (frv2_52063-cyt) were upregulated in all treatments, although R showed the most significant response. Similarly, amino acid metabolism (Fig. 8K) was mostly affected in rhizobial plants upon infection.

Metabolites, participating in the TCA (Fig. 9), such as citric acid, succinate, and oxaloacetate, showed exclusively lower levels in diseased plants than in healthy ones, except for malate which was similar in both of them.

RNA regulation (Fig. 8Q) and protein synthesis (Fig. 8E) comprises a large part of the infection response. In protein synthesis 2 subclusters can be distinguished. A remarkable set (subcluster E1), mainly ribosomal proteins (coloured violet; high in M) was exclusively depleted in M plants upon infection. However, the most common infection response (subcluster E2) consisted of an accumulation especially of ribosomal

proteins and EF2. Moreover, proteins involved in protein folding were significantly accumulating mainly in NS and R infected treatments.

A subset of proteins involved in protein degradation (Fig. 8R; frv2_53418-int, frv2_65095-int, frv2_83788-int) was specifically high in MR plants but levelled out in diseased plants. Proteins involved in protein folding, protein targeting or posttranslational modifications are combined in the functional group protein handling (Fig. 8S) which mostly comprises of chaperones (frv2_129745-cyt, frv2_81717-cyt), peptidyl-prolyl *cis*-trans isomerases (frv2_87460-cyt, frv2_50390-int), a heat shock protein (frv2_75932-cyt) and a patellin-2-like protein (frv2_49029-cyt). The majority of proteins in this category were found in the cytosol.

The infection affected 28 primary metabolites (Table S3) being visualised in Fig. 9. Briefly, among sugars, glucose and fructose were accumulated. Interestingly, we also noted a concomitant increase of several amino acids such as tryptophan, tyrosine, isoleucine, methionine, leucine, valine, alanine, and proline. Here, homoserine was poorly quantified. However, metabolic precursors for HOS (aspartate, asparagine) and its derivatives (isoleucine, methionine) were significantly upregulated (Fig. 9; amino acid metabolism). Co-inoculated plants (MR) showed eased or contrary response in these amino acids.

4. Discussion

This is, so far, the most comprehensive study of proteome and metabolome infection response to *D. pinodes* [48,59].

The main goal of our study was to reveal whether and how the different microbial treatments effect on plants metabolism and growth performance (4.1) and whether and how this would influence susceptibility of *P. sativum*'s response to the pathogenic induced ascochyta blight by *D. pinodes* (4.2). Differential symbiont treatments showed distinct effects on the plant metabolism with only little impact on growth as reflected by green area and dry matter production. But infected plants were strongly injured by the fungal disease.

4.1. Differential symbiont treatments show distinct effects on the plant metabolism with only little impact on the plants green area and dry matter production

Green area of pea plants depends on the addition and expansion of new leaves, pods or stem tissue and the senesences of older, dying leaves and maturing pods. Generally, the phenotypic host plant benefits in green area and dry matter production obtained from each microbial symbiont were sufficient and similar to the non-symbiotic treatment with synthetic fertilizer. As previously reported, metabolic exchanges from the microbial symbionts for host C have a stimulatory effect on leaf photosynthetic capacity [30], enhance green area growth and may also trigger systemic induced resistance against stress [12,60].

Plants inoculated with single AMF, however, showed mildly decreased dry matter compared to the other treatments. The distinct leaflet-proteome patterns that we found in response to different symbiotic treatments of *P. sativum* seemed, however, only slightly involved in growth performance. The AMF specific protein pattern was specifically striking for its higher levels of several proteins involved in protein regulation and synthesis. However, there was no clear indication of this AMF specific protein pattern to negatively regulate plant growth performance.

At least, the slightly enhanced dry matter of rhizobia treated plants matched well with the distinct accumulation of proteins involved in cell regulation such as tubulins (Fig. 7B; cell). Our results show that protein synthesis and RNA related proteins were dominant in the proteome of healthy plants inoculated with single rhizobia or AMF, but not with combined MR or synthetic fertilizer (NS). Among them the nascent polypeptide-associated complex subunit β was of great abundance. As reviewed by Rospert et al. [61], the nascent polypeptide-associated complex was suggested to shield nascent polypeptides deriving from the ribosome, and to be a negative regulator for translocation into the endoplasmic reticulum, but a positive one for translocation to the mitochondria. In addition, this complex was proposed to play a role in transcription rather than translation. Transcriptional changes were previously suggested to be highly controlled by the process of autoregulation of mycorrhisation or nodulation in *Glycine max* [62]. The higher levels of nascent polypeptide-associated complex subunit beta were accompanied by upregulation of a translation elongation factor-2 subunit in plants inoculated with single microbial symbionts. That is in agreement with Staudinger et al. [49], where rhizobia inoculated *Medicago truncatula* plants exhibited higher levels of elongation factor-2.

The tripartite association (MR) failed to show its synergistic effects on maximising both green area and dry matter production over the individual microbial symbionts (M or R). That might be mainly attributed to the lower effectiveness of the AMF symbiosis. A reduction of mycorrhizal benefit to host plants with increasing plant density has been reported by [63,64]. Furthermore, such lower root colonisation and nodulation in a combined MR situation might be attributed to competition between them for survival and multiplication, energy (C), nutrients and space on roots [65,66]. Nevertheless, the antagonistic effect that leads to lower colonisation observed for AMF and rhizobia of MR treated plants are consistent with other studies [32,37]. Similar results were reported previously, where AMF infection rate was lower in the tripartite symbiosis, but the number of nodules was not altered, however, nitrogenase activity was depressed [67]. A possible inhibition of N fixation and a negative effect on nodule development due to AMF colonisation of root nodules were reported [36–38]. Hence, our MR treatment results are in agreement with previous studies [32,37], but also contradicted others [68,69] which reported synergistic effects. The combination and efficiency of symbionts on plant species or even cultivars may be involved in the different findings.

It remains to be clarified, if this antagonistic effect is somehow linked to the distinct tripartite leaf-proteome pattern. Here, compared to the other treatments, the large number of highly accumulated proteins of the nitrogen- and secondary metabolism, stress, peroxidases and beta 1,3 glucan-hydratases pathways may be noticeable. These pathways are well known to be involved in stress response.

For instance, we found 12-oxophytodienoate reductase (frv2_110760) significantly higher (1.5 fold) exclusively in healthy tripartite plants compared to other treatments (M, R, NS). The 12-oxophytodienoate reductase catalyses the penultimate step in the JA synthesis. JA biosynthesis is known to be induced in barley [70] as well as in leaves of tomato associated with AMF [71]. Furthermore, a hypernodulating mutant of *Glycine* was shown to exhibit elevated levels of JA in the leaves indicating that this phytohormone is involved in the autoregulation of nodulation [72,73]. Others indicated that the degree of mycorrhisation reduces with lower levels of JA [74], which suggests a mediation of the plant–fungus interaction by this hormone. Kiers et al. [75] found that low levels of exogenously applied JA positively affect AMF colonisation, whereas high levels impede its colonisation. The suppressing characteristic of JA was recently affirmed in the interaction of *Populus* and *Laccaria bicolor* [76]. We also observed high abundance of metabolites and proteins related to flavonoid synthesis (phenylalanine, chalcone synthase/isomerase, isoflavon reductase) in MR treated plants that might play a role in the synthesis of Pisatin, the major phytoalexin in pea, known to be induced upon *D. pinodes* infection [77]. Changes in the composition of flavonoids in the shoot upon association with AMF were observed earlier in *Trifolium repens* [78]. In accordance with other studies, where glucan hydralases were found to be induced upon metal stress and JA signalling [79,80], we also observed high abundance of these proteins in MR plants.

We found a remarkable similarity between the induction of peroxidases of the tripartite treatment (MR) and the known general stress response mechanism in various plant species [81–83]. In MR plants, an accumulation of monodehydroascorbate reductase was observed, while their ascorbate level was equivalent to M, R and NS treatments.

This suggests that the glutathione ascorbate cycle is more active in tripartite plants and leads to the assumption that these peroxidases are mainly inactivating H_2O_2 within the cell by utilizing NAD(P)H. This is supported by the elevated levels of nicotinate, which serves as pyridine precursor [84] and mediates NAD synthesis [85]. It was proposed previously that enhanced resistance is accompanied by an increase of NAD, but not of ascorbate or glutathione in the leaves [86]. The demand for reduction equivalents can be affirmed by our observation of significantly increased *S*-(hydroxymethyl)-glutathione dehydrogenase, responsible for reduction of $NAD(P)^+$ to NAD(P)H, as well as the high abundance of phosphogluconate dehydrogenase and chloroplastic ferredoxin NADP oxidoreductase (Table S3) that both participate in the oxidative pentose pathway (OPP) providing NADPH. Our results suggest that aside the OPP, additional NADPH is produced through the Hatch-Slack pathway commonly known as C_4 photosynthesis.

Concerning microbial induced systemic stress alleviation, the proteomics data show further interesting overlaps with previous studies. In both, the single AMF and rhizobia treatments leaves showed an induction of metal handling proteins.

Heavy metal uptake and translocation was reported to be lower in mycorrhiza associated plants [87,88]. Even though the sets of induced metal binding proteins are different between the two symbiont treatments, in future it will be interesting to study if these are also involved in heavy metal stress alleviation for R treated plants. AMF protects plants against oxidative stress caused by heavy metals in the soil as reviewed by Schutzendubel and Polle [89]. Other authors thereon suggested a decreased need for reactive oxygen species (ROS) scavenging mechanisms in the shoot [79] and found, among other proteins, *S*-adenosylmethionine synthase (SAMS) downregulated in AMF associated plants (Table S3). *S*-adenosylmethionine (SAM) is a precursor of nicotianamine, which has been reported to play a role in metal ion homeostasis through chelation mechanisms and transport [90,91]. SAM additionally serves as a substrate for certain methylases regenerating glutathione (GSH) via the GSH-ascorbate-cycle.

In accordance to this, in tripartite (MR) plants, we found low levels of SAMS and aci-reductone dioxygenase (1,2-dihydroxy-3-keto-5-methylthiopentene), involved in methionine salvage. Affirmative to this, MR treated plants exhibited low ferritin pools, which are reported to protect cells against oxidative damage [92]. Altogether, these findings suggest a low demand for ROS scavenging mechanisms in mycorrhiza associated plants. However, the set of significantly altered proteins and metabolites (monodehydroascorbate reductase, peroxidases, galactose, nicotinate) in MR plants contradicts the assumption of lower activity in the ROS quenching machinery.

Overall, the relatively small number of proteins specifically accumulating in non-symbiotic plants emphasize significant microbial induced systemic leaf response to the symbiotic treatments as described previously [49,79,93].

4.2. Upon D. pinodes infection, a common pathogen response pattern is induced that superimposes most of the symbiont-acquired molecular response pattern

Infection and disease development depends on primary inoculum and on weather conditions which were optimum in this study. Consequently, our disease scores were similar to various previous studies such as [7]. We noted the severely negative effects of biotic (*D. pinodes*) stress on various aspects of plant growth such as green area and dry matter production as well as root nodulation and mycorrhizal colonisation. We also observed substantial changes in the composition of the proteome and metabolome.

The plasma membrane has been reported to play a key role in plants initial steps of defence signalling against pathogens [94]. The enrichment of the plasma membrane was satisfactory as suggested by an increased number of plasma membrane related proteins (Fig. S2). The greatest change upon pathogen infection was observed in the well characterized defence marker disease resistance protein Pi49 (PR10 like) [95,96], which is known to be induced by *D. pinodes* [97] and other pathogens [98] as well as by AMF and rhizobial symbionts [99].

Among proteins solely detected in the plasma membrane, a V-type H^+-ATPase subunit β was pathogen induced. This is well supported by the findings that plasma membrane H^+-ATPase are regulated by cytosolic Ca^{2+} [100] or bacterial pathogens [101]. The regulation of protein turnover upon pathogen infection [102] was also reflected in our results by upregulation of the proteasome subunit β, involved in protein degradation. Likewise, the *Arabidopsis* homologue (At5g19510) of the translational elongation factor 1 subunit β and a PfkB family carbohydrate kinase (At2g31390) were described to be responsive to bacterial pathogens and are confirmed to be localized in the plasma membrane (GO-annotation). Despite the relatively low yield of the plasma membrane fraction these proteins are evidenced key players and thus good marker proteins for plasma membrane localisation and pathogen attack. Nevertheless, further studies are necessary to clarify, if these markers are also involved in symbiont specific pathogen response or even improved pathogen resistance.

A common plant response against biotic stress was observed in the metabolome as well as in the proteome. Proline, which increased in diseased plants, was found to be induced by pathogen interaction triggering hypersensitive response [45,103] It is also known to be elevated upon environmental stress and reported to function in metal chelation, antioxidative defence as well as signalling [104].

We found that the primary metabolism contributes crucially to the plant's defence response in agreement with [25]. Above all, the TCA and amino acid metabolism (Fig. 8N and P) underwent fundamental regulations after pathogen infection. The most significant regulations in these pathways were present in rhizobial inoculated plants. The combined inoculated plants (MR), however, showed only minor response to infection (threonine, spermidine) what might be explained by the already affected concentrations in healthy MR plants. This applies also to certain functionally groups of proteins, which were elevated in MR plants, but levelled out in diseased plants (e.g. peroxidases, proteins associated to stress and secondary metabolism; Fig. 8B, I and A).

Upon pathogen infection, in all plants the TCA metabolites citric acid, succinate, and oxaloacetate exhibited low levels. This might derive from increased malate dehydrogenase synthesis, which was previously observed by Castillejo et al. [48] to be induced after *M. pinodes* infection. In all diseased plants, synthesis of PEPC was intensified. Exclusively in plants inoculated with rhizobia, increased levels of the NADP-dependent malic enzyme were observed. This indicates a need for stress alleviation. This enzyme was already shown to increase in *Pisum* upon infestation by *Fusarium oxysporum* [105]. The low levels of NADP-isocitrate dehydrogenase exhibited in R plants were significantly increased upon *M. pinodes* infection. This enzyme is mainly localized in the cytosol and was found to contribute to redox homeostasis and pathogen-response regulation in *Arabidopsis* [106].

There was an infection response in all treatments (M, R, MR, NS) in the ATP-citrate lyase which was emphasized in R and MR plants. ATP-citrate lyase was reported to be mainly localized in the cytosol and is responsible for the supply of Acetyl-Coenzyme A, which is required for the mevalonat pathway [107,108] and subsequently for isoprenoid synthesis. Isoprenoids are known to be produced upon fungal infection in rice [109]. However, our results showed a decrease of geranyl–geranyl diphosphate synthase (frv2_113098-cyt/int; Fig. 8C; secondary metabolism) in all diseased plants, which suggests a primary use of isoprenoids for gibberellin synthesis instead of higher terpenes. [110] reported higher resistance to the tobacco worm after silencing the geranyl-geranyl diphosphate synthase.

The importance of ROS is evident in the plant–pathogen interaction, which was expressed in our results via the upregulation of short chain dehydrogenases (SCD). Most of them are NAD(P) dependent oxidoreductases [111]. Short chain dehydrogenases are reported to regulate

PR1 gene expression in *Arabidopsis* [112] and thus mediate defence response.

