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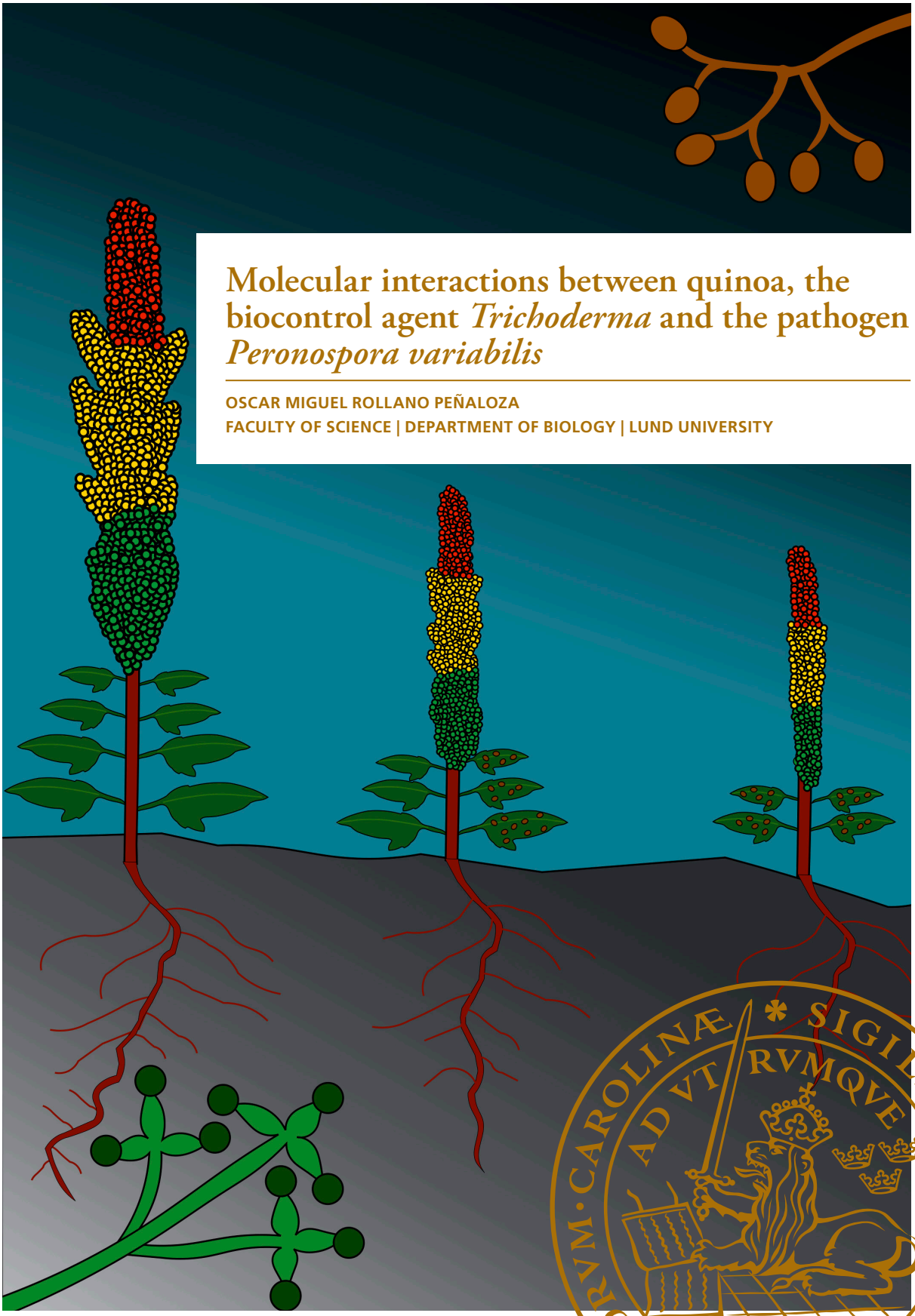

Molecular interactions between quinoa, the biocontrol agent *Trichoderma* and the pathogen *Peronospora variabilis*

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LUND UNIVERSITY

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Abstract

Plants have developed complex molecular mechanisms to recognize and respond to the different microorganisms present in their surroundings. The most studied response mechanisms are plant defense responses. These defense response mechanisms share many similar components with the plant response mechanisms to beneficial microbes. Therefore, studying the defense response mechanisms against pathogens can contribute to the understanding of compatible and incompatible interactions with beneficial microbes.

In this work, the molecular interactions of quinoa with the pathogen *Peronospora variabilis,* causal agent of the downy mildew disease, and the interactions of quinoa with the beneficial biocontrol fungi *Trichoderma harzianum* were studied. Experimental systems for interaction experiments were developed and used, followed by morphological, biochemical and transcriptomic analysis. We describe the response of two quinoa cultivars to the infection of *P. variabilis* under controlled conditions. The quinoa cultivar Kurmi was more tolerant to *P. variabilis* infection than the Real cultivar, despite the lack of hypersensitive response. The defense response observed in the Kurmi cultivar might be mediated by the jasmonic acid signaling pathway. Cultivars that can trigger hypersensitive response are more resistant to *P. variabilis* than Kurmi and therefore a better selection for agriculture.

Quinoa in the presence of *Trichoderma* had variable outcomes depending on the growth conditions. We observed that quinoa growth was promoted in regular soil experiments or by interaction with *Trichoderma* volatile compounds in axenic co-culture. However, the growth of two quinoa cultivars was significantly inhibited by *T. harzianum* in axenic co-culture and in steamed soil experiments. The transcriptomic data of the quinoa growth inhibition by *Trichoderma* suggests activation of molecular signaling very similar to the signaling observed during defense response against pathogens. Further, we observed a specific group of quinoa plant defensins to be more rapidly induced by *Trichoderma* in a resistant cultivar but not in a susceptible one. These plant defensins showed a recent evolutionary expansion and could play a major role in providing pathogen resistance to certain quinoa cultivars.

In order to protect quinoa from *P. variabilis* infections by *Trichoderma* application and enhance the quinoa yields in agricultural systems might be necessary to perform compatibility tests between the *Trichoderma* biocontrol agents and the quinoa cultivars. These compatibility tests should be performed in regular soil.

Key words *Chenopodium quinoa*, *Peronospora variabilis*, *Trichoderma harzianum*, Molecular interactions, qRT-PCR, RNA-seq, plant growth promotion

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The future is bio-based but needs scientists to make it real. The future needs scientist to play a stronger role in society.

To my beloved family, Mom, Dad & Andrés

A mi querida familia, Mamá, Papá y Andrés

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I. **Rollano-Peñaloza, O. M.**, Palma-Encinas, V. D., Widell, S., Rasmusson, A. G., and Mollinedo, P. A. 2019. The disease progression and molecular defense response in *Chenopodium quinoa* infected with *Peronospora variabilis*, the causal agent of quinoa downy mildew. *Submitted to PLOS One*. *bioRxiv,* 607465 DOI: 10.1101/607465.

II. **Rollano-Peñaloza, O. M.**, Widell, S., Mollinedo, P., and Rasmusson, A. G. 2018. *Trichoderma harzianum* T-22 and BOL-12QD inhibit lateral root development of *Chenopodium quinoa* in axenic co-culture. *Cogent Biology* 4:1-12.

III. **Rollano-Peñaloza, O. M.**, Widell, S., Rasmusson, A. G., and Mollinedo, P. A. 2019. Transcriptomic analysis of quinoa reveals a group of germin-like proteins induced by *Trichoderma*. Manuscript.

IV. **Rollano-Peñaloza, O. M.**, Palma-Encinas, V. D., Nogales-Ascarrunz, P., Widell, S., Rasmusson, A. G., and Mollinedo, P. A. 2019. First report of *Peronospora variabilis* causing downy mildew disease in cañahua (*Chenopodium pallidicaule*) in Bolivia. *Submitted to Plant Disease Notes*.

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Author contributions

I. O.M.R.P. and P.M. designed the study. O.M.R.P. P. M. and V.P. performed the experiments. O.M.R.P., V.P. and A.R. analyzed the data. O.M.R.P. wrote the paper with input from all authors who reviewed and approved the final manuscript.

II. A.R., P.M. and S.W. conceived the study. A.R. and O.M.R.P. designed the study. O.M.R.P. performed the experiments. O.M.R.P. and A.R. analyzed the data. O.M.R.P. and A.R. wrote the paper with input from all authors who reviewed and approved the final manuscript.

