PABLO SCHULMAN

A MICROBIAL FERMENTATION PRODUCT PROTECTION OF *Glycine max* AGAINST *Phakopsora pachyrhizi* THROUGH TRANSCRIPTOMICS, PROTEOMICS AND META-ANALYSIS

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PROTEÇÃO DE *Glycine max* CONTRA *Phakopsora pachyrhizi* POR UM PRODUTO DE FERMENTAÇÃO MICROBIANA ATRAVÉS DE TRANSCRIPTÔMICA, PROTEÔMICA E METANÁLISE

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APROVADA em 21 de Janeiro de 2021.

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RESUMO GERAL

A ferrugem asiática da soja (*Phakopsora pachyrhizi*) é a principal doença da soja no Brasil e seu manejo se baseia no uso de fungicidas sistêmicos e protetores. No entanto, o estreito leque de opções e a alta variabilidade do patógeno levaram à emergência de populações insensíveis, demandando outras opções de manejo. Dentre elas, tem sido proposta a indução de resistência. Um produto de fermentação microbiana é capaz de ativar respostas de defesa de plantas, mas ainda não se sabe os mecanismos pelos quais o produto funciona a nível molecular e sua efetividade considerando as diferentes realidades de produção de soja. Neste trabalho, objetivou-se avaliar os efeitos de um produto de fermentação microbiana (MFP) na proteção de *Glycine max* contra ferrugem através de estudos moleculares (transcriptômica, proteômica e metabolômica) e meta-análise de ensaios conduzidos em campo. No primeiro capítulo, abordamos uma revisão de eliciadores e seus receptores e nos dois capítulos restantes estudamos o efeito de um eliciador derivado de fermentação microbiana na proteção de soja contra *P. pachyrhizi*. *Glycine max* cv. Williams 82, quando tratada com o eliciador, aumentou a expressão dos genes *PR1*, *PR2*, *IPER*, *PAL* e *CHS*, particularmente quanto inoculados com *P. pachyrhizi*. Na análise de RNAseq, várias rotas associadas à defesa contra patógenos foram induzidas, incluindo interação planta-patógeno, rota de sinalização por MAPK, biossíntese de fenilpropanoides, metabolismo de glutationa, metabolismo de flavonoides e metabolismo de isoflavonoides. Em condições de campo, MFP também induziu aumento da concentração de compostos fenólicos e na atividade de peroxidase. No terceiro capítulo, apresentamos o resultado de uma meta-análise para sumarizar os efeitos de MFP na severidade da ferrugem e produtividade da soja. Usaram-se 24 entradas e realizaram-se metanálises de efeitos aleatórios separadas para severidade e produtividade com a transformação logarítmica da razão de resposta (L_s and L_y para severidade e produtividade, respectivamente). As estimativas foram usadas para se obter a eficiência de controle ($\bar{C}$) e resposta em produtividade ($\bar{Y}$) e seus respectivos intervalos de confiança (95% CI). $\bar{C}$ foi 21,1088 (95% CI: 33,1688 e 6,8818), enquanto $\bar{Y}$ foi 0,0798 (95% CI: 3,8004 e 13,0206). *P* valores para L_s e L_y foram 0,0051 e 0,0002, respectivamente. Uma metanálise de efeitos mistos foi então realizada para determinar os efeitos de diferentes moderadores (tipo de tratamento, pressão de doença, número de aplicações e época da primeira aplicação) no tratamento de MFP. Todos os moderadores com a exceção de pressão de doença para severidade afetaram $\bar{L}_s$ e $\bar{L}_y$. MFP teve melhor desempenho quando aplicado sozinho ($\bar{C}$ para sozinho foi 34,249 *vs.* 4,725 para misturado. $\bar{Y}$ para sozinho foi 14,717 *vs.* 4,019 para misturado) e quando aplicado mais cedo ($\bar{Y}$ foi 8,340 e 8,296 e $\bar{C}$ foi 24,799 e 8,561 para cedo e tardio, respectivamente). MFP demonstrou ativar as defesas de plantas de soja e as estratégias de posicionamento do produto para máxima eficiência foram determinadas. Espera-se que os resultados deste trabalho possam auxiliar no planejamento de futuros experimentos e na tomada de decisão da aplicação de MFP em condições de campo.

Palavras-chave: indução de resistência, MAMP, fermentação, ferrugem asiática da soja, RNAseq

GENERAL ABSTRACT

Asian soybean rust (*Phakopsora pachyrhizi*) is the main soybean disease in Brazil and its management is based on the use of systemic and protective fungicides. However, the narrow range of options and the high variability of the pathogen led to the emergence of insensitive populations, requiring other disease management options. Among them, resistance induction has been proposed. A microbial fermentation product can activate plant defense responses, but the mechanisms by which the product works at the molecular level and its effectiveness considering the different realities of soy production are still unknown. This work aimed to evaluate the effects of a microbial fermentation product (MFP) on the protection of *Glycine max* against rust through molecular studies (transcriptomics, proteomics and metabolomics) and meta-analysis of tests conducted in the field. In the first chapter, we discuss a review of elicitors and their receptors and in the remaining two chapters we study the effect of an elicitor derived from microbial fermentation (MFP) on soybean protection against *P. pachyrhizi*. MFP-treated *Glycine max* cv. Williams 82 had an increase in expression of the PR1, PR2, IPER, PAL and CHS genes, particularly when inoculated with *P. pachyrhizi*. During RNAseq, several plant pathways associated with pathogens defenses were induced including plant-pathogen interactions, MAPK signaling pathways, phenylpropanoid biosynthesis, glutathione metabolism, flavonoid metabolism and isoflavonoid metabolism. In field conditions, MFP also induced increase in phenol content and peroxidase activity. In the third chapter, we present the result of a meta-analysis to summarize the effects of MFP in Asian soybean rust severity and soybean yield. A total of 24 entries were used and separate random-effects meta-analysis for severity and yield were performed on the log-transformed ratios (L_s and L_y for severity and yield, respectively). The estimates were used to obtain control efficacy ($\bar{C}$) and yield response ($\bar{Y}$) and their respective confidence intervals (95% CI). $\bar{C}$ was 21.1088 (95% CI: 33.1688 to 6.8818), while $\bar{Y}$ was 8.3070 (95% CI: 3.8004 to 13.0206). *P* values for L_s and L_y were 0.0051 and 0.0002, respectively. A mixed effects meta-analysis was then performed to determine the effects of different moderators (type of treatment, disease pressure, number of applications, and timing of the first application) on MFP treatment. All moderators except disease pressure for severity affected $\bar{L}_s$ and $\bar{L}_y$. MFP had better performance when applied by itself ($\bar{C}$ for alone was 34.249 compared to 4.725 for mixed. $\bar{Y}$ for alone was 14.717 compared to 4.019 for mixed) and when applied earlier during the crop season ($\bar{Y}$ were 8.340 and 8.296 and $\bar{C}$ were 24.799 and 8.561 for early and late, respectively). MFP has been shown to trigger plant defenses in soybean and may potentially be used in a disease management system, together with other strategies, to ensure a sustainable agriculture. MFP has been shown to trigger soybean plant defenses and product application strategies for maximum efficiency have been determined. We hope our results can help in planning future trials and in the decision making of MFP application in field conditions.

Keywords: resistance induction, MAMP, fermentation, Asian soybean rust, RNAseq

SUMMARY

PART 1 ..... **8**

GENERAL INTRODUCTION **8**

REFERENCES **10**

PART 2 ..... **12**

REVIEW – PLANT INDUCED RESISTANCE: BIOTIC ELICITORS **12**

INDUCED RESISTANCE 13

BIOTIC ELICITORS FOR ACTIVATION OF PLANT DEFENSE RESPONSES 15

Proteins 15

Glycated Molecules 19

Lipids 23

Oligosaccharides 24

Nucleic Acids 27

CONCLUDING REMARKS **27**

REFERENCES **34**

MANUSCRIPT 1 – A MICROBIAL FERMENTATION PRODUCT INDUCES DEFENSE-RELATED TRANSCRIPTIONAL CHANGES AND THE ACCUMULATION OF PHENOLIC COMPOUNDS IN *Glycine max* **50**

INTRODUCTION 51

MATERIALS AND METHODS 52

RESULTS 58

DISCUSSION 67

REFERENCES **73**

SUPPLEMENTARY DATA **79**

MANUSCRIPT 2 – HOW MUCH DOES A FERMENTATION PRODUCT PROTECTS AGAINST *Phakopsora pachyrhizi* AND WHAT FACTORS POTENTIALLY INTERFERE WITH ITS EFFICACY? A META-ANALYSIS **85**

INTRODUCTION 87

MATERIALS AND METHODS 88

RESULTS 91

DISCUSSION 97

REFERENCES **101**

SUPPLEMENTARY DATA **105**

PART 1

GENERAL INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important agricultural commodities in the world. In Brazil, it is produced in almost all states, being important to the surplus of the nation's balance of trade. Diseases are a limiting factor to soybean production, demanding substantial care in the several producing regions (BATTISTI et al., 2018). It is estimated that 18% of crop losses are related to plant pathogens (ALMEIDA et al., 2005; HENNING et al., 2009; EMBRAPA, 2011). In Brazil, 46 diseases have been reported in soybeans, but this number could increase due to crop expansion and the selection pressure caused by the excessive application of chemical products and planting in monocropping (EMBRAPA, 2011).

Asian soybean rust (ASR), caused by the biotroph fungus *Phakopsora pachyrhizi*, occurred for the first time in Brazil in the 2001/2002 crop year in the states of Paraná and Rio Grande do Sul (DHINGRA et al., 2009), and can cause yield losses of up to 90% (GODOY et al., 2009). In later years, the disease spread significantly to other locations, leading to large losses in productivity to farmers. Currently, chemicals are the main method of disease control, including fungicides from the triazole, strobirulin, benzimidazole, carboxamide and dithiocarbamates groups. When a percentage of the pathogen population becomes resistant, the pesticide application program must be adjusted, either by changing the dose or by modifying the active ingredient (VAN DEN BOSCH et al., 2011). However, the intensive use of a few molecules, associated with large scale monoculture, has contributed to reduce the effectiveness of fungicide molecules, causing environmental impacts and exerting selection pressure on the pathogen (GODOY; CANTERI, 2004; YORINORI et al., 2005). In fact, there have been reports of insensitivity to demethylation inhibitors (DMI, triazoles) (SCHMITZ et al., 2014), quinone outside inhibitors (QoI, strobilurins) (KLOSOWSKI et al., 2016) and succinate dehydrogenase inhibitors (SDHI, carboxamides) (SIMÕES et al., 2018).

Thus, it is necessary to think about new disease management strategies to control ASR, such as resistance inducers. Concerning rusts, a moderate degree of success has already been achieved to control *Uromyces appendiculatus* in beans (DELGADO; DE FREITAS; STADNIK, 2013) with saccharin, *Uromyces pisi* in peas using beta-aminobutyric acid (BABA) (BARILLI; SILLERO; RUBIALES, 2010), *Hemileia vastatrix* in coffee using

acibenzolar-S-methyl (FERNANDES et al., 2013), and *Phakopsora pachyrhizi* in soy using saccharin (SRIVASTAVA et al., 2011) and acibenzolar-S-methyl (PEREIRA et al., 2009).

A proprietary microbial fermentation product (MFP) containing mannan oligosaccharides is primarily extracted from the cell wall of *Saccharomyces cerevisiae* and has been extensively studied as prebiotics in animal feed. Being a component of yeast cell wall, it is essentially a microbe-associated molecular pattern (MAMP), having an eliciting effect on different cultures. Plants also produce mannan polysaccharydes, which are deposited in the plant primary cell wall (SCHRÖDER; ATKINSON; REDGWELL, 2009), thus making it possible they are recognized as damage-associated molecular pattern (ZANG et al., 2019). There have already been some studies on the use of MFP to induce resistance in plants (COSTA, 2010; CABRAL, 2009), however, to date, there are no studies of the potential of MFP to control ASR. Thus, the goal of this work is a) to evaluate if MFP elicits defenses responses in soybean plants, and b) to assess overall protective effect of MFP against ASR in field conditions.

This dissertation is organized in three papers (1 review + 2 original research papers). The first paper is a review entitled “Plant Induced Resistance: Biotic Elicitors”, that discusses the diferent kinds of biotic elicitors of plant defenses and how they are recognized by plants. In the second paper, entitled “A microbial fermentation product induces defense-related transcriptional changes and the accumulation of phenolic compounds in *Glycine max*”, we assess the changes in gene expression in MFP-treated plants of *Glycine max* cv. Williams 82, with or without the presence of *Phakopsora pachyrhizi* through RT-qPCR and RNAseq. The third paper, entitled “How much does a fermentation product protects against *P. pachyrhizi* and what factors potentially interfere with its efficacy? A meta-analysis”, consists of a meta-analysis of the effects of MFP on the severity of asian soybean rust (ASR) and yield response during field trials.

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PART 2

REVIEW – PLANT INDUCED RESISTANCE: BIOTIC ELICITORS

Pablo Schulman[1], Flávio Henrique V. de Medeiros[1], Mário Lúcio V. de Resende[1]

Abstract: Plants rely on a two-tiered innate immune system to protect themselves from potential pathogenic microorganisms. This system is composed of a pattern-triggered immunity (PTI) and an effector-triggered immunity (ETI). PTI is associated with the perception of conserved molecules called microbe-associated molecular patterns (MAMPs). PTI is not specific and enhances defense against many pathogens, effectively protecting against most of the non-adapted microorganisms. MAMP elicitor recognition is done by pattern recognition receptors (PRR), which are either receptor kinases or receptor-like proteins. Given their ubiquity and importance in microbe-plants interactions, this review focuses on the already classified MAMP elicitors, their nature, recognition, and field applications. We highlight several classes of MAMPs to showcase their diversity. Algal molecular patterns are also included, given their similarity with fungal and oomycetes MAMPs.

Keywords: PAMP/MAMP, PTI, ISR, SAR

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INDUCED RESISTANCE

Plants rely entirely on innate immune responses for defense against potential pathogenic microbes or pests. They lack specialized immune cells or organs and each cell has the potential capacity to trigger immune responses autonomously. Innate immune perception triggers both local and systemic responses, allowing a plant to fight off pathogens both in a rapid and localized manner and on an extended scale of time and space. Plant innate immunity comes in two ways. It can be a pattern-triggered immunity (PTI), sometimes called basal resistance, or an effector-triggered immunity (ETI), an R-gene mediated resistance.

Typically, the ability to trigger ETI is pathogen strain or race-specific and is associated with programmed cell death (also called hypersensitive response, HR) and systemic acquired resistance (SAR) in the host (Thomma et al., 2011). Meanwhile, PTI is associated with the perception of conserved molecules that occur in organisms that interact with the plant. Those conserved molecules commonly are called pathogen-associated molecular patterns (PAMPs). Those patterns aren't exclusive to plant pathogenic organisms, therefore they are also referred as microbe-associated molecular patterns (MAMPs). Given the diverse and conserved nature of MAMPs, PTI is not specific, being advantageous because it can enhance defenses against multiple pathogens, effectively repelling most non-adapted microorganisms.

PTI involves two processes, PAMP/MAMP recognition, and signaling (Figure 1). Pattern recognition receptors (PRR) can be either receptor kinases, which possess an extracellular domain that is involved in ligand perception, a single-pass transmembrane domain, and an intracellular kinase domain, or receptor-like proteins that have an extracellular domain but lack an intracellular signaling domain (Couto and Zipfel, 2016).

Plant PRRs can also be divided based on their ligand-binding ectodomain. Leucine-rich repeat (LRR)-containing PRRs preferentially bind proteins or peptides, such as bacterial flagellin or elongation factor Tu (EF-Tu), or endogenous AtPep peptides. One such example is FLS2. Its extracellular domain has 28 LRR motifs. FLS2 also contains a cytoplasmic serine/threonine kinase domain which binds with BIK1, a receptor-like cytoplasmic kinase that causes a downstream MAPK cascade (Lu et al., 2010). FLS2 seem to be present and mostly conserved in all major groups of higher plants (Boller and Felix, 2009). Plant lysin-motif (LysM) domain proteins recognize N-acetylglucosamine (GlcNAc)-containing ligands, such as fungal chitin, bacterial peptidoglycan (PGN), or bacterial nodulation factors (NF), and have functions in symbiotic and immunity relations (Gust et al., 2012).

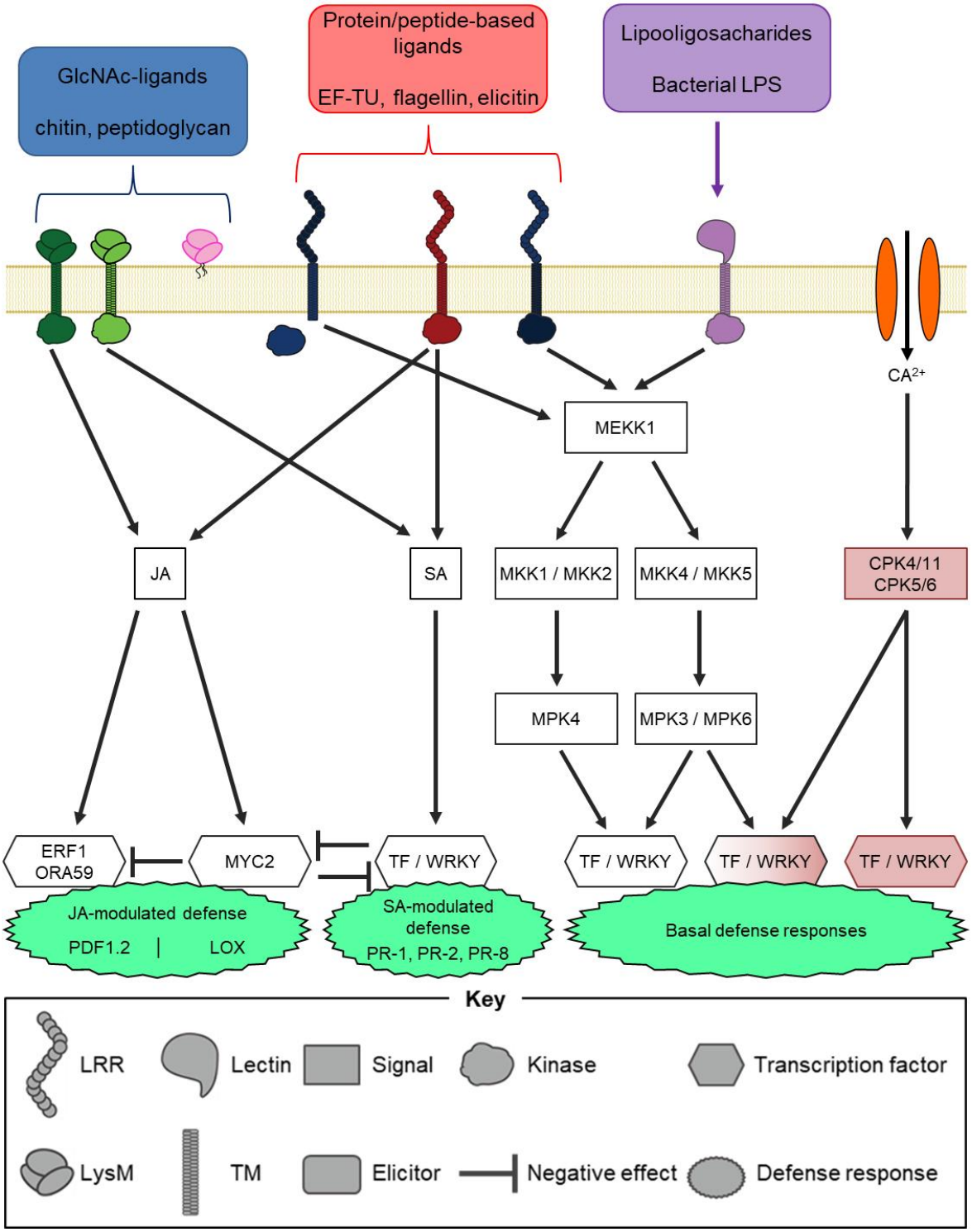

Figure 1. Plant perception of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) occurs via pattern recognition receptors (PRR) or receptor-like proteins (RLK). Receptors vary based on their ligand-binding ectodomain. Leucine-rich repeat (LRR)-containing receptors preferentially bind proteins or peptides, while lysin-motif (LysM) domain proteins recognize N-acetylglucosamine (GlcNAc)-containing ligands and lectin-type receptors bind lipooligosaccharides. After recognition, there is a signal cascade, which can be either mitogen-activated (MAPK), calcium-dependent, hormone- and reactive oxygen species-modulated. All those cascades may positively or negatively cross-talk to each other. At the end of signaling, transcription factors are activated and they modulate different responses. JA = jasmonic acid; SA = salicylic acid; TM = transmembrane domain.

As an example, two proteins have been reported as components of plant chitin receptors: CEBiP, a receptor-like protein, and CERK1, a receptor-like kinase (Shimizu et al., 2010).

After PRR protein activation comes signaling. Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling modules downstream of receptors that transduce extracellular stimuli into intracellular responses in eukaryotes (Meng and Zhang, 2013). A MAPK cascade typically consists of a modular complex that consists of a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK), which then phosphorylates a MAPK. It regulates the activity of diverse substrates, such as transcription factors and protein kinases (Dodds and Rathjen, 2010). Two MAPK cascades have been identified in *Arabidopsis thaliana*. The first one consists of the MAPKKs MKK4 and MKK5 upstream of the MAPKs MPK3 and MPK6, and leads to activation of WRKY-type transcription factors (Asai et al., 2002). The second one is composed of MEKK1, MKK1, MKK2 and MPK4 (Suarez-Rodriguez et al., 2007). Both are activated by flg22 treatment, but MPK3 and MPK6 are also activated by other PAMPs (Boller and Felix, 2009). Another alternative pathway is based on calcium-dependent protein kinases (CDPKs). Evidence shows that CDPK and MAPK signaling occur parallel to each other (Boudsocq et al., 2010). It was also demonstrated that there is a negative cross-talk between both signaling pathways mediated by ethylene, whose production is stimulated by CDPK (Ludwig et al., 2005). Further downstream signals include ion flux, oxidative burst, stomatal closure, hormone regulation, which leads to gene activation or silencing (Nicaise et al., 2009).

BIOTIC ELICITORS FOR ACTIVATION OF PLANT DEFENSE RESPONSES

Proteins

Flagellin

The protein flagellin is the building block of the motility organ flagellum present in some bacteria. Synthetic peptides corresponding to a highly conserved part of the flagellin N terminus act as potent elicitors at extremely low concentrations (Felix et al., 1999). The peptide flg22, 22 amino acids localized in the conserved region, is well-known for its eliciting responses in most plant species and is as active as the full-length flagellin (Boller and Felix, 2009). Flg22 induces rapid extracellular alkalinization, reactive oxygen species (ROS) production, activation of a mitogen-activated protein kinase (MAPK) cascade, up-regulation

of PR genes, callose deposition, ethylene production and seedling growth inhibition in *Arabidopsis* (Asai et al., 2002; Felix et al., 1999, Jeworutzki et al., 2010, Zipfel et al., 2004).

Flg22 is recognized by a Leu-rich repeat receptor kinase (LRR-RK) denominated FLS2 (Flagellin sensing). It consists of an extracellular domain with 28 LRR motifs, a transmembrane domain, and a cytoplasmic Ser/Thr kinase domain. FLS2 orthologs are present in all major groups of higher plants and seem to be quite conserved as evidenced in the genome of *Vitis vinifera, Populus trichocarpa, Ricinus communis, Lycopersicon esculentum, Arabidopsis thaliana, Oryza sativa,* and *Zea mays* (Boller and Felix, 2009). So far, FLS2 have been cloned from Arabidopsis, tomato, *Nicotiana benthamiana*, rice and grapevine (Gómez-Gómez and Boller, 2000, Hann and Rathjen, 2007, Robatzek et al., 2007, Takai et al., 2008, Trdá et al, 2014). Flg22 derived from pathogenic and non-pathogenic bacteria can create a differentiated response in the same plant. Trdá et al. (2014) assessed that the flg22 peptide from *P. aeruginosa* triggered early signaling events and the expression of defense genes in grapevine way strongly when compared with the flg22 fragment from the plant growth-promoting bacteria (PGPR) *Burkholderia phytofirmans*, suggesting a co-evolutionary effort between plant and the endophytic bacterium.