At metabolite level, the change of redox balance was observed in the linkage of galactose, nicotinate and ascorbate which were affected by infection. The lower levels of ascorbate suggest its presence in oxidized form (dehydroascorbate, monodehydroascorbate), or utilized for example in ET synthesis. In treatments with upregulated methionine (M, R), ascorbate showed no significant decrease, whereas treatments with no significant change in methionine (MR, NS) showed a depleted ascorbate pool. Methionine is processed in the yang cycle, which is essential for ethylene synthesis. Thus our results indicate differential synthesis of ET solely in plants inoculated with single microbial symbionts (M or R).

As reported by Howe et al. [113], changes in the redox balance imply regulation of calmodulin dependent protein kinases and therefore signal triggering. In accordance to this, we found calmodulin, calnexin and other proteins exhibiting calcium binding EF-hand increased in diseased plants. Calcium signalling is known to regulate defence mechanisms in fungal interaction [114–116]. We found modulation of the hormone system corresponding to the observed signalling response.

The 12-oxophytodienoate reductase (JA synthesis), ACC (ET synthesis), S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase and acyl-[acyl-carrier-protein] desaturase (SA synthesis) were all upregulated upon infection. Indications of elevated JA synthesis (accumulation of lipoxygenases) upon symbiont treatments were observed. However there was no indication for symbiont induced SA or ET synthesis in healthy plants. With respect to pathogen attacks, it is important to know, that infection induces SA [117]. However, JA and SA pathways are known to antagonistically affect each other [118]. Thus, the elevated JA levels upon symbiont treatments may not play a beneficial role for pathogen defence as also previously described [71]. In agreement with our findings, they reported that AMF treated tomato plants showed increased lipoxygenase activity compared to non-symbiotic plants, but unlike with controls, this did not increase further in response to pathogen attack. Moreover, ET and JA are reported to act synergistically upon infection with necrotrophic pathogens [119] and suppress the abscisic acid (ABA) pathway [118].

Furthermore, it was reported that ABA positively mediates vitamin B_1 synthesis [120]. In agreement to this, the levels of phosphomethylpyrimidin synthase and thiamine-thiazole synthase, required for vitamin B_1 synthesis, were low. Consistent with other studies heat shock proteins and ABA-responsive proteins were upregulated upon infection [1]. ABA-responsive proteins are reported to have similarity to PR proteins which are as well induced upon stress [121]. Additionally, we observed an increase in all treatments in chaperones, PR-thaumatin proteins, chitinases, and disease resistance responsive proteins, which was accompanied by changes in the cell wall associated proteome (pectin methylesterases, β-xylosidase/alpha-L-arabinofuranosidase-like protein, UDP-glucose 4-epimerase, UDP-D-glucuronate carboxy-lyase, glycoside hydrolase and a leucin rich repeat protein), indicating modifications and reinforcement, as previously reported [43,122,123].

The largest set of increased proteins involved in secondary metabolism was mainly related to flavonoid synthesis (chalcone synthase, chalcone/flavonone isomerase, isoflavone reductase, sophorol reductase) and points directly towards pisatin synthesis. The (+)-6a-hydroxymaackiain 3-*O*-methyltransferase, processing the final step in the pisatin synthesis, was binned separately in the category *O*-methyltransferases (Fig. 8A). Nevertheless, pisatin is ineffective on pathogens such as *D. pinodes* as it is inactivated through pisatin demethylase, a cytochrome P450 [124].

Thereby it becomes visible that upon infection, the single rhizobia inoculated plants exhibited significantly greater abundance of this protein than M, MR and NS treated plants. This observation might be the major reason for the significantly lower level of disease severity compared to the other treatments. This higher abundance of pisatin pathway proteins fits to the greater response of the TCA and amino acid metabolism in rhizobial treated plants. Whether this rhizobial induced response was stronger or happened to occur sooner remains to be elucidated. Generally, a systemic resistance conferred by beneficial microbes is not associated with substantial alterations of the transcriptome [125] and plants are primed for enhanced defence. Thus, defence responses are not per se activated, but accelerated upon attack [126].

The crucial enzymes phosphoenolpyruvate carboxylase (PEPC) and NADP-dependent malic enzyme (ME) were both significantly accumulating in infected plants. This alternate pathway serves to alleviate abiotic stress via provision of CO_2 (to counteract oxygenase activity of Rubisco) and Pi through the PEPC [127], but also NADPH by the NADP-dependent ME. NADPH is needed for the antioxidant system and for biosynthesis of lipids, amino acids, and secondary metabolites. We found the latter two being upregulated in concentrations or activated in synthesis upon infection. Moreover, the PEPC replenishes intermediates of the TCA and subsequently scaffolds for amino acid synthesis. We confirm this by altered levels of TCA intermediates (↓oxaloacetate; ↑citric acid) and associated amino acids (↓asparagine; ↑aspartate, isoleucine, threonine, leucine, valine). Glutamine was also lower in all symbiotic compared to non-symbiotic plants. That was concomitant with elevated levels of glutamate decarboxylase and its product γ-aminobutyric acid (GABA). As reviewed by Shelp et al. [128], GABA synthesis is involved in nitrogen storage, plant development, defence, and acts as an alternative pathway for glutamate utilization. In addition, GABA is suggested to increase the efficiency of symbiotic N_2 fixation in legumes [129,130].

As discussed before, stress related proteins were strikingly abundant in MR plants suggesting a systemic induced defence response upon the tripartite interactions. Indeed, also pathogenic related stress response proteins like chitinases and PR-proteins were significantly increased. However, these proteins were only partially matched to those activated upon *D. pinodes* infection. It is known from *Arabidopsis* that certain chitinases are expressed in most of the organs at all growth stages [131]. Pathogenesis related (PR)-1 protein, which is reported to be a robust stress marker for salicylic acid (SA) responsive gene expression [118], was not increased in MR plants rather than PR-4 proteins. These were found to have a chitin binding domain [132] and antifungal activity in *Theobroma cacao* [133]. In *Arabidopsis*, PR-4 is also associated with the JA induced resistance against the necrotrophic fungus *A. brassicicola* [134]. The additional upregulated PR thaumatin family protein was reported to be induced similarly upon the incompatible interaction with wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*, pathotype CY23) by JA [135].

5. Conclusions

There have been studies on the response of the *P. sativum* leaf proteome upon infection by *D. pinodes* but little is known about the effect of rhizobia and mycorrhiza on the leaf proteome and metabolome of healthy and diseased plants. Here, we found that root colonisation with symbiotic microorganisms (AMF and rhizobia) in bi- or tripartite association is able to satisfy plant N and P demand similar to synthetic fertilizer application, resulting in equivalent growth. Due to infection with the pathogen *D. pinodes*, plant growth is substantially impaired. Induction of symbiont specific molecular patterns in healthy leaves was characterized. Rhizobia or mycorrhiza inoculation severely affected leaf protein synthesis and RNA metabolism. Mycorrhizal inoculation also showed influence on metal handling and ROS quenching mechanisms. On the other hand, co-inoculated plants exhibited remarkable abundance of stress related proteins with a concomitant adjustment of proteins involved in jasmonate synthesis. The pathogen infection caused a common metabolic response (hormonal pathways, ROS scavenging, secondary metabolism and stress related proteins) that was more pronounced in rhizobial plants (TCA and amino acid metabolism, pisatin pathway), what concurred with fewer disease symptoms.

Hence, with this study we provide new insights into the symbionts induced systemic resistance of the leaf proteome as well as the specific early pathogen stress response, which emphasizes the importance of the plants interaction with microbial symbionts not only for nutrient acquisition. For further studies we suggest to compare cultivars in order to investigate different resistance levels derived from enhanced compatibility with symbionts.

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Transparency document

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3.3 Key metabolic traits of *Pisum sativum* maintain cell vitality during *Didymella pinodes* infection: Cultivar resistance and the microsymbionts´ influence

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The decision to follow up to the first publication with an experiment contrasting two genotypes emerged after being introduced to a new cultivar (Protecta) that was suggested to hold higher resistance against *D. pinodes*. Hence, our focus shifted slightly to the elucidation of a genotype induced variation of the immune response that is linked to higher resistance. Other studies previously analysed the response of different pea genotypes against *D. pinodes* in a proteomic approach. Here, like in our first study, we adapted experimental designs from others to allow comparability. An integrative approach joint with metabolome analysis and phenotypic characterisation allowed for conclusive interpretation and elucidation of possible traits conferring resistance that constitutively depend on the genotype but vary with the symbionts associated.

Author contributions

The manuscript was submitted to the Journal of Proteomics in the special issue 'International Plant Proteomics Organization' (2017). I did all parts concerning proteomics as well as data mining of the metabolite analysis. I largely wrote the manuscript with exceptions of parts related to phenotypic results.

Publication (submitted)

Key metabolic traits of *Pisum sativum* maintain cell vitality during *Didymella pinodes* infection: cultivar resistance and the microsymbionts´ influence

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1 Abstract

Ascochyta blight causes severe losses in field pea production and the search for resistance traits towards the causal agent *Didymella pinodes* is of particular importance for farmers. Various microsymbionts were reported to shape the plants´ immune response. However, regardless their contribution to resistance, they are hardly included in experimental designs. Here, we first delineate the bi-directional effect of the symbionts´ (rhizobia, mycorrhiza) and the leaf proteome/metabolome of two field pea cultivars with varying resistance levels towards *D. pinodes*. The pathogen infection showed higher influence on the interaction with the microsymbionts in the susceptible cultivar which was reflected in decreased nodule weight and root mycorrhiza colonisation. Vice versa, symbionts induced variation in the pathogen infection response, which, however, appeared to be overruled by the genotypic characteristics such as maintenance of photosynthesis and provision of sugars and carbon back bones to fuel secondary metabolism. An active sulphur metabolism, functionality of the glutathione-ascorbate hub and fine adjustment of hormone synthesis to suppress induced cell death appeared to support resistance. Thus, we conclude that sustainment of cell vitality through these complex metabolic traits is substantial for a more efficient infection response of the tolerant cultivar.

2 Keyword Index

Field pea, legume, *Mycosphaerella pinodes*, ascochyta blight, pathogen, rhizobia, mycorrhiza, proteomics, metabolomics

3 Significance

The infection response of two *Pisum sativum* cultivars with varying resistance levels towards *Didymella pinodes* was analysed most comprehensively at a proteomic and metabolomic level. Enhanced tolerance was linked to newly discovered cultivar specific metabolic traits such as hormone synthesis and presumably suppression of cell death.

4 Introduction

The contribution of legumes to a balanced human diet and sustainable agricultural systems was depicted elaborately during the international year of pulses in 2016. With a current production of 11 m tonnes (FAOSTAT, 2015), field pea (*Pisum sativum* L.) is a grain legume of global economic importance. At present, field pea production is restricted due to various abiotic and biotic stresses. This includes ascochyta blight, one of the most devastating disease complexes causing average yield losses of 50% [1, 2]. In this fungi complex, *Didymella pinodes* (formerly *Mycosphaerella pinodes*; anamorph *Ascochyta pinodes*) is responsible for the major damage [3, 4]. The disease control measures range from burying of infected residues, crop rotation, intercropping, or delayed sowing to the application of fungicides [5]. However, agricultural measures (e.g. delayed sowing date, intercropping) are not suitable for many farm situations, and isolates of *D. pinodes* show emerging insensitivity to relatively expensive fungicides [6]. Latest findings indicate that the non-specialized life style of *D. pinodes* allows its survival on alternative hosts complicating disease management [7, 8]. Therefore, host resistance against *D. pinodes* is most desired to facilitate sustainable cultivation of field pea.

Susceptibility to *D. pinodes* was previously associated with plant height, lodging and precocity [9, 10]. Hence, there is reason to infer that conditioned environment (e.g. less favourable microclimate due to upright habitus) limited pathogen survival. Up to now, there has been limited success in breeding for resistant cultivars with suitable agronomic traits [5, 11], and the substantial influence of symbiotic interactions on the immune response of pea against *D. pinodes* has so far been hardly included in experimental designs [12]. Specific traits that are chosen for marker assisted breeding, however, are expressed in developmental and environmental dependence [13], and microbial symbionts are known to crucially render the plants' immune response and resistance level [14-16]. Also, only a few studies covered the proteomic and metabolomic response of pea to *D. pinodes* [12, 17] and tolerant cultivars have so far not been examined in an integrative (same sample for proteome and metabolome study) approach.

In our previous study [12], we elaborated the impacts of mycorrhiza and rhizobia on the infection response of the susceptible field pea cv. Messire against *D. pinodes*. We found that particularly

the rhizobial symbiosis provoking higher tolerance against this pathogen via intensified activation of specific pathways (TCA, amino acid metabolism, secondary metabolism: pisatin). Given that nodulation intensity as well as the degree of mycorrhiza root colonisation varies between cultivars [18, 19], we here aim to (i) elucidate whether symbiotic influence and/or genotypic difference explain the asserted higher resistance of field pea cv. Protecta compared to Messire. Consequently, if higher resistance in cv. Protecta is present, our objective is to (ii) expose the mechanisms behind this resistance by assessing the proteome and metabolome in an integrative approach in order to draw conclusions relevant for breeding approaches. Hence, this study dissects and pinpoints mechanisms defining higher resistance against *D. pinodes*.

5 Materials and methods

5.1 Experimental design, growth conditions and phenotypic characterisation

A graphical overview of the experimental design and the analysis workflow is provided in figure S1. The experiment was carried out in a completely randomised design and comprised 2x2x4 factor levels including pathogen (infected and non-infected), genotype (cultivar Messire and Protecta) and microbial treatments: arbuscular mycorrhiza fungi (AMF) *Glomus mosseae* (M), *Rhizobium leguminosarum* bv. *viceae* (R), dual microbial symbionts of AMF and *Rhizobium* (MR) and without symbionts (NS). Four or three biological replicates were sampled to investigate whether a single or dual inoculation of AMF and rhizobia affect the phenotype the proteome and the metabolome.

In this study, soil material and plant nutrient solutions used as well as each procedure (i.e. seed treatment, planting, plant growth management, irrigation, greenhouse conditions, pathogen inoculum, infection tests) were carried out as described by Desalegn *et al*. [12]. Assessment of phenotypic characteristics such as plant disease severity, effectiveness of microbial symbionts including root colonisation by AMF and rhizobia (nodulation), and plant growth characters (i.e. green area and shoot dry matter production) were also adopted from Desalegn *et al*. [12].

5.2 Metabolomic and proteomic studies

5.2.1 Plant sampling and preparation

Leaflets were sampled and processed as previously described [12]. Briefly, modifications are explained in the following paragraphs. The sampling time point was 36 h after successful infection. Leaves from 4 plants per pot were pooled. After quenching and grinding in liquid nitrogen the samples were lyophilized and stored for further processing.

5.2.2 Integrative extraction of metabolites and proteins

Proteins and metabolites were extracted from the same leaf material as described previously [20]. About 10 mg dry weight was used for extraction with 1 ml freshly prepared and pre-cooled extraction buffer (MeOH:$CHCl_3$:H_2O, 2.5:1:0.5). With regular agitation, samples were kept on ice for 8 min with regular agitation before centrifugation (4 min, 14000 g, 4°C). The supernatant was mixed with 500 µl ultrapure water, shaken thoroughly and centrifuged (4 min, 14000 g, 4°C). The upper phase, containing polar metabolites, was dried in a vacuum concentrator and remaining leaf material was kept for protein extraction.

5.2.3 Derivatisation of metabolites and analysis with GC-MS

Vacuum dried metabolites were derivatised and analysed with GC-TOF (LECO Pegasus® 4D) as described previously [21]. Randomly queued samples were measured in batches of 10 with intermediate measurement of external standards in 5 different concentrations. Identification (based on MS-spectra match and retention time index) and quantification over the peak area was done with the software Chroma-TOF® (version 2.5, Company). Absolute metabolite quantities were calculated by normalisation to the slope of the external standard and to the fresh weight.