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IV. O.M.R.P. and P.M. designed the study. V.P. and P.N. performed the experiments. O.M.R.P., V.P. and P.N. analyzed the data. O.M.R.P. wrote the paper with input from all authors who reviewed and approved the final manuscript.

Abstract

Plants have developed complex molecular mechanisms to recognize and respond to the different microorganisms present in their surroundings. The most studied response mechanisms are plant defense responses. These defense response mechanisms share many similar components with the plant response mechanisms to beneficial microbes. Therefore, studying the defense response mechanisms against pathogens can contribute to the understanding of compatible and incompatible interactions with beneficial microbes.

In this work, the molecular interactions of quinoa with the pathogen *Peronospora variabilis,* causal agent of the downy mildew disease, and the interactions of quinoa with the beneficial biocontrol fungi *Trichoderma harzianum* were studied. Experimental systems for interaction experiments were developed and used, followed by morphological, biochemical and transcriptomic analysis. We describe the response of two quinoa cultivars to the infection of *P. variabilis* under controlled conditions. The quinoa cultivar Kurmi was more tolerant to *P. variabilis* infection than the Real cultivar, despite the lack of hypersensitive response. The defense response observed in the Kurmi cultivar might be mediated by the jasmonic acid signaling pathway. Cultivars that can trigger hypersensitive response are more resistant to *P. variabilis* than Kurmi and therefore a better selection for agriculture.

Quinoa in the presence of *Trichoderma* had variable outcomes depending on the growth conditions. We observed that quinoa growth was promoted in regular soil experiments or by interaction with *Trichoderma* volatile compounds in axenic co-culture. However, the growth of two quinoa cultivars was significantly inhibited by *T. harzianum* in axenic co-culture and in steamed soil experiments. The transcriptomic data of the quinoa growth inhibition by *Trichoderma* suggests activation of molecular signaling very similar to the signaling observed during defense response against pathogens. Further, we observed a specific group of quinoa plant defensins to be more rapidly induced by *Trichoderma* in a resistant cultivar but not in a susceptible one. These plant defensins showed a recent evolutionary expansion and could play a major role in providing pathogen resistance to certain quinoa cultivars.

In order to protect quinoa from *P. variabilis* infections by *Trichoderma* application and enhance the quinoa yields in agricultural systems might be necessary to perform compatibility tests between the *Trichoderma* biocontrol agents and the quinoa cultivars. These compatibility tests should be performed in regular soil.

Popular science summary

Plants have developed complex mechanisms to recognize and respond to the microorganisms present in their surroundings. The most studied response mechanisms are plant defense responses. Interestingly, the plant response to beneficial microbes shares many molecular compounds with the response to pathogenic microbes. Therefore, understanding plant defense responses against pathogenic microbes might contribute to a better understanding of plant responses to beneficial microbes. Thus, we could harvest the benefits from beneficial microorganisms and boost crop yields even further.

This work studied the quinoa plant defense responses triggered when infected with the fungus-like *Peronospora variabilis*, the pathogen causing the downy mildew disease in quinoa. We also studied quinoa responses to beneficial fungi of the genus *Trichoderma*. Finally, we did comparisons of the mechanisms activated during both interactions.

P. variabilis is a microbe which can not survive without a host plant (obligate biotroph). Therefore, new systems had to be designed to isolate *P. variabilis* and study quinoa-*Peronospora* interactions. The new systems allow studying the responses of different quinoa cultivars to the infection of *P. variabilis* under controlled conditions. Thus, we could observe that both cultivars were susceptible to the infection in a similar manner allowing *P. variabilis* to sporulate. Although the quinoa cultivar Kurmi was more tolerant to *P. variabilis* infection than the Real cultivar, Kurmi was not able to trigger programmed cell death, a defense mechanism that is normally activated in plants infected with obligate biotrophs. Quinoa within its large genetic diversity has quinoa varieties that can trigger programmed cell death against *P. variabilis*. The focus for breeding quinoa varieties resistant to *P. variabilis* should be on these varieties.

The *Trichoderma* genus has been known as a crop yield enhancer for decades and it is already commercially available. However, not all the interactions between *Trichoderma* and plants are beneficial. Certain cultivars have been reported to be negatively affected by the same *Trichoderma* strain that previously promoted growth in the same plant species. That is because outcome of the interactions between beneficial microbes and plants depends on their genetic compatibility and the environmental conditions.

In this work we have observed that *Trichoderma* can promote quinoa growth in regular soil. Such growth promotion was also observed when quinoa plants were exposed to volatile compounds from *Trichoderma* without physical interaction in sterile growth systems. However, the quinoa growth was severely inhibited when treated with *Trichoderma* in steamed soil and in experiments free from other organisms. The molecular mechanisms of the growth inhibition between *Trichoderma* and quinoa was studied by molecular global analysis. The results showed an activation of defense responses similar to responses observed against pathogenic microbes. Particularly a group of plant defense molecules with putative antimicrobial activity was induced in

the more resistant cultivar Kurmi. These molecules could be responsible for conferring resistance against pathogens into the resistant cultivars.

Trichoderma is already being applied in order to boost quinoa yields and prevent the downy mildew disease. However, those yields could be improved by performing compatibility tests between the quinoa cultivars of interest and the *Trichoderma* strains available. Our suggestion would be to perform these compatibility tests in regular soil.

Resumen de ciencia popular

Las plantas han desarrollado complejos mecanismos moleculares para reconocer y responder a los diferentes microorganismos con los que interactúan. Los mecanismos moleculares de respuesta más estudiados son los involucrados en la defensa contra patógenos. Sin embargo, éstos mecanismos moleculares comparten muchos componentes moleculares con los mecanismos de respuesta a organismos beneficiosos. Es por eso que entender los mecanismos moleculares de respuesta contra fitopatógenos puede contribuir a entender la compatibilidad o incompatibilidad que las plantas experimentan cuando interactúan con microorganismos beneficiosos.

En este trabajo, se estudiaron las interacciones moleculares de la quinua con el patógeno *Peronospora variabilis*, agente causante de la enfermedad del mildiu, y las interacciones de la quinua con el hongo biocontrolador *Trichoderma harzianum*. Sistemas experimentales para estudiar las interacciones fueron desarrollados y utilizados para realizar análisis morfológicos, bioquímicos y transcriptómicos. También se describen las respuestas de dos variedades de quinua a la infección con *P. variabilis* bajo condiciones controladas. La variedad Kurmi resultó más tolerante a la infección con *P. variabilis* que la variedad Real, a pesar de no poder generar muerte celular localizada en los tejidos que rodean al patógeno (respuesta hipersensitiva). Variedades de quinua que si puedan activar la respuesta hipersensitiva son mas resistentes al ataque de *P. variabilis* y serían la mejor opción para el cultivo de quinua en lugares propensos a la enfermedad del mildiu.

Trichoderma influyó el crecimiento de las plantas de quinua de varias formas dependiendo de las condiciones ambientales. En suelo común, *Trichoderma* promovió el crecimiento de las plantas de quinua. Ésta promoción de crecimiento también se observó cuando los compuestos volátiles emanados por *Trichoderma* interactuaron con las plantas de quinua en medios de cultivo estériles. Sin embargo, se observo que el crecimiento de las plantas de quinua fue significativamente inhibido por *T. harzianum* en medios de cultivos estériles y en suelo vaporizado. Los datos moleculares de la inhibición de quinua por *Trichoderma* sugieren que la planta de quinua está activando señales moleculares bastante similares a las señales observadas en la respuesta defensiva contra fitopatógenos. En especifico, se observo un grupo de proteínas inducidas por *Trichoderma* en el cultivo de quinua más tolerante a stress biótico que no fueron inducidas en el cultivo más susceptible. Éstas proteínas puede ser que tengan propiedades antibióticas, basados en estudios de su estructura molecular y podrían ser importantes en la promoción de resistencia contra patógenos en ciertos cultivos de quinua.

Trichoderma ya es utilizado para mejorar el rendimiento del cultivo de quinua y también como agente biocontrolador de la enfermedad del mildiu. Éstos rendimientos podrían ser mejorados realizando pruebas de compatibilidad entre las variedades de quinua utilizadas y agentes biocontroladores del género *Trichoderma*. Nuestra sugerencia sería realizar estas pruebas en suelo común.