Adapted and non-adapted bacteria having identical protein sequences can also differentially induce defense responses in nonhost plants, which suggests that other domains and/or posttranslational modifications of flagellin are also recognized (Taguchi et al., 2003, Takeuchi et al., 2007). One such example is the flgii-28 epitope. Flgii-28 was discovered by Cai et al. (2011) in a genome-based micro-evolutionary study of *Pseudomonas syringae* pv. *tomato*. Two non-synonymous mutations in the flagellin-encoding gene *fliC* allowed the identification of this epitope. Flgii-28 is recognized by the flagellin-sensing 3 (fls3) receptor, which seems to be present exclusively in some Solanaceae (Cai et al., 2011, Hind et al., 2016). Another epitome is CD2-1, identified in *Acidovorax avenae*. Katsuragi et al. (2015) observed that flg22 by *A. avenae* hardly generated any ROS in rice, whereas full *A. avenae* flagellin strongly did so. To find out what epitope was responsible for the response, the authors generated several fragments containing different domains of *A. avenae* flagellin. They found out that the C-Terminal fragment CD2- 1 generated defense responses even in *OsFLS2* mutants, which means CD2-1 is recognized differently than flg22 (Katsuragi et al., 2015).

Harpins

Harpins have been characterized as the first pathogen independent HR elicitor in plants (Wei et al., 1992), but their role as virulence factors have been elucidated as well which may characterize them as bacterial effectors. However, considering that when harpins are applied to plants directly or expressed in plant cells, these proteins trigger diverse beneficial responses such as induction of defense responses, we decided to describe them here.

Harpins are unique proteins that share common characteristics, which are distinct from other bacterial proteins. They carry a relatively high amount of glycine and serine residues, whereas they carry no or few cysteines and few aromatic amino acids. In terms of secondary structures, harpins are predicted to have several regions of α-helices. Third, harpins are very acidic, based on their theoretical isoelectric points, with the exception of HopAK1 and HpaXm. Finally, harpins are heat stable, probably due to the lack of obvious tertiary structures stabilized by cysteine bridges (Choi et al., 2013).

Spray treatment with HrpN of *Erwinia amylovora* in Arabidopsis induced expression of PR1 genes and activated systemic acquired resistance to *Hyaloperonospora arabidopsidis* and *P. syringae* pv. *tomato* (Dong et al., 1999). In tomato, foliar application of HrpN reduced diseases caused by *Phytophthora infestans* and *Botrytis cinerea* (Fontanilla et al., 2005a, 2005b). Foliar application of the Hpa1 from *Xanthomonas* spp. and its fragment induced strong resistance in rice to *X. oryzae* pv. *oryzae*, *Thanatephorus cucumeris* and *Magnaporthe grisea* in greenhouse and field conditions (Chen et al., 2008). Meanwhile, PopW of *Ralstonia solanacearum* provided protection against *Tobacco mosaic virus* (Li et al., 2011).

There have been some commercial products based on harpins. Messenger® is a product formulation that contains 3% harpin protein, formulated as a water-dispersible granule. ProAct® is an end-use product which contains 1% harpin αβ protein, a protein that consists of four different harpin fragments produced in transformed *Escherichia coli*. (Copping and Duke, 2007). These products have shown promising results in crops such as tomato, orange, and strawberries (Lucon et al., 2010, Tomazeli et al., 2016, Zhu and Zhang, 2016). Harpin αβ- and harpin-treated strawberry plants infected by *B. cinerea* had significantly lower levels of aborted flowers and rotten fruits compared with mock-treated plants. They also had fewer mites than ASM-treated plants, showing that harpin induced resistance in a JA/ET-dependent manner (Tomazeli et al., 2016).

Siderophores

Siderophores are low molecular mass organic compounds produced by microorganisms and plants growing under low iron conditions which chelate Fe3+ from different habitats and make it available for microbial and plant cells. Meziane et al. (2005) assessed that purified pseudobactin, a siderophore from the PGPR *Pseudomonas putida* strain WCS358, moderately reduced symptoms from *Pseudomonas syringae* pv. *tomato* in *Arabdopsis*. It also moderately induced resistance against *Botrytis cinerea* and *Colletotrichum lindemuthianum* in common bean (*Phaseolus vulgaris*) and *B. cinerea* in tomato. Mutants lacking the production of siderophores also lost their ability to induce ISR (Meziane et al, 2005, Press et al., 2001).

Resistance mediated by siderophore-producing rhizobacteria is affected by iron content in the environment. ISR by *Serratia marcescens* 90-166 in cucumber and *Pseudomonas* spp. in radish were significantly reduced under high iron availability (Leeman et al., 1996, Press et al., 2001). Given that siderophores are produced by organisms growing under low iron conditions, higher iron content inhibits their production highlighting their importance to induce ISR in plants.

Enzymes

Proteins with enzymatic activity such as xylanase, cellulose, and proteases have shown their ability to induce resistance in plants. Xylanases induced ethylene biosynthesis and hypersensitive responses in tobacco plants (Bailey et al., 1991, Rotblat et al., 2002). In melon cotyledons, cellulases from *T. longibrachiatum* have been able to activate salicylic acid and ethylene pathways (Martinez et al., 2001). Proteases can also function as resistance elicitors. These enzymes were found to be involved in the induction of PR proteins and phytoalexin synthesis (Morán- Diez et al., 2009, Djonović et al., 2006). Another class of enzymes that participate in resistance induction is chitinases. They have shown effect in apple and cotton plants against *Venturia inaequalis* and *R. solani*, respectively (Faize et al., 2003, Kumar et al., 2009).

Small Proteins

Small proteins are well-described as microbial molecules involved in early signaling (Templeton et al., 1994). They include hydrophobins (proteins related to root surface

adherence) and expansin-like proteins (related to cell wall development), both playing an important role in the interaction between symbiotic fungi and their host plants (Yu and Li, 2014). Djonović et al. (2006) identified a small protein (SM1) with hydrophobin-like properties in *Trichoderma virens* strains which protected cotton seedlings from *Colletotrichum* sp. Brotman et al. (2008) reported that swollenin, an expansin-like protein from *Trichoderma,* has a cellulose binding domain (CBD domain) similar to CBD domains of *Phytophthora* species capable of stimulating local defense responses in cucumber roots and leaves. In that sense, swollenin (more specifically the CBD domain) may be recognized by the plant as a MAMP in the *Trichoderma*-plant interaction.

Another class of fungal compounds with resistance-inducing capabilities is peptaibols. They are short-chain length (≤20 residues) peptides produced by non-ribosomal peptide synthase. They have a high content of α-aminoisobutyric acid and generally have both N-terminal (mainly acetyl groups) and C-terminal modifications in the form of amino alcohol groups rather than amino acids (de la Fuente-Núñez et al., 2013). Peptaibols demonstrate strong antimicrobial activity against gram-positive bacteria and fungi and are also an important class of elicitors released largely by *T. virens* and *T. atroviride* strains (Mukherjee et al., 2012). Alamethicin induced typical HR responses such as deposition of callose, production of phenolic compounds, and transcription of defense genes in *Arabidopsis thaliana* (Rippa et al., 2010) and elicited JA and SA biosynthesis in lima bean (Engelberth et al., 2001). Other two peptaibol molecules, trichokonin and trichovirin II, had their resistance-inducing responses described, the first in tobacco and the second in cucumber (Luo et al., 2010, Viterbo et al., 2007).

Glycated Molecules

Lipopolysaccharides

Lipopolysaccharides (LPS) are the main component of the outer membrane of gram-negative bacteria and acts as a PAMP in dicots and monocots (Newman et al., 2007). They contain a long-chain polysaccharide of repeating units, called O-antigen, that is highly variable regarding composition, length, and branching of its carbohydrate subunits. In contrast, the oligosaccharide core and the lipid A, which form the sheet of the membrane, are highly conserved in different bacteria (Nicaise et al., 2009). Those conserved areas explain why LPS from many different bacteria induce plant defenses.

In *Arabidopsis*, LPS from *Pseudomonas* spp. and *Xanthomonas campestris* were detected via a bulb-type lectin S-domain-1 receptor-like kinase LORE (lipooligosaccharide-specific reduced elicitation), which recognizes the Lipid A moiety. *A. thaliana* mutants were impaired in LPS-triggered PTI in response to infection with *Pseudomonas syringae* and transient expression of LORE conferred sensitivity to LPS onto tobacco, showcasing the role of LORE in LPS detection (Ranf et al., 2015). Phylogeny of LORE-like SD-RLKs suggests that LORE-mediated sensing of LPS is restricted to the plant family Brassicaceae. In contrast, Meziane et al. (2005) found that the O-antigen alone was enough to reduce the severity of *Colletotrichum lindemuthianum* and *B. cinerea* in common bean and tomato, respectively.

LPS have induced several responses in plants, including induction of oxidative burst and HR (Desaki et al., 2006), cell wall alterations and deposition of callose (Keshavarzi et al., 2004), induction of β-1,3- glucanases and PR proteins (Newman et al., 1995, Silipo et al., 2005), phosphorylation of MAPK and other proteins (Piater et al., 2004). Most of these responses have been localized, but, in some cases, the effects of LPS application are seen systemically. Non-pathogenic rhizobacteria were able to trigger ISR against fungi, bacteria, and viruses in *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato when applied to the roots of plants (van Loon et al., 1998). The majority of the rhizobacteria that induce ISR belongs to the group of fluorescent *Pseudomonas* spp. but systemic effects are also seen with LPS from other bacteria. As an example, LPS of *Rhizobium etli* strain G12 induced systemic resistance to infection by the cist nematode *Globodera pallida* when applied in potato roots (Reitz et al., 2000).

Rhamnolipids

Rhamnolipids (RLs) are glycolipids produced by various bacteria species including some *Pseudomonas* sp. and *Burkholderia* sp. RLs are amphiphilic molecules composed of 3-hydroxy fatty acids linked through a beta-glycosidic bond to mono- or di-rhamnoses (Vatsa et al., 2010). They are involved in the uptake and biodegradation of poorly soluble substrates and are essential for surface motility and biofilm development (Abdel-Mawgoud et al., 2010). Rhamnolipids possess antibiotic effect against both oomycetes and fungi. A rhamnolipid-based biosurfactant formulation PRO1 inhibited spore germination of *Phytophthora cryptogea* and controlled disease in witloof chicory (De Jonghe et al., 2005). It also inhibited spore germination and mycelium growth of *Botrytis cinerea* (Varnier et al., 2009).

RLs have been characterized as new MAMPs involved in non-specific immunity in plants. It was demonstrated that RLs from *P. aeruginosa* and *Burkholderia plantarii* trigger strong defense responses in grapevine including early events of cell signaling like Ca2+ influx, ROS production, and MAP kinase activation. The chitinase (*chit4c*), glucanase (*gluc*), protease inhibitor (*pin*), stilbene synthase (*sts*), 9-lipoxygenase (*lox*) and phenylalanine ammonia lyase (*pal*) genes were upregulated 24 hours after rhamnolipid application. RL treatment conferred resistance against *Botrytis cinerea* (Varnier et al., 2009). In *Arabidopsis*, treatment by RL protected against *B. cinerea*, *P. syringae* pv. *tomato* DC3000 371 and *Hyaloperonospora arabidopsidis*, pathogens with different lifestyles (Sanchez et al., 2012).

Peptidoglycans

Peptidoglycan (PGN) consists of glycan strands cross-linked by peptide bridges and provides rigidity and structure to the cells of both Gram-positive and Gram-negative bacteria. PGN is found as a thick layer in Gram-positive bacteria, whereas only a thin layer is present in Gram-negative bacteria. The carbohydrate part of PGN is conserved in all bacteria and consists of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) linked by β-1,4-glycosidic bonds. The peptides moieties, however, can be considerably variable PGN is a molecule never found in eukaryotes, which makes it a perfect target for eukaryotic innate immune systems (Erbs and Newman, 2012).

Chen et al. (2014) found out that peptidoglycans from the fermentation process of *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, and *Staphylococcus aureus* were able to elevate transcription of plant defense genes *STS* (stilbene synthase), *CHIT-4c* (an acidic class IV chitinase), and *PR2* (a β-1,3-glucanase) in grapevine cells. PGN was also able to greatly reduce *Plasmopara viticola* colonization in detached leaves. PGN from *Xanthomonas campestris* pv. *campestris* and *Agrobacterium tumefaciens* elicited the transcription of the defense gene *PR1*, oxidative burst, medium alkalinization, and formation of callose in *Arabidopsis thaliana* (Erbs et al., 2008).

The perception of GlcNAc-containing microbial molecules by plants involves receptor kinases or receptor-like proteins that carry lysin motif (LysM-domain-containing extracellular domains) (Gust et al., 2012). Genetic evidence suggests that CERK1, a LysM-containing chitin receptor, alongside LYM1 and LYM3, also recognizes bacterial PGN (Willman et al., 2011), indicating that CERK initiates immunity in response to bacterial PGN and fungal chitin in *Arabidopsis*. Two rice LYM proteins, LYP4 and LYP6, homologs of AtLYM1 and

AtLYM3, have been ascribed a role in immune responses to PGN (Liu et al., 2012). In the same work, the authors suggest OsCERK1 may be involved in PGN and chitin perception.

Cyclic lipopeptides

Microbial surfactants are amphiphilic compounds that confer on the organism the ability to accumulate between fluid phases thus reducing surface and interfacial tension. These compounds consist of a cyclic heptapeptide is linked to a long hydrophobic alkyl chain (Bonmatin et al., 2003). They are produced by several bacteria including species from the *Bacillus* and *Pseudomonas* genera, well-known biocontrol agents.

Bacillus spp. produce surfactin, fengycin, and iturin. These compounds have exhaustively shown antibiotic activity against other microorganisms (Peypoux et al., 1999, Vanittanakom et al., 1986). They have been also shown to induce resistance in plants. Ongena et al. (2007) assessed that pure surfactin and fengycin were able to reduce symptoms of *B. cinerea* in tomato and bean, with similar levels compared to the producing bacteria. Moreover, LP-overproducing mutants of *B. subtilis* 168, a strain which is not able to synthesize these compounds and is not active on plants, generated a significant protective effect in treated plants. In lettuce, surfactin-deficient mutants of *Bacillus amyloliquefaciens* subsp. *plantarum* failed to induce plant defense responses toward *Rhizoctonia solani*, while the wild-type enhanced *PR1*, *PDF1.2* and *LOX* responses (Chowdhury et al., 2015). Treatment of potato tuber cells with purified fengycins resulted in the accumulation of plant phenolics involved in or derived from the phenylpropanoid metabolism (Ongena et al., 2005).

Pure massetolide A from *Pseudomonas fluorescens* provided significant control of *Phytophthora infestans* in tomato both locally or systemically via induced resistance. Resistance induction was independent of salicylic acid signaling, since *nahG* transgenic tomato plants, which are unable to accumulate SA, had a similar lesion area to the wild-type tomato cultivar (Tran et al., 2007).

Ascarosides

The role of effectors in nematode-plant interactions is pretty well-established, but little is known about how nematode-associated molecular patterns (NAMPs) influence those interactions. In recent years, however, a breakthrough has been made, with the discovery of one molecule class with eliciting activity.

Ascarosides are small molecules that act as pheromones in the social behavior of several nematodes. Their biosynthesis and signaling are highly conserved among nematodes (Choe et al., 2012). Structurally, ascarosides are glycolipids that consist of the dideoxy sugar ascarylose linked to a fatty acid-derived lipophilic side chain and are secreted in the nematode's surroundings (Ludewig and Schroeder, 2013). Manosalva et al. (2015) found several ascarosides in samples of plant pathogenic nematodes (*Meloidogyne javanica*, *M. hapla*, *M. incognita*, *Pratylenchus brachyurus* and *Heterodera glycines*) metabolite extracts, including Ascr#18, a compound featuring an 11-carbon side chain. Ascr#18-treated plants had increased expression of six defense gene markers in *Arabidopsis thaliana*. Moreover, Ascr#18 perception made *Arabidopsis*, tomato, potato and barley less susceptible to a broad range of pathogens, which included viruses, bacteria, oomycetes, fungi, and nematodes.

Lipids

Ergosterol

Ergosterol, a 5,7-diene oxysterol, is the most abundant sterol found in fungal cell membranes (As'wad et al., 2011). Ergosterol biosynthesis has never been reported in plants (which mostly accumulate stigmasterol, β-sitosterol, and campesterol) and is thus recognized as 'non-self' by a plant cell (i.e. an indicator of an alien cell) (Sanabria et al., 2010). Interestingly enough, mycorrhizal fungi, which establish symbiotic relationships with plants, do not produce ergosterol but sterols, which closely resemble phytosterols (Siebers et al., 2016). The specific mechanism behind ergosterol recognition by plant cells is not known thus far, but it activates a signal pathway including mobilization of internal calcium (Kasparovsky et al., 2003, Vatsa et al., 2011). Ergosterol elicited oxidative burst in tobacco cells (Kasparovsky et al., 2004) and protected grape against *Botrytis cinerea* infection (Laquitaine et al., 2006).

Sphingolipids

Sphingolipids are complex fatty acids that are found in membranes of eukaryotic organisms. Both plants have two classes of them: phosphoinositol sphingolipids and glucosylceramides (GlcCer) (Siebers et al., 2016). What differentiates between plant and fungal sphingolipids is the presence of a C-9 methyl group on the long chain sphingoid base (Warnecke and Heinz, 2003). C-9 methylated sphingolipids and especially fungal GlcCer are

important for fungal pathogenesis and may induce different plant defense mechanisms. It was reported that cerebrosides A and C (two glycosphingolipids) isolated from *Magnaporthe oryzae* induce hypersensitivity response and defense in rice plants (Koga et al., 1998, Umemura et al., 2000). Also, sphingolipids induce resistance in a non-specific manner: cerebroside B from *Rhizoctonia* sp. was able to induce resistance against *Sclerospora graminicola* in pearl millet (Deepak et al., 2005).

Eicosapolyenoic Acids

Arachidonic acid (AA) and eicosapentaenoic acid (EPA) are 20-carbon, all-*cis* PUFAs found in oomycete pathogens that are not present in higher plants and do not contain AA and EPA. Nonetheless, plants are exposed to these fatty acids during infection (Walley et al., 2013). Eicosapolyenoic acid effects on plants were discovered when mycelial extracts of *Phytophthora infestans* induced synthesis of sesquiterpenoid phytoalexins, lignin deposition and cell death in potato tissue, reactions similar to an HR to incompatible races of the pathogen. Purification and analysis of all active fractions in these extracts identified AA and EPA either free or esterified to other molecules (Bostock et al., 1981, 1982).

Eicosapolyenoic acids induce systemic resistance in potato as well as in other plant species to various pathogens. Seedling roots previously treated with AA showed less colonization by *P. cinnamomi* (Romero-Correa et al., 2014). Pearl millet seedlings were also greatly protected against *Sclerospora graminicola* infection when treated with AA or EPA, in contrast to non-treated seeds (Amruthesh et al., 2005).

The effect of eicosapolyenoic acids seems to vary depending on dose and plant species. AA-induced resistance in potato leaves to *Alternaria solani* with levels of SA and a PR1-like protein elevated in the AA-treated leaves (Coquoz et al., 1995). In tomato leaves, AA-treatment induced accumulation of transcripts for *P4* (Fidantsef et al., 1999), a PR-1 family member and SAR marker in tomato (van Kan et al., 1992). In contrast, Savchenko et al. (2010) found that treatment of tomato and *Arabidopsis* leaves with AA increased JA levels, reduced SA levels, and increased resistance to *Botrytis cinerea*. Transformed EP-producing *Arabidopsis* plants had constitutively elevated levels of JA and JA-marker gene expression and reduced levels of SA and SA-marker gene expression relative to wild-type.

Oligosaccharides

Chitin and Chitosan

Two of the best studied fungal-derived elicitors are polysaccharides: chitin, a β-1,4-linked polymer of *N*-acetyl-d-glucosamine that is a major component of fungal cell walls and the exoskeletons of arthropods (Rinaudo, 2006), and chitosan, a deacetylated derivative of chitin. Both have been well described as active components that increase resistance to bacterial and fungal pathogens in several plant species including crop plants such as cucumber (El-Ghaouth et al., 1994), grape (Reglinski et al., 2010), rice (Li et al., 2013), pinus (Fitza et al., 2013) and cowpea (Berger et al., 2016). Defense responses enhanced by chitosan include the increase in H+ and Ca2+ influx into the cytosol, activation of MAP kinases, callose apposition, oxidative burst, HR, as well as the synthesis of abscisic acid, jasmonates, phytoalexins, and PR-proteins (Amborabé et al., 2008).

Two membrane proteins have been reported as components of plant chitin receptors: CEBiP, a receptor-like protein, and CERK1, a receptor-like kinase. In rice, OsCEBiP and OsCERK1 are required for chitin perception and signaling (Shimizu et al., 2010). *Arabidopsis* spp. have two homologous proteins, AtCEBiP and AtCERK1, but only AtCERK1 was required for both ligand perception and signaling (Shinya et al., 2012). After rice and *Arabidopsis*, several CEBiP and CERK1 analogs have been reported from other plant crops including barley, wheat and tomato (Tanaka et al., 2010, Lee et al., 2014, Zeng et al., 2012), showcasing that chitin oligosaccharides recognition is universal in both monocotyledons and dicotyledons (Yin et al, 2016). CERK1 is a member of the LysM receptor kinase family. LysM receptors seem to recognize a GlcNAc-X-GlcNAc motif, X being either GlcNAc (N-acetylglucosamine) or MurNAc (N-acetylmuramic acid), with further specificity determined by secondary factors (Mesnage et al., 2014).

Oomycetes oligosaccharides

Oligosaccharide elicitors derived from the β-glucans of pathogenic oomycetes have been very well characterized. Oomycete cell wall composition essentially contain β -1,3-glucan polysaccharides, whereas chitin is absent in the Peronosporales (Mélida et al., 2012). A doubly-branched hepta-β-glucoside generated by partial acid hydrolysis from *Phytophthora sojae* glucan was shown to induce glyceollin biosynthesis in soybean cotyledon cells (Sharp et al., 1984). A partial hydrolysate of the *P. sojae* β-glucan also acts as an elicitor on various

plant cells of other plants of the Fabaceae family such as *Medicago truncatula* and *Lotus japonicus*, indicating the presence of similar receptors in these plants (Côté et al., 2000).

Algae oligosaccharides

One of the most important sources of polysaccharides for plant defense induction is certainly algae. Algal polysaccharides are one of the most abundant organic molecules in the oceans and have a great molecular biodiversity, depending on the species they derive from.

A λ-carrageenan protected against *Sclerotinia sclerotiorum*, while a ι-carrageenan increased disease severity (Sangha et al., 2010). The authors demonstrated that the resistance was due to an increase in oxalate oxidase activity and related to the expression of jasmonic acid-associated genes like AOS, PDF1.2, and PR3. In another work, different types of oligo-carrageenans protected tobacco plants against *Pectobacterium carotovorum*, but some types generated a higher degree of protection against *B. cinerea* (Vera et al., 2012).

Fucoidans are ingredients of most seaweed-based biostimulants and fertilizers, but studies about their eliciting effects are still scarce. *Ascophyllum nodosum* extracts have been shown to increase peroxidase activity and phytoalexin synthesis in pepper plants, thus helping control *Phytophthora capsici* (Lizzi et al., 1998). In another work, Klarzynski et al. (2003) described the eliciting activity of a fucan obtained from *Pelvetia canaliculata* which induced the synthesis of scopoletin and PR proteins in tobacco leaves. Tobacco plants treated with this extract systemically accumulated SA and acidic PR-1, and developed SAR against TMV.

Laminarans are structurally similar with β-linked glucose polysaccharides from oomycetes and fungi cell walls. Laminarin elicits defense responses such as medium alkalization, H2O2 production, SA accumulation and PR gene induction in plants such as tobacco, grapevine, alfalfa and rice (Aziz et al., 2003, Inui et al., 1997, Klarzynski et al., 2000, Kobayashi et al., 1993).