5.2.4 Integrative protein extraction

Protein extraction and measurement of peptides was done according to Desalegn *et al*. [12]. Briefly, proteins were extracted from the leaf material left from metabolite extraction. The samples were sonicated in TRIzol® (80 µL) and after phase separation with 160 µL chloroform, the phenolic phase was transferred and precipitated overnight (-20°C, 0.5% β-mercaptoethanol/acetone).

5.2.5 Digestion and nano ESI LC-MS/MS analysis

The protein pellet was dissolved in urea buffer (8 M urea, 50 mM HEPES, pH 7.8) and the protein content was determined with Bradford assay. Per sample, 100 µg protein was digested with Lys-C (1:100 vol/vol, 5 h, 30 °C, Roche, Mannheim, Germany) and trypsin (1:10, vol/vol, overnight, 37°C, Applied Biosystems, Darmstadt, Germany). Peptides were desalted with C18-SPEC 96-well plates (Varian, Darmstadt, Germany) and graphite according to manufacturer's instructions (Thermo scientific, Pierce® graphite spin columns). Graphite and C18 eluates were combined, split into two technical aliquots and dried in a vacuum concentrator. Peptides of 3 biological replicates were dissolved in 100 µL 2% ACN, 0.1% FA and 1 µg of each sample was applied in random order on a C18 column (15 cm x 50 µm column, PepMap®RSLC, Thermo scientific, 2 µm particle size) and separated during a 90 min gradient with a flow rate of 300 nl min^{-1} for subsequent measurement on an LTQ-Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) with the following settings: Full scan range 350-1800 m/z, max. 20 MS2 scans (activation type CID), repeat count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 60 s, charge state screening enabled with rejection of unassigned and +1 charge states, minimum signal threshold 1000.

5.2.6 Protein identification and label free quantification

The *Pisum sativum* genome remains to be sequenced. Therefore, a database was assembled as previously presented [22], with additional transcript information from Alves-Carvalho *et al*. [23]. Identification and quantification was done with MaxQuant v1.5 [24] with following parameters: first search peptide tolerance 20 ppm, main search tolerance 4.5 ppm, ITMS MS/MS match tolerance 0.6 Da; maximum 3 of the following variable modifications: oxidation of methionine and acetylation of the N-term; maximum two missed cleavages allowed; best retention alignment function was determined in a 20 min window and identifications were matched between runs in a 0.7 min window. A FDR cut-off at 0.01 (at PSM and protein level) was set with aid of a revert decoy db. A minimum of 6 amino acids was required for identification of a peptide and at least two peptides were required for protein identification. For label free quantification (LFQ) at least one MS2 scan was present with a minimum ratio of 2. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [25] partner repository with the dataset identifier PXD005861.

5.2.7 Data analysis and statistics

Proteins were functionally classified with the Mercator tool [26]. Data processing and statistics for proteome/metabolome analysis was computed in R [27]. Proteins/metabolites present in more than half the observations of any group were statistically assessed. If less than half the observations of a group were missing, the values were estimated via k-nearest neighbour algorithm, otherwise, a minimum value (half of the lowest value multiplied with a random value between 0.1 and 1) of the respective protein/metabolite was imputed. Proteomic/metabolomic data was log2 transformed and tested for normal distribution with Shapiro Wilkins test and for homogeneity of variances with Leven´s test. Significance between symbiotic treatments was tested with Kruskal-Wallis and posthoc Dunn´s test. Significance between infected and uninfected plants (regardless the symbiotic treatment) was tested with pairwise Wilcoxon (proteins) and ANOVA/Tukey HSD (metabolites). Group averaged intensities were used to calculate the ratios between infected and non-infected treatments. Significant proteins additionally required a minimum fold change of ≥2. The independent component analysis of log2 transformed metabolite/protein intensities was done in R with the 'ipca' function of the mixomics package [28, 29]. Mode deflation was set for estimating the unmixing matrix with 200 maximum iterations to perform.

6 Results

Microsymbionts such as mycorrhiza and rhizobia influence plant nutrient uptake and the defense response to pathogens. To investigate whether (i) the pea cultivars Messire and Protecta are differently affected by these root symbionts and to (ii) elucidate if, how and why the cultivars differ in their pathogen resistance levels, we assessed various phenotypic aspects as well as the leaf metabolome and proteome.

6.1 Phenotypic characterisation of microsymbiont associated pea genotypes

By adjusting and optimising essential nutrient supply for each treatment, symbiotic interactions were facilitated and nutrient deficiencies minimised. Thereby, similar growth conditions among plants in both pea cultivars with varying symbiont associations were established (Fig. S2).

6.1.1 Nodulation

Root nodulation intensity (i.e. nodule number and fresh weight) at the respective time point of metabolite/protein sampling (after growth of 30 days, at ~ 8 internodes) indicated that the number of nodules was similar between cultivars (Fig. 2-A). However, nodules of cv. Messire tended to be heavier than those of cv. Protecta at day 30 but not at day 42 (Fig. 2-B; fig. S2-C, D). While in cv. Messire the number of nodules and the average nodule fresh weight increased until day 42 (Fig. 2-B), in cv. Protecta the average nodule fresh weight increased significantly (Fig. 2-A&B), but the number of nodules remained at initial levels (Fig. 2-A). As shown in fig. 3-A, at the time point of flowering, both cultivars showed lower nodule dry weight (NDW) in infected compared to non-infected plants.

6.1.2 Root mycorrhiza colonisation

The cultivars were different in root mycorrhizal colonisation (RMC; Fig. 3-B) at BBCH 65. Here, the overall RMC reductions due to *D. pinodes* infection were about 40% and 20% in Messire and Protecta, respectively. The highest RMC was recorded in Protecta, although both non-infected field pea genotypes have similar mean RMC.

6.2 Symbionts effect on the leave metabolism of non-infected plants

The assessment of the symbiotic influence on the metabolism comprised the identification and quantification of 39 primary metabolites (Tab. S1) and 1107 proteins (Tab. S2). An ICA of the identified metabolome (Fig. 3-A) and proteome (Fig. 4-A) clearly discriminated between cultivars. The symbiotic treatments showed an effect on the leaf metabolome in each cultivar (Tab. S2, Fig. 3-A): Protecta inoculated with mycorrhiza (PrMh) showed significantly low levels of sugars (sucrose, glucose, fructose, galactose, maltose, threitol, threonate) and some amino acids (glycine, tyrosine, valine), while mycorrhiza inoculated plants of cv. Messire (MeMh) showed significantly low levels of ornithine compared to other symbiotic associations and to cv. Protecta (Tab. S3, Fig. 3-B). The rhizobia associated plants of non-infected cv. Messire (MeRh) showed significant accumulation of glutamine in comparison to other symbiotic associations, what was also visible as trend in cv. Protecta.

An ICA (Fig. 4-A) of the non-infected leaf proteome separated the cultivars on IC1 and the symbiotic association (R, M, MR, NS) on IC2. Mycorrhiza associated individuals of both cultivars were

similarly located on the negative axis of IC2. In order to extract the symbionts effects, a loading cut off at IC2 was set (Fig. 4-B) and protein loadings of each biological category were averaged (Fig. 4-C). ICA loadings, ratios and p-values are provided in table S4. Symbionts predominantly influenced the leaf metabolism of non-infected plants in vitamin synthesis (thiamine biosynthesis protein ThiC), DNA synthesis (histone proteins), TCA metabolism (NADP-dependent malic enzyme, aconitate hydratase), signalling (G-proteins, GA protein, LRR receptor-like kinase), C_1-metabolism (ribonuclease E inhibitor, glycine dehydrogenase, folate processing), transport (ADP/ATP carrier protein), photosynthesis (glycine dehydrogenase, chlorophyll *a-b* binding protein, CP12-2, ribulose bisphosphate carboxylase small chain 3A), protein post-translational modification (tyrosine phosphatases, serine/threonine phosphatase) and RNA processing. Beside these mentioned biological functions some others contributed to symbiotic separation to less degree (oxidative pentose phosphate pathway, glutathione S-transferases (GST), short chain dehydrogenase, abscisic acid metabolism, metal handling, cell organisation, N-metabolism, peroxidases, ethylene metabolism).

6.3 Microsymbiont influence on leaf metabolism upon pathogen infection

The metabolite intensities of pathogen infected leaves displayed on an ICA (IC1 Fig. 3-C) showed clear separation of the cultivars. In cv. Messire, the symbiotic effects were more visible and rhizobia associated plants were most similar to cv. Protecta. By comparing the metabolites with the highest and lowest loadings (Fig. 3-D) with those of non-infected plants (Fig. 3-B), it becomes visible that the amino acids methionine, tyrosine and lysine gained of importance during the infection as these explained just a small part in the variance of the non-infected plants´ metabolome. Similarly, pyruvate and TCA intermediates such as citrate and succinate gained loading weight.

The proteome of infected leaves showed a separation of the cultivars on IC1, while symbiont related treatments separated on IC2 (Fig. 4-D). After setting a loading cut off at IC2 (Fig. 4-E), the functional categories contributing to the separation of symbionts (IC2) were related to functional categories such as abscisic acid metabolism (zeaxanthin epoxidase), GST, glycolysis (phosphoglycerate mutase), redox (aldo/keto reductase family oxidoreductase), photosynthesis (primarily chlorophyll binding proteins, photosystem II, cytochrome b6 and glycerate kinase), stress responsive proteins,

jasmonate/ethylene synthesis (cystathionine Beta-synthase), vitamin metabolism (thiamine biosynthesis protein, riboflavin synthase), TCA-cycle (aconitase) and development (Fig. 4-F). To lesser extent contributing to separation of symbionts rather than cultivars were the following categories: RNA processing, biodegradation of xenobiotics, tetrapyrrole synthesis, lipid metabolism, secondary metabolism, transport, peroxidases, C1-metabolism, oxidative pentose phosphate pathway, and major CHO metabolism (Fig. 4-F).

6.4 Immune response specific for the examined genotypes

6.4.1 Metabolites

Although the clustering of non-infected leaf replicates indicated differences between symbionts and cultivars (Fig. 3-A, C), the direct comparison (ANOVA, Tukey-HSD, $p<0.05$) of single metabolites did not allow for differentiation between cultivars (Tab. S5). However, the ANOVA on the IC1 position of each cultivar in the ICA (Fig. 3-A, C) showed a significant difference between the metabolome of cultivars in non-infected and infected status (i.e., non-infected: $p=0.000518$; infected: $p=0.000508$). There was a strong common reaction upon infection in both cultivars (Fig. 5) which comprised an increase of sugars, sugar alcohols and glycolysis/TCA intermediates. Other pools (galactose, raffinose, maltose, threitol, galactinol, melibiose, fructose, pyruvat) accumulated upon infection in cv. Protecta. Amino acid pools showed similar regulation in both cultivars (Fig. 5). However, phenylalanine depleted significantly in cv. Messire, whereas isoleucine, valine and proline accumulated. In cultivar Messire, the spermine precursors ornithine and putrescine significantly accumulated.

An ICA of the proteome showed that the cultivars were responsible for the greatest separation (Fig. 4-D). Therefore, protein LFQ intensities were compared between cultivars in non-infected and infected plants (Prh vs. Meh, Pri vs Mei) to analyse initial as well as disease response differences. For clear arrangement, most decisive biological functions are presented in figure 6 and less critical functions are found in the supplements (Fig. S4).

6.4.2 Photosynthesis and disease severity

Isoforms of RuBisCO small chain (Fig. 6-A: RBS3_PEA, C6SVL2_SOYBN) and two types of the chlorophyll *a-b* binding proteins (Fig. 6-A: CB2D_SOLLC, CB215_PEA) accumulated in Protecta after

infection. At the same time, this cultivar exhibited significantly lower mean values in disease severity (DS) compared to cv. Messire (Fig. 7). After 3, 6 and 10 days of *D. pinodes* infection, the DS of leaflets was significantly higher in cv. Messire by about 30%, 60% and 53%, respectively (Fig. 7-A). In cv. Messire, the DS increased by about 60% between day 3 and day 10, whereas it increased by approximately 35% in cv. Protecta during this period. Between day 3 and day 10, DS also increased by about 85% on stipules of cv. Messire (Fig. 7-B). Overall, the increase in DS was more intense on stipules compared to leaflets. Although DS was higher in cv. Messire, the abundance of photosystem I reaction centre subunit III (PSAF_ARATH) was constitutively more abundant. Coupled to the reaction in PSI is the subsequent transfer of electrons to ferredoxin (Fig. 6-B: FENR2_PEA), which also accumulated in cv. Messire. Cv. Protecta showed constitutively higher levels of chloroplastic transketolase (Fig. 6-B: TKTC2_ARATH), an enzyme regulating carbon allocation by participating in the Calvin cycle and the oxidative pentose phosphate pathway.

6.4.3 Growth and green area

The cultivars' difference was noted in growth and morphology. Cv. Protecta exhibited a taller, less branched habitus (Fig. S2). Dry matter production (Fig. S3) was significant higher in Protecta independent of infection. Infected and non-infected Messire plants had similar mean shoot dry matter production. Likewise, the total green area was significantly higher in cv. Protecta (Fig. S5). Overall, pathogen infection reduced the mean green area of cv. Messire by about 90%. In contrast, the effect of *D. pinodes* infection on green area reduction of was less than 25% in cv. Protecta.

6.4.4 Protein-protein regulation

In Messire, proteins involved in protein degradation were more abundant (Fig. 6-D). These were mainly serine proteases (CBP22_HORVU, V7B7G0_PHAVU, UPI00032A9A84). On contrary, cv. Protecta showed constitutively higher abundance of a cysteine proteinase inhibitor (CYT5_ARATH), which is involved in defense responses. The protein content (Fig. S6) was similar between cultivars in non-infected and infected leaves, and was significantly increased in infected leaves.

6.4.5 Amino acid metabolism

Amino acid (AA) pools were similar between cultivars in non-infected and infected leaves (Tab. S3, Tab. S5), and both cultivars responded alike to infection (Fig. 5): Phe pools decreased, whereas Gly, Tyr, Val, Leu and Ile accumulated. To this, the methylmalonate semialdehyde dehydrogenase (G7JU35_MEDTR), involved in Ile synthesis, was more abundant in non-infected cv. Messire compared to non-infected cv. Protecta and Ile accumulation after infection was also more significant in cv. Messire (Fig. 5). Besides Ile, the AA Lys, Asp and Met gained higher loadings in the metabolite ICA of infected leaves (Fig. 3-C) compared to the metabolite ICA of non-infected leaves (Fig. 3-A). Regarding Met pools, S-adenosyl-methionine-synthetase (Fig. 6-G: METK_MEDTR, H6UJ33_CAJCA) was increased upon infection in both cultivars with significantly higher levels in infected Messire leaves. This enzyme links sulphur metabolism with the biosynthesis of ethylene and polyamines. Another key player in the polyamine synthesis is the glutamate acetyltransferase (Fig. 6-G: arginine biosynthesis bifunctional protein A0A072VLB2_MEDTR) which was constitutively more abundant in cv. Protecta. Glutamate pools were similar among all plants, however, in non-infected leaves of cv. Protecta its products proline and glutamine tended to accumulate (Fig. 3-A, B; Tab. S3). Glutamate decarboxylase (Fig. 6-G: DCE_SOLLC), forming γ-amino-butyric acid (GABA), and aspartate aminotransferase (Fig. 6-G: G7JCN3_MEDTR) accumulated in non-infected cv. Messire. Upon infection, both cultivars responded with accumulation of chorismate synthase (Fig. 6-G: UPI00032AB5DB); however, infected cv. Messire showed significantly higher levels. This was in line with significant depletion of Phe in cv. Messire upon infection.