Glossary

Axenic	Free from undesired living organisms.
Variety	A group of plants occurring in nature with a determined phenotype.
Cultivar	A plant variety selected and cultivated in large scale.
Landrace	A plant variety that only grows in a certain region.
Pathogen	An organism that causes disease into another organism.
Resistance	The capacity of an organism to prevent a disease.
Tolerance	The capacity of an organism to live with a disease.
Biotroph	An organism that feeds on living cells.
Obligate biotroph	A biotroph that can not survive without its host
Necrotroph	An organism that feeds on dead tissue.
Compatible interaction	A plant-pathogen interaction that benefits the pathogen growth.
Incompatible interaction	A plant-pathogen interaction that stops the pathogen growth.
Hypersensitive response	A programmed cell death in plants triggered upon pathogen recognition.
SA	Salicylic acid
JA	Jasmonic acid
ET	Ethylene
GLP	Germin-like protein
Genome	The entire genetic code found in an organism.
Transcriptome	The entire collection of expressed genes in an organism under specific conditions.

1. Introduction

Plants provide construction material, fuel, medicine and energy in the form of food and they are therefore one of the most important biological resources for humanity. Plant-related activities contribute significantly to human impact on the planet and the disruption of the fragile balance of nature (Mancini et al. 2016). Hence, good plant management strategies, as improvement of agricultural yields and diversification of our staple crops, will potentially stop the need of agricultural land extension, thus decreasing the actual deforestation rates and reducing our ecological footprint. Increasing the food supply worldwide is not going to be an easy task, given that The United Nations has estimated the population of our planet in the year 2050 to be almost 10 billions i.e. four times the world population in 1950 (UN 2015). According to the Food and Agriculture Organization (FAO) we will need to increase global food production of today by 70% to fulfill the food demand of 2050 (FAO 2016).

One of the proposed solutions to increase food production is to diversify our staple crops (Massawe et al. 2016). Across the world there are more than 50,000 edible plant species, yet just 15 of them account for 90% of the world food energy intake (Ji et al. 2013). The lack of diversity in edible species poses a risk to food security worldwide because all plant crops are constantly threatened by climate change and new pest emergences (Ordonez et al. 2015). Therefore, there is a need to increase the diversity of crops resistant to difficult environmental conditions. This situation has led us to work with quinoa, a plant ancestrally cultivated by the Native American population in the Andean plateau. Quinoa (*Chenopodium quinoa* Willd.) is widely studied for its interesting nutritional properties and resistance to harsh environments, thoroughly described for salinity and drought (Bertero 2003; Bhargava and Srivastava 2013; Jacobsen et al. 2003; Raney et al. 2014; Ruiz et al. 2014). Quinoa has gluten-free seeds with a high protein content that contains all essential amino acids, plus vitamins, antioxidants, fatty acids and minerals (Repo-Carrasco et al. 2003; Ruales and Nair 1992; Vega-Gálvez et al. 2010; Yao et al. 2014).

The United Nations designated 2013 as the international year of quinoa, to show the great potential of this crop. Although quinoa is resistant to abiotic stresses like salinity and drought, the crop may still suffer severe losses due to pathogen infections, especially due to downy mildew disease. It has been reported that farmers using susceptible quinoa cultivars (e.g. *C. quinoa* cv. Utusaya) in the Andean plateau can lose up to 90% of the crop yield (Danielsen et al. 2000). Downy mildew disease in quinoa is caused by the oomycete *Peronospora variabili*s, which is an obligate biotroph, *i.e.* in contrast to necrotrophs it can only take nutrients from living plant cells. This pathogen

grows and reproduces on quinoa leaves, reducing heavily the photosynthetic activity and inducing precocious flowering. Overall, *P. variabilis* causes noteworthy decreases in quinoa seeds yield (Choi et al. 2010; Choi et al. 2008; Danielsen and Munk 2004).

In countries that have decided to produce quinoa organically, plants are heavily affected by downy mildew disease (Danielsen et al. 2003). However, losses on the organic quinoa production can be reduced by application of beneficial microorganisms that can decrease the detrimental effects of the pathogens (Ortuño et al. 2013). Similar diseases, like downy mildew in grape caused by *Plasmopara viticola* (Perazzolli et al. 2012) and in snapdragon caused by *Peronospora antirrhini* have been successfully prevented by the application of beneficial fungi of the genus *Trichoderma* (Harman 2000). *Trichoderma* is a genus of common ascomycete fungi widely studied because their ability to antagonize plant-pathogenic fungi, bacteria, oomycetes, and nematodes. *Trichoderma* has also been reported to enhance plant growth in the absence of pathogens, enrich the nutrient availability in soils and induce plant systemic resistance (Druzhinina et al. 2011; Harman et al. 2008; Vinale et al. 2008; Vos et al. 2015). However, the mechanisms governing the beneficial effects of *Trichoderma* on plants have not been fully elucidated and especially for quinoa, little is known.

In this work we have studied the interactions of quinoa with *P. variabilis*, the downy mildew disease causal agent, and the quinoa interactions with the beneficial fungi *T. harzianum* BOL-12 and *T. afroharzianum* T22. Our aim is to find and understand the molecular responses of quinoa towards pathogenic and beneficial microorganisms. The understanding of these possibly common mechanisms can allow us to predict the outcome of different quinoa cultivars in response to certain beneficial and pathogenic microorganisms. Thus, we could generate strategies based on plant-microbe interactions to increase the yields of quinoa in agricultural systems.

2. Plant – microbial interactions

2.1. Plant-microbe recognition

Plants throughout its lifetime have to deal effectively with all kinds of microorganisms in their natural habitats. The outcome of interactions between plants and microorganisms can be very variable, from beneficial to detrimental. Therefore, plants have developed mechanisms to recognize microbial signatures that contain information about the microorganisms in their vicinity. These signatures are known as microbial associated molecular patterns (MAMPs) sometimes also called pathogen associated molecular patterns (PAMPs) in case they originate specifically from pathogens (Jones and Dangl 2006). Common examples of MAMPs are chitin from cell walls of fungi like *Rhizoctonia solani*, flagellin from bacteria like *Pseudomonas syringae* or B-glycans from cell walls of oomycetes like *Phytophtora infestans* (Wan et al. 2008).

A plant can distinguish beneficial microbes from pathogens because the plant has a large variety of molecular receptors on the plant plasma membrane surface that recognize type, concentration and cellular localization of MAMPs (Zipfel and Robatzek 2010). The structure and function of microbe recognition receptors of plants can be similar between symbiont and pathogen-response detection systems. For example, the symbiotic recognition receptor NOD FACTOR RECEPTOR1 (LjNFR1) in *Lotus japonicus* and the pathogen recognition receptor CHITIN ELICITOR RECEPTOR KINASE1 (AtCERK1) in *A. thaliana* are proteins with LysM domains and intracellular kinase domains to trigger their respective signaling cascades. In addition, both recognition receptors have to form homo- or heterodimers in order to recognize, either the Nod factor or chitin, respectively (Miya et al. 2007; Oldroyd 2013).

Plants have developed complex mechanisms to surveil and control the interaction with different microorganisms. For example, symbiotic colonization starts with plant roots releasing molecular signals that easily diffuse over short distances, e.g. strigolactones for attracting mycorrhizal fungi (Akiyama et al. 2005) and flavonoids for *Rhizobium* bacteria (Long and Staskawicz 1993; Peters et al. 1986). Target symbiont microbes recognize these signals and secrete MAMPs in response. In the case of mycorrhizal fungi, these diffusible signals are denoted as “Myc factors” (Bonfante and Genre 2010). The plants that have sent the initial signal recognize these MAMPs/Myc factors and activate signaling cascades to allow the recognized symbiont microbes to proceed with colonization of the tissue, usually roots in interactions with mycorrhizal fungi.

Interestingly, plant responses to beneficial microorganisms share many components with the defense response to pathogens (Contreras-Cornejo et al. 2016; Zeilinger et al. 2016). Some of this shared components include recognition receptors (Rey et al. 2015), transcription factors (Plett and Martin 2018) and proteins related to pathogenesis (Alizadeh et al. 2013). Given that plant defense responses to pathogens have been studied for a longer time, this knowledge is helping to understand the mechanisms of communication between plants and beneficial microorganisms.

In this work plant responses to microorganisms are classified according to the response time and type of molecular signaling into pre-existing or inducible responses.

2.2. Plant pre-existing responses mechanisms

Plant pre-existing response mechanisms can be defined as the plant response mechanisms triggered by microorganisms before the induction of gene expression. Pre-existing response mechanisms are usually activated within one day from microbe recognition and involve components already present in cells (e.g. production of reactive oxygen species) (Fig. 1).