As with others MAMPs, β-glucans seem to be recognized by receptors. A β-1,3, β-1,6 heptagluconic (β-1,6 glucose with two β-1,3 glucose ramifications) receptor was cloned on legume crops (Mithöfer et al., 2000). In another work, the authors infer that CERK1 functions as an immune co-receptor for linear 1,3-b-D-glucans (Mélida et al., 2018). Recognition depends on the nature of glucan and host. For example, rice cells responded to a pentaglucan obtained from *Pyricularia oryzae*, but not to hexaglucans. That same pentaglucan wasn't able to induce responses in soybean cotyledon cells (Yamaguchi et al., 2000). Tobacco was shown to react to laminaran, but not to β-1,3, β-1,6 heptaglucan (Klarzynski et al., 2000).

Ulvan has shown promising results against diseases such as Glomerella leaf spot on apple (Araújo and Stadnik, 2013" por "Araújo et al., 2008), powdery mildew on wheat and barley (Paulert et al., 2010, Jaulneau et al., 2011), anthracnose on common bean (de Freitas and Stadnik, 2012). Responses varied but included phytoalexins biosynthesis and an increased post-infection activity of glucanase and peroxidase. Ulvan has also shown the ability to prime defenses. Paulert et al. (2010) found that ulvan itself did not change the production of hydrogen peroxide in suspension-cultured wheat or rice cells, but its previous addition enhanced chitin- and Chitosan-elicited oxidative burst.

Nucleic Acids

Antiviral immune concepts are generally excluded from plant innate immunity models but Mandadi and Scholthof (2013) attempted to integrate antiviral immune concepts and definitions in the current plant immunity models. Several findings may validate this idea. Most R proteins against viruses share structural similarities with antibacterial and antifungal R proteins and elicit typical ETI-based immune responses. Second, viral PAMPs activate typical PTI responses through immune co-receptors of plant PTI (Kørner et al., 2013). Lastly, a viral Avr factor that triggers ETI in resistant genotypes has been shown to suppress of PTI, thus following the zigzag model (Gouveia et al., 2017).

Double-stranded RNAs (dsRNAs) are conserved molecular patterns associated with virus replication. Niehl et al. (2016) applied *in vitro*-generated dsRNAs, dsRNAs purified from virus-infected plants and poly(I:C) (polyinosinic-polycytidylic acid, a dsRNA analog) in *Arabidopsis thaliana* and found that these molecules induced typical PTI responses dependent on co-receptor SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), but independent of Dicer-like (DCL) proteins. This finding suggests that dsRNAs represent genuine PAMPs in plants, which induce a signaling cascade involving SERK1 and a specific dsRNA receptor (NIEHL et al., 2016).

CONCLUDING REMARKS

Induced resistance strategies take advantage of plant innate immunity and can be an important tool to a sustainable agriculture. Although several bacterial and fungal elicitors have been well-established (Table 1), there is plenty of room for others to be studied. We've seen some advances regarding nematode and viral elicitors in recent years, but there is still a

lot of ground to cover. Understanding the role of molecular patterns can help us not only unveil how plants interact with microorganisms but find economically viable options for mass production of resistance inducers, like the use of leftovers from the industry, such as any industry that uses microorganisms in their daily routine (like in fermentation processes) or the fishing industry, which is the case of chitosan and chitin.

Table 1. Biotic Elicitors derived from microorganisms and algae

ELICITOR	ORIGIN	PLANT	EFECT	REFERENCES
		PROTEINS		
Acyl homoserine lactones (AHL)	*Serratia liquefaciens*, *P. putida*	*S. lycopersicum*	ISR	Schuhegger et al., 2006
Cold shock protein (CSP) and epitope: csp22 and csp15	*Staphylococcus aureus*, *Ralstonia solanacearum*	*N. tabacum*, *S. lycopersicum*	PTI induction ROS and ethylene production	Felix and Boler, 2003 Wang et al., 2016 Wei et al., 2018
EF-Tu	*Escherichia coli*, *A. avenae*	*A. thaliana*, *Brassica Alboglabra*, *B. oleracea*, *Sinapis alba*, *O. sativa*	ROS and ethylene production, H_2O_2 production and callose deposition	Kunze et al., 2007 Furukawa et al., 2014
Flagellin and epitopes flg22, flgii-28 and CD2-1	*Pseudomonas syringae* pv. *tabaci*, *P. syringae* pv. *tomato*, *Acidovorax avenae*	*Arabidopsis thaliana*, *Solanum lycopersicum*, *S. peruvianum*, *S. tuberosum*, *Nicotiana tabacum*, N. *benthamiana*, *Oryza sativa*	K^+ efflux, ROS production, stomatal closure, PTI induction	Asai et al., 2002 Cai et al., 2011 Felix et al., 1999 Jeworutzki et al., 2010 Katsuragi et al., 2015 Zipfel et al., 2004
Harpin	*Erwinia amylovora*, *Ralstonia solacearum*	*N. tabacum*, *A. thaliana*, *Fragaria x ananassa* , *S. lycopersicum*, *Oryza sativa*	SA production; HR	Chen et al., 2008 Dong et al., 1999 Li et al., 2011 Tomazeli et al., 2016 Wei et al., 1992 Zhu and Zhang, 2016
Nep1-like protein (NPL) and epitope: nlp20	*Streptomyces coelicolor*, *Bacillus halodurans*	*A. thaliana*, *N. benthamiana*	Necrosis and ethylene production	Böhm et al., 2014 Oome et al., 2014 Qutob et al., 2002
Siderophore	*Pseudomonas fluorescens*, *Pseudomonas putida*	*A. thaliana*, *Raphanus sativus*, *S. lycopersicum*, *Phaseolus Vulgaris*	SA production; ISR	Leeman et al., 1996 Meziane et al., 2005 Press et al., 2001
Superoxide dismutase (SOD)	*Xanthomonas campestris*, pv. *campestris*, *E. coli*	*N. tabacum*	H_2O_2 production	Watt et al., 2006
Xanthine/uracil permease and epitope xup25	*P. syringae*	*A. thaliana*	POX induction	Mott et al., 2016
Cellulase	*Trichoderma viridae*, *Rhizoctonia solani*	*A. thaliana*, *Capsicum annuum*	Cellular death; Phytoalexin accumulation	Ma et al., 2015a Watson and Brooks, 1984
Cerato-platanin	*Ceratocystis fimbriata* f. sp. *platani*	*N. tabacum*, *Platanus* × *acerifolia*	Necrosis induction;	Pazzagli et al., 1999

Table 1 (continued)

Cutinase	*Sclerotinia sclerotiorum*	*A. thaliana*, *Brassica napus*, *Glycine max*, *N. benthamiana*, *N. tabacum*, *O. sativa* *Triticum aestivum*, *Zea mays*	HR, H_2O_2 accumulation, and expression of defense-related genes	Zhang et al., 2014
Cyclodipeptides	*Eupenicillium brefeldianum*	*N. tabacum*	Extracellular alkalinization and H_2O_2 production	Chen et al., 2015
Endopolygalacturonase	*Botrytis cinerea*	*A. thaliana*, *V. vinífera*	CA^{2+} , ROS and phytoalexin accumulation;	Poinssot et al., 2003 Zhang et al., 2013
Ethylene-inducing xylanase (EIX)	*T. viride*	*A. thaliana*, *N. tabacum*	Ethylene and xylanase activity	Benschop et al., 2007 Fuchs et al., 1989
Hydrophobin	*Trichoderma longibrachiatum* strain MK1	*S. lycopersicum*	ISR	Ruocco et al., 2015
Hypersensitive response-inducing protein (HRIP)	*Alternaria tenuissima*	*N. tabacum*	HR, PR genes and SAR induction	Kulye et al., 2012
Nascent polypeptide-associated complex (NAC) α-polypeptide	*A. tenuissima*	*N. tabacum*	SAR induction	Mao et al., 2010
Necrosis- and Ethylene-inducing protein 1 (Nep1)	*Fusarium oxysporum* f. sp. *Erythroxyli*	*A. thaliana*, *Erythroxylum Coca*	Necrosis and ethylene production	Bailey, 1995 Keates et al., 2003
Necrosis-inducing protein1 (NP1)	*Rhynchosporium commune*	*Hordeum vulgare*	Necrosis induction	Rohe et al., 1995
PemG1	*M. grisea*	*A. thaliana*, *O. sativa*,	SAR induction; PAL gene expression, proline increase	Peng et al., 2011 Qiu et al., 2009
Peptaibols	*T. virens* and *T. atroviride*	*Arabidopsis thaliana*, *Phaseolus lunatus*, *N. benthamiana*, *Cucumis sativus*	callose, phenolic compounds production, transcription of defense genes, JA and SA biosynthesis	Engelberth et al., 2001 Luo et al., 2010 Mukherjee et al., 2012 Rippa et al., 2010 Viterbo et al, 2007
Rapid alkalinization factor (RALF)	*F. oxysporum* f. sp. *lycopersici*	*N. benthamiana*, *S. lycopersicum*	ROS production, alkalinization and mitogen-activated protein kinase activation	Thynne et al., 2017
Serine protease (AsES)	*Acremonium strictum*	*A. thaliana*, *Fragaria×ananassa*	ROS and callose accumulation	Chalfoun et al., 2013
Cellulose-binding elicitor lectin (CBEL) and epitope CBD2synth	*Phytophthora parasitica* var. *nicotianae*	*A. thaliana*, *N. tabacum*	Hydroxyproline accumulation, necrosis and CA^{2+} changes	Gaulin et al., 2006 Séjalon et al., 1995

Table 1 (continued)

Elicitin	*Phytophthora cryptogea*, *Phytophthora capsici*	*N. tabacum*	Necrosis induction	Ricci et al., 1989
Glycoside hydrolase (XEG1)	*Phytophthora sojae*	*G. max*, *S. lycopersicum*, *C. annum*, *N. benthamiana*	PTI induction	Ma et al., 2015b
NLP	*Pythium aphanidermatum*, *Hyaloperonospora arabidopsidis*, *P. parasítica*	*A. thaliana*, *N. tabacum*, *Arabids alpina*, *Thlaspi arvense*, *Draba rigida*, *Lactuca Sativa*	Necrosis, callose deposition, ethylene production	Veit et al., 2001 Oome et al., 2014 Böhm et al., 2014
Transglutaminase GP42 and epitopes Pep-13 and Pep-25	*Phytophthora megasperma*	*Petroselinum crispum*	H^+/Ca^{2+} influxes, K^+/CL^- effluxes, ROS and phytoalexin production	Nürnberger et al., 1994
GLYCATED MOLECULES				
Cyclic lipopeptides	*Bacillus subtilis* *B. amyloliquefaciens*	*A. thaliana*, *P. vulgaris* *Gossypium hirsutum*	PTI induction; synthesis of plant phenolics	Han et al., 2015 Ongena et al., 2005
Lipopolysaccharides (LPSs)	*P. fluorescens* *X. campestris* *Burkholderia cepacia*	*A. thaliana*, *Dianthus Caryophyllus*	Phytoalexins accumulation and increased of POX activity Callose deposition	Dow et al., 2000 Keshavarzi et al., 2004 Madala et al., 2012 Meziane et al.,2005 Silipo et al., 2005 van Peer and Schippers, 1992
Peptidoglycans (PGNs)	*S. aureus*	*A. thaliana*	Alkalinization, nitric oxide and camalexin production, induction of MAPK activities	Gust et al., 2007
Rhamnolipids	*Pseudomonas aeruginosa*	*A. thaliana*, *Vitis vinífera*	PTI induction	Sanchez et al., 2012 Varnier et al., 2009
Invertase and epitope gp8c	*Saccharomyces cerevisiae*	*S. lycopersicum*	Ethylene production and PAL activation	Basse et al., 1992
Ascarosides	*Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne hapla*, *Pratylenchus brachyurus*, *Heterodera glycines*	*A. thaliana*, *S. lycopersicum*, *S. tuberosum*, *H. vulgare*	PTI induction	Manosalva et al., 2015
OLIGOSACCHARIDES				
Lipoteichoic acid (LTA)	*S. aureus*	*A. thaliana*	PTI induction	Zeidler et al., 2004
Chitin	*P. syringae*	*A. thaliana*,	PTI induction	Truernit et al., 1996
Oligochitosan	*Fusarium solani*	*A. thaliana*, *P. sativum*	PAL and H_2O_2 production; Phytoalexin production	Cabrera et al., 2006 Hadwiger and Beckman, 1980

Table 1 (continued)

β-1,3-glucan	*M. grisea*	*O. sativa*	Phytoalexins production	Yamaguchi et al., 2000
Glucan-chitosaccharides	*Aphanomyces euteiches*	*Medicago truncatula*		Nars et al., 2013
Heptaglucoside	*P. megasperma* f. sp. *Glycinea*	*G. max*	Phytoalexins production	Sharp et al., 1984
Carrageenans	Red algae (Rhodophyta)	*A. thaliana* *N. tabacum*	Upregulation of JA-linked genes, PAL activation, phenylpropanoid accumulation	Sangha et al., 2010 Vera et al., 2012
Fucoidans	*Ascophyllum nodosum* *Pelvetia canaliculata*	*C. annuum* *N. tabacum*	POX activity, phytoalexin and PR proteins synthesis, SA accumulation	Lizzi et al., 1998 Klarzynski et al., 2003
Laminarans	*Laminaria digitata* *Eisenia bicyclis*	*V. vinifera* *O. sativa* *N. tabacum* *Medicago sativa*	alkalization, H_2O_2 production, SA accumulation and PR genes expression	Aziz et al., 2003 Inui et al., 1997, Klarzynski et al., 2000 Kobayashi et al., 1993
Ulvans	*Ulva fasciata* *Ulva armoricana*	*Malus domestica* *T. aestivum* *H. vulgare* *P. vulgaris*	phytoalexins biosynthesis, glucanase and peroxidase upregulation, defense priming	Araújo and Stadnik, 2013 Paulert et al., 2010 Jaulneau et al., 2011 de Freitas and Stadnik, 2012
		LIPIDS		
cis-11-methyl-2dodenoic acid (DSF)	*X. campestris* pv. *campestris*	*A. thaliana*, *N. benthamiana*, *O. sativa*	HR symptoms, autofluorescent compounds, H_2O_2 production, expression of PR-1 gene	Kakkar et al., 2015
Ergosterol	*C. fulvum*	*S. lycopersicum*	Extracellular alkalinization	Granado et al., 1995
Sphingolipids	*Magnaporthe oryzae*, *Rhizoctonia sp.*	*O. sativa*, *Pennisetum glaucum*	HR and phytoalexin accumulation	Koga et al., 1998 Deepak et al., 2005
Arachidonic acid (AA)	*P. infestans*	*A. thaliana*, *S. tuberosum*	Necrosis and fungitoxic sesquiterpenes production	Bostock et al., 1981 Savchenko et al., 2010
Eicosapentaenoic acid (EPA)	*Phytophthora infestans*	*S. tuberosum*	Necrosis and fungitoxic sesquiterpenes production	Bostock et al., 1981
		NUCLEIC ACIDS		
dsRNA	*Oilseed rape mosaic virus*	*A. thaliana*	PTI induction	Niehl et al., 2016

HR = Hypersensitive response; ISR = induced systemic resistance; PAL = phenylalanine ammonia-lyase; POX = peroxidase; PTI = pattern-triggered immunity; ROS = reactive oxygen species; SA = salicylic acid; SAR = systemic acquired resistance; JA = jasmonic acid.

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MANUSCRIPT 1 – A MICROBIAL FERMENTATION PRODUCT INDUCES DEFENSE-RELATED TRANSCRIPTIONAL CHANGES AND THE ACCUMULATION OF PHENOLIC COMPOUNDS IN *Glycine max*

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Abstract

With the progressive loss of fungicide efficacy against *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust (ASR), alternative methods to protect soybean crops are needed. Resistance induction is a low impact alternative and/or supplement to fungicide applications that fortifies innate plant defenses against pathogens. Here, we show that a microbial fermentation product (MFP) induces plant defenses in soybean and transcriptional induction is enhanced with the introduction of ASR. MFP-treated plants exhibited 1,011 and 1,877 differentially expressed genes (DEGs), 12 and 60 hours after treatment (hat), respectively, compared to water controls and 2,401 DEGs were observed with MFP treatment followed by pathogen exposure at 48 h and analysis at 60 h. The plant defense genes *PR1*, *PR2*, *IPER*, *PAL*, *CHS* were specifically induced with MFP application and induction was enhanced with ASR. Enriched pathways associated with pathogen defense included plant-pathogen interactions, MAPK signaling pathways, phenylpropanoid biosynthesis, glutathione metabolism, flavonoid metabolism and isoflavonoid metabolism. While MFP induces defense responses in laboratory-grown soybean, further studies are necessary to identify best practices for MFP application alone or in combination with fungicides for effective disease management in field-grown soybean.

INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important commodities in the world, being used as food source, animal feed and biofuel. Brazil and the United States are major producers, accounting for *ca*. 65% of the world's total production in 2019/2020 (USDA – FAS, 2020). A significant threat to soybean production is an aggressive obligate fungus, *Phakopsora pachyrhizi*, potentially causing yield losses of up to 90% (Godoy et al. 2009). Originally found in Taiwan, this fungus was reported in South America in 2001 (Yorinori et al. 2005) and subsequently in North America (Schneider et al. 2005). In places with mild winter temperatures, such as Brazil and the southern U.S., *P. pachyrhizi* overwinters due to numerous plant species that can serve as a host; such year-round growing seasons increase the fungal inoculum as well as disease pressure. To date, 158 plant species representing 54 genera have been infected by this pathogen in the Americas (Kelly et al. 2015).

Triazole, strobirulin, benzimidazole, carboxamide and dithiocarbamates fungicides have traditionally served as the first line of defense against Asian soybean rust. Indeed, the intensive use of such fungicides has contributed to fungicide resistance and reduced fungicide efficacy (Godoy and Canteri 2004; Yorinori et al. 2005). Specific examples include *P. pachyrhizi* insensitivity to demethylation inhibitors (DMI, triazoles) (Schmitz et al. 2014), quinone outside inhibitors (QoI, strobilurins) (Klosowski et al. 2016) and succinate dehydrogenase inhibitors (SDHI, carboxamides) (Simões et al. 2018).

An alternative approach is to induce a plant's biological defenses conferring greater plant protection against pathogens. Indeed, the focus of this study is to probe how a microbial fermentation product (MFP), essentially composed of microbial-associated molecular patterns (MAMPs), can induce plant defenses. Such signals or molecular patterns can activate localized and/or systemic defense responses (Schulman et al. 2018). Plants recognize such signals via pattern recognition receptors (PRR). PRRs can be either receptor kinases, that contain an extracellular domain involved in ligand binding, a single-pass transmembrane domain and an intracellular kinase domain, or receptor-like proteins that have an extracellular domain but lack an intracellular signaling domain (Couto and Zipfel 2016). The nature of the ligand includes polypeptides, lipids, oligosaccharides and nucleic acids.

Understanding how MAMPs function can provide biochemical clues as to how to induce plant defenses on demand (Schulman et al. 2018). Thus far, microbial fermentation products (MFPs) based on *Saccharomyces sp.* have been effective in activating plant defenses in wheat (Twamley et al. 2019), cocoa (Costa et al 2010; Pereira et al. 2013) and melon

(Cabral 2009). The cell wall of this yeast is mostly composed of three polysaccharide molecules: *β*-glucan (*ca.* 60% of the cell wall dry mass), mannose (*ca.* 40%) and chitin (ca. 2%) (Aguilar-Uscanga and Francois 2003). The perception of GlcNAc-containing chitin by plants is by receptor kinases or receptor-like proteins containing a lysine motif (LysM) domain (Gust et al. 2012). Glucans are polysaccharides composed of glucose subunits linked through several chemical linkages. In the case of β-linked glucans, the glucose subunits are mostly linked by (1,3)-β, (1,4)-β, or (1,6)-β glycosidic bonds (Stier et al. 2014), with β-1,3-glucan being the most abundant (Fesel and Zuccaro 2016). The elicitor effect of β-glucan has been characterized, showing that β-1,6-glycosidic linkage is vital for elicitor activity (Sharp et al. 1984a; Sharp et al. 1984b), but so far, no putative sequence and domain structure for the plant β-glucan receptor was found (Fesel and Zuccaro 2016). Mannan oligosaccharides (MOS) are major components of the yeast outer cell wall, more specifically forming mannoproteins, where they represent 50 to 95% of the structure weight (Lipke and Ovalle 1998). As a component of yeast cell wall, MOS are essentially MAMPs, possibly eliciting effect on different cultures.

To the best of our knowledge, there have been no studies on the effects of MOS-based MFP on soybean plant defenses or its role in protection against *Phakopsora pachyrhizi*. Thus, the goal of this work was to verify the changes on gene expression effected by a proprietary MFP (Alltech; Lexington, KY) on *Glycine max* cv. Williams 82 prior and after *Phakopsora pachyrhizi* infection.

MATERIALS AND METHODS

Soybean growth and treatments

Soybean plants (*Glycine max* cv. Williams 82) were grown from seed in 4'' diameter plastic pots containing potting mix (Sunshine LC1, Sun Gro Horticulture, Santa Maria, CA). One week after germination, seedlings were osmocote-treated (14-14-14 NPK, 1g; Scotts-Sierra Horticultural Products Co, Marysville, OH) (Pawlowski et al. 2016). Plants were kept in a growth room at 20-25ºC with a 16 h photoperiod (high-pressure sodium and metal halide lighting) and watered to maintain field capacity.

Microbial fermentation product (MFP) provided as a concentrated liquid by Alltech (Lexington, KY) was stored at room temperature until being dissolved in water (2500 ppm) and placed in an atomizer for leaf applications. The solution was used within 1 h of being

prepared. *P. pachyrhizi* urediniospores were provided by the Soybean-Maize Germplasm, Pathology & Genetics Research Laboratory (USDA-ARS; Urbana, IL). They were stored at -80 °C until 2 h before inoculation. Spores were thawed in a water bath (39 °C) for 1 min and resuspended in a sterilized aqueous solution containing 0.04% Tween 20 (Yamanaka et al. 2017); the solution was diluted to $1x10^5$ spores per mL with a hematocitometer. The observed germination rate under a light microscope was approximately 30%.

MFP was applied to the aerial portion of the plant as recommended by the manufacturer (500 mL ha^{-1}). A hectare was considered to have a plant population of 400,000 plants and spray volume to be 200 L ha^{-1}, resulting in *ca*. 1.25 µL MFP per plant. For ASR plant exposure, the *P. pachyrhizi* spore solution was applied with a sterilized cotton swab to the abaxial side of the second trifoliate leaf. Plants were then placed in black plastic bags for 12 h to allow for pathogen germination and infection. Plants were initially treated 42 days after planting (V5 growth stage) and arranged in the growth room in a completely randomized design with three biological replicates, each composed by three plants per pot.

Reverse-Transcription quantitative PCR (RT-qPCR) analysis

The second trifoliate leaf and roots of three plants per replicate were pooled at 0, 12, 24, 48 and 72 hours after treatment (hat), flash-frozen in liquid nitrogen and stored at -80 ºC. Total RNA was extracted (RNeasy Plant Mini kit; Qiagen, Germantown, MD) with a supplementary DNA digestion (RNase-Free DNase Set, Qiagen, Germantown, MD). Complementary DNA was then synthesized (qScript cDNA Supermix, Quantabio, Beverly, MA) and products were diluted 3X. RNA and cDNA were stored at -80 ºC until use.

Reverse-Transcription quantitative PCR (RT-qPCR) was performed using a 96-well plate format (7500 Real-Time PCR system; Applied Biosystems, Foster City, CA) with three technical replicates. PerfeCTa SYBR Green Supermix, Low ROX (Quantabio, Beverly, MA) was used to quantify cDNA. The expression of six defense-related genes was assessed; *CONS7* was chosen as the reference gene (Table 1). All primers were previously reported in the literature (Libault et al. 2008; Zhong et al. 2014) and *in silico*-tested with Primer-BLAST (Ye et al. 2012). Primer sets were added to obtain a 1 µM final concentration for each primer. The thermal profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min. Normalization was performed with the equation ΔCt = Ct (reference; *CONS7* gene) – Ct (target gene) and calibration with the formula ΔΔCt = ΔCt (replicate) – average ΔCt (mock-treated). Gene expression differences were analyzed by t-test ($p \leq 0.05$, $n = 3$).