6.4.6 Sulphur, redox and hormone associated proteins

Cultivars differed significantly in sulphur compound handling proteins: The initial protein of sulphur assimilation ATP-sulfurylase 1 (Fig. 6-F: APS1_ARATH, chloroplastic) was constitutively more abundant in cv. Protecta together with a set of redox and defense related proteins including GST (Fig. 6-G: GSTL1_ARATH) and lactoylglutathion lyase (Fig. 6-H: G7L865_MEDTR, A0A072V303_MEDTR). Non-infected cv. Protecta showed increased levels of aminocyclopropane-1-carboxylate oxidase (Fig. 6-I: G8A030_MEDTR) participating in ethylene synthesis. However, after

infection levels were the same. Also the 12-oxophytodienoic acid reductase (Fig. 6-K: UPI00032AACAF), participating in jasmonate synthesis, showed higher abundance in infected cv. Messire.

6.4.7 Peroxidases and cell wall associated proteins

In infected plants, peroxidases (Fig. 6-L: PER15_IPOBA, PER73_ARATH, G7KFM2_MEDTR) were more abundant in Messire. The sequences of all peroxidases were analysed in the peroxibase database [30] what allowed classification to class III peroxidases. This class of peroxidases is heme binding and has most versatile functions such as cell wall elongation, stiffening and protection against pathogens. Cell wall modifying proteins (Fig. 6-M) were more abundant in infected leaves of cv. Messire. Among these is pectinesterase (PME21_SOLLC), polygalacturase inhibitor (PGIP_PYRCO), β-xylosidase (A0A072UQA9_MEDTR), and expansin-B1-like protein (A0A072UPE6_MEDTR).

6.4.8 Secondary metabolism and stress related proteins

In non-infected Protecta leaves, two enzymes, participating in flavonoid synthesis, were more abundant (Fig. 6-N): chalcone isomerase (CFLI1_MEDSA) and isoflavone reductase (IFR_PEA). More abundant in non-infected cv. Protecta (Fig. 6-O) were chitinases (CHIX_PEA, DR206_PEA, CHI2_PEA), a PR-thaumatin like protein (A0A072TVX9_MEDTR), and a V-type ATPase (Q9M7D8_PEA).

7 Discussion

In our previous work [12] we revealed a rhizobial enhanced pathogen response mechanism with reduced disease severity on pea leaves that is likely mediated by induced systemic resistance. Messire has been described as susceptible pea cultivar (Fondevilla et al., 2011). Here, our data provides evidence for an increased resistance of cv. Protecta against *D. pinodes* reflected in lower disease severity on stipules and leaflets (Fig. 7). The lower disease severity on stipules than on leaflets might be attributed to stipule architecture, which may contribute to create less favourable humidity and temperature

conditions for spore germination. Still, pathogen infection hampered plant-microbe interactions in both cultivars.

Decreased nodule number, nodule fresh weight, and lower root mycorrhiza colonisation upon *D. pinodes* infection was recently described by Ballhorn *et al.* [31]. They showed lower symbiotic interaction due to the host immune response against the pathogen, which was accompanied by higher chitinase and polyphenol oxidase activity, but they excluded the effect of decreased leaf area on the interaction with the symbionts. Higher chitinase accumulation upon infection in cv. Messire supports the finding of Ballhorn *et al*. [31] (Fig. 6-O). This suggests that synthesis of chitinases is induced upon infection and is responsible for suppression of nodule formation, which was more visible in susceptible cv. Messire. However, chitinase accumulation does apparently not increase resistance to the pathogen.

Different root nodule phenotypes and temporal varying nodulation patterns could indicate higher nitrogenase activity in cv. Protecta at later stages of development. This is supported by the overall highest shoot dry weight of rhizobia treated plants and the increased glutamine level of cv. Protecta leaves. A variation of nitrogen fixation with proceeding plant development among pea cultivars was described before [32]. Nevertheless, our data do not allow final conclusions on the influences of the nodulation phenotype and/or possibly relation of N-fixation efficiency with the enhanced pathogen resistance of cv. Protecta.

Furthermore, antagonistic effects of the double inoculates confirm our previous finding [12] and show similarity between the cultivars. The associations with AMF and/or rhizobia affect primary metabolism (Fig. 3) possibly through their growth promoting impact and their influence on nitrogen and phosphorus acquisition [33]. However, the general symbionts influence on the primary metabolism seems not to provoke specifically enhanced resistance in cv. Protecta. Hence, other genotypic factors must explain resistance in cv. Protecta. Some genotype specific mechanisms have been described before [17]. By disentangling cultivar from symbiont related effects and by using a more efficient shotgun MS approach that integrates protein and metabolite analysis, we gained further insights into genotypic related mechanisms that are leading to higher pathogen resistance.

7.1 In non-infected plants, activation of immune related metabolism is cultivar specific and varies with symbiotic treatment

In Medicago, symbionts affect non-infected plants metabolic functions related to defense response [34]. This was confirmed for *P. sativum* in our previous study [12]. In addition, our data show that the outcome of microsymbionts´ influence depends on the interacting cultivar (Fig. 3 & 4; sugars and amino acids, GST, zeaxanthin metabolism, peroxidases, ethylene metabolism), and leaf metabolism of susceptible cv. Messire is more affected by mycorrhiza and rhizobia (Fig. 4-A, B).

7.2 Symbionts directed immune response

Besides hormonal adjustment during symbiont and pathogen interaction, the plants´ redox status received vigorous attention in recent years [35-37]. After mycorrhiza was reported to affect redox balance in *Medicago* [38], we here showed in addition, that mycorrhiza modulated redox balance during infection by *D. pinodes*. Disease infected mycorrhiza associated plants of both cultivars exhibited higher abundance of L-galactose dehydrogenase (acc.: carvalho24141), an enzyme catalysing the penultimate step of ascorbate synthesis. It remains to be elucidated whether increased levels of ascorbate, rather than increased levels of NAD, are responsible for enhanced resistance, as shown for *Nicotiana tabacum* and the tobacco mosaic virus [39]. However, here mycorrhiza associated plants with increased L-galactose dehydrogenase abundance did not show lower disease severity.

Also, cystathionine β-synthase (acc.: frv2_52070), influencing the redox balance via regulation of thioredoxin [40], showed symbiont dependent accumulation in leaves of cv. Messire (Ri > Mi/MRi > NSi) upon infection. As cv. Protecta did not show cystathionine accumulation after infection, we conclude that this is connected with the cultivars´ varying intensity of symbiotic interaction. The finding that cystathionine β-synthase is induced in the roots upon rhizobia/mycorrhiza infection [41] suggests a systemic response. With regard to redox balance, this systemic effect of the symbionts on the cultivars' infection response (isoflavone synthesis, glycine-betaine synthesis) was most intense in cv. Messire MRi leaves, but not visible in cv. Protecta.

7.3 A network of metabolic traits defines the cultivar specific defense response

We recently exemplified the symbionts influence on the defense response of pea cv. Messire and showed delayed disease progress in rhizobia associated plants [12]. Nonetheless, symbionts are just capable to exploit the host´s pre-defined defense arsenal by accelerating or intensifying a defense response [14]. Additionally to the analysis of the specific symbionts influences on the host cultivar, it is therefore necessary to dissect the cultivars' inherent defense response.

Evidently, during *D. pinodes* infection, the leave proteome of *P. sativum* undergoes severe modifications [12, 17]. Increasing protein accumulation upon infection has been described before [42] and is confirmed here (Tab. S7; Messire: ↓14 ↑44; Protecta: ↓4 ↑46). It remains to be elucidated whether accumulation of serine proteases in Messire promotes susceptibility while accumulation of the cysteine protease inhibitor protein in Protecta confers resistance, as reported for pearl millet [43]. This would support the concept of protein protection as general stress mitigation strategy as previously described for improved drought tolerance in pea [44].

7.3.1 Hormone metabolism induced hypersensitive response in cv. Messire connected to susceptibility

The homeostasis of amino acids forms the immune response basis via mediating synthesis of hormones (e.g. ET, SA), secondary metabolites, or polyamines. Moreover, they represent a precious nitrogen source for pathogens [45]. One key player is Ile, which conjugates with jasmonate to its active form [46]. Augmented synthesis of Ile in non-infected cv. Messire was here indicated in accumulated levels of methylmalonate semialdehyde dehydrogenase and a significant Ile increase upon infection. However, Ile is reported to confer resistance against a biotrophic pathogen of Arabidopsis [47]. Additionally, the catabolism of Ile influences the crosstalk of SA and JA [48]. Our results indicate that in Messire the balance is located on the side of JA signalling as the oxophytodieonate reductase (JA) and the ACC oxidase (ET) were accumulated. This notion is encouraged by elevated levels of S-adenosyl methionine synthase. The indication of amplified JA/ET signalling in cv. Messire suggests augmented occurrence of programmed cell death which was reviewed to promote virulence of necrotrophs [49]. However, in case of the *P. sativum* interaction with *D. pinodes*, reports are

controversially. Carillo *et al*. [50] found that occurrence of epidermal cell death was associated with lower lesion size (48 hai), but the number of established colonies (24 hai) was unchanged or even higher. With our findings, we confirm a susceptible response of cv. Messire and establishment of colonies that is associated with programmed cell death mediated via JA/ET signalling and induction of a hypersensitive response. As indicated by Carillo *et al*. [51], epidermal cell death occurs to less extent in their tested tolerant accession P665. This encourages the reasoning that sustainment of cell vitality in cv. Protecta and suppression of cell death is associated with higher tolerance.

7.3.2 Enhanced sugar levels reflect higher tolerance in cv. Protecta

Besides providing carbon and energy to plants, sugars were reported to enhance resistance through various ways and gave rise to the idea of a 'sweet immunity' [52, 53]. Certainly, for pathogens pervading the apoplast, sugars are a precious resource. During *Cladosporum fulvum* infection, plant sucrose levels were first found increased while declining at later stages when glucose and fructose start to increase [45, 54]. Although sucrose was previously thought to be degraded by fungal secreted invertases for subsequent import as glucose/fructose [55], latest findings indicate an important role of sucrose transporters in fungal membranes [56]. Our data showed sucrose depletion in both cultivars but only Protecta showed a significant increase in fructose, pyruvate and citrate, suggesting that Protecta pushes glycolysis to fuel the TCA cycle.

Other sugars being of particular interest during defense, are trehalose, galactose and raffinose. Latter is known to be of great abundance in legumes [57]. All these sugars are proposed to be signalling molecules [58] and, furthermore, to have ROS scavenging properties [59]. Galactose, additionally, is reported to stimulate the accumulation of defense related gene transcription in tobacco and subsequently enhances resistance against *Botrytis* [60]. Infected cv. Messire exhibited significant higher levels of trehalose, while cv. Protecta´s levels remained unchanged. The report that trehalose induces phenylalanine ammonium lyase and peroxidase activity in wheat [61] matches remarkable well with our findings of depleted phenylalanine pools and increased abundance of peroxidases in cv. Messire. To this, cv. Messire accumulated chorismate synthase. The finding that cv. Protecta, in contrast, manages to preserve its phenylalanine levels, may be due to accumulated aspartate amino transferase,

which is reported to have prephenate aminotransferase activity and thus represents a key step in the synthesis of aromatic AA [62, 63].

The sugar availability for the pathogen seems pivotal during infection and Joosten *et al.* [54] indicated that during a tolerant defense response sugar levels remain high until the end of infection. They suggested that in an immune response against necrotrophs, resistance is enhanced if cell death is not triggered, because the fungi cannot feed on released sugars. Protecta´s accumulation of sugar pools may indicate higher tolerance and slowed down infection, because sugars were not yet metabolised by the pathogen. Supportive for a decelerated infection process was the constitutively more abundant actin depolymerising factor in Protecta, as it was reported that penetration is restrained in non-host interaction of tobacco cell cultures due to an actin related defense mechanism inducing cell wall fortification and callose deposition [64].

7.3.3 Enhanced resistance through maintaining the linkage from photosynthesis to secondary metabolism

Programmed cell death includes the global downregulation of photosynthetic genes [65]. Accordingly, RuBisCO and chlorophyll *a-b* binding protein levels decreased in Messire after infection. Similar observations were made for this cultivar after *Uromyces pisi* and *D. pinodes* attack [17, 66]. The maintenance of these protein´s abundance in cv. Protecta is presumably due to the lower disease severity that is connected to stable green area. Contradictory to this is the constitutively higher abundance of photosystem I in Messire. Together with the elevated ferredoxin levels in this cultivar, we suggest a higher occurrence of cyclic electron flow. This might result in the sole provision of ATP, but lower reducing potential, which is necessary for pathogen defense [67].

In various stresses, required reducing equivalents are provided through the oxidative pentose phosphate pathway. A key metabolic hub for sugar conversion is transketolase (TKL), an enzyme also participating in the Calvin cycle. Substrates for shikimic acid and phenylpropanoid pathway (erythrose-4-phosphate), as well as for thiazole biosynthesis (glyceraldehyde-3-phosphate: precursor of thiamin) are provided by TKL. The constitutive higher abundance of TKL (acc.: carvalho29613, TKTC2_ARATH) in Protecta might promote tolerance to *D. pinodes*, which is also reflected in the

maintained levels of phenylalanine. This interpretation is supported by other reports where a decrease of plastidic TKL resulted in decreased levels of intermediates and products of the phenylpropanoid metabolism [68], which leads to synthesis of lignin and/or flavonoids. To this, Protecta showed constitutive higher abundance of chalcone-flavon isomerase and isoflavon reductase, involved in flavonoid biosynthesis. Thus, maintenance of photosynthesis and sustainment of TKL activity might enable continuance of phenylpropanoid synthesis, such as flavonoids and lignins. The accumulation of flavonoid synthesis related proteins in non-infected cv. Protecta further indicates increased ROS scavenging ability. Messire, on the other hand, accumulated these proteins after infection suggesting a delayed and thus less effective response, while cv. Protecta might have been able to quench pathogen derived H_2O_2.

7.3.4 Evidence for importance of sulphur and glutathione metabolism in promoting resistance

Sulphur availability and its usage in plants to enhance resistance against various pathogens has been under continuous discussion. However, the exact mode of action by which Sulphur supply affects defensive forces is not yet fully understood [69]. Sulphur application was found to contribute to higher resistance against hemibiotrophs and necrotrophs in species such as maize, potato and tomato [70-73]. Our results indicate decisive differences between cultivars in sulphur assimilation: Protecta showed significant higher accumulation of ATP-sulfurylase, which catalyses the reduction of sulphate to adenosine 5´-phosphosulfate (APS). Hence, increased ATP-sulfurylase abundance during pathogen infection suggests maintained or even increased sulphur assimilation rates. This points towards a cultivar difference in glutathione (GSH) metabolism. During infection, GSH is reviewed to be involved in stress signalling, detoxification of xenobiotics, transport and storage of reduced Sulphur [74]. Its further usage is the detoxification of methylgyoxal, which evolves during spontaneous non-enzymatic elimination of the phosphate group of glycerinaldehyd-3-phosphate or dihydroxyacetone-phosphate. Protecta showed greater potential of methylglyoxal detoxification by constitutive increased abundance of lactoylglutathione lyase, which catalyses conjugation of GSH and methylglyoxal. During this process GSH is regenerated and lactate is formed [75]. Increased usage of glutathione in Protecta is also

reflected in constitutive higher abundance of GST that is capable to bind GSH to xenobiotics to provoke their degradation. Our the data further suggest higher activity of the ascorbate-glutathion pathway that accounts for restoration of ascorbate [76]. Matching to this is the higher accumulation of galactose dehydrogenase (ascorbate synthesis) upon infection in Protecta.