The pre-existing response mechanisms activated after microbe recognition, both beneficial and pathogenic, are very similar. They usually involve the early production of reactive oxygen species (ROS), the activation of mitogen-activated protein kinase (MAPK) cascades and calcium-dependent protein kinase (CDPK) signaling pathways (Fig. 1). These responses will ultimately trigger gene expression and synthesis of new proteins that will induce specific responses against beneficial or pathogenic microbes.

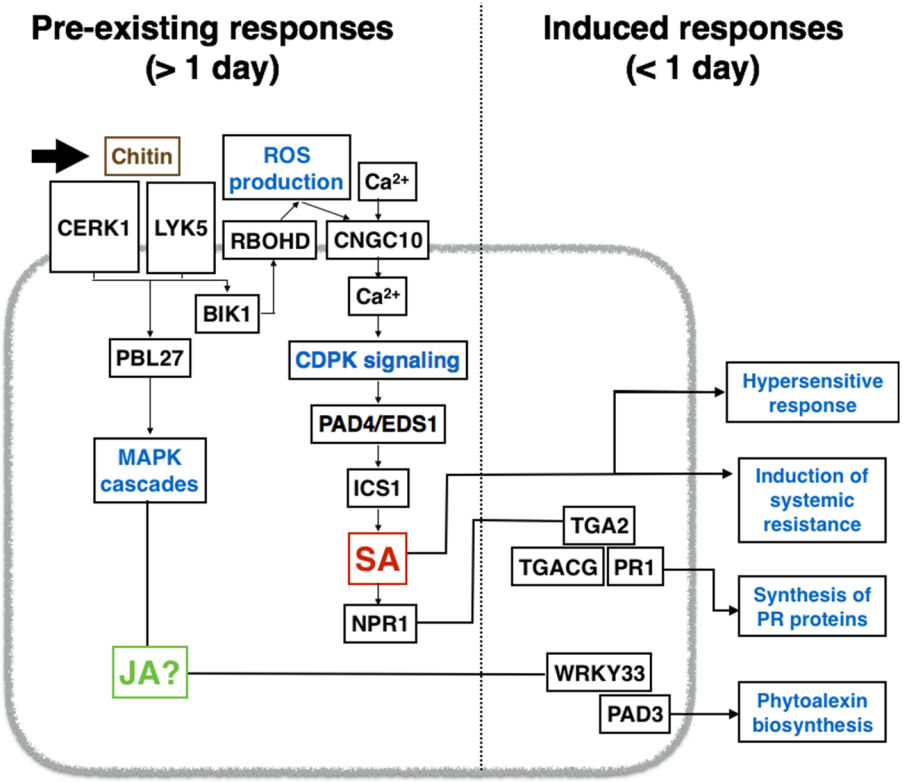

Figure 1 Plant responses to a fungal microbe in *Arabidopsis thaliana*.
The model plant *A. thaliana* responses to microorganisms can be classified according to the response time and type of molecular signaling into pre-existing or inducible responses. Pre-existing responses are triggered shortly after microbe recognition (e.g. chitin from fungi) and involve components already present in cells (e.g. MAMP-recognition proteins, plasma membrane protein kinases, cytoplasmic kinases, CDP-Kinases, MAP-Kinases and enzymes). Inducible responses are triggered by pre-existing responses and usually involve gene expression by activation of transcription factors and orchestrate complex defense response mechanisms (e.g. hypersensitive response, induction of systemic resistance, synthesis of PR proteins and biosynthesis of phytoalexins). CERK1, Chitin elicitor receptor kinase 1; LYK5, LysM-containing receptor-like kinase 5; PBL27, PBS1-like kinase 27; BIK1, *Botrytis*-induced kinase; MAPK, Mitogen-activated protein kinase; JA, Jasmonic acid; PAD4, Phytoalexin deficient 4; EDS1, Enhanced Disease Susceptibility 1; RBOHD, Respiratory Burst Oxidase homologue protein D; CNGC10, cyclic nucleotide-gated channel 10; CDPK, Calcium dependent protein kinase; ICS1, Isochorismate synthase; SA, Salicylic acid; NPR1, nonexpresser of PR genes 1; TGA2, transcription factor; PR1, Pathogenesis-related protein 1; WRKY33, transcription factor WRKY33. Sources: (Boudsocq et al. 2010; Caarls et al. 2015; Cao et al. 2014; Couto and Zipfel 2016; Kadota et al. 2015; Kawasaki et al. 2017; Meng and Zhang 2013; Oldroyd 2013; Pieterse et al. 2009; Zhang et al. 2010).

2.2.1. Early ROS production

Plant pre-existing response mechanisms are activated by recognition of MAMPs in the plasma membrane by MAMP receptors (e.g. *AtCERK1*). These membrane-associated proteins feature a kinase domain on the cytoplasmic side that allows the receptor to phosphorylate cytoplasmic kinases like *Botrytis* induced kinase 1 (*AtBIK1*). Cytoplasmic kinases then phosphorylate plasma membrane NADPH oxidases (e.g. *AtRBOHD*) that starts producing ROS extracellularly (Nanda et al. 2010). *Arabidopsis*

mutants lacking *AtRBOHD* have an impaired plant immune response, especially against biotrophic pathogens (Kadota et al. 2015).

Early ROS production in plants has been proposed to be the first response of plant defense because it is triggered within minutes of the initial recognition (Torres et al. 2006). ROS are directly toxic to microorganisms (Lambeth 2004). Early ROS production has been observed after recognition of pathogenic, symbiotic and opportunistic microorganisms (Kadota et al. 2015; Torres 2010; Tsuda and Katagiri 2010). However, the duration of ROS production after the initial interaction will differ between beneficial or pathogenic microorganisms. For example, *Arabidopsis* roots produce nitric oxide (NO) only for 10 min after being in contact with *Trichoderma asperelloides,* whereas the NO production continues for up to 120 min in response to the soil pathogen *Fusarium solani* (Gupta et al. 2014).

Early ROS production is also known as an inducer of calcium ion transport into the cell. Calcium ion concentration increase in the cytoplasm will act as a positive feedback regulator on late ROS production and will also lead to the activation of downstream signal components (Dubiella et al. 2013; Kadota et al. 2015; Torre et al. 2013) (Fig. 1).

2.2.2. CDPK signaling

Calcium-dependent protein kinases (CDPKs) are kinases which respond to elevated calcium ion concentrations. They phosphorylate transcription factors involved in plant response to both beneficial and pathogenic microorganisms (Figure 1). Most transcription factors phosphorylated by CDPKs are involved in regulating the synthesis of salicylic acid (SA), a hormone that plays an important role in plant defense against biotrophic pathogens (Coca and San Segundo 2010). Mutants of *Arabidopsis* with loss of CDPK function are more susceptible to pathogens than its wild-type counterpart (Boudsocq et al. 2010; Dubiella et al. 2013). Additionally, overexpression of a major CDPK (*AtCPK1*) leads to an overall accumulation of SA, turning plants resistant to a broad diversity of pathogens (Coca and San Segundo 2010). Intracellular oscillations in calcium ion concentration are used by plants to detect both symbiotic and pathogenic microorganisms (Ehrhardt et al. 1996; Vadassery and Oelmüller 2009; Walker et al. 2000). Oscillation of free cytoplasmic calcium concentration is also known as calcium spiking and it has been described as a central signaling component to monitor plant colonization in symbiotic interactions (Ehrhardt et al. 1996; Oldroyd 2013; Ranf et al. 2011; Thor and Peiter 2014).

2.2.3. MAPK cascades

Alongside ROS production and CDPK signaling, MAPK cascades takes place as one of the earliest responses after MAMP recognition (Meng and Zhang 2013). MAMP receptors can directly phosphorylate cytoplasmic kinases that will phosphorylate a

diverse set of initial MAPKK kinases that eventually will start the different MAPK cascades (Figure 1). For example, *AtCERK1* recognizes chitin and phosphorylates the cytoplasmic PBS1-like kinase 27 (*AtPBL27*). *AtPBL27* will then phosphorylate MAPKKK 5 and trigger the MAPK3/6 cascade (Kawasaki et al. 2017; Shinya et al. 2014). Genes are activated by the MAPK cascades through transcription factors or enzymes, that can reprogram gene expression or can control synthesis of different defense molecules. For example MAPK cascades activate the transcription factor *AtWRKY33* that eventually activates the cytochrome P450 enzyme (*AtPAD3*) that catalyzes the last two steps in camalexin biosynthesis (Lemarié et al. 2015; Meng and Zhang 2013).

MAPKs and CDPKs target transcription factors that activate the plant inducible responses.