Table 1. Real time PCR primers used on qRT-PCR. Taken from Libault et al. (2008) and Zhong et al. (2014)

Gene	Forward/Reverse Primers	Target	Description	Reference
PR1	5' TGTTGTGTTTGTTAGGGTTAGTCA *5' TGTTGGTGAGTCTTGAGCATACG*	AF136636	PR1a precursor antimicrobial protein	Zhong et al. (2014)
PR2	5' GTCTCCTTCGGTGGTAGTG 5' ACCCTCCTCCTGCTTTCTC	M37753	Beta-1,3-Endoglucanase	Zhong et al. (2014)
CHS	5′ AGGCTGCAACTAAGGCAATC 5′ TAATCAGCACCAGGCATGTC	X53958	Chalcone synthase	Zhong et al. (2014)
PAL	5′ GTGCAAGGGCTGCTTATG 5′ CCCAGTCCCTAATTCCTCTC	X52953	Phenylalanine ammonia-lyase	Zhong et al. (2014)
AOS	5′ CCTCTGTCTCCGAGAAACC 5′ CCTTCAAGGGACCGATCAC	DQ288260	Allene oxide synthase	Zhong et al. (2014)
IPER	5' CTCTCAGGTGCTCATACATTCG 5' TGGATCAGGTTTGCCAGTTC	AF007211	Basic peroxidase precursor	Zhong et al. (2014)
CONS7	5′ ATGAATGACGGTTCCCATGTA 5′ GGCATTAAGGCAGCTCACTCT	AW310136	Metalloprotease	Libault et al. (2008)

RNA library preparation and sequencing

Libraries for RNAseq were prepared from 1 µg total RNA using TruSeq Stranded mRNA kit (Illumina, San Diego, CA) based on the manufacturer's instructions. The quality of the libraries were assessed using TapeStation (Agilent, Palo Alto, CA) and quantified with Qubit dsDNA high sensitivity (HS) assay kit for Qubit 2.0 Fluorometer (Invitrogen, Life technologies, Grand Island, NY). Libraries were then normalized to 10 nM before pooling. The samples were sequenced using a SP flow cell, 2x50 bp, paired-end sequencing on NovaSeq 6000 (Illumina, San Diego, CA).

Quality control and genome mapping

RNAseq resulted in 275,735,938 paired-end reads, with 50 bps per read. RNAseq libraries were inspected with the FastQC software to evaluate PHRED quality scores, adapter sequences and duplication level. Trimmomatic v0.39 (Bolger et al. 2014) was used to discard all fragment sequences with a PHRED score below 20 and fragments with less than 30 bps. The remaining fragments had their adapters removed. 239,773,670 sequenced fragments (ca. 87% of the total) were uniquely mapped to the *Glycine max* genome (Schmutz et al. 2010) available at PLAZA 4.0 Dicots (Van Bel et al. 2018) with the software STAR v. 2.7.2b

(Dobin et al. 2013). The alignment files were sorted and checked for duplications with the Picard toolkit (Broad Institute 2019). The number of fragments mapped to exons in the genome was quantified with the script htseq-count (Anders et al. 2015).

Differential Gene Expression Analysis

The *edgeR* package (Robinson et al. 2010) was used to identify differentially expressed genes (DEGs). All pairwise comparisons consisted of similar-treated plants with and without the MFP treatment. Genes with average expression values lower than 25 fragments per library were removed. Genes with $\geq$ 2x fold change ($\log_2 FC \geq 1$ and $\log_2 FC \leq -1$) and false discovery rate (FDR; Benjamini and Hochberg 1995) < 0.05 were considered DEGs. Gene Ontology (GO) terms were enriched by comparing the ontology terms of DEG with the annotated terms of *Glycine max Wm82.a2.v1* assembly with the online tool agriGO2 (Tian et al. 2017). The selected statistical method was Fisher's exact test and terms were considered significant when their FDR was lower than 0.05. Redundant terms with a semantic similarity score ≥ 0.5 (based on the semantic similarity score simRel) were removed with REVIGO (Supek et al. 2011). KEGG pathway enrichment was performed with the web-based tool ShinyGO (Ge et al. 2020) using the Ensembl annotation for *Glycine max* (FDR < 0.05).

Field trial setup, treatment and sampling

A field trial was set up at the Centro de Desenvolvimento Científico e Tecnológico em Agropecuária-Fazenda Muquém/UFLA (Lavras, Brazil; coordinates 21°12'18.8"S, 44°58'51.9"W) on January 15th, 2020, a period in central Brazil when ASR inoculum pressure is relatively high. The soybean cultivar M6410 IPRO was planted for a field density of 300,000 plants ha^{-1} with rows spaced 0.6 m apart. The soil was fertilized with 400 kg ha^{-1} of NPK (8-28-16) upon sowing followed by top dressing fertilization with 150 kg ha^{-1} of potassium chloride (KCl) 15 days after sowing. Herbicides glyphosate (Roundup Original™; Monsanto, Brazil), clethodim (Cartago™; Alta, Brazil) and pyrethroid insecticide (Hero; FMC, Brazil) were applied 45 days after sowing.

The experiment was carried out in a randomized block design with four plot replicates per treatment. Each plot consisted of seven rows with a length of 6.5 m (total plot area = 27.3 m^2). Treatments consisted of three applications of MFP (500 mL ha^{-1}) or water alone 14 days apart, starting when plants reached the V5 growth stage. Applications were made with a hand-held sprayer, pressurized by CO_2 and adjusted to 200 L ha^{-1}. Severity assessments began with

the appearance of the first symptoms of ASR and were carried out through plant senescence. ASR severity was assessed based on the diagrammatic scale published by Godoy et al. (2006). Three low, medium and upper trifoliate leaves were evaluated in plants located in the 5 central rows of each plot. Grades were used to calculate McKinney's disease index (McKinney 1923). For yield, an area of 9.6 m² was harvested from each plot on day 115. Grains were weighted with an electronic scale and their moisture content was assessed with a moisture tester (Al-102 Eco; Agrologic, Brazil). Weight was corrected by standardizing moisture content to 12%. ASR severity and grain yield were tested for normality (Shapiro-Wilk test; $p > 0.05$) and homogeneity of variances (Bartlett's test; $p > 0.05$), prior to a t-test in the R environment with default parameters (R Core Team 2018).

Enzyme activity and protein analysis

For protein, soluble phenol and lignin analysis, leaves were collected a week after the third MFP application. The leaves were frozen in liquid nitrogen and stored at -80 °C. Subsequently, samples (0.2 g) were ground with a mortar and pestle in liquid nitrogen. The ground tissue was homogenized in 100 mM potassium phosphate pH 7.0 with 2% (p/p) polyvinylpyrrolidone (1.2 mL) and centrifuged (14,000 rpm) at 4 °C, for 5 min. Total protein of each plant extract was measured according to Bradford (1976).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by inhibition of nitrotetrazole blue (NBT) photoreduction (Beauchamp and Fridovich 1971) in an incubation medium with 100 µL of 100 mM potassium phosphate pH 7.0, 40 µL of 70 mM methionine, 10 µL of 0.1 mM EDTA, 15 µL of 1 mM NBT, 2 µL of 1 mM riboflavin, and 2 µL of plant extract. The mixture was incubated for 15 min with a 30 W fluorescent lamp. Absorbance (560 nm) difference between control and treatment was calculated and a unit of superoxide dismutase was considered to be the amount of enzyme able to inhibit 50% of NBT photoreduction under the assay conditions.

Peroxidase (POX; EC 1.11.1.7) activity was determined based on the oxidation of guaiacol, according to the methodology of Urbanek et al. (1991). Plant extracts (40 µL) were mixed to 160 µL of a solution containing 100 µL of 100 mM potassium phosphate pH 7.0, 30 µL of 50 mM guaiacol, and 30 µL of 125 mM hydrogen peroxide. After incubating the mixture at 30 °C for 10 min, absorbance was measured at 480 nm, and the molar extinction coefficient of 1.235 mM^{-1} cm^{-1} was used to calculate the peroxidase activity (Chance and Maehly 1955).

Poliphenol oxidase (PPO; EC 1.10.3.1) activity was measured following the methodology of Monteiro et al. (2016) with changes. Plant extract (20 µL) was added to 140 µL of 70 mM potassium phosphate pH 7.0 and 40 µL of 20 mM catechol. After incubation at 30 °C for 10 min, absorbance was measured at 410 nm. The molar extinction coefficient of 1.235 mM^{-1} cm^{-1} was used to calculate PPO activity (Chance and Maehly 1955).

Pheylalanine ammonia-lyase (PAL; EC 4.3.1.24) activity was quantified by a modified method of Guo et al. (2007): Plant extract (5 µL) was combined with 50 mM Tris-HCl buffer (pH 8.8) (145 µL) and 50 µL of 50 mM L-phenylalanine. After incubation at 37 °C for 20 min, absorbance was measured at 280 nm. The molar extinction coefficient of 10^4 mM^{-1} cm^{-1} (Zucker 1965) was used to calculate PAL activity.

Total soluble phenols and soluble lignin analysis

Ground samples were freeze-dried and an aliquot (30 mg) was combined with 80% methanol (1.2 mL). The mixture was prepared in the dark at room temperature, agitated on an orbital shaker for 16 h and centrifuged at 12,000×g for 7 min. The supernatant was used to determine soluble phenols based on a modified method of Spanos and Wrolstad (1990). At room temperature, a supernatant aliquot (150 µl) was mixed with Folin-Ciocalteau reagent (150 µL, 0.25 N) for 5 min, homogenized with Na_2CO_3 (150 µL, 1 M) for 10 min and diluted with distilled water (1 mL) for 1 h. Phenol amount was calculated based on a catechol standard curve (absorbance at 725 nm). The precipitate was used to determine lignin content based on a modified method of Doster and Bostock (1988). Distilled water (1.2 mL) was added to the precipitate and centrifuged at 12,000×g for 7 min at room temperature. The precipitate was oven-dried at 45 ºC for 16 h and homogenized with 1.2 mL of 2 M thioglycolic acid-HCl (1:9) mixture. Samples were placed in a water bath at 100 ºC for 4 h prior to centrifugation at 12,000×g for 7 min. The resulting precipitate was washed with distilled water, resuspended in NaOH (1.2 mL, 0.5 M) and agitated on an orbital shaker for 16 h at room temperature. The mixture was then centrifuged at 12,000×g for 7 min, the supernatant combined with ACS HCl (200 µL) and kept at 4 ºC for 4 h prior to centrifugation at 14,000×g for 7 min at room temperature. The supernatant was discarded and the precipitate dissolved in NaOH (1.2 mL, 0.5 M). Absorbance was measured at 280 nm.

Measurements and statistical analysis

Absorbance measurements for all assays were performed using a 96-well microplate format in the PowerWave XS Microplate Spectrophotometer (BioTek, Winooski, VT) running Gen 5 v. 1.05 software. Three technical replicates were run for each sample. Data was tested for normality (Shapiro-Wilk test; $p > 0.05$) and homogeneity of variances (Bartlett's test; $p > 0.05$) and outliers were removed (Dixon's Q test; $p \leq 0.05$) prior to a t-test in the R environment with default parameters (R Core Team 2018).

RESULTS

Reverse-Transcription quantitative PCR (RT-qPCR)

To identify the kinetics of defense gene induction with MFP treatment, transcripts for several classic plant defenses were monitored in soybean. In leaves, induction maxima were observed for *PR1*, *PR2*, *IPER* and *PAL* between 12 and 24 h post treatment. *IPER*, the gene encoding for basic peroxidase, was differentially induced throughout most of the sampling times, whereas the other genes had a narrower response window of differential expression (Figure 1B). With *P. pachyrhizi* leaf infection 48 h after MFP treatment, *PR1*, *IPER*, *PAL* and *CHS* were induced within 12 h of infection. *PAL* and *CHS* had the highest expression 12 h after infection, while IPER and PR1 induction was highest at 72 and 96 h, respectively (Figure 1C). Only *AOS* (allene oxide synthase) was not induced at any of the time points monitored. Neither MFP nor pathogen exposure elicited gene expression changes in the roots (Figures 1B and 1C) for the genes tested.

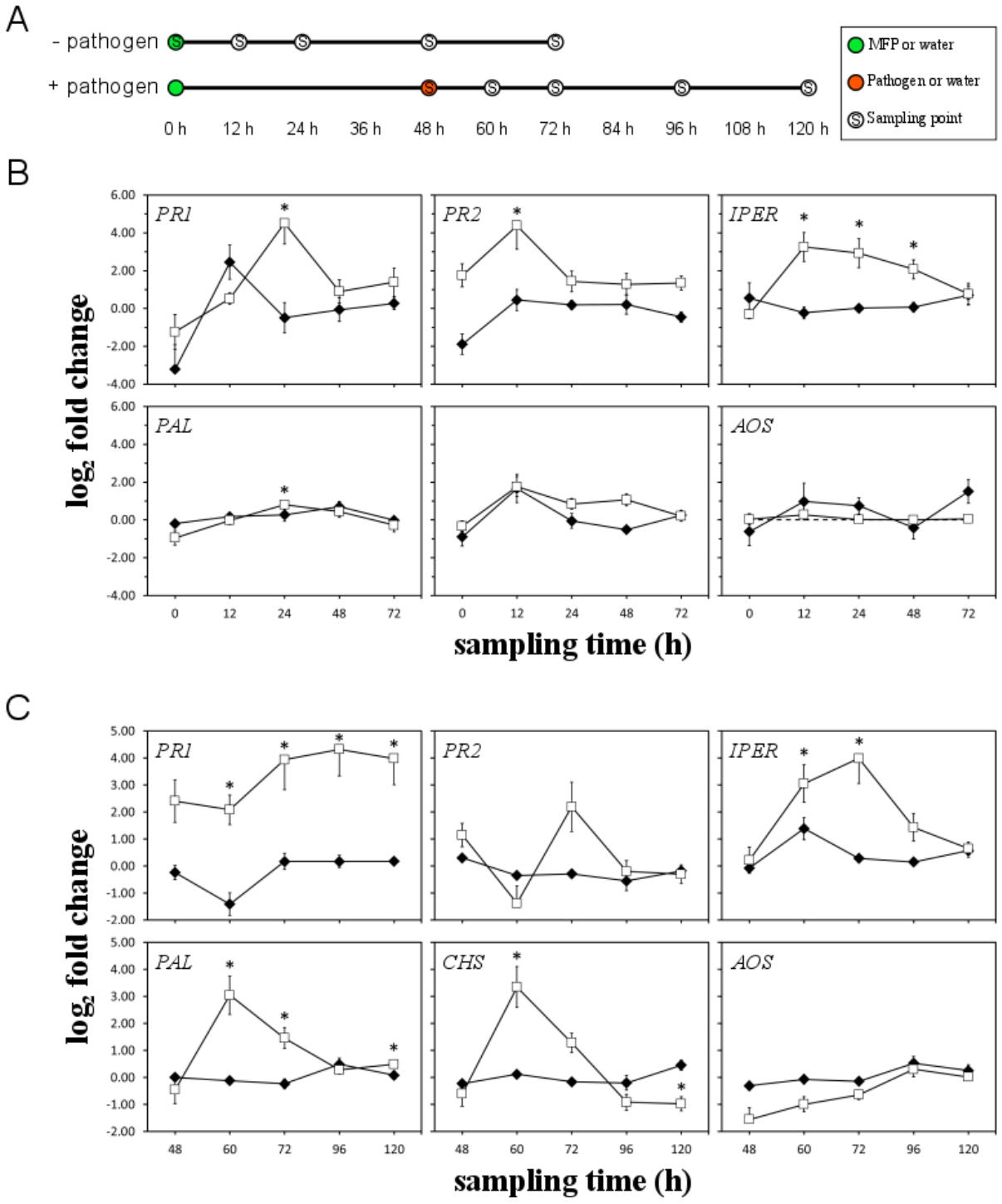

Figure 1. Timeline of MFP application, inoculation and sampling (A). Expression profile of 6 pathogenesis-related genes, including: *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*), *PATHOGENESIS-RELATED PROTEIN 2* (*PR2*), *BASIC PEROXIDASE* (*IPER*), *PHENYLALANINE AMMONIA-LYASE* (*PAL*), *CHALCONE SYNTHASE* (*CHS*), and *ALLENE OXIDE SYNTHASE* (*AOS*) in soybean leaves (white) and roots (black) at 0, 12, 24, 48 and 72 hours after MFP treatment (B) or MFP treatment followed by *Phakopsora pachyrhizi* inoculation (C). Points represent log2 of fold change of genes in elicited plants relative to the control (mock treated, sprayed with water). Points headed with asterisks are statistically different to mock treatment (t-test, $p \leq 0.05$, $n = 3$). Error bars = pooled standard error of the mean.

RNAseq Differential Gene Expression Analysis

In order to evaluate what processes may be involved in MFP-activated defense against *P. pachyrhizi*, cDNA-sequencing (RNAseq) was performed to verify the transcriptional profile of soybean responses to MFP. Given the pattern found during RT-qPCR trials, RNAseq analysis was run with leaf samples collected 12 h after initial exposure to MFP and/or pathogen. Three pairwise comparisons were generated: MFP versus water 12 hat (A12); MFP versus water 60 hat (A60); and MFP versus water with *Phakopsora pachyrhizi* inoculation 48 h after treatment and plants sampled 12 h later (for a total of 60 hat) (AP60). The number of DEGs in the leaves for the three treatment comparisons totaled 5,289. A12 had 89 down-regulated and 922 up-regulated DEGS (1,011 in total), A60 had 504 down- and 1,373 up-regulated (1,877), and AP60 had 662 down- and 1,739 up-regulated (2,401) (Figure 2).

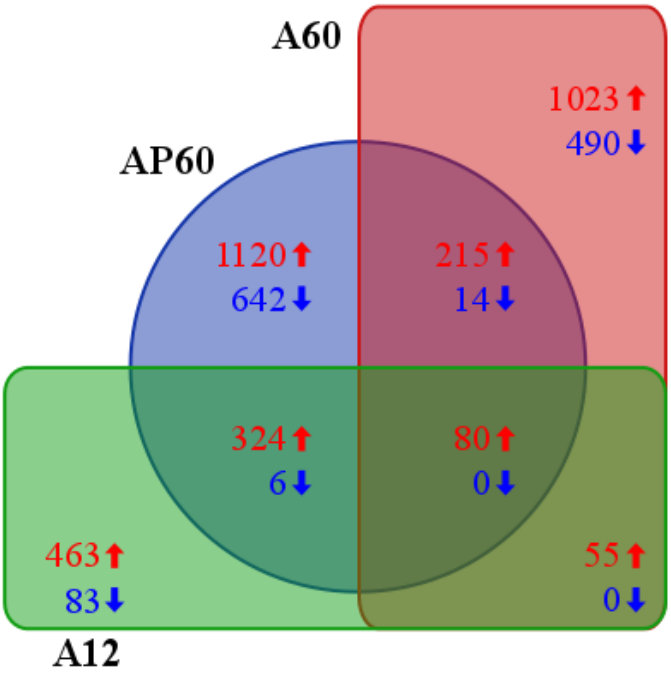

Figure 2. Number of down- (down arrow) and up-regulated (up arrow) differentially expressed genes in soybean (*Glycine max* cv. Williams 82) 12 (A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (A60) or inoculated with *Phakopsora pachyrhizi* (AP60) 48 hours after MFP treatment.

Gene ontology enrichment

DEGs were enriched in 47 pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Supplementary Table S1). Several enriched pathways are associated with plant defenses against pathogens including plant-pathogen interactions, MAPK signaling pathways, phenylpropanoid biosynthesis, glutathione metabolism, flavonoid metabolism and isoflavonoid metabolism (Figure 3).

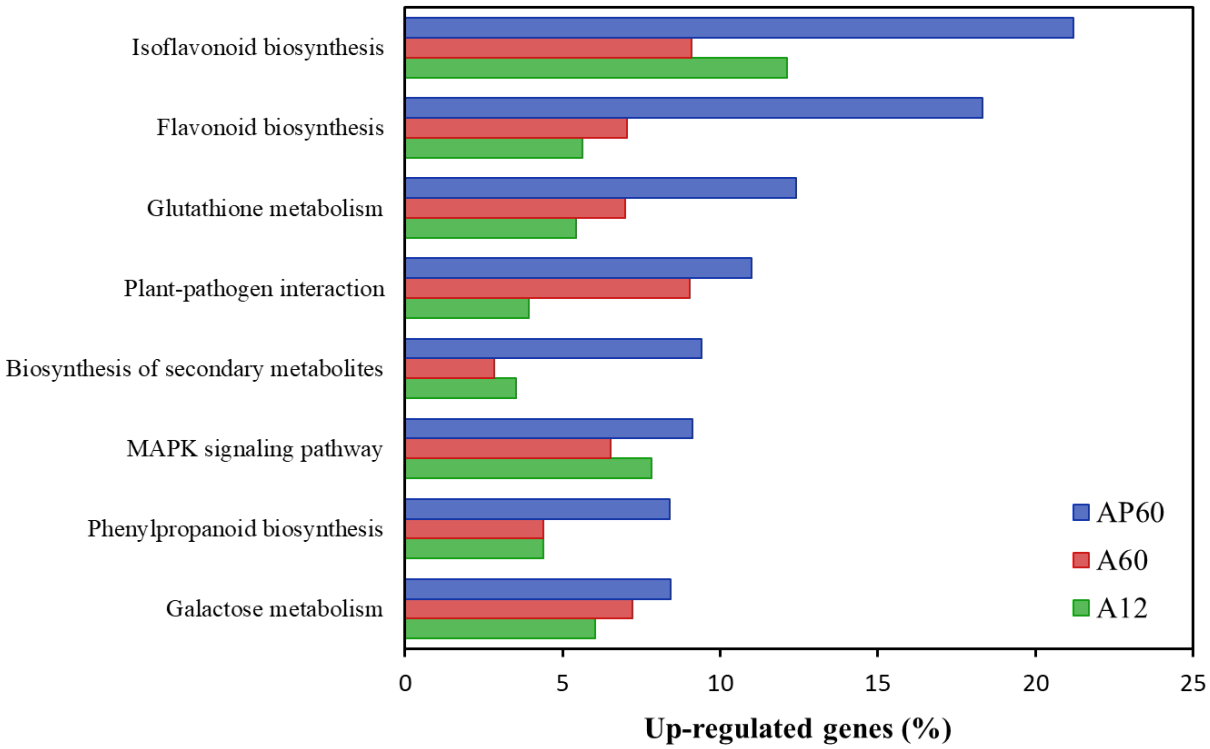

Figure 3. Percentage of up-regulated genes in enriched KEGG pathways (FDR < 0.05) in soybean leaves (*Glycine max* cv. Williams 82) 12 (A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (A60) or inoculated with *Phakopsora pachyrhizi* (AP60) 48 hours after MFP treatment. Only pathways enriched in all comparisons are shown.

DEGs were also annotated and enriched for gene ontology (GO) terms and categorized in biological processes, molecular function and cellular components. There was only enrichment for the down-regulated genes in AP60: transcription initiation factor activity and sigma factor activity, for molecular function; and photosynthetic membrane, photosystem II, photosystem, thylakoid part, thylakoid, oxygen evolving complex and extrinsic to membrane, for cellular components (data not shown). Concerning biological enrichment for up-regulated genes, although A60 has a higher number of DEGs than A12, the number of enriched biological process terms was lower (Table 2). Most enriched terms in A60 were related to downstream processes: biological regulation, regulation of cellular process, protein folding, protein modification by small protein conjugation or removal and protein ubiquitination. In A12, they were related to response to external stimuli and signaling. MFP, in the presence of the pathogen, had a greater impact on the primary metabolism. The only commonly enriched term in all comparisons was response to biotic stimulus (Table 2).

Table 2. Biological process gene ontology enrichment (FDR < 0.05) of up-regulated genes in leaves of soybean (*Glycine max* cv. Williams 82) 12 (A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (A60) or inoculated with *Phakopsora pachyrhizi* 48 hours after MFP treatment (AP60). Enrichment was processed with AgriGO and redundant GO terms were removed with REVIGO. Process = name of biological process; Hits (%) = percentage of up-regulated genes in process.