The disposal of H_2O_2 in plants is achieved through oxidation of ascorbate to monodehydroascorbate by means of class I peroxidases. Leaves of infected cv. Messire accumulated class III peroxidases. Class III peroxidases are secreted in order to facilitate cell wall elongation. Notable, cell wall modifying proteins were exclusively more abundant in the susceptible cv. Messire after infection. During the first hours of infection (1-12 h), increased cell wall modification (e.g. callose deposition, active pectinesterases) was reported as effective defense mechanism [77]. However, such changes in the apoplast might be induced by the pathogen in order to either feed on sugars derived from cell wall degradation [56], or to promote easier proliferation of hyphae. The accumulation of expansin (causing cell wall relaxation) in Messire supports this conclusion.

S-adenosyl methionine synthase accumulation in cv. Messire points towards synthesis of polyamines (e.g. spermine or spermidine), for which S-adenosyl methionine is required as methyl donor. Polyamines are reported to constitute a source of H_2O_2 during pathogen infection [78]. The significant accumulation of ornithine and putrescine as precursors of spermine in the susceptible cv. Messire (Fig. 5) is in accordance to this.

8 Conclusions

Our previous study [12] showed the rhizobia and mycorrhiza impact on leaf metabolism and pinpointed the rhizobial symbiosis as central element for augmented resistance of the susceptible pea cultivar Messire against the pathogen *D. pinodes*. The resistance promoting effect of rhizobia resembled an induced systemic resistance and was characterised by increased synthesis of proteins involved in TCA pathway and secondary metabolism. In the present study, we compared the susceptible cultivar Messire with the more tolerant cultivar Protecta and confirmed the importance of pisatin synthesis during defense response. However, the previous reported positive impact of rhizobia on the defense response seems to rely on the cultivars´ temporal varying interaction with the symbionts. Thus, cultivar-

symbiont compatibility should be progressively considered in breeding programs. This study revealed novel defense traits promoting tolerance against *D. pinodes* (Fig. 8). Reduced disease severity in cultivar Protecta was associated with the ability to enhance cell wall fortification, suppress hypersensitive response, and maintain photosynthesis, flavonoid synthesis and glutathione-ascorbate pathway. Since the infection process is mostly determined by readiness of the host´s defense response, future studies may aim to elucidate the temporal onset of the here presented pathways.

9 Highlights

- The effects of rhizobia and mycorrhiza on plants above ground parts are genotype dependent
- Higher genotype susceptibility leads to enhanced repulsion of below ground symbionts after infection
- Symbionts induced variation of infection response is overruled by the genotypic setup
- Higher tolerance is pinpointed to maintenance of a functional glutathione-ascorbate pathway, provision of sugars for secondary metabolism, and fine regulation of hormone synthesis

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11 References

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Figure captions

Fig. 1: (A) Nodule number and fresh weight at the time point of metabolome/proteome sampling (30 d and 42 d); n=12. (B) Average fresh weight per nodule for each time point (n=12).

Fig. 2: Effect of *Rhizobia* inoculation on nodule dry weight (A) and inoculation with arbuscular mycorrhiza fungi on root colonisation (B) of field pea genotypes (*P. sativum*) infected with *D. pinodes*. Genotypes and symbionts: Messire (Me), Protecta (Pr), mycorrhiza (M), rhizobia (R), no symbionts (NS); values are means (n = 4); error bars indicate standard error. Means followed by the same letter were not significantly different according to Tukey's multiple range test. Statistical significance was defined at $p < 0.05$.

Fig. 3: (A, C) Independent component analysis (ICA) of metabolite concentrations in non-infected (A, explained variance IC1 13.5%, IC2 10.5%) and infected leaves (C, explained variance IC1 15.9%, IC2 8.1%) with the respective loadings of each metabolite on IC1 and IC2 (B, D).

Fig. 4: (A, D) ICA of log2 protein LFQ intensities of non-infected (A, explained variance IC1 15.3%, IC2 8.7%) and infected leaves (D, explained variance IC1 14.2%, IC2 9.8%) with the respective loadings of each protein on IC1 and IC2 (B, E). The corresponding histogram of IC2 loadings are visualised to the right of each ICA-plot: Black dots and bars in the loading-plot indicate proteins which were binned in biological functions in C and F.

Fig. 5: Log2 ratios of infected vs. non-infected metabolite concentrations for cv. Messire (Me) and Protecta (Pr), n=12. High ratios were levelled to ±3 in order to visualise lower values. Asterisks indicate p-values after ANOVA and Tukey-HSD: *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 6: Log2 LFQ intensities of proteins showing significant differences (Kruskal Wallis test, p<0.05, 2 fold) in abundance between cultivars in non-infected or infected leaves (n=12). Error bars indicate standard error. If the ratio fell below the threshold of 2, the fold change was indicated above the error bars.

Fig. 7: *Didymella pinodes* severity scores (0-6) ten days after infection on (A) leaflets and (B) stipules of P. sativum. Values are means (n = 4) and error bars indicate standard error. Means followed by the same letter were not significantly different according to the Tukey's multiple range test and statistical significance was defined at p < 0.05.

Fig. 8: Condensed model of a susceptible (blue: cv. Messire) and tolerant (red: cv. Protecta) infection response. Arrows outline functional connections but not complete pathways. While cell wall modifications (expansin, peroxidase) and augmented signalling via JA (jasmonate) and ET (ethylene) delineate an ineffective response, the sustainment of a functional ascorbate GSH (glutathione) hub and the sugar to secondary metabolism linkage confer enhanced tolerance. GST: glutathion S-transferase; TKL: transketolase; SAM: S-adenosyl methionine.

Fig. S1: Workflow

Fig. S2: Shoot and root morphology of cv. Messire (A, C) and cv. Protecta (B, D).

Fig. S3: *Didymella pinodes* effect on shoot dry matter yield of field pea (*Pisum sativum* L.) genotypes. Genotype and symbiont: Messire (Me), Protecta (Pr), mycorrhiza (M), rhizobia (R), no symbionts (NS); values are means (n = 4) and error bars indicate standard error. Means followed by the same letter were not significantly different according to the Tukey's multiple range test and statistical significance was defined at p < 0.05.

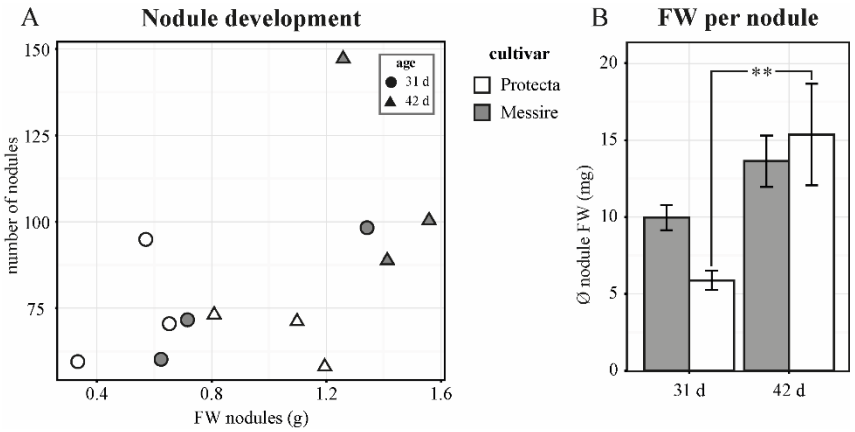
Fig. S4: Supplementary material to figure 6.

Fig. S5: *Didymella pinodes* effect on green area production of field pea (*Pisum sativum* L.) genotypes. Genotype and symbiont: Messire (Me), Protecta (Pr), mycorrhiza (M), rhizobia (R), no symbionts (NS);

values are means (n = 4) and error bars indicate standard error. Means followed by the same letter were not significantly different according to the Tukey's multiple range test and statistical significance was defined at $p < 0.05$.

Fig. S6: Protein content of non-infected and infected leaves for each cultivar (n=12). Asterisks indicate p-values after ANOVA and Tukey-HSD: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 1



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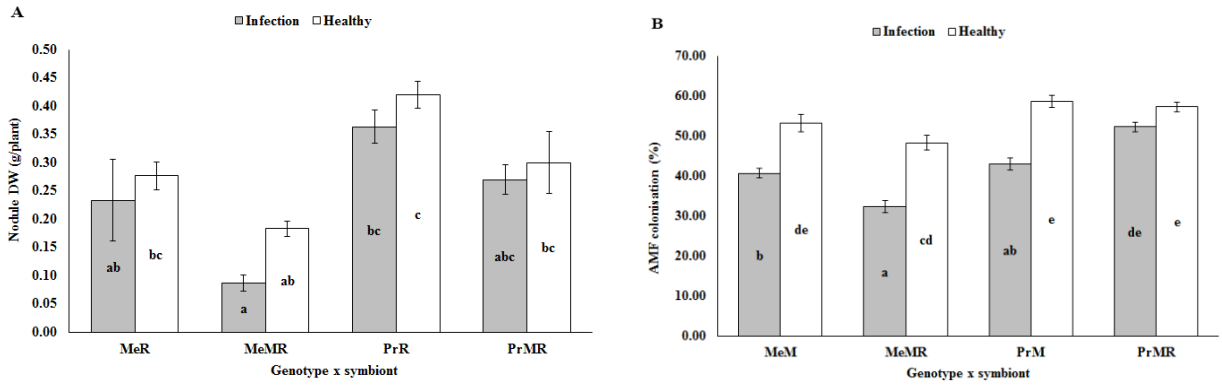


Figure 3

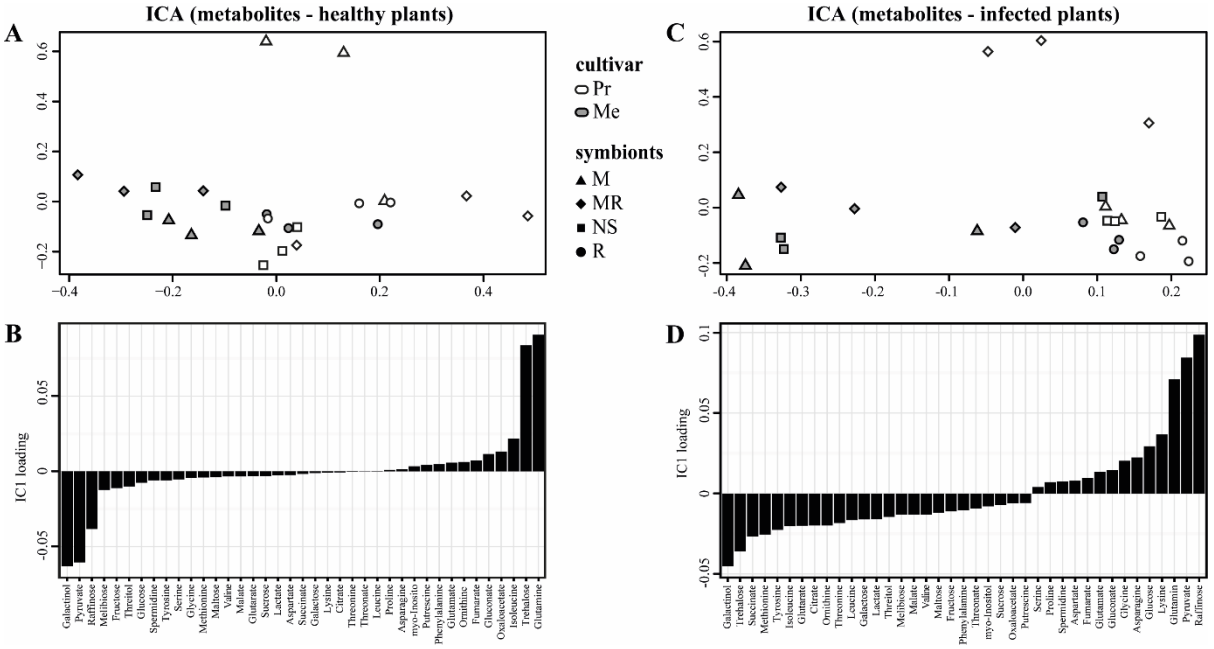


Figure 4

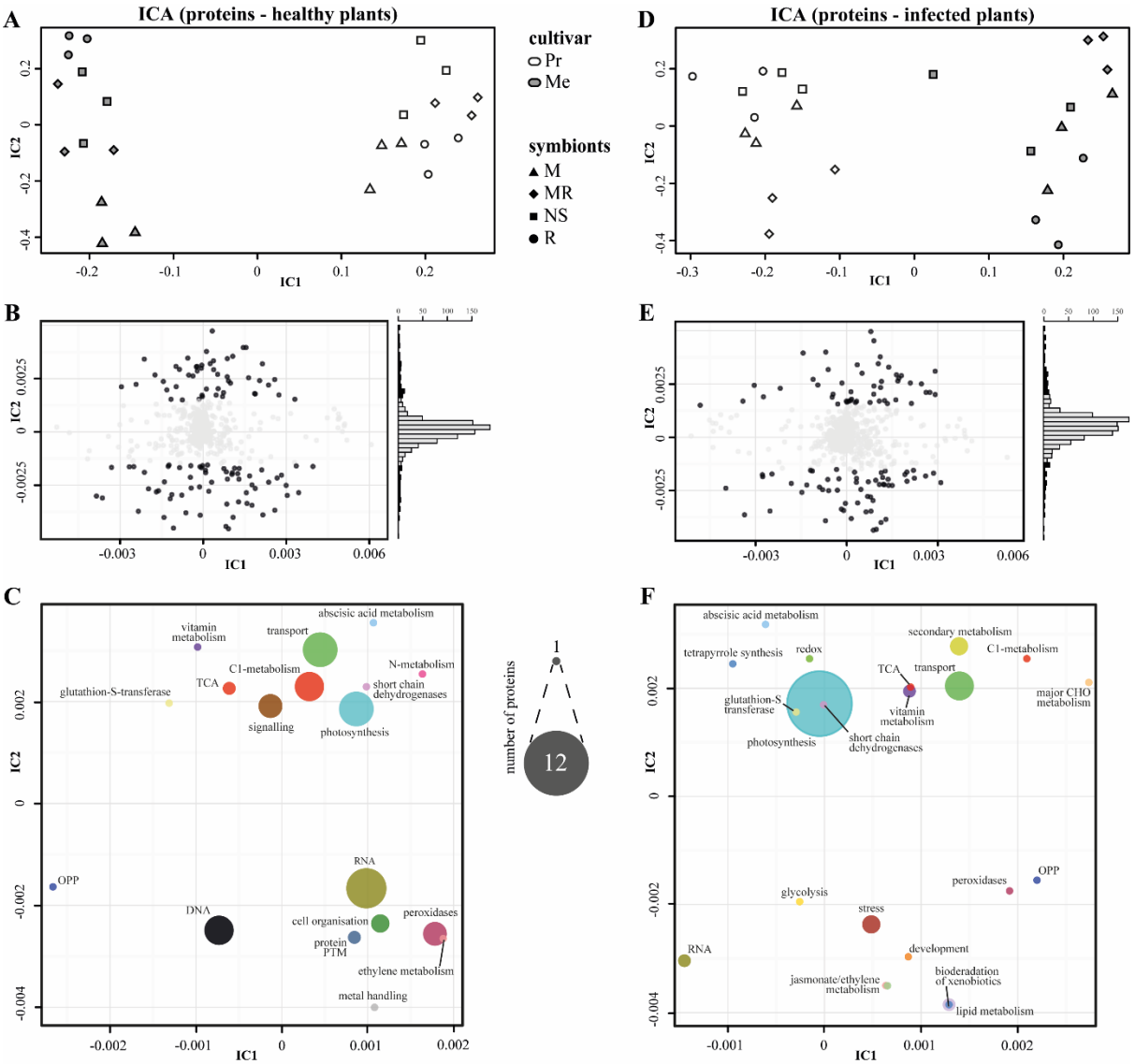

A
ICA (proteins - healthy plants)
IC2
IC1
cultivar
Pr
Me
symbionts
M
MR
NS
R
D
ICA (proteins - infected plants)
B
E
C
vitamin metabolism
transport
abscisic acid metabolism
N-metabolism
C1-metabolism
TCA
glutathion-S-transferase
short chain dehydrogenases
signalling
photosynthesis
RNA
OPP
DNA
cell organisation
peroxidases
protein PTM
ethylene metabolism
metal handling
number of proteins
1
12
F
abscisic acid metabolism
secondary metabolism
tetrapyrrole synthesis
redox
C1-metabolism
TCA
transport
major CHO metabolism
glutathion-S transferase
vitamin metabolism
photosynthesis
short chain dehydrogenases
peroxidases
OPP
glycolysis
stress
RNA
development
bioderadation of xenobiotics
jasmonate/ethylene metabolism
lipid metabolism