2.3 Plant inducible response mechanisms

Inducible plant responses mechanisms can be defined as response mechanisms activated by pre-existing response mechanisms that will trigger gene expression and orchestrate complex response mechanisms (e.g. programmed cell death). Inducible responses usually occur one day after microbe recognition (Fig. 1). Plants will trigger different types of inducible responses depending on the potential damage associated with the microorganism detected. Mild pathogen infection and beneficial microorganism colonization will usually trigger responses like cell wall modification, synthesis of plant defense compounds and synthesis of systemic immune signals. Stronger pathogen infection may additionally trigger the induction of host cell death at the infection site, a response also known as hypersensitive response (HR) (Couto and Zipfel 2016; Shinya et al. 2014; Yamaguchi et al. 2013).

2.3.1. Cell wall modification

Cell wall modification is known as the first inducible response (Collinge 2009). Plant defense against microorganisms usually implies the creation of cell wall appositions (CWA) named papilla. CWA are physical barriers created for the plant to avoid microbe penetration (Doehlemann and Hemetsberger 2013). Callose is one of the major components of the cell wall and its deposition can be induced in response to detrimental fungi through callose synthases like *AtPMR4* (Bellincampi et al. 2014). *AtPMR4* overexpression enhances resistance against penetration of *Alternaria brassicicola* (causal agent of powdery mildew) in *A. thaliana* (Ellinger et al. 2013). Lignin may also play a key role for plant defense against microbes because lignin biosynthesis impairment negatively affects plants resistance towards powdery mildew in monocots (Bhuiyan et al. 2009). Modifications of the cell wall like callose depositions have also

been observed when the beneficial fungi *Trichoderma* has attempted to colonize plant roots (Yedidia et al. 1999).

2.3.2. Synthesis of plant defense compounds

Besides compounds that strengthen the cell walls, plants also synthesize a wide variety of other defense compounds upon microbe recognition. The largest groups are the secondary metabolites know as phytoalexins (Hammerschmidt 1999) and the pathogenesis-related (PR) proteins (van Loon et al. 2006).

Phytoalexins are low molecular weight compounds with different chemical structures that are produced by different plants families and that inhibit the growth of microbes, usually by disrupting cell membranes (Hammerschmidt 1999). Examples of popular phytoalexins are capsidiol and capsaicin from chilli peppers (Maldonado-Bonilla et al. 2008) and resveratrol from grapevines.

One of the most studied phytoalexin is camalexin, a tryptophan-derived secondary metabolite synthesized by plants in the Brassicaceae family that includes the model plant *A. thaliana*. Studies have found a higher camalexin concentration in *A. thaliana* ecotypes resistant to *Hyaloperonospora arabidopsidis* (Ws-3) than susceptible ecotypes (Col-0) (Mert-Türk et al. 2003). The concentration of camalexin can increase upon exposure to pathogen elicitors (Jeandet et al. 2010; Rogers et al. 1996). Furthermore, camalexin biosynthesis has been described to be induced by the beneficial fungi *Trichoderma* (Contreras-Cornejo et al. 2011; Kottb et al. 2015).

The most studied group of phytoalexins in the Cariophyllales order are the betalains, which confers the reddish color to beetroot, amaranth, cactus and quinoa (Jarvis et al. 2017; Kujala et al. 2001; Tang et al. 2015). Phytoalexins from the Cariophyllales order can also be flavonoids like betavulgarin and betagarin which have antigfungal properties and are know to be induced by fungal infection (Martin 1977).

PR proteins are polypeptides categorized into 17 types, having molecular masses between 5 and 75 kDa. PR proteins have different biological functions which are well detailed by Sels et al. (2008). Some PR proteins are enzymes targeting components of the microbe cell walls or cell membranes and they can be partially redundant. For example, when PR-10 was silenced in Medicago truncatula other PR proteins were induced and the pathogen tolerance was even increased (Colditz et al. 2007). PR proteins are usually induced upon pathogen challenges (van Loon et al. 2006). Further, PR proteins can be induced (Perazzolli et al. 2012) or repressed upon interaction with beneficial fungi as well (Morán-Diez et al. 2012).

2.3.3. Hormones and systemic defense signaling

Infected plants rapidly synthesize diffusible signals in the form of hormones that trigger defense at systemic level and prevent upcoming attacks. Plants after recognizing

MAMPs/PAMPs can transmit signals from different tissues to all parts of the plant, *i.e.,* the signals are systemic. The systemic defense signals that are mostly described in the literature are the plant hormones ethylene, salicylic acid (SA) and jasmonic acid (JA) (Pieterse et al. 2009).

SA has been described to have a central role in plant disease resistance against biotrophic pathogens (e.g. *H. arabidopsidis* Noco infecting *A. thaliana Col-0*) (Delaney et al. 1994). The impairment of SA production makes plants susceptible to biotrophs because such plants are less able to trigger programmed cell death, and thus halt the growth of the pathogen. On the contrary, impairment of SA production does not increase the susceptibility of plants against necrotrophic pathogens (e.g. *Botrytis cinerea* infecting *A. thaliana* Col-0)(Thomma et al. 1998). These are instead primarily mediated by JA signaling (Thomma et al. 1998). JA signaling induces expression of several types of defense proteins with antimicrobial properties (e.g. chitinases, glucanases, defensins) which contribute to counteract the growth of necrotrophic pathogens but do not trigger HR (Pieterse et al. 2009).

Recently it has been shown that defense responses against biotrophic pathogens, traditionally known to be mediated only by the SA response pathway, can also induce synthesis of the JA hormone and genes involved in the JA-defense response pathways during the first 24 hours after infection (e.g. grapevines infected with the biotroph *Plasmopara viticola*) (Guerreiro et al. 2016). Further, certain genes (e.g. *VvWRKY33*) that were known traditionally to confer protection only against necrotrophic pathogens are now known to confer resistance also to biotrophs (Merz et al. 2015).

The regulation between the activation of the SA- and the JA-mediated defense response pathways to trigger an optimal immune response can vary between different cultivars of the same species, as it has been described for wild grapes (Yin et al. 2017).

2.3.4. Hypersensitive response

The localized programmed plant cell death at the infection site of a plant is known as hypersensitive response (HR). This is a common plant response after biotrophic pathogen attacks and it confines the pathogen by isolating it from the living tissues that are essential for the survival of the pathogen (Jellouli et al. 2008). HR is not effective against necrotrophic attacks because necrotrophs thrive on dead tissues. Consistently, the experimental impairment of plant enzymes involved in HR have been shown to increase plant resistance against necrotrophs (Marino et al. 2012).

The initiation of HR is complex and highly regulated. For example, in Vitis cell lines, it has been observed that the activation of HR to trigger cell death demands the activation of MAPKs along with the presence of the plant phytoalexin resveratrol (Chang et al. 2011). The late ROS response also plays a role in the activation of the HR. The late ROS response is prolonged compared to the early ROS response and creates the right environment to start HR. ROS and SA also diffuse to adjacent cells to further

trigger cell death (Tsuda and Katagiri 2010; Zurbriggen et al. 2014). Ultimately, when all the conditions to induce HR are fulfilled, certain plant proteases (metacaspases) are induced and start the protein degradation that eventually leads to the plant cell death (Coll et al. 2011).

2.4 Acquired and induced systemic resistance

Induced defense responses can orchestrate the activation of defense responses at systemic level to prevent upcoming attacks of pathogens in plant tissues distal from the infection site. The induction of systemic resistance can be triggered by pathogenic and nonpathogenic microbes. When the systemic resistance is activated by a pathogenic microbe signal, this phenomenon is known as systemic acquired resistance (SAR) (Durrant and Dong 2004). For example, cucumber plants can activate a systemic defense response after being attacked by the hemibiotrophic microbe (e.g. *Colletotrichum lagetarium*) and simultaneously trigger HR at the infection site. Therefore, the subsequent infections by *C. orbiculare*, at sites distal from the initial infection (e.g. upper part leaves) will have reduced impact and minor damages on the plant (Metraux et al. 1990). The protection against subsequent infections provided by SAR can be against pathogens different from the pathogen that did the first infection (Smith and Métraux 1991). Another form of systemic resistance is activated by nonpathogenic microbe signals and is known as induced systemic resistance (ISR) (Pieterse et al. 2014).