A12		A60		AP60	
Process	Hits (%)	Process	Hits (%)	Process	Hits (%)
response to biotic stimulus	18.75	response to biotic stimulus	12.50	aminoglycan metabolism	33.33
defense response	11.86	protein folding	9.94	aminoglycan catabolism	33.33
cell wall macromolecule metabolic process	10.00	protein ubiquitination	8.46	aromatic amino acid family biosynthetic process	32.14
steroid biosynthetic process	9.74	protein modification; small protein conjugation / removal	8.33	chorismate metabolic process	31.25
steroid metabolic process	9.49	response to oxidative stress	7.46	chlorophyll biosynthesis	26.09
cell recognition	7.41	regulation of transcription, DNA-templated	4.53	dicarboxylic acid metabolism	25.64
multi-organism process	7.33	biological regulation	3.82	NADPH regeneration	20.51
Reproduction	7.25			response to biotic stimulus	20.31
reproductive process	7.25			amine biosynthetic process	18.99
response to chemical	5.46			aromatic compound biosynthetic process	18.18
lipid metabolic process	4.30			alkaloid metabolic process	17.31
oxidation-reduction process	4.24			nicotinamide metabolism	17.31
transmembrane transport	3.77			cellular amide metabolism	15.79
response to stimulus	3.67			cell wall macromolecule metabolic process	14.29
protein phosphorylation	3.65			steroid biosynthetic process	14.29
post-translational protein modification	3.58			steroid metabolic process	13.92
phosphorus metabolic process	3.46			monosaccharide metabolism	13.37
macromolecule modification	3.40			tetrapyrrole metabolism	13.10
				defense response	12.71
				cellular nitrogen compound metabolic process	11.46
				amine metabolic process	11.26
				cofactor metabolic process	10.82
				cellular aromatic compound metabolic process	10.69
				cellular ketone metabolism	9.59
				generation of precursor metabolites and energy	9.38
				nucleobase-containing small molecule metabolic process	8.93
				oxidation-reduction process	8.43
				small molecule metabolism	8.17
				catabolic process	7.82
				carbohydrate metabolism	5.68

For molecular function, peroxidase activity and antioxidant activity were the only commonly enriched terms. A12 and A60 had pattern binding, polysaccharide binding and transcription factors; sequence-specific DNA binding in common; A60 and AP60 shared calcium ion binding, peroxidase activity; A12 and AP60 shared transferase activity, catalytic activity, steroid dehydrogenase activity and endopeptidase inhibitor activity. Noterworthy terms in AP60 alone were chitinase activity and ammonia-lyase activity (Table 3). Few cellular component GO terms were enriched: extracellular region part and extracellular matrix for A12; extracellular region and apoplast, for A60; mitochondrial part, mitochondrial envelope and mitochondrion, for AP60 (data not shown).

Table 3. Molecular function gene ontology enrichment (FDR < 0.05) of up-regulated genes in leaves of soybean (*Glycine max* cv. Williams 82) 12 (A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (A60) or inoculated with *Phakopsora pachyrhizi* 48 hours after MFP treatment (AP60). Enrichment was processed with AgriGO and redundant GO terms were removed with REVIGO. Function = name of molecular function; Hits (%) = percentage of up-regulated genes in function.

A12		A60		AP60	
Function	Hits (%)	Function	Hits (%)	Function	Hits (%)
pattern binding	10.67	chaperone binding	17.65	ammonia-lyase activity	58.33
polysaccharide binding	10.67	xyloglucan:xyloglucosyl transferase activity	14.29	intramolecular lyase activity	50.00
steroid dehydrogenase activity	10.27	UDP-N-acetylmuramate dehydrogenase activity	11.11	ketol-acid reductoisomerase activity	38.46
endopeptidase inhibitors	9.78	unfolded protein binding	11.11	chitinase activity	33.33
peroxidase activity	7.14	pattern binding	10.67	transferases, acyl into alkyl	28.57
oxidoreductases; acting on peroxide as acceptor	7.14	polysaccharide binding	10.67	oxidoreductases; acting on NADPH, oxygen as acceptor	27.78
carbohydrate binding	6.69	antioxidant activity	8.75	ligases; nitrogen-metal bonds and coordination complexes	23.08
antioxidant activity	6.06	calcium ion binding	8.27	ligases; nitrogen-metal bonds	23.08
oxidoreductases; acting on CH-OH donors	5.95	peroxidase activity	7.94	NADP binding	17.65
oxidoreductases; CH-OH donors, NAD or NADP as acceptor	5.95	oxidoreductases; acting on peroxide as acceptor	7.94	steroid dehydrogenase activity	15.07
heme binding	5.36	sequence-specific DNA bind	5.63	O-methyltransferase activity	13.83
tetrapyrrole binding	5.33	oxidoreductases; acting on CH-OH donors	5.49	oxidoreductases; acting on aldehyde or oxo donors	13.64
iron ion bind	5.26	transcription factors; sequence-specific DNA bind	5.46	transferases; alkyl or aryl (other than methyl) groups	13.25
oxidoreductase activity	4.05	(old) transcription regulators	5.34	endopeptidase inhibitors	11.96
oxidoreductases; paired donors, incorporating or reducing molecular oxygen	3.83	coenzyme binding	5.09	oxidoreductases; acting on CH-OH donors	11.67
transcription factors; sequence-specific DNA bind	3.75	hydrolases; glycosyl bonds	5.01	peroxidase activity	10.71
protein kinase activity	3.63	hydrolases; O-glycosyl compounds	4.96	oxidoreductases, acting on peroxide as acceptor	10.71
(old) transcription regulators	3.48	oxidoreductase activity	3.83	calcium ion binding	9.60
nucleotide binding	3.19	metal ion binding	3.72	lyase activity	9.45
Transferases; phosphorus-containing groups	3.15	cation binding	3.71	cofactor binding	9.31
transferase activity	2.93	ion binding	3.71	antioxidant activity	9.09
catalytic activity	2.69			tetrapyrrole binding	8.47
				heme binding	8.38
				iron ion binding	8.02
				oxidoreductase activity	8.02
				transferases; acyl groups	7.91
				electron carrier activity	7.41
				oxidoreductases; paired donors, incorporating or reducing molecular oxygen	6.30
				cation binding	5.48
				ion binding	5.48
				catalytic activity	4.93
				transferase activity	4.82

Field trial variables

In field conditions, there was no significant difference between water- and MFP-treated plants for either yield or ASR severity ($p \leq 0.05$) (Figure 4).

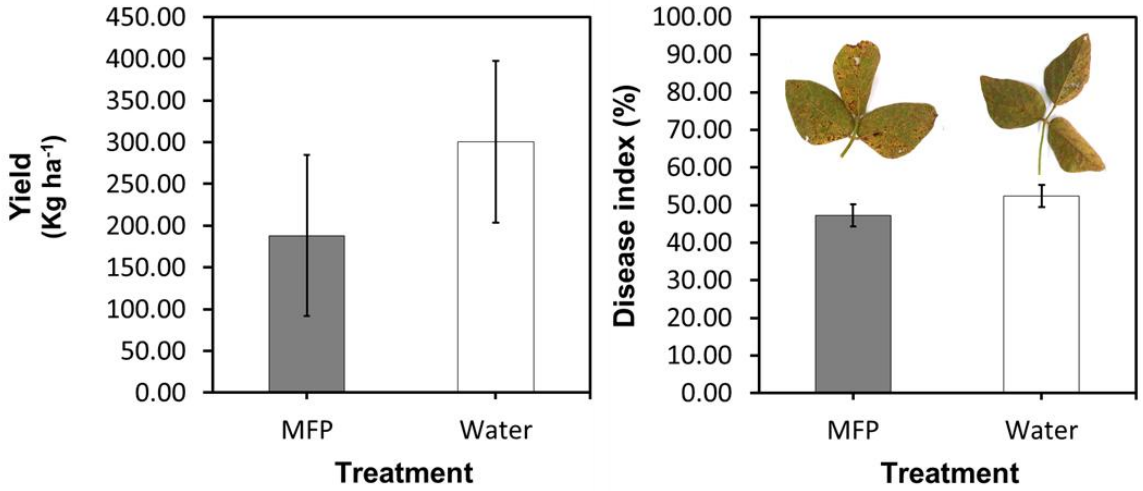

Figure 4. Yield (kg ha^{-1}) and Asian soybean rust (ASR) Mckinney's disease index of mock- and MFP-treated soybean (*Glycine max* cv. Williams 82) plants in field conditions. Pictures inside show representative leaves with ASR symptoms for each treatment.

To verify the treatments effects in a practical scenario with high inoculum pressure, leaves of mock- and MFP-treated plants were collected a week after the third spray application of MFP or water. Three enzymes related to antioxidant metabolism were tested: peroxidase (POX), superoxide dismutase (SOD) and polyphenol oxidase (PPO). POX activity in MFP-treated plants was 240,335.79 U mg^{-1} of protein, while mock-treated plants had 109,577.78 U mg^{-1} of protein ($p \leq 0.01$). Water-treated plants had higher phenylalanine ammonia-lyase (PAL) activity than MFP-treated plants at the sampled time (282.66 and 104.61 U mg^{-1} of protein, respectively) ($p \leq 0.01$). There was no difference in soluble lignin content, but MFP-treated plants had a higher amount of total soluble phenols than mock-treated plants ($p \leq 0.05$) (Figure 5).

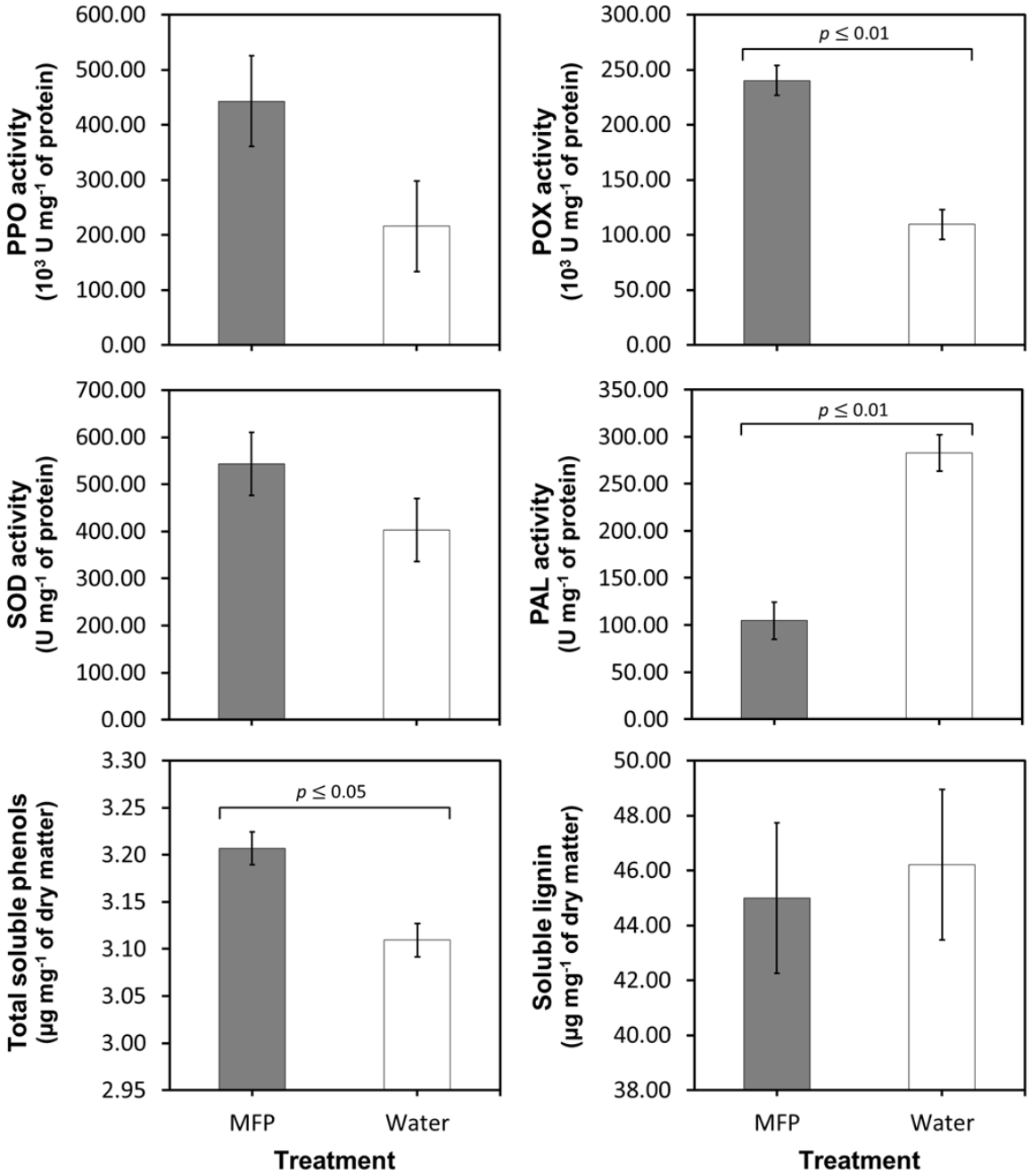

Figure 5. Enzymatic specific activity (U mg $^{-1}$ of protein) of polyphenol oxidase (PPO), peroxidase (POX), superoxide dismutase (SOD) and phenylalanine ammonia-lyase (PAL); and total soluble phenols and lignin content in (µg mg^{-1} of dry matter) in leaves of mock- and MFP-treated soybean (*Glycine max* cv. Williams 82) plants in field conditions. Bars linked by brackets are statistically different (t-test, n = 4). Error bars = pooled standard error of the mean.

DISCUSSION

GO enrichment

Most of the enriched biological processes GO terms in A60 were related to processes such as biological regulation, regulation of cellular process, protein folding, protein modification by small protein conjugation or removal and protein ubiquitination. In A12, they were related to response to external stimuli and signaling. MFP, in the presence of the pathogen, had a greater impact on the primary metabolism (Table 2). The only commonly enriched term for DEGs in all comparisons was response to biotic stimulus. Meanwhile, defense response and cell wall macromolecule metabolism were enriched in both AP60 and A12, but not in A60. Included in the GO term cell wall macromolecule metabolism are endo-glucanases, such as laminarinase (EC 3.2.1.39), which are thought to be important against fungal pathogens (Kirubakaran and Sakthivel 2007). They catalyze the hydrolytic cleavage of the (1,3)-b-D-glucosidic linkages in (1,3)- b-glucans and act primarily on glucans of fungal cell walls (Gupta et al. 2013). Small molecule metabolism, a term that was only enriched in AP60, includes endochitinases and chitinases, important responses against fungi and belonging to either the PR-3 or PR-4 groups of pathogenesis-related proteins (Ali et al. 2018).

Several genes associated with pollen recognition were up-regulated in the A12 comparison (reproductive process; table 2). The up-regulated genes in said biological process encode for receptor-like protein kinases and several were annotated as having a S-locus glycoprotein-domain and a D-mannose binding lectin-domain, both constituents of G-type lectin receptor-like kinase (Bellande et al. 2017). The S-locus glycoprotein-domain has a role in self-incompatibility in flowering-plants (thus the enrichment of this biological process) (Sherman-Broyles et al. 2007). D-mannose binding was initially considered as a mannose-specific lectin, but some of them exhibit a strong affinity toward oligomannosides and high-mannose *N*-glycans (Bellande et al. 2017). Since MFP is composed of mannan oligosaccharides, which are also present in higher plants and seaweeds as structural and storage polysaccharides (Ojima 2013), this might explain the up-regulation of genes related to these receptor-like kinases. This is also in agreement with the enrichment of the molecular function term polysaccharide binding (Table 3). A12 also had the enrichment of the GO term transferases; transferring phosphorus-containing groups, which encompasses all kinases. This might be an indication that MFP may help plants respond faster to posterior pathogenic attacks by improving (MAPK) cascade signaling. Some enriched molecular function GO

terms directly related to defenses include peroxidase activity (A12, A60, AP60), chitinase and ammonia-lyase activity (AP60). Ammonia-lyase activity includes *PHENYLALANINE AMMONIA-LYASE* (*PAL*), which was also up-regulated during RT-qPCR, more specifically 12 hours after pathogen inoculation (correspondent to AP60).

For cellular components, the results imply soybean plants recognize MFP in the extracellular space (GO terms extracellular region part and extracellular matrix; A12) and up-regulates to stop invasion (extracellular region and apoplast; A60), which is a known basal response to infection (Lygin et al. 2010). Given our results, we expect the deposition of suberin, lignin and compounds with antimicrobial activity in the apoplast. In AP60, enriched cellular component GO terms were mitochondrial part, mitochondrial envelope and mitochondrion, for AP60. Mitochondria have been proposed as target sites for the signaling molecules produced during plant-pathogen interactions. Furthermore, they are an important source of reactive oxygen species (ROS), influencing the behavior of the whole cell, which may lead to several responses including programmed cell death (Colombatti et al. 2014). AP60 had also enrichment for down-regulated genes involving plant chloroplast (photosynthetic membrane, photosystem II, photosystem, thylakoid part, thylakoid, oxygen evolving complex). Several of the down-regulated genes were associated with calcium ion binding. It is possible that overregulation of cytoplasm calcium binding genes related to plant-pathogen interaction (as discussed below) caused competition for calcium, resulting in down-regulation of chloroplast calcium binding genes.

Activation of the plant-pathogen interaction pathway

Concerning plant-pathogen interaction pathways, there has been an overexpression of genes related to calcium binding, such as *RBOH* (respiratory burst oxidase) and *CaCML* (calcium-binding protein CML) in all comparisons (Supplementary Figure S1). Another CA+ related gene (CDPK; CA^{2+}-dependent protein kinase) was overexpressed in the comparisons at 60 hat (A60 and AP60), but not at 12 hat (A12). Another interesting finding was the overexpression of *FLS2* in A12. That result was not expected given the MFP-product used in this study is yeast-based and *FLS2* is responsible for the perception of elicitor-active epitopes of bacterial flagellin (FLG22) (Göhre et al. 2008). Looking at the literature, we found that salicylic acid can regulate microbial pattern receptor (PRR) kinase levels in a positive feedback loop, although Tateda et al. (2014) and Landi et al. (2017) found increased levels 24-48 hat, whereas we found at 12 hat. An increased number of PRRs may speed up defense responses

by enhancing the plant's ability to detect MAMPs. MAPK3/6 was up-regulated at 12 h, but not at 60 hat. The transcription factors *WRKY33* was up-regulated in all comparisons, *PTI6* was up-regulated in A12 and A60, and *WRKY22* was only up-regulated in AP60.

PR1 was up-regulated in A12 and AP60, but not in A60 (Supplementary Figure S1). PR-1 proteins are among the most abundantly produced polypeptides defense responses and have been reported to constitute around 2% of the total leaf protein in pathogen-infected tobacco (Alexander et al. 1993). *PR1* expression is enhanced by salicylic acid and is therefore used as a marker for salicylic acid-dependent induction. The role of PR1 family proteins is not well established, but it is suggested it includes antimicrobial properties and the amplification of defense signals (Breen et al. 2017). Despite the lack of a clear *in vivo* mode of action evidence, it is certainly an important defense pathogenesis-related protein given it seems to be targeted by pathogen effectors (Breen et al. 2016; Lu et al. 2014; Zhang et al. 2012). The finding that *PR1* was overexpressed in AP60 but not in A60 might be explained as a response to a biotic stimulus that must be continually applied (as in the case of A60) otherwise the plants might revert to a basal state of *PR1* expression.

Lignin accumulation

The phenylpropanoid pathway, besides being the precursor pathway for both the flavonoid and isoflavonoid pathways, also plays a role in lignification. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) is the first step in the phenylpropanoid pathway and *PAL* genes were differentially expressed in A12 and AP60 but not in A60. Up-regulation for lignin production was a process that appeared early (12 h) and kept building up until 60 h. Lignin, a major component of cell walls of vascular plants, is also deposited in cell-wall appositions (papillae) that plants assemble in an attempt to hinder the penetration of biotrophic fungi, such as *P. pachyrhizi*, thus being considered a first line of defense (Bhuiyan et al. 2009).

One must consider that lignin formation encompasses two processes: monolignol biosynthesis and monolignol polymerization (Xie et al. 2018), so the up-regulation of monolignol biosynthesis does not necessarily mean lignin formation. Monolignol biosynthesis-related DEGs in all comparisons included cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) (both up-regulated in the three comparisons) and cinnamoyl-CoA reductase (CCR) (EC 1.2.1.44) (down-regulated in A12; up-regulated in A60 and AP60). DEGs in A60 and AP60 included genes that codify for enzymes shikimate O-hydroxycinnamoyltransferase (EC 2.3.1.133) and 4-coumarate--CoA ligase (*4CL*, EC 6.2.1.12) (the latter was down-regulated in

A60 while up-regulated in AP60). DEGs exclusively in AP60 included trans-cinnamate 4-monooxygenase (EC 1.14.14.91), caffeate *O*-methyltransferase (EC 2.1.1.68) and caffeoylshikimate esterase (EC 3.1.1.-). Monolignol polymerization, on the other hand, is performed by laccases or peroxidases (Wang et al. 2013). The latter use peroxide produced by superoxide dismutase proteins (SOD) and NADPH oxidase as co-substrate to make oxidative radicalization of phenols (Wang et al. 2013). Peroxidase (EC 1.11.1.7) was up-regulated in all comparisons (Supplementary Figure S2). Peroxidases are part of the PR-9 family group and are important in several plant defense processes (van Loon et al. 2006), including the strengthening of cell walls to create a physical barrier against pathogen invasion in host tissues, the production of ROS (which causes several physiological changes), and antimicrobial compounds (Almagro et al. 2008).

In field conditions, there was no difference in soluble lignin content between mock and MFP-treated plants (46.22 and 45.00 µg mg^{-1} of dry matter, respectively), even though peroxidase specific activity was higher in MFP plants (240,335.79 vs 109,577.78 U mg^{-1} of protein). Interestingly, PAL activity was higher in mock-treated plants (Figure 5). Considering that PAL, POX and lignin represent different stages of the phenylpropanoid pathway, one can speculate that lignin formation may still increase, with accumulation being faster in MFP-treated plants. Regardless, previous studies have shown that lignin accumulation is slow (or non-existent) in susceptible and partially resistant cultivars (Juliatti 2018; Lygin et al. 2009), showing that rather than the amount, the speed in what it is produced might be one of the key factors to inhibit the pathogen.

Phenolic compounds content

In regard to up-stream genes of the flavonoid biosynthetic pathway, chalcone isomerase was up-regulated in all comparisons, but chalcone synthase was only up-regulated in A12 and AP60 (Supplementary Figure S3). The first and second committed enzymes of the flavonoid pathway are, respectively, chalcone synthase (CHS, EC 2.3.1.74), which catalyzes the production of 2′,4,4′,6′-tetrahydroxychalcone (THC), and chalcone isomerase (CHI, EC 5.5.1.6), which catalyzes chalcone into flavanone (Waki et al. 2020), showing a metabolic commitment to flavonoid production. This commitment became clearer at 60 hat (A60 and AP60), when there was the up-regulation of genes that codify for flavonol synthase, a downstream enzyme responsible for galangin, kaempferol, quercetin and myricetin production. In the isoflavonoid pathway, the greatest MFP effect was observed in AP60, with

the up-regulation of genes that codify for isoflavone 7-O-methyltransferase (EC 2.1.1.150), 2-hydroxyisoflavanone dehydratase (EC 4.2.1.105) and glyceollin synthase (EC 2.5.1.36), leading respectively to isoformononetin and prunetin, daidzein, and glyceollin (Supplementary Figure S4).

In soybeans, flavonoid and isoflavonoid compounds play a significant role in plant defense. For example, kaempferol has been reported to have a deleterious effect on spore germination of *Pyricularia oryzae* (Padmavati et al. 1997), while quercetin can inhibit conidia germination of *Neurospora* spp. (Peer and Murphy 2006). Lygin et al. (2009) reported that quercetin, kaempferol, alongside the isoflavonoids formonetin and glyceollin, reduced *P. pachyrhizi* spore germination *in vitro*. Lygin et al. (2013) found that transgenic plants for the production of chalcone synthase were more susceptible to both *Phytophthora sojae* and *Macrophomina phaseolina*. Dhawale et al. (1989) also found an increase in chalcone synthase mRNA in pathogen-inoculated soybean plants; the highest increase was in between 8 to 10 h after vacuum infiltration of leaves with *Pseudomonas syringae* pv. *glycinea.* This trend was also similar to our RT-qPCR results, where the highest induction was around 12 h and it only decreases from there (Figure 1). Although we did not directly measure a specific phenol compound, MFP-treated plants accumulated more total soluble phenols than mock-treated plants in field conditions (note that soluble lignin was measured in a different assay) (Figure 5).