Figure 5

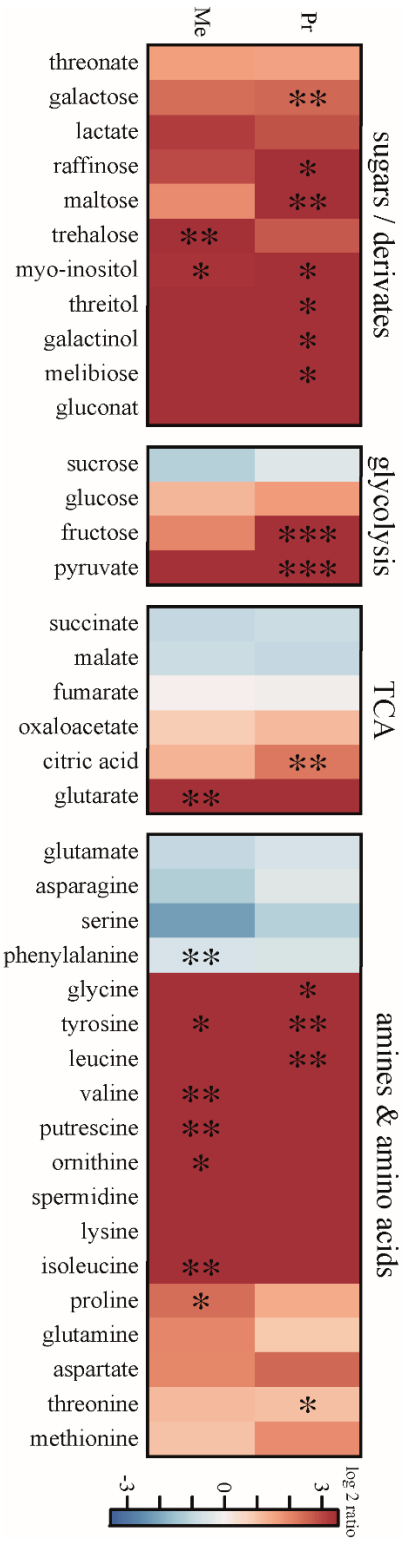

Me
Pr
threonate
galactose
lactate
raffinose
maltose
trehalose
myo-inositol
threitol
galactinol
melibiose
gluconat
sugars / derivates
sucrose
glucose
fructose
pyruvate
glycolysis
succinate
malate
fumarate
oxaloacetate
citric acid
glutarate
TCA
glutamate
asparagine
serine
phenylalanine
glycine
tyrosine
leucine
valine
putrescine
ornithine
spermidine
lysine
isoleucine
proline
glutamine
aspartate
threonine
methionine
amines & amino acids
-3
0
3
log 2 ratio

Figure 6

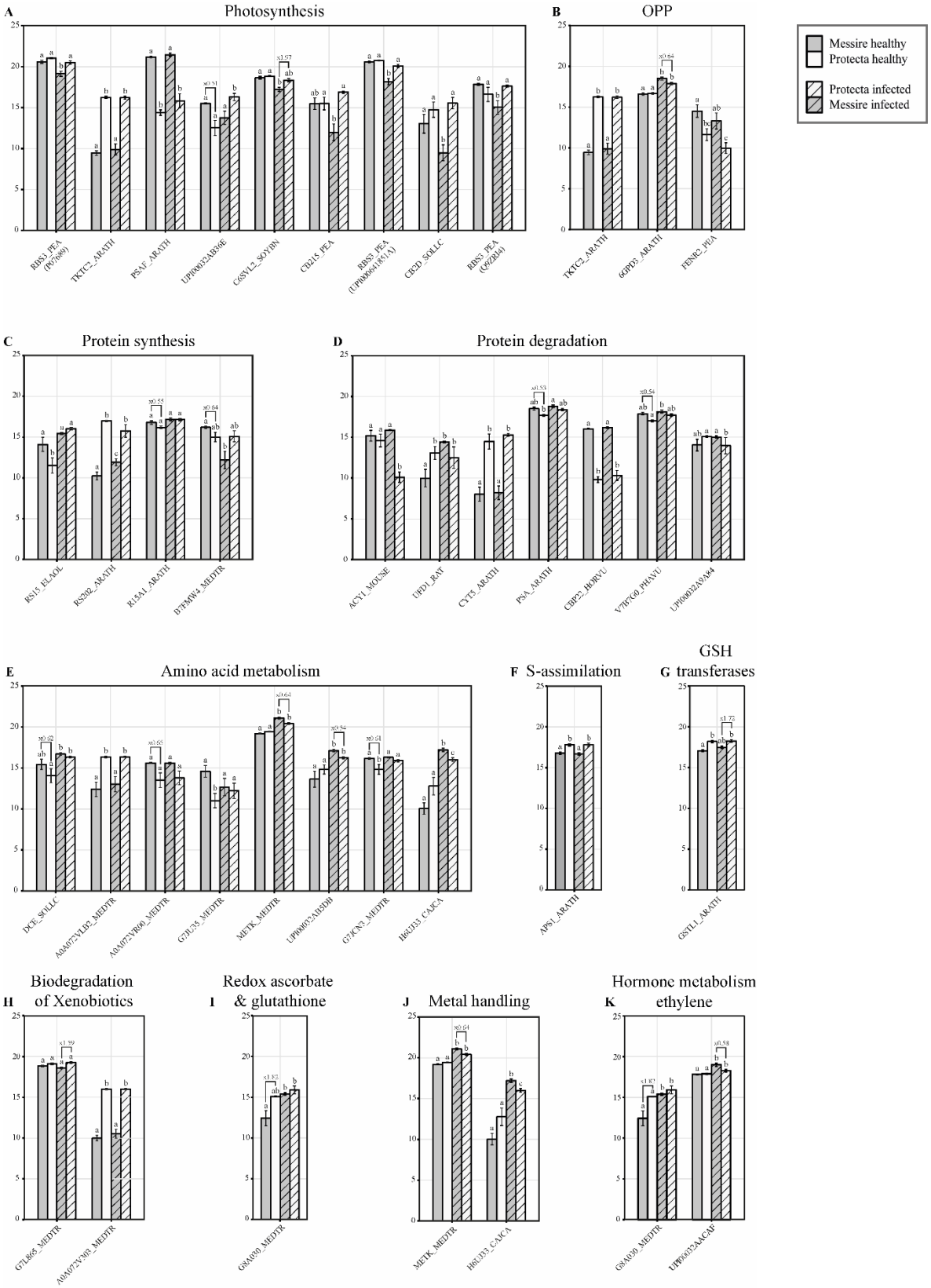
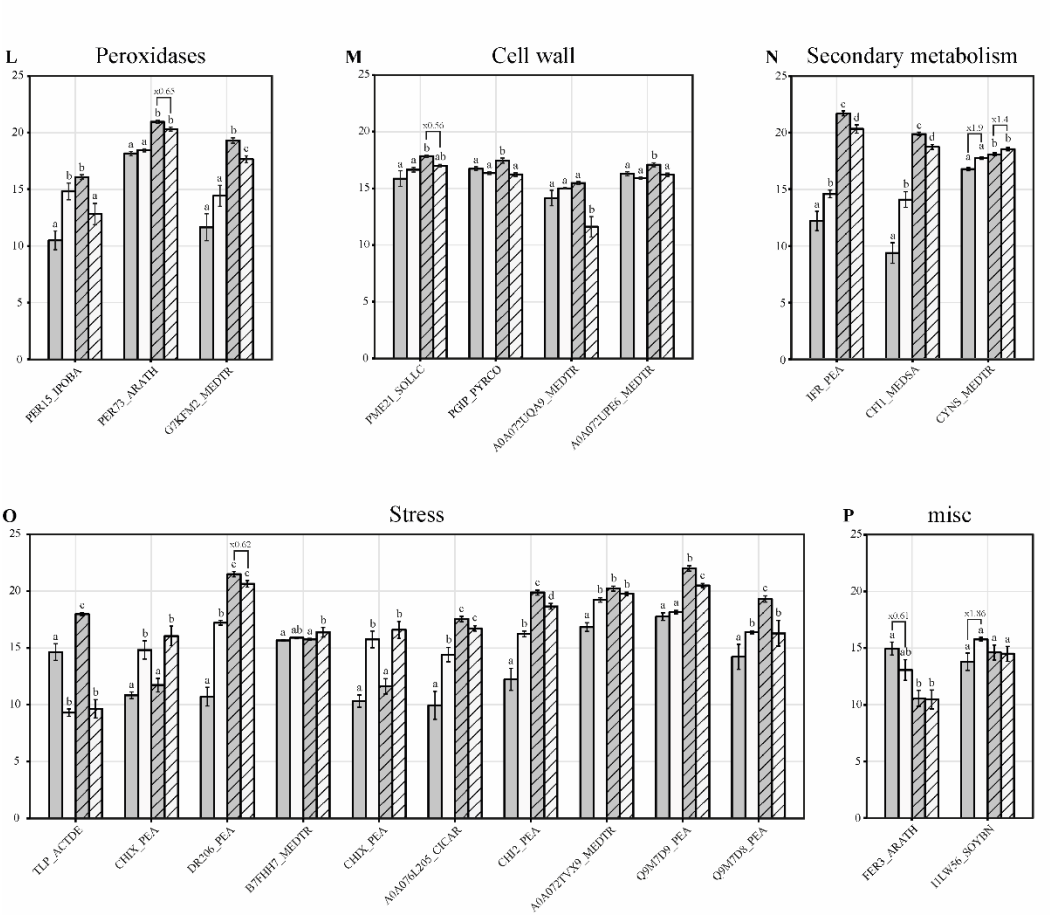

A Photosynthesis
B OPP
C Protein synthesis
D Protein degradation
E Amino acid metabolism
F S-assimilation
G GSH transferases
H Biodegradation of Xenobiotics
I Redox ascorbate & glutathione
J Metal handling
K Hormone metabolism ethylene
Messire healthy
Protecta healthy
Protecta infected
Messire infected

Figure 6


L
Peroxidases
PER15_IPOBA
PER73_ARATH
G7KFM2_MEDTR
M
Cell wall
PME21_SOLLC
PGIP_PYRCO
A0A072VQA9_MEDTR
A0A072UPE6_MEDTR
N
Secondary metabolism
IFR_PEA
CTI1_MEDSA
CYNS_MEDTR
O
Stress
TLP_ACTDE
CHIX_PEA
DR2A6_PEA
B7FHH7_MEDTR
CHIX_PEA
A0A076L295_CICAR
CHI2_PEA
A0A072TVX9_MEDTR
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Q9M7D8_PEA
P
misc
FER3_ARATH
I1LW56_SOYBN