ISR can provide future protection against several pathogens (Pieterse et al. 2014). ISR was first described to be promoted by bacteria (Van Peer et al. 1991; Wei et al. 1991) and later on by several other microbes (Van Loon et al. 1998). Among them the beneficial fungi *Trichoderma harzianum* T39 which was able to induce ISR that is active against *Botrytis cinerea* on bean (*Phaseolus vulgaris*) (Bigirimana et al. 1997). Further, molecules isolated from nonpathogenic microbes alone have been shown to activate ISR. For example, infiltration of cellulases isolated from *Trichoderma longibrachiatum* enhanced melon (*Cucumis melo*) resistance against powdery mildew disease (caused by *Sphaerotheca fuliginea)* (Martinez et al. 2001). ISR has become an important research area in the quest to successfully prevent diseases in agriculture by activation of natural plant defenses (Pieterse et al. 2014).
The molecular mechanisms behind ISR signaling are not yet completely understood. However, the mechanisms resemble that of signaling pathways triggered by pathogenic microbes. Though ISR generally has been linked to JA and ethylene signalling (Walters 2011), ISR activation in cucumber occurred after an increase of the JA and SA hormone concentrations observed 6 hours after the treatment with *Trichoderma asperellum* T34 (Segarra et al. 2007). Therefore, it is likely that both the SA and JA-modulated defense pathways can be involved in the activation of plant ISR (Van Wees et al. 2000).

Induced resistance by either elicitor molecules or microorganisms has been proposed to be inherited by the progeny. ISR induced by B-aminobutyric acid (BABA) in *Solanum physalifolium* (a weed relative to potato) against *Phytophthora infestans* was transmitted to the S2 generation by non-genetic inheritance (Lankinen et al. 2016). ISR induced by *Trichoderma atroviride* in tomato plants against plant-parasitic nematodes has also been reported to be inherited to the second generation (Medeiros et al. 2017). Nevertheless, the type of plant response differs with every microorganism species that interacts. The plant response to the pathogenic genus *Peronospora* and to beneficial *Trichoderma* fungi will be described below with a special focus on quinoa plants.

3. Plant - *Peronospora* interactions

3.1 Biology of *Peronospora variabilis*

Peronosporaceae is a family of oomycetes (protist water molds) comprising 19 genera. *Peronospora*, *Plasmopara* and *Hyaloperonospora* are the three most studied groups, all of which contain only obligate biotrophs. They parasite specific host plants, causing the so-called downy mildew disease, characterized by heavy sporulation in the basal part of the leaves (Thines and Choi 2016).

One of the most studied oomycetes that causes downy mildew disease is *Hyaloperonospora arabidopsidis* (Peronosporaceae). *H. arabidopsidis* is a natural pathogen of *A. thaliana* (Coates and Beynon 2010; Kamoun et al. 2015). Given that the biology of the species in the Peronosporaceae clade is very similar, most of the *P. variabilis* knowledge is based on studies of *H. arabidopsidis* (Thines and Choi 2016).

P. variabilis is thought to have two main phases in their life cycle, the asexual phase, in which sporangia are quickly propagated on leaves, and the sexual phase, in which oospores are produced to ensure reproduction. The infection normally begins in crop fields with oospores that have survived the winter in the soil. Once oospores sense favorable conditions and signals from the host plant, they germinate and colonize the plant through its roots (Kamoun et al. 2015). The new hyphae colonizes the plant roots, travels through the hypocotyl via the xylem, and reach the leaves where they begin their establishment (Holub 2006; Yadeta and Thomma 2013). Hyphae grow and develop in the leaves until they produce sporangiophores and thus, start the asexual life cycle.

The beginning of the asexual life cycle in *P. variabilis* features enormous production of sporangia (Figure 2). Sporangia that land on leaves will start germination under high humidity conditions (>80%). Sporangia germinate consistently within 12 h (Choudhury and McRoberts 2018). Thereafter, germinated sporangia will start forming hyphae until developing appressoria. The appressorium is a specialized structure which penetrates the plant cuticle, allowing hyphae to grow inside the leaf intercellular spaces (Koch 1990). Hyphae will continuously grow and develop haustoria, which are structures for feeding and host defense suppression (Catanzariti et al. 2007), until they reach the abaxial cuticle and come out through the stomata. Then they proceed to develop reproductive structures called sporangiophores that produce the asexual sporangia (Figure 2).

Depending on the environmental signals, hyphae can also differentiate into male (paragynous anteridia) or female (oogonia) gamete structures for sexual reproduction. Then, differentiated hyphae will fuse to produce sexual oospores. The oospores have the capability to survive a winter or dry season and thus wait quiescently for the next season to start a new life cycle (Koch 1990; Thines and Choi 2016).

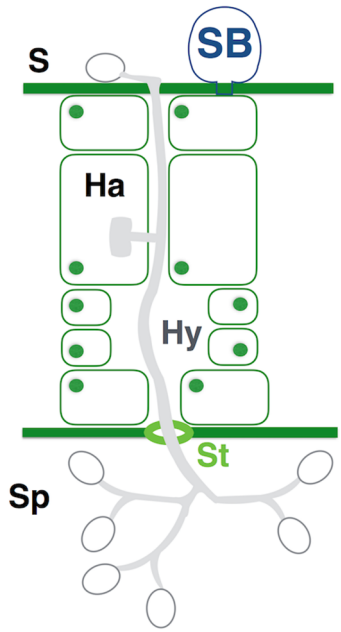

Figure 2 Model of the growth of *P. variabilis* in quinoa leaves in compatible interactions.
Proposed model of growth of *P. variabilis* in compatible interaction with quinoa after the landing of sporangia on the upper side of the leaf until the development of new asexual sporangia. S, sporangia; SB, salt bladder; Ha, haustorium; Hy, hypha; St, Stoma; Sp, mature sporangiophore.

3.2 Plant responses to Peronosporaceae pathogens

The response of plants to Peronosporaceae pathogens is highly variable, even within the same species. Plant varieties that allow pathogens to complete their life cycle inside the plant host are known as susceptible varieties (i.e. hosts in compatible interactions). Plants that have the ability to impede the life cycle of the pathogen are known as resistant varieties (incompatible interactions). For example, *A. thaliana* Col-0 can be successfully colonized by *H. arabidopsidis* isolate Waco9 or Noco2 allowing sporulation in less than 4 days, as an example of a compatible interaction (Asai et al. 2014). In contrast, varieties of *A. thaliana e.g.,* Wassilewskija (Ws) or Niederzenz (Nd) will trigger immediate HR, and thus halt the growth and colonization of *H. arabidopsidis,* thereby being an incompatible interaction (Holub et al. 1994).

During incompatible interactions, resistant plants trigger defense responses usually involving HR and this appears to be mediated by the SA defense response pathway. Supporting this evidence, molecular markers for the SA defense response pathway are induced at 1 dpi during incompatible interactions (*AtPR1, AtPAD4)*. However, other genes that are known to be part of the standard SA defense response pathway are not always significantly changed 1 dpi (e.g. *AtNPR1*, *AtEDS1, AtGRX480)* (Asai et al. 2014).

The defense response behind compatible interactions with necrotrophs has been traditionally known to be mediated by the JA pathway (Pieterse and Van Loon 1999). However, the defense responses during compatible interactions with biotrophs has been less studied, because most focus has been on defense responses to biotrophic pathogens in incompatible interactions. Studies show that the defense response during compatible interactions in general takes longer time to be activated than during incompatible interactions (Pritsch et al. 2000; Venisse et al. 2002). Defense responses during compatible general interactions usually involve production of antimicrobial compounds and chlorosis without triggering HR and might be mediated by the JA signaling pathway. The activation of the JA signaling pathway is supported by the observation that external JA application can enhance the plant tolerance against biotrophic pathogens in compatible interactions (Cohen et al. 1993). Further, genes involved in the JA pathway (*AtHSP90*, *AtWRKY33*, *AtPR4* and *AtPDF1.2)* are also significantly changed in *A. thaliana* plants during compatible interactions with biotrophic pathogens but not changed during incompatible interactions (Asai et al. 2014). Similarly, quinoa genes (*CqHSP83*, *CqWRKY33* and *CqPR4)* involved in the JA defense response pathway were significantly induced during a compatible interaction with *P. variabilis* (**Paper I**).

The JA response observed in compatible interactions with biotrophs might be sporadic because it was induced only at 3 dpi and not at 1 dpi or 5 dpi in a thorough transcriptomic study of the interactions between *A. thaliana* and *H. arabidopsidis* (Asai et al. 2014). This observation could explain why the literature is inconsistent about the activation of the JA defense pathway during compatible interactions with biotrophs. The SA defense response pathway seems to be activated also during compatible interactions, although with a significantly delayed response, as compared to during incompatible interactions (3 dpi vs 1 dpi, respectively) (Asai et al. 2014). Our data and bibliographic revision suggest that the defense response against biotrophic pathogens in compatible interactions might be mediated by both the JA and the SA signaling cascades.