Severity and yield in field conditions

Although MFP activated defenses in controlled conditions, it did not increase ASR control efficacy in field conditions (Figure 4). According to Walters et al. (2013), several factors may influence efficacy including the environment (climate and soil conditions), plant genotype and life history, and frequency and timing of elicitor application. To this list, we can add the timing of infection: often, controlled assays inoculate the pathogen simultaneously or not long after the elicitor treatment, when the eliciting effect is stronger (approach we followed for our qRT-PCR and RNAseq essays). In field conditions, we cannot control when infection will happen. There is evidence that MFP may have a priming effect (Twamley et al. 2019), but more studies are required to verify if this effect is pathogen- and/or crop-dependent.

There was no difference in yield between mock- and MFP-treated plants during the field trial, although yield was smaller in MFP plants due to large variation (Figure 4). One possible explanation could be damage by brown stink bug (*Euschistus heros*), which causes

shriveled seeds and thus reduction in yield. More pod damage was observed but not measured in MFP- than in water-treated plots. Our data indicate that MFP activates the salicylic acid pathway (as evidenced by *PR1* induction, a well-known SAR marker, see figure 1), possibly with a detriment of the jasmonic acid/ethylene pathway given their negative crosstalk (Wei et al. 2014), which might have a deleterious effect on herbivory defenses. Given that MFP applications went all the way to pod development, where the stink bug causes damage, it is possible MFP-treated plants suffered more from brown stink bug attack.

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SUPPLEMENTARY DATA

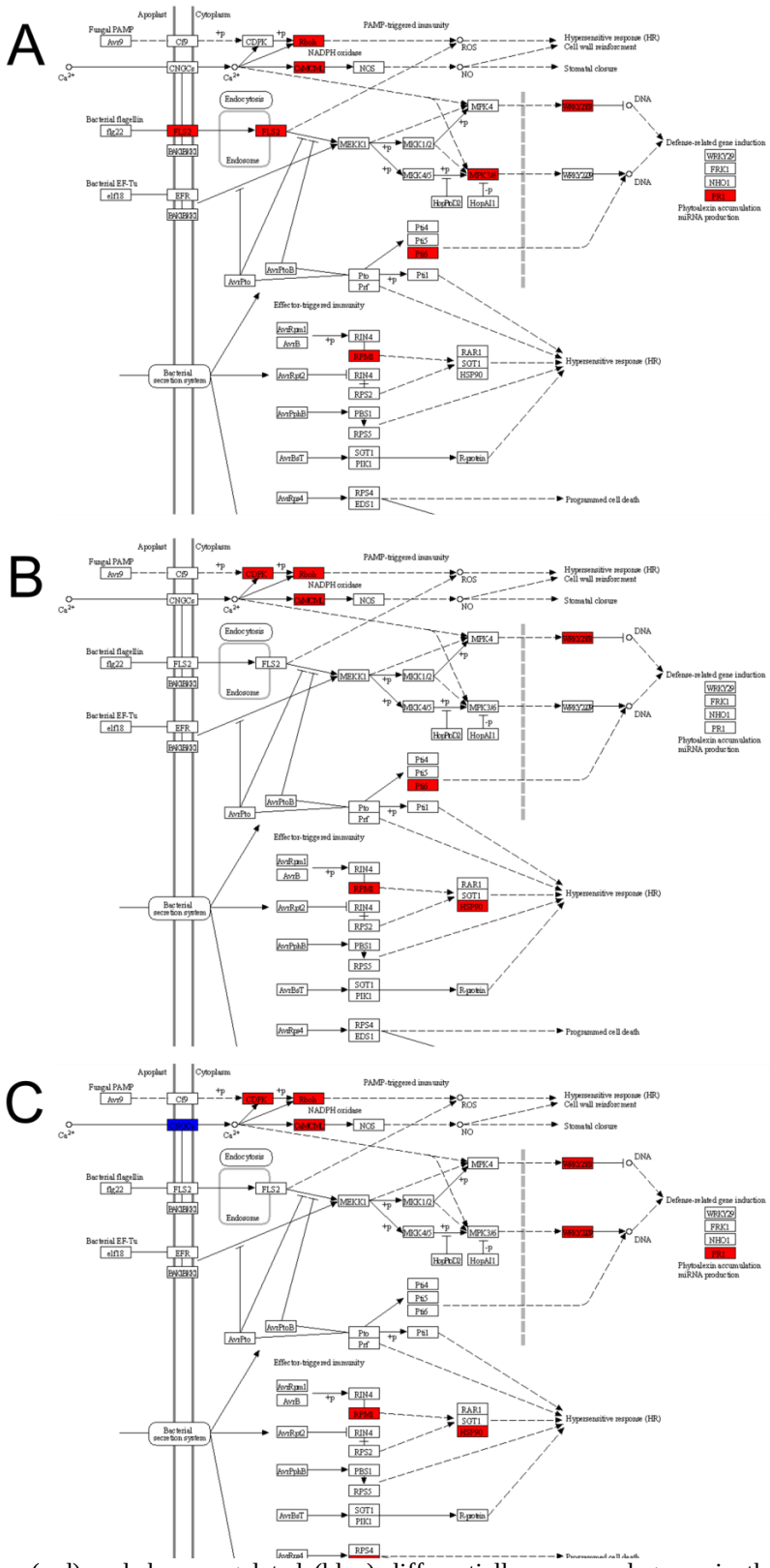

Figure S1. Up- (red) and down-regulated (blue) differentially expressed genes in the plant pathogen interaction KEGG pathway in soybean leaves (*Glycine max* cv. Williams 82) 12 (A; A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (B; A60) or inoculated with *Phakopsora pachyrhizi* (C; AP60) 48 hours after MFP treatment.

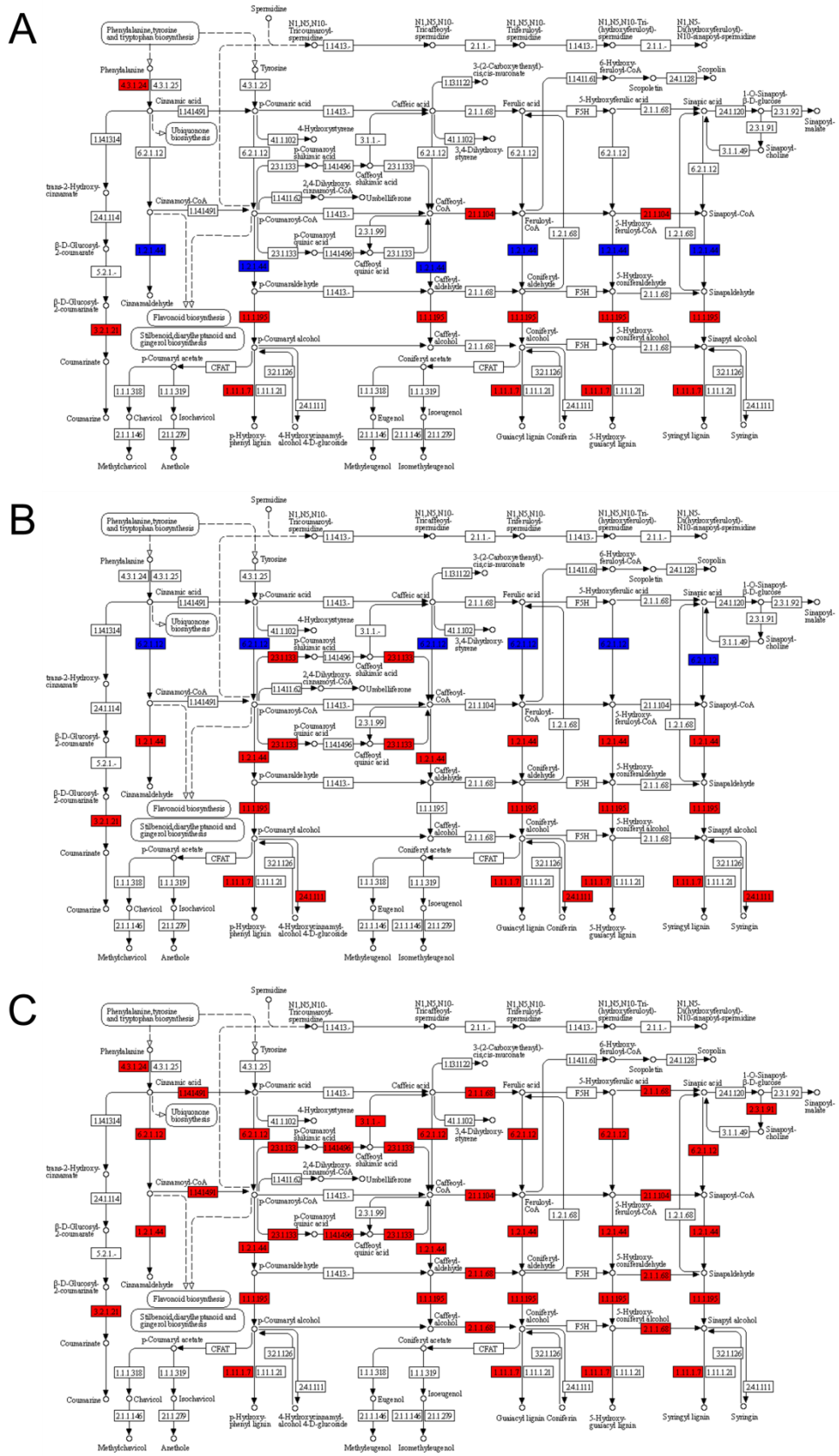

Figure S2. Up- (red) and down-regulated (blue) differentially expressed genes in the phenylpropanoid KEGG pathway in soybean leaves (*Glycine max* cv. Williams 82) 12 (A; A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (B; A60) or inoculated with *Phakopsora pachyrhizi* (C; AP60) 48 hours after MFP treatment.

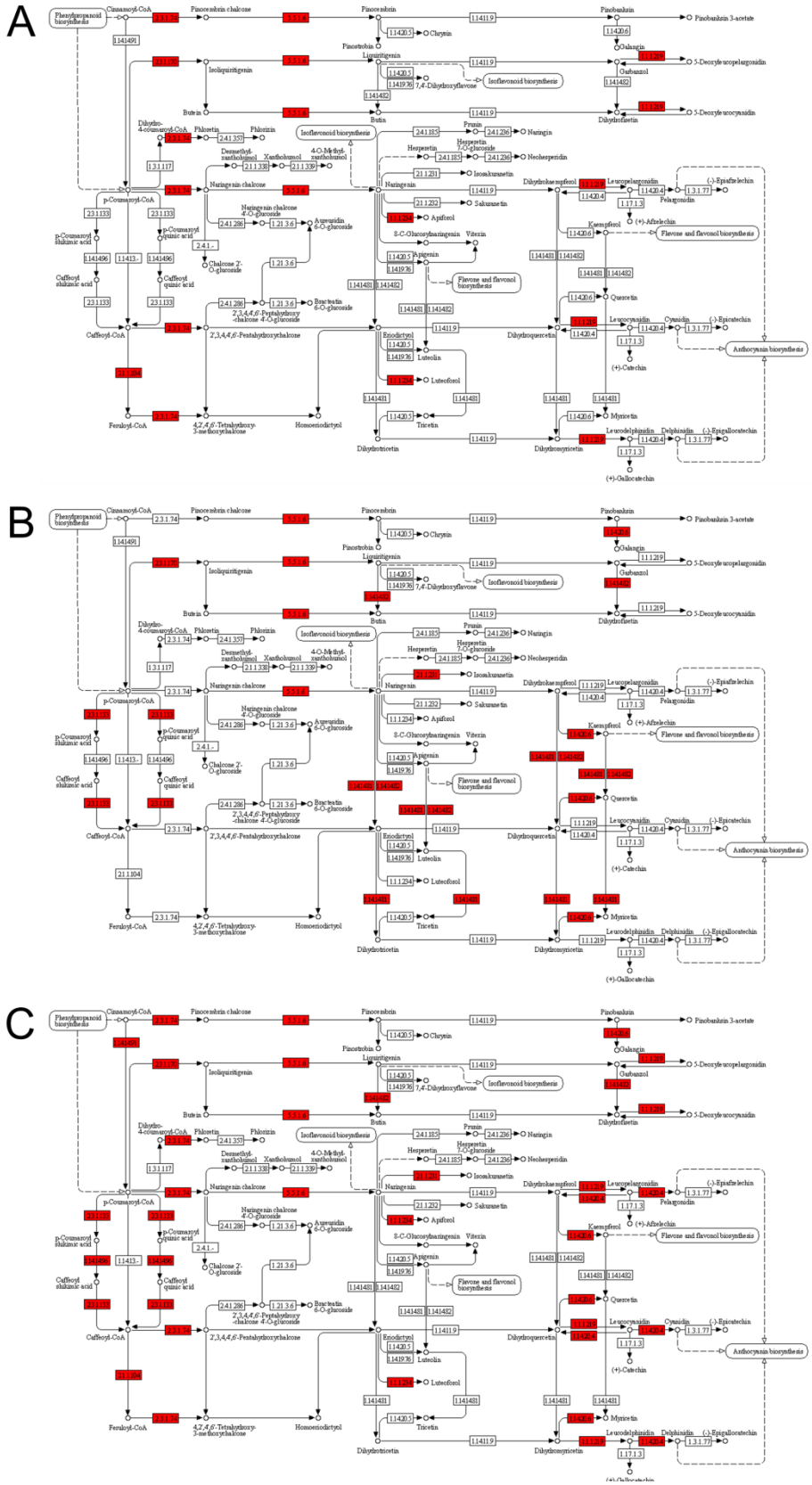

Figure S3. Up- (red) and down-regulated (blue) differentially expressed genes in the flavonoid KEGG pathway in soybean leaves (*Glycine max* cv. Williams 82) 12 (A; A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (B; A60) or inoculated with *Phakopsora pachyrhizi* (C; AP60) 48 hours after MFP treatment.

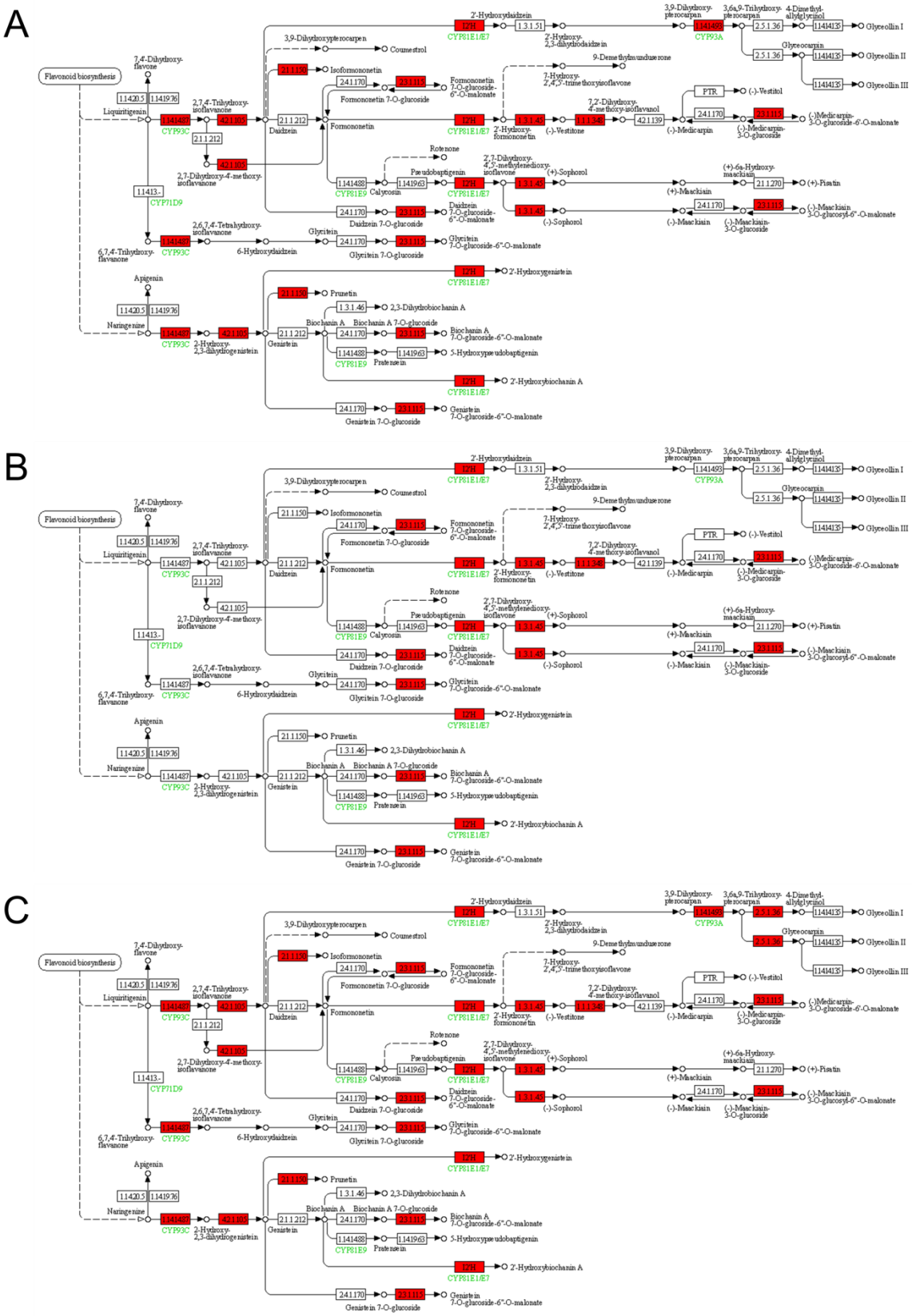

Figure S4. Up- (red) and down-regulated (blue) differentially expressed genes in the isoflavonoid KEGG pathway in soybean leaves (*Glycine max* cv. Williams 82) 12 (A; A12) and 60 hours after treatment (hat) with MFP compared to mock-treated plants. Plants collected 60 hat were either mock (B; A60) or inoculated with *Phakopsora pachyrhizi* (C; AP60) 48 hours after MFP treatment.

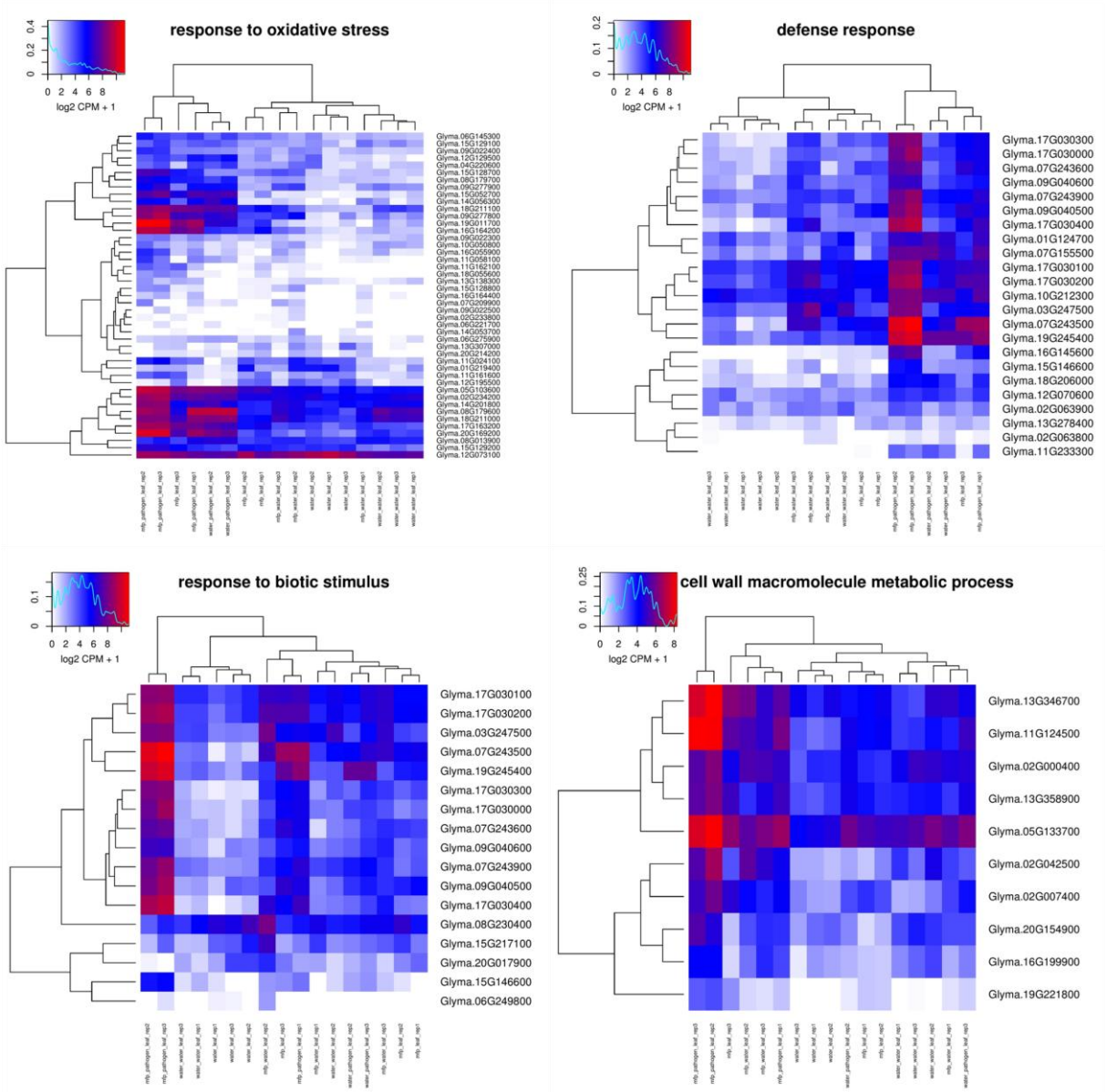

Figure S5. Heatmap and hierarchical clustering of up-regulated genes in selected biological processes of MFP- and mock-treated soybean leaves (*Glycine max* cv. Williams 82) collected 12 (mfp_leaf or water_leaf) or 60 hours after treatment (hat). Plants collected 60 hat were either mock (mfp_water or water_water) or inoculated with *Phakopsora pachyrhizi* (mfp_pathogen or water_pathogen) 48 hours after MFP treatment.