Figure 7

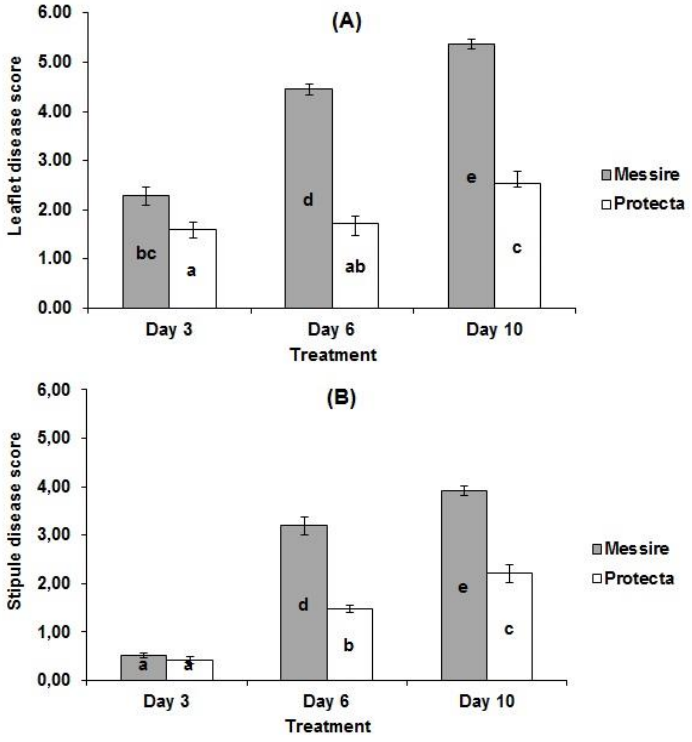


Figure 8

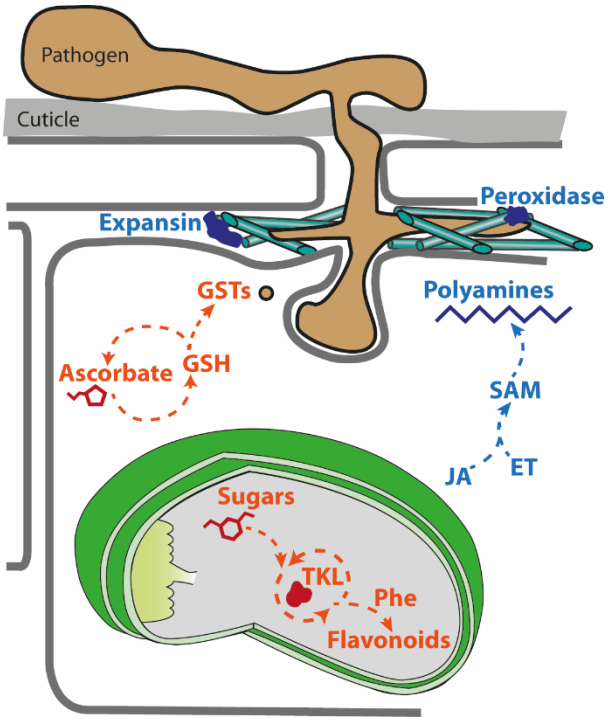


Figure S1

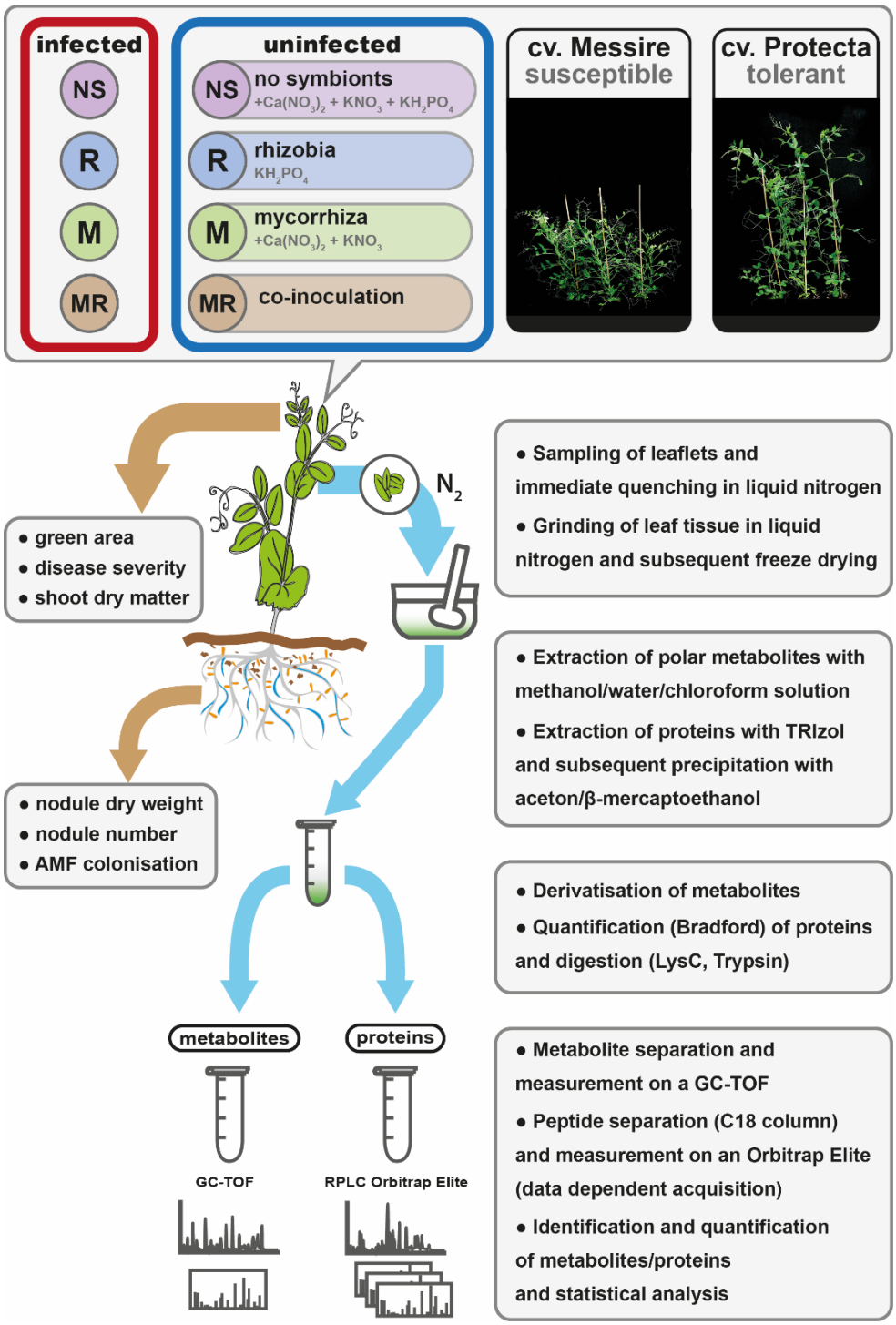

infected
NS
R
M
MR
uninfected
NS
no symbionts
+Ca(NO3)2 + KNO3 + KH2PO4
R
rhizobia
KH2PO4
M
mycorrhiza
+Ca(NO3)2 + KNO3
MR
co-inoculation
cv. Messire
susceptible
cv. Protecta
tolerant
N2
• green area
• disease severity
• shoot dry matter
• nodule dry weight
• nodule number
• AMF colonisation
• Sampling of leaflets and immediate quenching in liquid nitrogen
• Grinding of leaf tissue in liquid nitrogen and subsequent freeze drying
• Extraction of polar metabolites with methanol/water/chloroform solution
• Extraction of proteins with TRIzol and subsequent precipitation with aceton/β-mercaptoethanol
• Derivatisation of metabolites
• Quantification (Bradford) of proteins and digestion (LysC, Trypsin)
metabolites
proteins
GC-TOF
RPLC Orbitrap Elite
• Metabolite separation and measurement on a GC-TOF
• Peptide separation (C18 column) and measurement on an Orbitrap Elite (data dependent acquisition)
• Identification and quantification of metabolites/proteins and statistical analysis

Figure S2

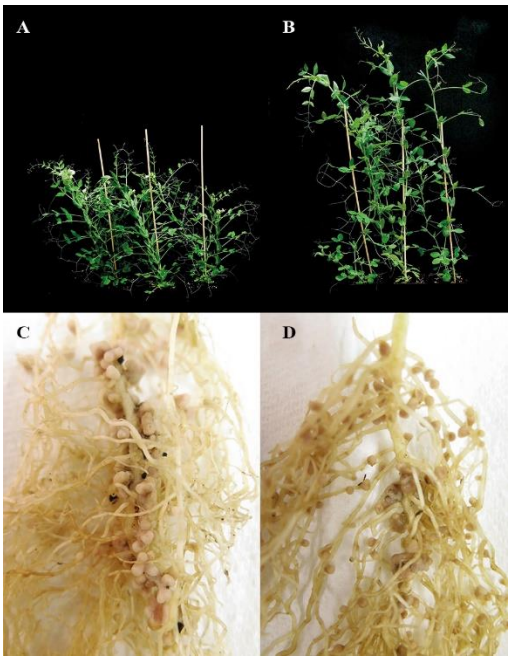


Figure S3

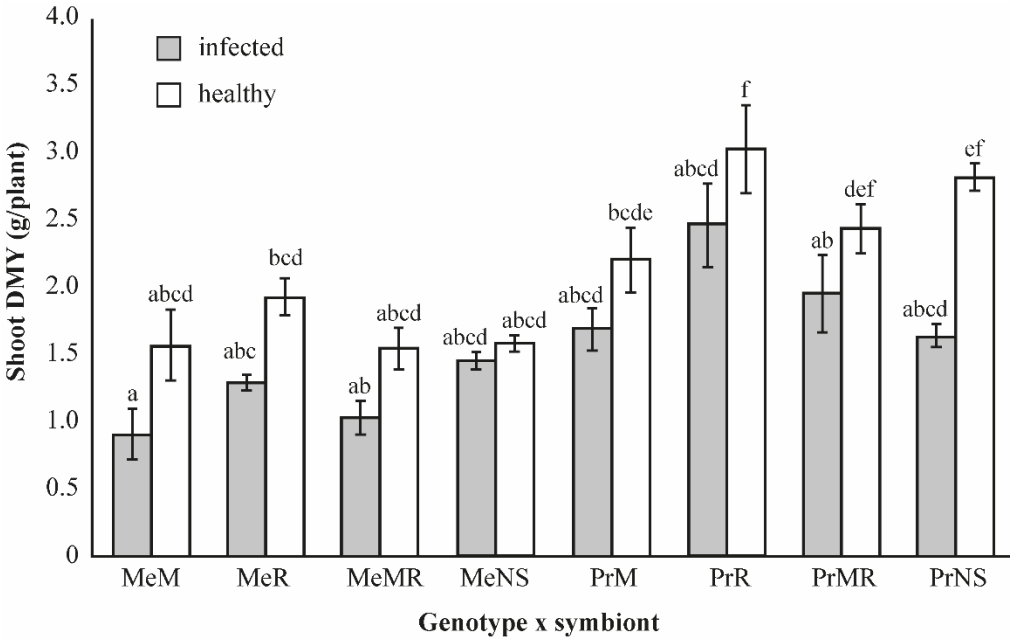


Figure S4

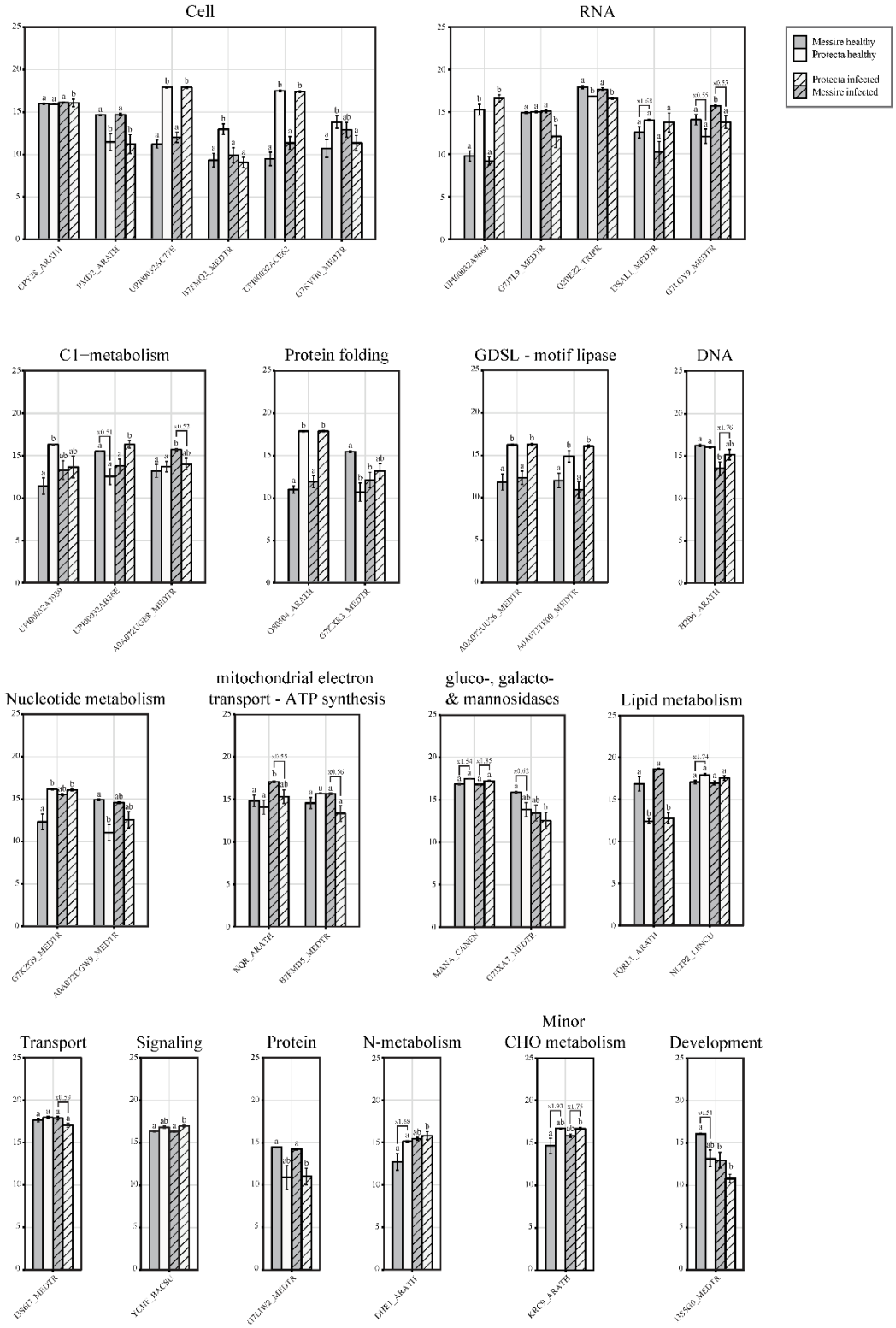

Cell
RNA
Messire healthy
Protecta healthy
Protecta infected
Messire infected
C1−metabolism
Protein folding
GDSL - motif lipase
DNA
Nucleotide metabolism
mitochondrial electron transport - ATP synthesis
gluco-, galacto- & mannosidases
Lipid metabolism
Transport
Signaling
Protein
N-metabolism
Minor CHO metabolism
Development

Figure S5

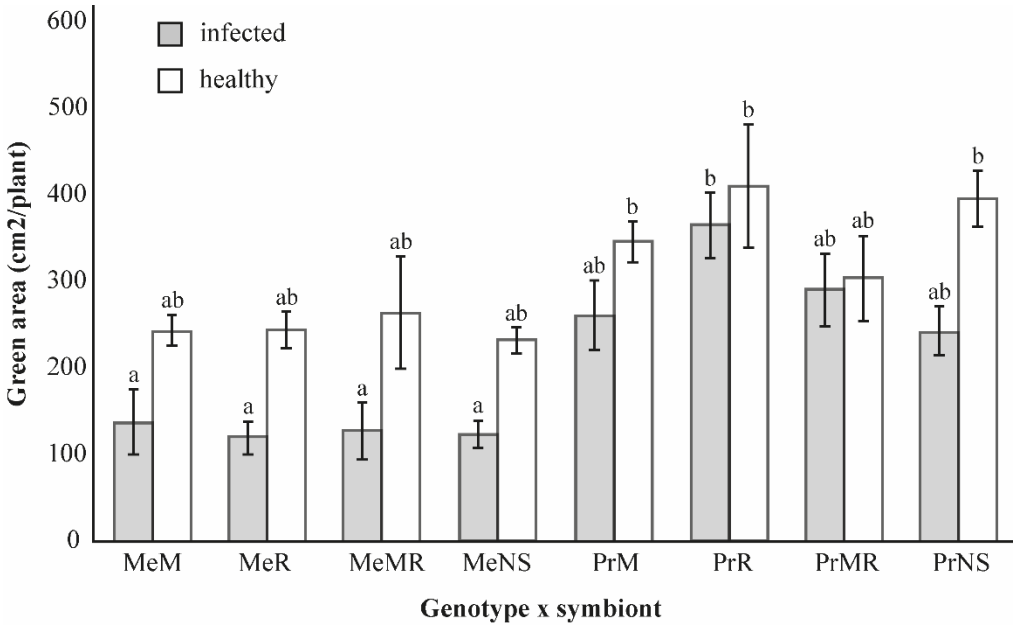
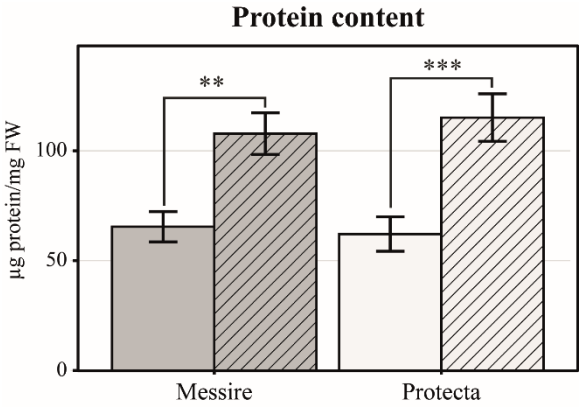


Figure S6



4 Concluding discussion and future perspectives

Within this cumulative doctoral thesis method optimisation was an integral part at all stages. In the first instance, to facilitate shotgun proteomic experiments, data collection (sequence information) and processing was of utmost importance, and alongside database assembly, LC-MS optimisation and MS data evaluation was the basis work for all publications.