Plant cultivars which are resistant to pathogen infections (incompatible interaction) are economically important for agriculture. However, for quinoa, there are not detailed descriptions of the response of the different cultivars against *P. variabilis*, thus the compatibility of the cultivars with the pathogen is lacking (Julio et al. 2012; Khalifa and Thabet 2018). The lack of data on *Peronospora*-quinoa interactions might be due to the biotrophic nature of *P. variabilis*, which makes the isolation of the pathogen more complicated than for organisms that can be cultivated *in vitro*. Therefore, we developed

methodology to collect, describe and maintain *P. variabilis,* which should facilitate the study of downy mildew disease in quinoa. Of special importance was the inclusion of a fungicide in the infection protocol. We describe the responses of two cultivars under controlled conditions. We found that none of the quinoa cultivars studied was able to trigger HR when infected with *P. variabilis*. Despite the lack of HR, one of the cultivars (Kurmi) showed a higher tolerance than another (Maniquena Real) to the infection. This might be due to the expression of defense-related genes that might be involved in the JA defense pathway (**Paper I**).

Although the Kurmi cultivar has higher tolerance to the infection than Real it can still suffer major losses on the fields. Therefore, quinoa-breeding programs are focusing on breeding cultivars that can trigger HR. Quinoa cultivars that have the ability to trigger HR against *P. variabilis* have been observed in quinoa fields (Danielsen and Ames 2000)(Gabriel Julio, *pers. comm,* Proinpa, Cochabamba, Bolivia). Such landraces could be used to breed cultivars that can trigger HR against *P. variabilis*.

We have found HR responses in Chenopodiaceae against *P. variabilis* in cañahua (*Chenopodium pallidicaule,* Aellen), a cultivated close relative of quinoa (**Paper IV**). Cañahua wild landraces can be found in fields where quinoa is cultivated in the Andes. Further, the cañahua genome is one of the ancestral diploid genomes that was part of the hybridization that quinoa have undergone to become a tetraploid millions of years ago (Jarvis et al. 2017; Kolano et al. 2016). Due to its simpler diploid genome, we believe cañahua may be a good model for quinoa to study *P. variabilis* infections at molecular level in the Chenopodiaceae subfamily.

4. Plant - *Trichoderma* interactions

4.1. *Trichoderma* development in agriculture

Trichoderma was first reported as a fungal parasite of other fungi (Weindling 1932) and was successfully utilized for control of the phytopathogen *Rhizoctonia solani* on citrus seedlings a few years later (Weindling and Fawcett 1936). Nevertheless, the agricultural potential of *Trichoderma* remained unexploited until the late 70s when other *Trichoderma* strains were shown to antagonize several fungal phytopathogens (Elad et al. 1980; Hadar 1979). The application of *Trichoderma* spores to crop seeds to control soil-borne diseases in replacement of harmful pesticides has become an important agriculture alternative. This has created a demand for bioproducts based on *Trichoderma* that boosts the development of new strains with biocontrol properties. One new strain was produced by protoplast fusion of two *Trichoderma harzianum* agents (T12 & T95). The new strain was denominated as *Trichoderma harzianum* T22 and induced significant increase in the yields of several crops like cotton, cucumber, pea, snap bean, sweet corn and wheat (Harman et al. 1989; Stasz et al. 1988). Recently, T22 was claimed to belong to a different species taxon within the same *T. harzianum* clade and to be named *Trichoderma afroharzianum* T22 (Chaverri et al. 2015). *Trichoderma* can contribute to agricultural improvements by plant-growth promotion, induction of systemic resistance and by direct phytopathogen antagonism (Contreras-Cornejo et al. 2016). Antagonism is one of the most studied aspects of *Trichoderma* due to its potential in agriculture and its ease to study it in isolation.

4.2. Phytopathogen antagonism

Several species of *Trichoderma* have the ability to antagonize detrimental microorganisms and nematodes by nutrient competition, mycoparasitism and antibiosis (Contreras-Cornejo et al. 2016). Antagonism ability depends on the capacity to dynamically secrete extracellular enzymes and a highly diverse set of secondary metabolites (Druzhinina et al. 2011). Extracellular enzymes (*e.g.* proteases, chitinases, beta-glucanases, etc.) are usually secreted for cell wall/membrane degradation of the prey (Kubicek et al. 2011; Lorito et al. 1993). Secondary metabolites are the chemical arsenal for attack and defense during antibiosis (e.g. harzianic acid, trichodermin, gliotoxin, peptaibols etc.). The most studied class of *Trichoderma* secondary metabolites are peptaibols, *i.e.,* peptides of 500 – 2200 Da produced by non-ribosomal

synthesis and typically containing non-standard amino acids like alpha-aminoisobutyric acid and an C-terminal amino-alcohol (Montesinos 2007). There are more than 300 peptaibols described so far (http://peptaibol.cryst.bbk.ac.uk/home.shtml) from different *Trichoderma* species. Most peptaibols have antibiotic properties targeting the plasma membrane of the prey. For some peptaibols, the functions remain unknown, but they might be involved in endogenous processes like conidiation and signaling during symbiosis (Kubicek et al. 2007; Whitmore and Wallace 2004). By antagonizing undesired microorganisms, *Trichoderma* aids in pest management of pathogen-infested fields, thus allowing healthy growth of different crops (Harman 2011). For example, *T. harzianum* can antagonize the corn disease causal agent *Fusarium graminearum* up to 96% during *in vitro* tests and completely remove the aboveground disease symptoms in soil trials (Saravanakumar et al. 2017).

The ability of *Trichoderma* to antagonize pathogens observed in lab experiments (e.g. plate confrontation assays) shows the potential of *Trichoderma* to increase production on agricultural fields. However, the pathogen antagonizing efficiencies in soil are yet difficult to assess and therefore have not been estimated to the same extent.

4.3. Plant-growth promotion

A plentitude of plant crop species have been reported to show enhanced growth when treated with *Trichoderma* in agricultural fields (Harman et al. 1989; Maag et al. 2013; Tucci et al. 2011; Yedidia et al. 2001), including quinoa crops (Ortuño et al. 2013; Ortuño et al. 2016). One of the most studied *Trichoderma* strains that stimulate significant plant growth of several crops is *T. harzianum* T22 (later renamed to *T. afroharzianum*) (Chaverri et al. 2015; Harman 2011; Harman et al. 1989).

Three mechanisms behind *Trichoderma*-promoted plant growth have been proposed. The first one is that the fungus enhances solubilization of nutrients in the soil to make them more accessible for plant nutrition (Altomare et al. 1999). However, strains that can solubilize phosphate and produce siderophores do not always enhance growth (Contreras-Cornejo et al. 2009; Hoyos-Carvajal et al. 2009).

The second proposed mechanism is the activation of plant growth-regulating signals by fungal metabolites. Molecules obtained from *Trichoderma* that are claimed to be the responsible for plant growth promotion are secreted analogue compounds to phytohormones like indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), and indole-3-ethanol (IEt)) (Contreras-Cornejo et al. 2016). However, strains that produce analogue compounds of phytohormones do not usually promote plant growth (Hoyos-Carvajal et al. 2009). The activation of plant growth-regulating signaling by fungal metabolites was thought to be the most likely mechanism of action because of results from axenic systems, where *Trichoderma* was able to induce plant growth promotion in media cultures rich in nutrients (Contreras-Cornejo et al. 2009). However, plant growth

promotion in axenic conditions was only achieved in short co-cultivation times and when *Trichoderma* was placed at a considerable far distance from the seedlings (Contreras-Cornejo et al. 2009) (Figure 3). Axenic co-cultures of quinoa and *Trichoderma* for longer than a week and with *Trichoderma* placed as near as to be on top of the roots, showed instead a significant inhibition of quinoa seedlings growth (**Paper II** and Figure 3). This growth inhibition was even shown by the commercially available biocontrol strain T22 (**Paper II**). Further revision of the literature revealed other *Trichoderma* strains that cause growth inhibition during longer co-culture times, especially in axenic hydroponic cultures (Alonso-Ramírez et al. 2014; Nogueira-Lopez et al. 2018; Pelagio-Flores et al. 2017). One of the possible explanations for the growth inhibition in axenic co-cultures was suggested by Pelagio-Flores et al. (2017) during studies of the interaction between *Trichoderma atroviride* and *A. thaliana*. The authors reported that acidification produced by *Trichoderma* inhibits plant growth and buffering the system can reverse growth inhibition but they have not measured *Trichoderma* effects in co-cultivations longer than 4 days (Pelagio-Flores et al. 2017). We have observed that pH in the media is acidified by *Trichoderma* the first days and then goes back to near neutral (pH = 6) after 5 days, when *Trichoderma* starts to conidiate (**Paper 2**; Suppl. Figure 1). Therefore, activation of plant growth-regulating signaling by fungal metabolites should be studied in systems with growth conditions more closely related to soil.