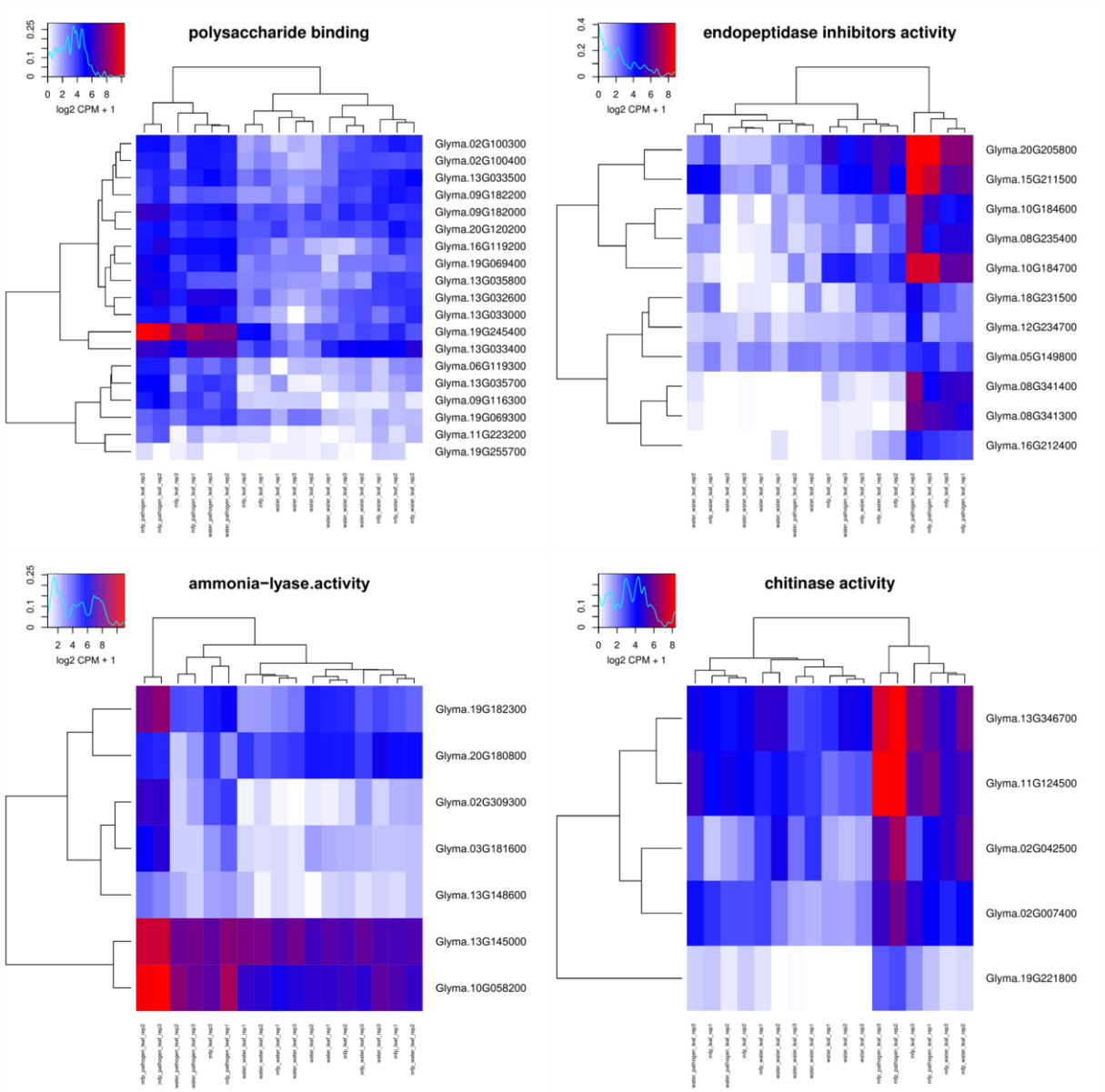

Figure S6. Heatmap and hierarchical clustering of up-regulated genes in selected molecular functions of MFP- and mock-treated soybean leaves (*Glycine max* cv. Williams 82) collected 12 (mfp_leaf or water_leaf) or 60 hours after treatment (hat). Plants collected 60 hat were either mock (mfp_water or water_water) or inoculated with *Phakopsora pachyrhizi* (mfp_pathogen or water_pathogen) 48 hours after MFP treatment.

MANUSCRIPT 2 – HOW MUCH DOES A FERMENTATION PRODUCT PROTECTS AGAINST *Phakopsora pachyrhizi* AND WHAT FACTORS POTENTIALLY INTERFERE WITH ITS EFFICACY? A META-ANALYSIS

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Abstract

A meta-analysis of non-peer reviewed studies conducted in Brazil was performed to summarize the effect of a microbial fermentation product (MFP) inducer in Asian soybean rust (ASR) severity and soybean yield. Response ratios were initially calculated for 24 entries and separate random-effects meta-analysis for severity (k = 22) and yield (k = 24) were performed on the log-transformed ratios (L_s and L_y for severity and yield, respectively). The estimates were used to obtain control efficacy ($\bar{C}$) and yield response ($\bar{Y}$) and their respective confidence intervals (95% CI). L_s ranged from –1.0033 to 0.9255, with 64% being negative (i.e. better ASR control). $\bar{C}$ was 21.1088% (95% CI: 33.1688 to 6.8818%). L_y ranged from –0.4688 to 0.3315, with 67% being positive (i.e. better yield). $\bar{Y}$ was 8.3070% (95% CI: 3.8004 to 13.0206%). P values for L_s and L_y were 0.0051 and 0.0002, respectively. A mixed effects meta-analysis was then performed to determine the effects of type of treatment (alone, k = 10 for severity and 11 for yield; mixed, k = 12 for severity and 13 for yield), disease pressure (low, k = 9 for severity and 10 for yield; medium, k = 5, and high, k = 8 for severity and 9 for yield), number of applications (low, k = 6 for severity and 7 for yield; medium, k = 11 for severity and 12 for yield; and high, k = 5) and timing of the first application (early, k = 16 for severity and 17 for yield; late, k = 6 for severity and 7 for yield) on MFP treatment. All moderators except disease pressure for severity affected $\bar{L}_s$ and $\bar{L}_y$. For type of application, $\bar{L}_s$ and $\bar{L}_y$ were only different from zero in MFP alone (p < 0.0001). $\bar{C}$ for alone was 34.249% compared to 4.725% for mixed. $\bar{Y}$ for alone was 14.717% compared to 4.019% for mixed. Pairwise differences in $\bar{L}_s$ and $\bar{L}_y$ between combinations of disease pressure did not differ from zero. $\bar{Y}$ were 10.650, 9.647 and 5.781% for high, medium and low disease pressure, respectively. $\bar{C}$ in all disease pressures were similar. For number of applications, $\bar{L}_y$ and $\bar{L}_s$ were only different from zero in medium (4 applications), with p values of 0.001 and < 0.0001, respectively. $\bar{C}$ were 13.116, 30.573, and 7.012% for high, medium, and low, respectively. $\bar{Y}$ were 2.942, 11.706, and 5.485%, in that same order. P values for early and late were 0.004 and 0.602, for severity, and 0.002 and 0.062, for yield. $\bar{Y}$ were 8.340 and 8.296% and $\bar{C}$ were 24.799 and 8.561% for early and late, respectively. Results can help in planning future trials and in the decision making of MFP application in field conditions.

INTRODUCTION

Plants are challenged by pathogens in virtually all natural and agronomic ecosystems, therefore developing a suit of constitutive and induced defense mechanisms to thrive in their environments. As their names suggest, constitutive defense is always present in the plant, while induced defense is activated in response to an extrinsic stimulus (Vale et al. 2001). Evolution-wise, plants may have evolved inducible defense mechanisms in order to minimize the fitness cost that comes with constitutive defenses: by synchronizing defense resource investment with pathogen attack, plants avoid defense-related costs under enemy-free conditions (Cipollini and Heil 2010; Herms and Mattson 1992).

Resistance induction is a disease control strategy that exploits the natural, inducible mechanisms that plants have developed by exposing them to an extrinsic stimulus prior to plant pathogen infection (Vale et al. 2001). Its use in integrated pest management can lessen the number and/or dosage of chemical sprayings, which may help achieve a more sustainable agriculture. However, while there is strong scientific evidence of the reliability of resistance induction under controlled conditions, there have been mixed results in field trials (Gozzo and Faoro 2013). Factors that may influence efficacy include the environment, plant genotype and life history, and frequency and timing of elicitor application (Walters et al. 2013).

While experiments may provide important information related to the disease management in specific experimental conditions, to draw any conclusion about the viability and implementation of resistance induction as a pest management tool based on a single work is a risky endeavor. However, conflicting or inconclusive results across individual researches are also a challenge to uncover best evidence (Panesar et al. 2009).

Meta-analysis is a methodology that allows researchers to combine the results of several independent trials with the broad aim of synthetizing existing evidence (Sutton and Higgins 2008) and it is built on the idea that science is a cumulative process with results of individual studies contributing to the total (Madden and Paul 2011). Meta-analysis also permits to model or quantify the effects of study-specific factors (Edwards Molina et al. 2019), which are in-built in individual studies and thus, not isolated.

Phakopsora pachyrhizi is an obligate fungus that causes the Asian soybean rust (ASR). Particularly aggressive, it can potentially cause yield losses of up to 90% in soybean (Godoy et al. 2009). Originally found in Taiwan, this fungus was reported in 2001 in South America (Yorinori et al. 2005) and 2005 in North America (Schneider et al. 2005). The main ASR control strategies aim to reduce initial inoculum (a yearly soybean-free period and early

planting) and reduce both the rates of infection and disease progress (protectant and most importantly, systemic fungicides) (Furlan et al. 2018; Godoy et al. 2016). However, disease control is getting increasingly harder due to higher rates of fungicide-resistant isolates (Godoy et al. 2016).

In field trails carried out in Brazil, a proprietary microbial fermentation product (MFP) has been observed to protect soybean plants against *P. pachyrhizi*, with variable results. Since product performance seems to vary, and the factors that influence the performance of the product are yet unknown, the goal of this work is to use meta-analysis to i) determine a general control efficacy and yield response to MFP application and ii) identify factors that may affect the efficacy of the tested product.

MATERIALS AND METHODS

The initial database for the meta-analysis consisted of efficacy trial reports contracted by the manufacturer of the proprietary MFP (15) and trials carried out at Universidade Federal de Lavras (4), spanning four crop years (2016-2017 through 2019-2020). The following criteria were used to select the data to be included in the study: 1) The tests had to have a negative control; 2) MFP had to be applied with a dosage of 0.500 L ha^{-1}, either alone or mixed with other products; 3) if MFP was applied in tandem with other products, the tests had to have a correspondent fungicide-only treatment, 4) the trial reports had to report information regarding yield and ASR severity for the negative control.

Based on the criteria, 13 out of 19 trial reports were included in the meta-analysis, resulting in 11 entries where the proprietary MFP was used by itself and 13 entries where it was used in tandem with other fungicides. The trials were performed in the following cities: Campo Verde (MT), Jaboticabal (SP), Lavras (MG), Londrina (PR), Luís Eduardo Magalhães (BA), Planaltina (DF), Rio Verde (GO), Tangará da Serra (MT) (Table 1).

Table 1. Location of trials where MFP was used (A) alone or (B) mixed with chemical fungicides per crop year. Trials mostly occurred in the South, Southeast and Midwest regions from Brazil.

Crop year	Location	
	MFP	Mixed
2016-2017	Jaboticabal	Jaboticabal
	Lavras	Lavras
	Londrina	Londrina
	Luís Eduardo Magalhães	Luís Eduardo Magalhães
		Tangará da Serra
2017-2018	Jaboticabal	Jaboticabal
	Lavras	Lavras
	Londrina	Londrina
	Planaltina	Planaltina
2018-2019	Lavras	Lavras
	Rio Verde	Rio Verde
		Campo Verde
2019-2020	Lavras	Lavras

Quantitative synthesis of MFP effect across trials

The levels of disease and yield in MFP-treated plots relative to the levels in check plots were used to evaluate the efficacy of MFP treatment in each study. Mean severity and yield from MFP-treated plots and control plots were collected from each study and used to find the effect size, estimated as the response ratio (*R*):

$$R = \frac{\bar{X}_{MFP}}{\bar{X}_{check}}$$

, where $\bar{\bar{X}}_{MFP}$ is mean from plots treated with MFP, and $\bar{X}_{check}$ is mean from check plots. For trials where MFP was used alone, $\bar{X}_{check}$ was considered the mock-treated (negative) control. For trials where MFP was used in tandem with other fungicides, $\bar{X}_{check}$ was considered the correspondent fungicide-only treatment in order to isolate the contribution of MFP to severity and yield. Subscripts are added to *R* to refer specifically to severity and yield results (R_s and R_y, respectively).

Overall severity and yield response

As explained by Paul et al. (2007), a log-transformation was applied to the R_s and R_y values prior to the meta-analysis. Subscripts are added to L to refer specifically to log severity and yield results (L_s and L_y, respectively). A random-effects meta-analysis was performed to estimate the overall mean effect sizes ($\bar{L}_s$ and $\bar{L}_y$) and to determine the variability in effect

sizes among studies. Study was considered a random effect, and separate meta-analyses were performed for L_s and L_y. The amount of heterogeneity (i.e., τ^2), was estimated using the restricted maximum-likelihood estimator (Viechtbauer 2005). The within-study variance (V) for severity or yield for these trials was estimated from the coefficient of variation (CV) of an analysis of variance of the effects of treatment on disease or yield, by first estimating the standard deviation (*SD*). When only the means were available, V was estimated for a single study as the pooled standard deviation from all other available *SD*. Studies were weighted in inverse proportion to their sampling variances (within-study variances). Studentized residuals and Cook's distances were used to examine whether entries may be outliers and/or influential in the context of the model (Viechtbauer and Cheung 2010); any possible outlier and overly influential was excluded and another random-effects meta-analysis was performed.

The analysis was carried out using R (version 4.0.2) (R Core Team 2018) and the metafor package (version 2.4.0) (Viechtbauer 2010). Overall mean percent control efficacy ($\bar{C}$), yield response ($\bar{Y}$), and their corresponding 95% were estimated with the formulas $\bar{C} = [1 - exp(\bar{L}_s)]$ x 100 and $\bar{Y} = \left[\exp\left(\bar{L}_y\right) - 1\right]$ x 100 , by back-transforming $\bar{L}_s$, $\bar{L}_y$, as well as their lower and upper bound 95% confidence intervals (CI_{LB} and CI_{UB}) (Dalla Lana et al. 2018; Paul et al. 2008).

Moderator variables

A mixed-effects meta-analysis was performed to evaluate the influence of type of application (alone or mixed application), disease pressure, number of applications and timing of the first application on the overall mean log ratios ($\bar{L}_s$ and $\bar{L}_y$). A separate random-effect meta-analysis was performed for each effect size and for each moderator variable, with k for each level of the moderators varying between each effect size. The number of entries (k) for the severity and the yield mixed-effects meta-analyses were 22 and 24, respectively. Using disease pressure, studies were classified into three groups based on the disease severity of the untreated check (S_{mock}) (Scherm et al. 2009): low ($S_{mock} < 40\%$, k = 9 for severity and 10 for yield), medium ($40\% \leq S_{mock} \leq 70\%$, k = 5), and high ($S_{mock} > 70\%$, k = 8 for severity and 9 for yield). The disease pressure variable reflects the overall favorability for disease development taking in consideration factors such as location, weather, and planting date (Scherm et al. 2009). Treatment-oriented moderator variables included type of application, number of applications (up to 6), and timing of the first application. Studies were

categorized as alone (k = 10 for severity and 11 for yield) or mixed application (k = 12 for severity and 13 for yield) based on the type of application. For number of applications, studies were grouped into three sets: low (3 applications, k = 6 for severity and 7 for yield), medium (4 applications, k = 11 for severity and 12 for yield) and high (5 or 6 applications, k = 5). For timing of the first application, two groups were assigned: early (up to V5, k = 16 for severity and 17 for yield) and late application (V8 and up, k = 6 for severity and 7 for yield).

Overall mean percent control efficacy ($\bar{C}$), yield response ($\bar{Y}$), and their corresponding 95% CI were estimated as described above.

RESULTS

ASR severity, yield and corresponding response ratios

Across the 24 trials, severity in the MFP-treated entries (S_{MFP}) ranged from 3.3 to 80% (median: 29.585%), with values 10% being most common (Figure 1A). Disease severity in check entries (S_{check}) ranged from 0.88 to 83.631% (median: 33.335%) (Figure 1C). Yield ranged from 188.141 to 6735.000 kg ha^{-1} (median: 3597.545 kg ha^{-1}) and 300.677 to 6331.8 kg ha^{-1} (median: 3666.605 kg ha^{-1}), for MFP-treated and check plots, respectively (Figures 1B and 1D). Separate frequency distributions of ASR severity and yield for MFP alone and MFP + fungicide and their corresponding checks are shown in Supplementary Figure S1. Response ratios for disease severity (R_s) varied from 2.5 (an increase on severity) to 0.1811 (i.e., a decrease by 81.88% relative to check); 62.5% of R_s values were below 1 (i.e. MFP improved ASR control). Most frequently, R_s values were between 0.6 and 1.1 (Figure 1E). R_y ranged from 0.625 (a decrease by 37.5% relative to check) to 1.393 (yield increase by 39.3%). 66.66% of R_y values were above 1 (i.e. MFP improved yield). R_y values were most common in the 0.92-1.16 range (Figure 1F).

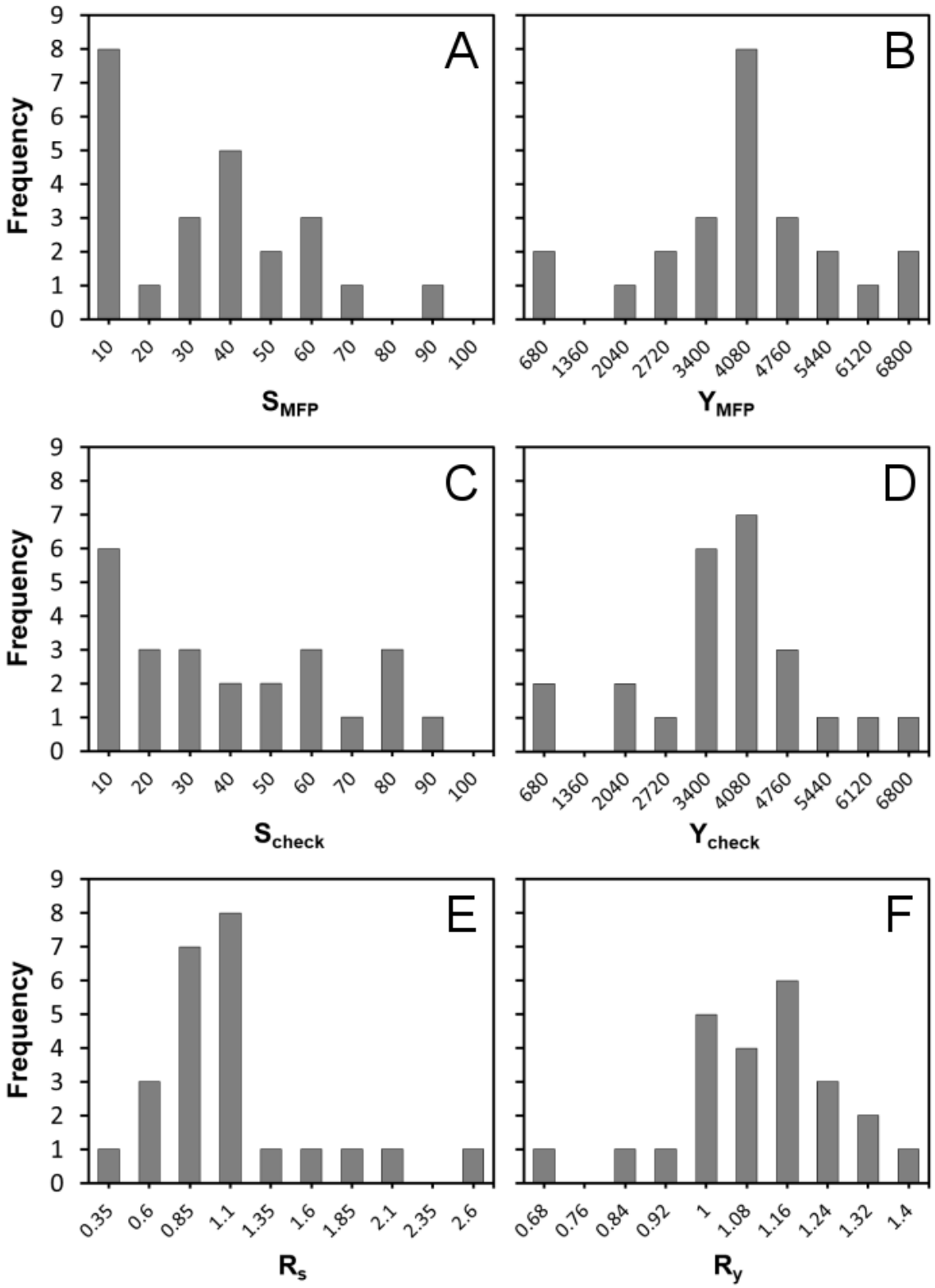

Figure 1. Frequency distributions of Asian soybean rust severity (A and C), yield (B and D) and response ratio for disease severity (R_s; E) and yield (R_y; F) based on 24 entries conducted in Brazil from 2016-2017 to 2019-2020. Value distribution of MFP-treated (S_{MFP} or Y_{MFP}) and untreated checks (S_{check} or Y_{check}) entries are shown in A-B and C-D, respectively. Response ratio is defined as the disease severity or yield of a given MFP-treated entry divided by the disease severity or yield of the corresponding untreated check. Smaller values of R_s and larger values of R_y indicate better control efficacy and yield response, respectively.

Overall severity and yield response

A total of k = 22 entries were included in the severity meta-analysis after removal of outliers and overly influential entries. The observed log response ratios ranged from –1.0033 to 0.9255, with the majority of estimates being negative (64%) (i.e. improved ASR control). The estimated average response ratio based on the random-effects model was $\hat{\mu} = -0.2371$ (95% CI: –0.4030 to –0.0713), which corresponds to an overall mean percent control efficacy ($\bar{C}$) of 21.1088% (95% CI: 33.1688 to 6.8818). For yield, k = 24 entries were included in the analysis. Log response ratio from –0.4688 to 0.3315, with the majority of estimates being positive (67%) (i.e. improved yield). The estimated average response ratio based on the random-effects model was $\hat{\mu} = 0.0798$ (95% CI: 0.0373 to 0.1224), corresponding to an overall mean yield response ($\bar{Y}$) of 8.3070% (95% CI: 3.8004 to 13.0206). Probability levels for severity and yield were 0.0051 and 0.0002, respectively (Table 2). The range of estimated response ratios for all entries after analysis can be seen in figure 2.

Table 2. Log of response ratio (effect size), mean effect, and corresponding statistics for the effect of a microbial fermentation product (MFP) on Asian soybean rust severity and soybean yield in field conditions

Variable	k[a]	Effect Size[b]						Mean effect[c]		
		$\bar{L}$	$se(\bar{L})$	CI_{LB}	CI_{UB}	Z value	p value	$\bar{E}$	CI_{LB}	CI_{UB}
Severity	22	-0.2371	0.0846	-0.4030	-0.0713	-2.8020	0.0051	21.1088	33.1688	6.8818
Yield	24	0.0798	0.0217	0.0373	0.1224	3.6784	0.0002	8.3070	3.8004	13.0206

[a] Total number of entries used in each analysis after removing outliers and overly influential entries.
[b] $\bar{L}$ = Log-transformed response ratio (L_s and L_y for severity and yield, respectively); $se(\bar{L})$ = standard error of $\bar{L}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\bar{L}$; Z value = (standard normal) statistic from the meta-analysis of the effect of MFP on yield and severity; p value = probability value (significance level).
[c] Mean effect ($\bar{E}$) and lower (E_{LB}) and upper (E_{UB}) limits of the 95% confidence interval for $\bar{E}$. For severity, $\bar{E} = \bar{C} = [1 - exp(\bar{L}_s)]$ x 100. For yield, $\bar{E} = \bar{Y} = \left[\exp(\bar{L}_y) - 1\right]$ x 100.

Based on Wald-type chi-square tests, all the moderator variables evaluated in this study, with the exception of disease pressure for severity, affected $\bar{L}_s$ and $\bar{L}_y$ ($p < 0.05$). Both effect sizes were influenced by type of application (P < 0.001). Although the type of application as a whole was statistically significant, $\bar{L}_s$ and $\bar{L}_y$ values were only different from zero in MFP alone (Table 3). $\bar{L}_s$ and $\bar{L}_y$ for alone differed ($p < 0.05$) from mixed with corresponding higher estimated mean effects ($\bar{E}$, either $\bar{C}$ for severity and $\bar{Y}$ for yield). $\bar{C}$ for alone was 34.249% (95% CI: 46.608 to 19.031) compared to 4.725% (95% CI: 23.264 to –17.916) for mixed (a difference in percent control of nearly 30%). $\bar{Y}$ for alone was 14.717%

(95% CI: 7.939 to 21.921) compared to 4.019% (95% CI: –1.094 to 9.396) for mixed (Table 3).