Rather than pursuing a specific research question in pathology (e.g. mechanisms of action between elicitors/effectors and receptors), our publications followed the idea of a systemic view that is best answered with an unbiased approach. The studies in this thesis aimed to present a systemic view of the pathogen response that is influenced by factors such as symbiotic association of genotype variation.

The plethora of data obtained through 'omics' experiments requires technical skills in data management and mining. Another challenge is the availability of resources such as genomic sequence information that are limited for non-model organisms. The initial publication presents a workflow to facilitate accurate identification and quantification for non-model organisms in proteomic experiments and is not only restricted to the project´s purpose, but is applicable in any proteomic experiment and open to a large scientific community. With an integrative approach, I managed to answer biological questions in a systemic extent. Reasonable visualisation of complex data allowed for pinpointing symbiotic influences that partially resemble defense responses. The separation of plasma membrane and cytosolic compartments facilitated deeper insights to the symbionts influence and the infection response at a level that has not been achieved before. Hence, the results contribute to research of plant microbe interaction and plant pathogen response by picturing the effects at various cellular processes. Although initially, the primed state mounted through symbiosis remained hidden, our shotgun proteome analysis enabled ISR visualisation after infection in form of stronger activation of primary pathways and defense responses (Desalegn 2016). View studies before elucidated the plant response of *P. sativum* against *D. pinodes* on a proteomic level, ignoring the influence of symbiotic influences. By assembling of a comprehensive database, applying high-throughput shotgun proteomics, integrating metabolomic analysis and genotype phenology our publications contributed crucially to the understanding of the host metabolic response during the infection process.

During my studies I identified numerous target proteins being induced upon symbiotic association or responsible for higher tolerance against *D. pinodes*. This collection is shared by uploading our obtained data to online repositories (i.e. PRIDE) to ensure highest usability. The studies in this thesis, however, represent just a snapshot of the proteome and metabolome at a specific time point after infection. Nevertheless, an infection outcome strongly depends on the timing of the host defense events and single time point analysis allow only limited conclusions. At present, our target protein collection enables us to construct a proteotypic peptide library for a selected reaction monitoring approach. For future investigations, we aim to analyse the course of infection in a time series experiment and strive for most accurate quantification of target peptides on a triple quadrupole instrument. Additionally, we will focus on the specific host response at tissue level as e.g. the epidermis is the initial barrier where pathogens are sensed and the apoplast the first site of combat [126, 330].

5 Zusammenfassung

Biotischer Stress in Form von Mikroorganismen ist für Pflanzen allgegenwärtig, jedoch verfügen nur wenige Pathogene über das notwendige biochemische Arsenal um in eine Wirtspflanze einzudringen. Im evolutionären Verlauf führten gegenseitige Anpassung von Pathogen und Wirt zu spezialisierten Beziehungen der Organismen die ähnlich einem Schlüssel Schloss Prinzip interagieren. Andererseits verfügen manche Krankheitserreger über die Fähigkeit eine Vielzahl an Pflanzenspezies infizieren zu können. Die Brennfleckenkrankheit (*Ascochyta* blight) wurde ursprünglich als erbsenspezifisch betrachtet, ist nun aber als Breitbanderreger von Leguminosen bekannt. Dennoch ist Erbse der Hauptwirt und jährlich werden weltweit erhebliche Einbußen auf diese Krankheit zurückgeführt. Der Haupterreger in diesem Krankheitskomplex, der aus mehreren Pilzspezies besteht, ist *Didymella pinodes*. Die Mechanismen, mit denen sich der hemibiotrophische Pilz *D. Pinodes* Zutritt zu den Wirtszellen verschafft, sind nur teilweise bekannt. Ebenso beschränkt ist das Wissen über die biochemische Stressantwort der Pflanze die über unzählige Stoffwechselwege passiert. Genauso umfangreich wie der Einfluss eines Krankheitserregers gestaltet sich auch die Auswirkung von nützlichen Mikroben auf den Stoffwechsel einer Pflanze. Im Fall von Leguminosen ist es die essentielle Beziehung mit Rhizobien, aber auch mit den weit verbreiteten Mycorrhiza Pilzen, die den Stoffwechsel der Pflanze beeinflusst. Hier kann man ebenso von einer spezialisierten Beziehung sprechen, da beherbergte Mikroben das Immunsystem der Pflanze beeinflussen um eine Abwehrreaktion zu unterdrücken. Vorhergehende Studien hatten gezielte Untersuchungen zu möglichen Erreger-Wirts, oder Wirts-Mikroben Interaktionen durchgeführt mit Fokus auf Komponenten des Immunsystems der Pflanze. Mit Hochdurchsatz Methoden verschafft Systembiologie einen Gesamteindruck sowohl über die metabolische Auswirkung der Symbionten Interaktion, als auch über die Stressantwort gegenüber *D. Pinodes*. In den hier beinhalteten Publikationen wurden daher das Proteome und das Metabolome als wichtigste Elemente des Stoffwechsels analysiert.

Grundlegend für das Generieren der proteomischen Ergebnisse war die Sammlung von Sequenzdaten und Erstellung einer umfangreichen Proteindatenbank, die überdies kultivarspezifische Sequenzdaten beinhaltet, welche über *de novo* Sequenzierung von MS/MS Daten gewonnen wurde. Die Arbeitsschritte

wurden detailliert in einer Publikation dargestellt um sie einer größtmöglichen Gruppe zur Verfügung zu stellen. Im ersten Experiment wurde das *D. Pinodes* empfindliche Kultivar Messire mit verschiedenen Kombinationen von Symbionten beimpft (Mykorrhiza, Rhizobien, Co-Inokulation, ohne Symbionten), um zu sehen wie sich die Symbiose auf den Stoffwechsel der Blätter auswirkt und ob die Interaktion die Abwehrreaktion der Pflanze beeinflusst. Wir konnten dadurch zeigen, dass sowohl Mykorrhiza als auch Rhizobien Änderungen im RNA Metabolismus und in der Proteinsynthese herbeiführen. Mykorrhiza alleine beeinflusste Stoffwechselfunktionen die mit dem Umgang von Metallen beschäftigt sind und mit der Unschädlichmachung von reaktiven Sauerstoffspezies. Co-Inokulation mit Rhizobien und Mykorrhiza bewirkte die Synthese von Proteinen die mit einer Stressantwort in Verbindung stehen. Wie erwartet, war die allgemeine Abwehrreaktion gegen *D. Pinodes* geprägt von hormoneller Abstimmung (Jasmonat, Ethylen), Beseitigung von reaktiven Sauerstoffspezies und Aktivierung des sekundären Stoffwechsels. Die Leguminosen innewohnende Interaktion mit Rhizobien zeigte bei Pathogeninfektion eine verstärkte Aktivierung des Citratzyklus, des Aminosäurestoffwechsels und des Sekundärstoffwechsels, was die Synthese von Pisatin inkludiert. Wir weisen darauf hin, dass es sich hierbei um eine induzierte systemische Resistenzreaktion handeln könnte.

In einem zweiten Experiment wurde gezeigt, dass die Interaktion mit Symbionten stärkeren Einfluss auf ein *D. pinodes* empfindliches als auf ein tolerantes Kultivar (Protecta) hat. Umgekehrt hatte die Infektion mit *D. pinodes* eine stärkere Auswirkung auf die symbiontische Interaktion des empfindlichen Kultivares, was sich im Knöllchengewicht und Mykorrhiza-Besiedlung bemerkbar machte. Die effizientere Abwehrreaktion des toleranten Kultivares zeichnete sich aus durch Aufrechterhaltung der Photosynthese, sowie der Versorgung mit Zuckern und Kohlenstoffgerüsten aus, welche den Sekundärmetabolismus speisen. Schwefel Stoffwechsel, die Funktionsfähigkeit des Glutathion-Ascorbat Zyklus sowie Feineinstellung der Hormonsynthese um den induzierten Zelltod zu verhindern scheinen die Resistenz zu fördern.

6 Abstract

Biotic stress caused by microorganisms is omnipresent, however just a few pathogens possess the biochemical arsenal to invade a host. Evolution lead to mutual adaptations and some pathogens evolved specialised strategies for host invasion analogues to a key-lock principle. Other pathogens are able to infect several plant species. *Ascochyta blight* was long time thought to be a host specific disease of *Pisum sativum*, but is now known to infect many alternative legume hosts. Still, pea is the main host and significant yield losses are recorded globally every year. The causing agent in this fungi complex is known to be *Didymella pinodes*. The components this hemibiotrophic pathogen uses to access the host are partly known. Similarly limited is the current knowledge about the biochemical stress response of the plant. Like the multitude of effects a pathogen has on its host, are the great influences of beneficial microbes on the plant metabolism. In case of legumes it is the essential relation with rhizobia, but also mycorrhiza. This relation is much specialised as rhizobia alter the plants immune system to suppress a defense response. Previous studies focused mainly on components of the host plant interacting with pathogens or beneficial microbes. By applying high-throughput methods, systems biology provides an overall picture of the metabolic impact of the symbionts on the host, as well as of the stress response against *D. pinodes*. Hence, in the here included publications, the proteome and the metabolome as integral parts of metabolism were analysed.

Essential for the generation of the here presented proteomic results was the collection of sequence data and the compilation of a comprehensive protein database, which moreover contains genotypic sequence variations that was obtained through *de novo* sequencing of MS/MS data. The workflow was presented in detail in the here included publication and is available for scientists facing related issues.

In our first experiment, the susceptible pea cultivar Messire was inoculated with different combinations of symbionts (mycorrhiza, rhizobia, co-inoculation, no symbionts) to examine in what way symbionts influence leaf metabolism and to see whether symbiosis affects the plant defense response against *D. pinodes*. With our obtained results we could show that mycorrhiza and rhizobia alter leaf RNA metabolism and protein synthesis. Mycorrhiza alone influenced metabolic functions handling metals and disposal of reactive oxygen species. Co-inoculation with rhizobia and mycorrhiza caused accumulation of proteins

related to a stress response, however, during infection the defence response was dampened. The general defense response against *D. pinodes* was characterised by accumulation of proteins modulating hormone levels (jasmonate, ethylene), disposal of reactive oxygen species and activation of secondary metabolism including proteins of the *P. sativum* typical pisatin pathway (i.g. 6a-hydroxymaackiain 3-O-methyltransferase). The legumes inherent interaction with rhizobia lead to enhanced activation of the citric acid cycle, amino acid metabolism and secondary metabolism including proteins involved in the synthesis of pisatin. The data indicate that this is mediated by the phenomena of induced systemic resistance.

A second experiment showed that symbiont interaction influences a susceptible cultivar´s defense response heavier than a tolerant (Protecta) ones. This implied that the infection with *D. pinodes* had a stronger impact on the symbiotic interaction in the susceptible cultivar, which was noticed in nodule weight and mycorrhiza colonisation. The more effective defense response of the tolerant cultivar distinguished from the susceptible cultivar through sustainment of photosynthesis, provision of sugars and carbon skeletons supplying secondary metabolism. In addition, sulphur metabolism, functioning of the glutathione-ascorbate hub and hormonal adjustment to avoid induced cell death seem to promote tolerance.

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9 Curriculum vitae

Reinhard Turetschek

Education

2013 - present	PhD student at the Department for Ecogenomics and Systems Biology, University of Vienna
2006 - 2013	Master in education for the school subjects biology and geography at the University of Vienna; Master thesis: 'Rhizobial symbiosis related response of *Medicago truncatula* to salt and drought stress.' at the department of Ecogenomics and Systems Biology
2011 - 2012	Erasmus studies at the University of Manchester, Life Science
2000 - 2005	High school diploma at 'technologisches Gewerbe Museum, Höhere technische Lehranstalt - biomedizinische Technik', Vienna

Recent Training

Dec 2016	Doctoral School (Vienna): Proposal writing for postdoc funding applications
Nov 2016	ThermoFisher Scientific (Vienna): Metabolomics seminar
Sep 2016	Mixomics Workshop (Toulouse): Exploration and Integration of Omics data
Mar 2016	Peaks Training Workshop (Hamburg): De Novo sequencing and data validation of MS data
Jan 2016	Training Course 'LTQ Orbitrap Elite Mass Spectrometer' (Vienna)

Skills

Languages	German	native language
	English	fluent
Programming	R	

Teaching

Proteomics in systems biology (University of Vienna)
Practical course in the summer semesters 2014 - 2016: I supervised students in practical lab work and data analysis of proteomic results and gave a workshop on de novo sequencing and proteomic analysis with the software MaxQuant

Ecosystems and biochemistry laboratory – Techniques in Systems Biology (University of Vienna)
Practical course in the summer semesters 2015 – 2016: I supervised students in practical lab work and gave a lecture on basic chromatographic methods.

Supervision of master students

Tamara Epple	'Metabolomic analysis of low- and partial resistant cultivars of *Pisum sativum* to *Didymella pinodes*'
Stephan Holzbach	'Subcellular Fractionation'

Publications

2016 Microbial symbionts affect *Pisum sativum* proteome and metabolome under *Didymella pinodes* infection, G. Desalegn, **R. Turetschek**, H.-P. Kaul, S. Wienkoop, Journal of Proteomics, Vol 143, Food and Crop Proteomics: p. 173-187.

2015 A Proteomic Workflow Using High-Throughput De Novo Sequencing Towards Complementation of Genome Information for Improved Comparative Crop Science. In: Proteomics in Systems Biology: Methods and Protocols. Ed. Reinders J. **Turetschek R**, Lyon D, Desalegn G, Kaul H-P, Wienkoop S. New York, NY: Springer New York; 2016. p. 233-43.

2012 Possible role of nutritional priming for early salt and drought stress responses in *Medicago truncatula*, C. Staudinger, V. Mehmeti, **R. Turetschek**, V. Egelhofer, S. Wienkoop, Frontiers in Plant Science, Vol 3, 285.

Conferences

Aug 2016 **12th European Nitrogen Fixation Conference** (Budapest, Hungary)
Poster: 'Rhizobia Inoculation Reduces Didymella pinodes Impacts on Photosynthetic Efficiency'

April 2016 **COST FA1036** (Copenhagen, Denmark)
Presentation: 'Microbes shape defense response of *Pisum sativum* against *Didymella pinodes*'

Nov 2015 **miCROPe** (Vienna, Austria)
Poster: '*Pisum sativum´s* cultivar specific taste for symbionts'

June 2015 **COST FA1306** (Gatersleben, Germany)
Poster: '*Didymella pinodes* impact on shoot and nodule development in *Pisum sativum*'

Feb 2015 **COST Workgroup Omics** (Lissabon, Portugal)
Poster/Presentation: 'Cultivar specific symbiotic teamwork in *Pisum sativum*'

Aug 2014 **1st INNPO Worl Congress** (Hamburg, Germany)
Poster: 'High throughput deNovo sequencing for improved cultivar specific protein isoform identification of *Pisum sativum*'

Jun 2013 **20th Congress of the Austrian Society of Plant Biology** (Lunz, Austria)
Poster: 'Symbiont´s mutual impact on Pisum sativum cause immune system priming'

Sep 2013 **Tropentag 2013** (Stuttgart, Germany)
Poster: '*Rhizobium* and mycorrhiza inoculation affect yield components in *Pisum sativum* L.', Poster price awarded

Feb 2013 **Vienna International Plant Conference Association** (Vienna, Austria)
Poster: 'Nutritional priming studies of Pisum sativum cv. Sirius to Rhizobium and mycorrhiza inoculation'