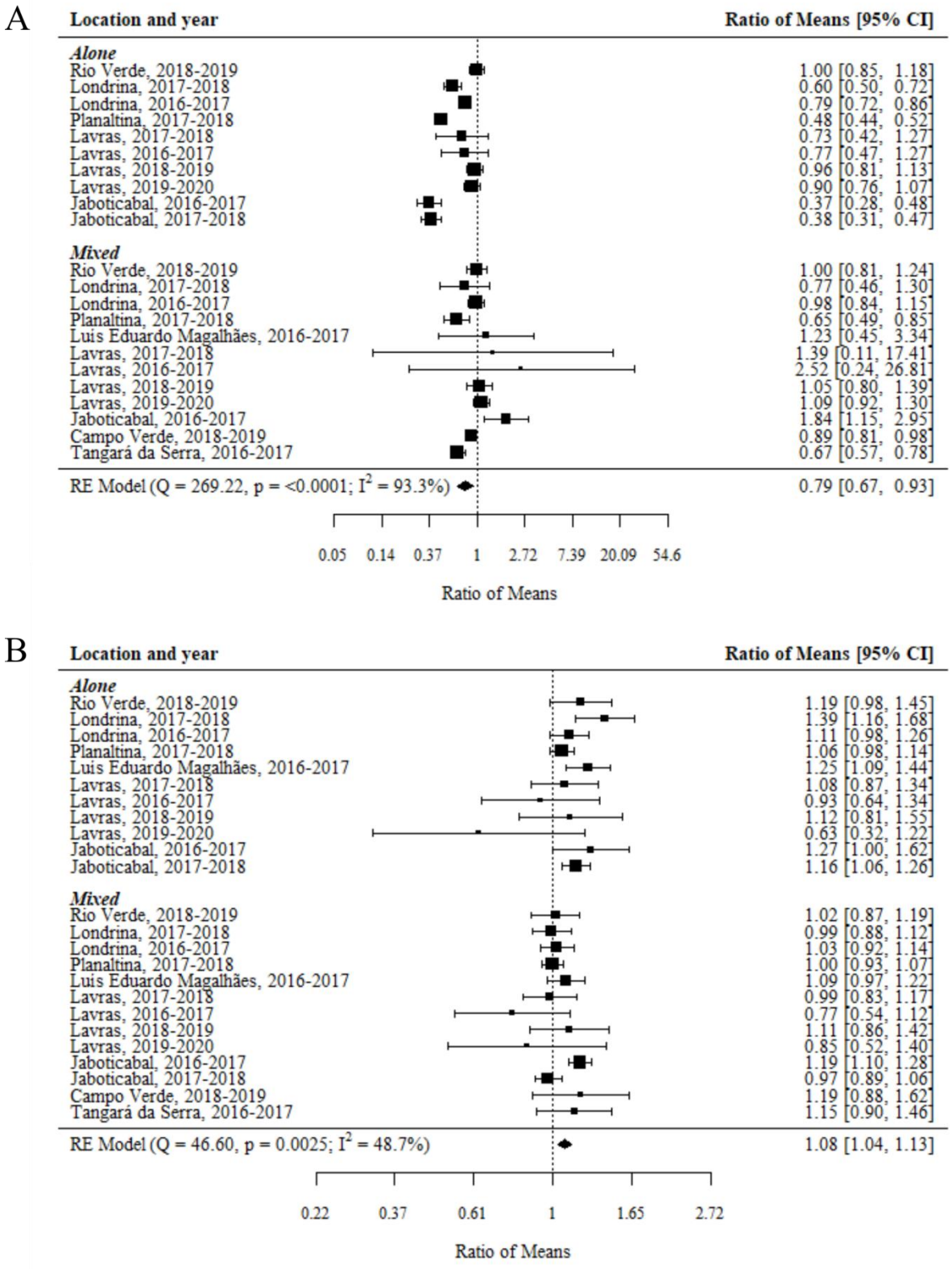

Figure 2. Observed response ratios (ratio of means, *R*) and the estimate of the random-effects model of a microbial fermentation product (MFP) effect on (A) Asian soybean rust severity (k = 22) and (B) soybean yield (k = 24) when applied alone or mixed with chemical fungicides. For severity, $R < 1$ means severity reduction.

For yield, $R > 1$ means yield increase.

Table 3. Log of response ratio (effect size), mean effect, and corresponding statistics for the influence of the type of treatment on a microbial fermentation product (MFP) effect on Asian soybean rust severity and soybean yield in field conditions

Variable	Treatment Type[a]	k[b]	Effect Size[c]					Mean effect[d]		
			$\bar{L}$	$se(\bar{L})$	CI_{LB}	CI_{UB}	p value	$\bar{E}$	CI_{LB}	CI_{UB}
Severity	Alone	10	-0.419	0.106	-0.628	-0.211	< 0.0001	34.249	46.608	19.031
	Mixed	12	-0.048	0.110	-0.265	0.165	0.661	4.725	23.264	-17.916
Yield	Alone	11	0.137	0.031	0.076	0.198	< 0.0001	14.717	7.939	21.921
	Mixed	13	0.039	0.026	-0.011	0.090	0.125	4.019	-1.094	9.396

[a] Type of treatment in each analysis. MFP applied alone (Alone) or in tandem (Mixed) with chemical fungicides. Chemicals used in all trials included azoxystrobin + benzovindiflupyr, epoxiconazole + fluxapyroxad + pyraclostrobin; picoxystrobin + cyproconazole; prothioconazole + trifloxystrobin; azoxystrobin + cyproconazole; propiconazole + difenoconazole; pyraclostrobin + fluxapyroxad; and tebuconazole + picoxystrobin.
[b] Total number of entries in each analysis after removing outliers and overly influential entries.
[c] $\bar{L}$ = Log-transformed response ratio (L_s and L_y for severity and yield, respectively); $se(\bar{L})$ = standard error of $\bar{L}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\bar{L}$; p value = probability value (significance level).
[d] Mean effect ($\bar{E}$) and lower (E_{LB}) and upper (E_{UB}) limits of the 95% confidence interval for $\bar{E}$. For severity, $\bar{E} = \bar{C} =$ $[1 - exp(\bar{L}_s)]$ x 100. For yield, $\bar{E} = \bar{Y} = \left[\exp(\bar{L}_y) - 1\right]$ x 100.

Only yield was affected by disease pressure (p < 0.01). Only $\bar{L}_y$ for high disease pressure in yield was different from zero (p < 0.01) (Table 4). Pairwise differences in $\bar{L}_s$ and $\bar{L}_y$ between combinations of disease pressure did not differ from zero (p > 0.05). $\bar{Y}$ for high and medium disease pressure were higher than for low disease pressure: 10.650% (95% CI: 3.417 to 18.388), 9.647% (95% CI: -3.014 to 23.961) and 5.781% (95% CI: -1.005 to 13.021) for high, medium and low, respectively. $\bar{C}$ in all disease pressures were almost the same (Table 4).

Table 4. Log of response ratio (effect size), mean effect, and corresponding statistics for the influence of Asian soybean rust disease pressure levels on a microbial fermentation product (MFP) effect on Asian soybean rust severity and soybean yield in field conditions

Variable	Disease Pressure[a]	k[b]	Effect Size[c]					Mean effect[d]		
			$\bar{L}$	$se(\bar{L})$	CI_{LB}	CI_{UB}	p value	$\bar{E}$	CI_{LB}	CI_{UB}
Severity	High	8	-0.234	0.138	-0.505	0.037	0.091	20.872	39.655	-3.769
	Medium	5	-0.236	0.177	-0.583	0.110	0.181	21.054	44.150	-11.605
	Low	9	-0.235	0.158	-0.544	0.074	0.137	20.927	41.958	-7.724
Yield	High	9	0.101	0.035	0.034	0.169	0.003	10.650	3.417	18.388
	Medium	5	0.092	0.063	-0.031	0.215	0.141	9.647	-3.014	23.961
	Low	10	0.056	0.034	-0.010	0.122	0.097	5.781	-1.005	13.021

[a] Final severity in mock-treated plot (S_{mock}) for each entry. Low: $S_{mock} \leq 40\%$; Medium: $40\% \leq S_{mock} \leq 70\%$; High: $S_{mock} > 70\%$.
[b] Total number of entries in each analysis after removing outliers and overly influential entries.
[c] $\bar{L}$ = Log-transformed response ratio (L_s and L_y for severity and yield, respectively); $se(\bar{L})$ = standard error of $\bar{L}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\bar{L}$; p value = probability value (significance level).
[d] Mean effect ($\bar{E}$) and lower (E_{LB}) and upper (E_{UB}) limits of the 95% confidence interval for $\bar{E}$. For severity, $\bar{E} = \bar{C} =$ $[1 - exp(\bar{L}_s)]$ x 100. For yield, $\bar{E} = \bar{Y} = \left[\exp(\bar{L}_y) - 1\right]$ x 100.

Both yield and severity were affected by number of applications ($p < 0.01$). $\bar{L}_y$ and $\bar{L}_s$ were only different from zero in medium (4 applications), with p values of 0.001 and < 0.0001, respectively (Table 5). Pairwise differences in $\bar{L}_s$ and $\bar{L}_y$ between combinations of number of applications were not different from zero ($p > 0.05$). $\bar{C}$ values were 13.116 (95% CI: 39.498 and –24.770), 30.573 (95% CI: 44.434 to 13.247) and 7.012% (95% CI: 31.326 to –25.898) for high (5 or 6 applications), medium (4 applications) and low (3 applications), respectively. For yield, mean effect size values ($\bar{Y}$) were 2.942% (95% CI: –5.522 to 12.165), 11.706% (95% CI: 5.654 to 18.105) and 5.485% (95% CI: –3.815 to 15.673) (Table 5).

Table 5. Log of response ratio (effect size), mean effect, and corresponding statistics for the influence of the number of applications on a microbial fermentation product (MFP) effect on Asian soybean rust severity and soybean yield in field conditions

Variable	Application number[a]	k[b]	Effect Size[c]					Mean effect[d]		
			$\bar{L}$	$se(\bar{L})$	CI_{LB}	CI_{UB}	p value	$\bar{E}$	CI_{LB}	CI_{UB}
Severity	High	5	-0.141	0.185	-0.503	0.221	0.446	13.116	39.498	-24.770
	Medium	11	-0.365	0.114	-0.588	-0.142	0.001	30.573	44.434	13.247
	Low	6	-0.073	0.155	-0.376	0.230	0.638	7.012	31.326	-25.898
Yield	High	5	0.029	0.044	-0.057	0.115	0.508	2.942	-5.522	12.165
	Medium	12	0.111	0.028	0.055	0.166	< 0.0001	11.706	5.654	18.105
	Low	7	0.053	0.047	-0.039	0.146	0.257	5.485	-3.815	15.673

[a] Number of applications of MFP during trials in each entry. Low: 3; Medium: 4; High: 5 or 6.
[b] Total number of entries in each analysis after removing outliers and overly influential entries.
[c] $\bar{L}$ = Log-transformed response ratio (L_s and L_y for severity and yield, respectively); $se(\bar{L})$ = standard error of $\bar{L}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\bar{L}$; p value = probability value (significance level).
[d] Mean effect ($\bar{E}$) and lower (E_{LB}) and upper (E_{UB}) limits of the 95% confidence interval for $\bar{E}$. For severity, $\bar{E} = \bar{C} = [1 - exp(\bar{L}_s)]$ x 100. For yield, $\bar{E} = \bar{Y} = \left[\exp(\bar{L}_y) - 1\right]$ x 100.

Both yield and severity were affected by the timing of the first application as a whole ($p < 0.01$). Probability levels (p value) for early timing (up to V5) and late timing (V8 and up) were 0.004 and 0.602, for severity, and 0.002 and 0.062, for yield. Pairwise comparisons of $\bar{L}_s$ and $\bar{L}_y$ between early and late timing were not different from zero ($p > 0.05$). Overall mean yield response ($\bar{Y}$) were nearly equal: 8.340% (95% CI: 2.912 to 14.042) and 8.296% (95% CI: -0.389 to 17.739) for early and late, respectively. Overall mean control efficacy ($\bar{C}$) were 24.799% (95% CI: 37.954 to 8.853) and 8.561% (95% CI: 34.688 and -28.018) for early and late, in that order (Table 6).

Table 6. Log of response ratio (effect size), mean effect, and corresponding statistics for the influence of the timing of the first application on a microbial fermentation product (MFP) effect on Asian soybean rust severity and soybean yield in field conditions

Variable	Timing[a]	k[b]	Effect Size[c]					Mean effect[d]		
			$\bar{L}$	$se(\bar{L})$	CI_{LB}	CI_{UB}	p value	$\bar{E}$	CI_{LB}	CI_{UB}
Severity	Early	16	-0.285	0.098	-0.477	-0.093	0.004	24.799	37.954	8.853
	Late	6	-0.090	0.172	-0.426	0.247	0.602	8.561	34.688	-28.018
Yield	Early	17	0.080	0.026	0.029	0.131	0.002	8.340	2.912	14.042
	Late	7	0.080	0.043	-0.004	0.163	0.062	8.296	-0.389	17.739

[a] Timing of the first application during trials in each entry. Early: up to the V5 growth stage; Late: V8 onward.
[b] Total number of entries in each analysis after removing outliers and overly influential entries.
[c] $\bar{L}$ = Log-transformed response ratio (L_s and L_y for severity and yield, respectively); $se(\bar{L})$ = standard error of $\bar{L}$; CI_{LB} and CI_{UB} = limits of the 95% confidence interval around $\bar{L}$; p value = probability value (significance level).
[d] Mean effect ($\bar{E}$) and lower (E_{LB}) and upper (E_{UB}) limits of the 95% confidence interval for $\bar{E}$. For severity, $\bar{E} = \bar{C} = [1 - exp(\bar{L}_s)]$ x 100. For yield, $\bar{E} = \bar{Y} = [\exp(\bar{L}_y) - 1]$ x 100.

DISCUSSION

P. pachyrhizi is one of the biggest threats for soybean crops in tropical conditions, due to its aggressiveness, causing yield losses of up to 90% (Godoy et al. 2009) and the fact it can survive and multiply in places with mild winter conditions due to their large host range (Kelly et al. 2015). Chemical fungicides have been the main method for ASR control, but there have been reports of insensitivity to the main fungicide groups used against *P. pachyrhizi* (Klosowski et al. 2016; Schmitz et al. 2014; Simões et al. 2018). In a meta-analysis study on the historical performance of fungicides against soybean rust, the authors found a significant decline in the performance of all evaluated commercial fungicides but one over a period as short as 4 years (Dalla Lana et al. 2018).

In this light, resistance inducers are often seen as an option to further protect soybean crops. In our study, we investigated the effect of a mannan oligosaccharide-based MFP in reducing disease severity and improving yield. The estimated R_s values were mostly between 0.37 and 1 (Figure 2A), a relative disease reduction of between 0 and 63% in response to MFP treatment (median: 27%). For yield, most estimated $R_y$values were positive and between 1 and 1.39 (Figure 2B), a relative yield increase of 0 to 39% (median: 13%).

There were significant associations between R_s or R_y and type of treatment (Table 3). Probability levels (p values) for L_s were < 0.0001 and 0.661 for alone and mixed, respectively; while for $L_y$were < 0.0001 and 0.125, in the same order. The estimated R_s and R_y (and corresponding $\bar{C}$ and $\bar{Y}$) for MFP alone was higher (p < 0.05) than mixed. For MFP alone, $\bar{C}$ and $\bar{Y}$ were 34.249% and 14.717%, respectively. Those values were on par with the efficacy of single active ingredient fungicides found by Dalla Lana et al. (2018) at the last

year of their analysis for both $\bar{C}$ and $\bar{Y}$, but below the one found for active ingredient mixtures, which is recommended and used against soybean rust.

Since the idea was to isolate the effect of MFP, when MFP was used in tandem with other fungicides, check plots considered the correspondent fungicide-only treatment. The results suggest that MFP, when applied with chemical fungicides, did not contribute to improve plant protection or yield. Although Dalla Lana et al. (2018) reported that fungicides have been losing efficacy, all treatments in the included trials were only applied twice, whereas in the current meta-analysis they were applied a minimum of three times. Currently, uniform field trials are evaluating ASR control with 3 or 4 fungicide applications, finding for premix fungicides a control of at least 50% (Godoy et al. 2018; Godoy et al. 2019). Our results are in agreement with other works that did not find a significant enhancement of ASR control when using resistance inducers in a fungicide-heavy control program (3+ fungicide applications) (Barros 2011; Oliveira et al. 2015).

Disease pressure (i.e. the final severity in mock-treated plots; S_{mock}) did not affect the effect of MFP on severity, but affected on yield. $\bar{L}_y$ values were similar in all disease pressure: -0.234 (95% CI: 0.037 to 0.091), -0.236 (95% CI: 0.110 to 0.181) and -0.235 (95% CI: 0.074 to 0.137) for high, medium, low pressure respectively (Table 4). For yield, although $\bar{L}_y$ values on high ($S_{mock} > 70\%$) and medium disease pressure ($40\% \leq S_{mock} \leq 70\%$) were similar, only $\bar{L}_y$ for high pressure was different from zero ($p < 0.01$) (Table 4). Our results were similar to Scherm et al. (2009) and Delaney et al. (2018) in that the increment in yield was more positive than in ASR severity at higher levels of disease pressure.

Among the factors that influence resistance induction efficacy are frequency and timing of elicitor application (Walters et al. 2013). Number of applications (i.e. frequency) affected the effect of MFP on both yield and severity ($p < 0.01$), although $\bar{L}_y$ and $\bar{L}_s$ were only different from zero when applied 4 times (medium number of applications) (Table 5). A small number may not provide protection given that, in natural conditions, it is uncertain when infection will occur. In that case, a higher number of applications would be necessary given that, often times, the effect of elicitors in plants is transient (Barros 2011). Although there is evidence that MFP have a priming effect on wheat (Twamley et al. 2019), this is not yet known for soybeans. As mentioned above, the number of fungicide applications to properly control ASR has increased overtime, with uniform field trials now evaluating fungicide efficacy based on 3 or 4 applications (Godoy et al. 2019), whereas earlier trials were tested with only two applications. Four applications are not unreasonable, considering that some

fields may receive up to 6 fungicide applications (Godoy et al. 2016); however this number should be weighed against the level of protection given by the applied product.

Protection against soybean rust is especially crucial during the reproductive period (Scherm et al. 2009), and normally takes place around the R1 (beginning flowering) or R2 (full flowering) reproductive stages (Godoy et al. 2009; Nascimento et al. 2018). In most cases, applications during the vegetative period are considered ineffective and discouraged (Juliatti et al. 2017; Miles et al. 2003). In the current study, we found that the timing of the first application affected the effect of MFP on yield and severity ($p < 0.01$). However, we found that early first applications (beginning on early vegetative stages) had a greater effect than late applications (late vegetative stage and first reproductive stage) (p values for early timing and late timing, respectively: 0.004 and 0.602 for severity, 0.002 and 0.062 for yield). There are two possible explanations for that. First, it takes some time for downstream regulation after resistance induction by elicitors, which means early applications that precede inoculum deposition are more effective in reducing disease severity (Cavalcanti and Resende 2005; Sharathchandra 2004). Additionally, Panthee et al. (2009) found that soybean gene expression in response to *P. pachyrhizi* is growth stage-dependent. The authors report that plants in the V4 vegetative stage that were inoculated with *P. pachyrhizi* had roughly five times more defense-related differentially expressed genes than R1 plants. Most early application entries had first applications near the V4 growth stage, which may have resulted in maximum defense induction. Another explanation is that, in order to maximize disease pressure, all trials included in the meta-analysis were planted in late November or early December, while the soybean sowing window normally starts in late September. Consequently, fields sown later receive a greater amount of inoculum and suffer an early onset of the disease (Godoy et al. 2016). That could mean that early applications roughly coincided with inoculum deposition, while late applications were done when severity was already high, compromising protection (Scherm et al. 2009).

While defense induction products have been used successfully for plant disease control under laboratory and greenhouse conditions, field results have not always been successful (Walters and Fountaine 2009). Field conditions are highly variable, and lack of knowledge of biotic and abiotic factors that influence elicitation may affect the efficacy of elicitor application. To our knowledge, this is the first meta-analysis studying the protective effect of a fermentation elicitor product in field trials. While it may be based on few studies, it it is our

hope this study can serve as the basis to design a management program that includes MFP to protect crops and increase yield in a highly variable agricultural setting.

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SUPPLEMENTARY DATA

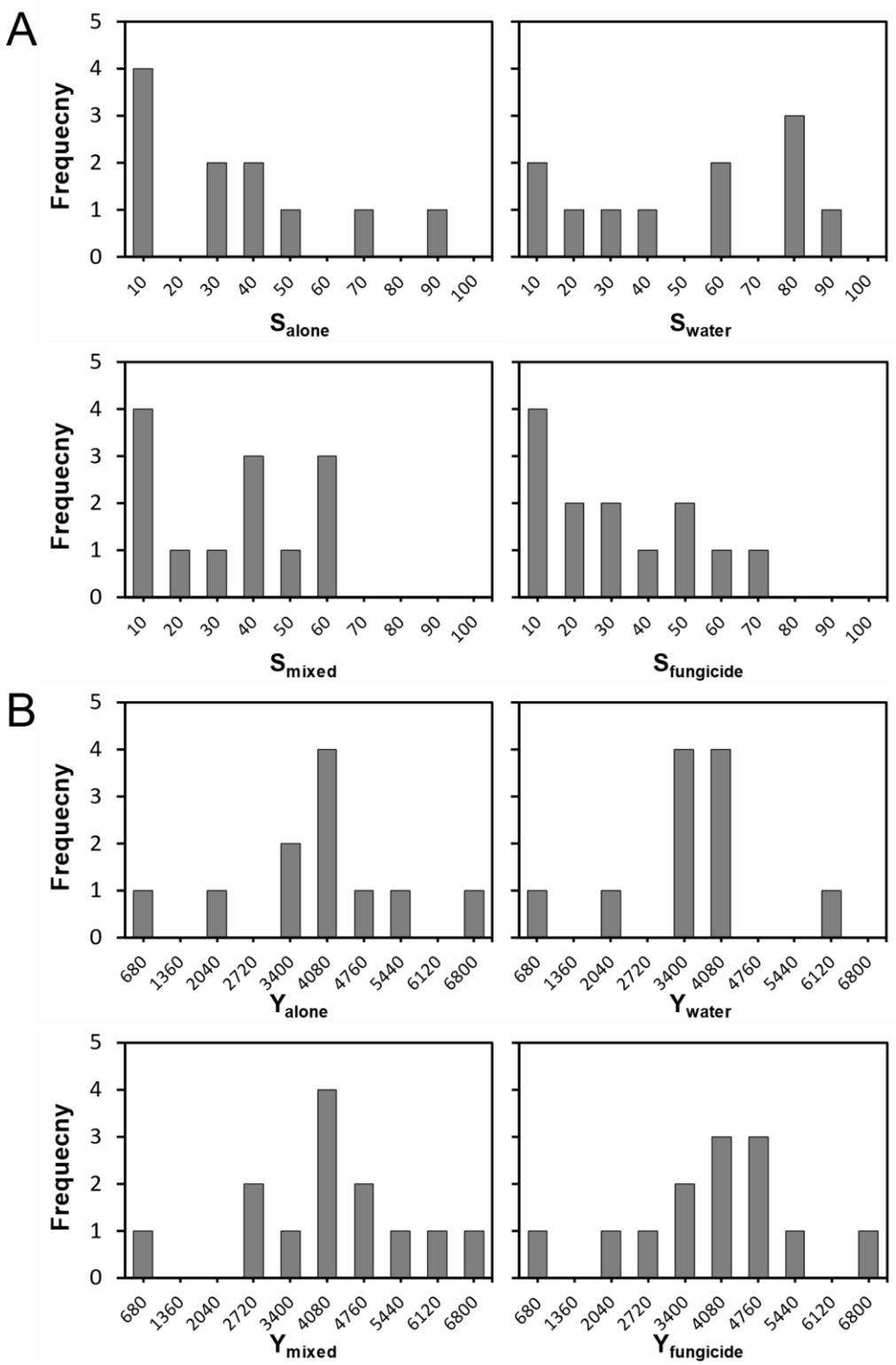

Figure S1. Frequency distributions of Asian soybean rust severity (A) and yield (B) of plots treated with water, MFP (alone), fungicide and MFP + fungicide (mixed) based on 24 entries conducted in Brazil from 2016-2017 to 2019-2020.