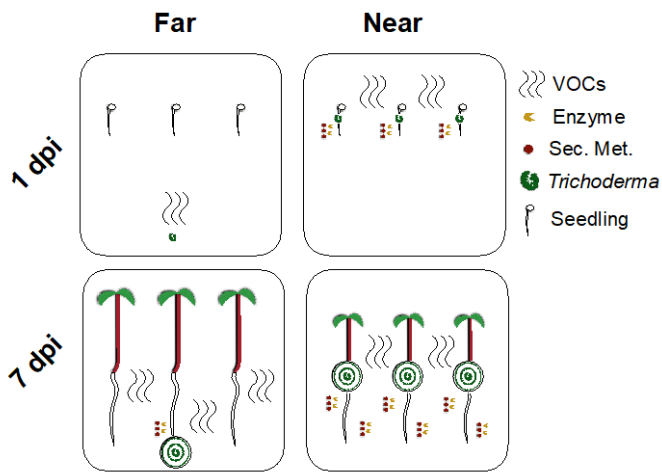

Figure 3 The time and proximity of *Trichoderma* to plants generate different growth outcomes in axenic co-cultures.
Plant growth promotion in axenic co-cultures can be achieved by placing *Trichoderma* at a considerable distance from the plant seedlings. Because of the far distance, the growth promoting VOCs from *Trichoderma* are the first compounds to interact with the seedlings. Further, the physical interactions are only achieved after a few days when the roots seedlings reach *Trichoderma* mycelium and only a few enzymes and secondary metabolites interact. On the contrary, if *Trichoderma* is interacting with the roots from the beginning of the co-cultivation, it will release enzymes and secondary metabolites that may negatively affect the roots from the beginning of the interaction, resulting in growth inhibition under axenic conditions.

The third and most recent mechanism of action described is the plant growth promotion by volatile organic compounds (VOCs) synthesized by *Trichoderma*. Even single VOCs (e.g. 1-octen-3-ol) produced by *Trichoderma* have been shown to promote plant growth (Hung et al. 2013). Other VOCs, like six-pentyl-2*H*-pyran-2-one have been shown to promote plant growth to different plants e.g. *A. thaliana* (Contreras-Cornejo et al. 2014) and tomato (Lee et al. 2016). In preliminary experiments (Figure 4), volatile compounds from *T. harzianum* BOL-12 resulted in stimulated growth in quinoa in tow cases out of four tested, whereas application of BOL-12 directly on the root resulted in quinoa growth inhibition (**Paper 2**).

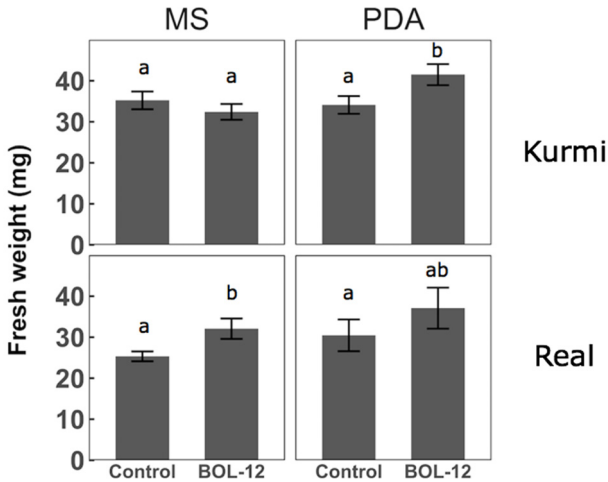

Figure 4 Quinoa growth promotion mediated by Trichoderma VOCs in axenic media. Volatile compounds were released from *Trichoderma* culture plates physically separated from the plants in a system that only allowed gas-phase interchange (*n* = 9). BOL-12 was grown in two different culture media 0.1X MS (Murashige & Skoog) and Potato Dextrose Agar (PDA). Quinoa plants were grown in 0.1X MS culture medium supplemented with agar [0.8 g/L]. Data shows means ± SE per treatment. Statistically significant differences are denoted with different letters.

In these experiments, the effects of *T. harzianum* BOL-12 on the plants where thus different depending on the culture conditions under which the fungus was growing. Volatiles from *Trichoderma* are variable depending on its growth culture media (Nieto-Jacobo et al. 2017), meaning that the effect produced by *Trichoderma* depends on the nutrient conditions available for the fungi. Nonetheless, no correlation between a single VOC of *Trichoderma* and plant growth promotion of *A. thaliana* has been observed when comparing the VOC profiles of 20 *Trichoderma* isolates (Lee et al. 2016). Therefore, the combination and concentration of VOCs released by *Trichoderma* might be the key to understand its plant growth promotion effects.

4.4 Induced systemic resistance

The plant response to *Trichoderma* can have different outcomes that will vary from growth promotion to growth inhibition depending of the environmental conditions. One of the more recently described outcome of the interaction between plants and *Trichoderma* is the ISR. De Meyer et al. (1998) showed that the application in soil of *T. harzianum* T39 spores to the roots of tomato, bean, lettuce, pepper and tobacco conferred protection against the necrotropic pathogen *B. cinerea* in the leaves. Later, it was observed that the induction of ISR in *A. thaliana* by *T. atroviride* was mediated by the SA- and JA-mediated defense response pathways at the same time (Contreras-Cornejo et al. 2011). The expression of marker genes of both SA and JA pathways peaked after 96 h and conferred protection against the necrotroph *B. cinerea* and the hemibiotroph *Pseudomonas syringae* for at least two weeks (Salas-Marina et al. 2011).

ISR is a complex process with multiple compounds acting simultaneously. For instance, *Trichoderma* mutants lacking cellulases (Saravanakumar et al. 2016) or mutants lacking the proteinaceous elicitor SM2 (Crutcher et al. 2015) have each partially lost the ability to induce systemic resistance in maize, showing that multiple fungal components might be involved in the induction of ISR in the plant.

Single compounds isolated from secretions of *Trichoderma* such as the small protein Sm1 from *T. virens* induced systemic resistance in cotton and maize (Djonović et al. 2006; Djonović et al. 2007). Studies on the Sm1 and Epl1 from *T. atroviride* gave a glance of the high abundance of cerato-platanins in *Trichoderma*, which are secreted elicitors found only on fungi, beneficial and pathogens alike (Baccelli 2014; Lamdan et al. 2015; Seidl et al. 2006).

The induction of systemic resistance can be triggered by compounds isolated from the secretome of *Trichoderma*. For example, cellulase from *Trichoderma viride* but not from another species was shown to confer resistance to plants against the otherwise harmful peptaibol alamethicin (Aidemark et al. 2010). However, studies of global gene expression on the induction of SR in plants by a single secreted *Trichoderma* compound are so far lacking.

Trichoderma VOCs have also been shown to be inducers of plant systemic resistance against different pathogens. For example, 6-pentyl-α-pyrone (6PP) can induce systemic resistance against *Alternaria brassicicola* and *B. cinerea* in low concentrations but at higher concentrations instead inhibits plant growth (Kottb et al. 2015).

4.5 Plant-*Trichoderma* compatibility

The beneficial outcome of treating plants with *Trichoderma* has been shown to be dependent on the plant cultivar. Studies in tomato (Tucci et al. 2011) and maize (Harman 2006; Nogueira-Lopez et al. 2018) have shown growth promotion and inhibition by the same *Trichoderma* strain. Similar variation in the outcome holds for the compatible and incompatible interactions observed between plants and pathogens (Bell et al. 1986). Further, the *A. thaliana sid2* mutant which is impaired in the SA-defense pathway displays severe growth inhibition when treated with *Trichoderma*. These plants also exhibit *Trichoderma* colonization in leaf vascular tissues as well as root rot, symptoms typical of infections with pathogenic fungi (Alonso-Ramírez et al. 2014). Boosting the plant immune system by application of SA to *A. thaliana* plants delays the colonization of *T. harzianum* T78 but the application of JA instead promotes colonization reaching vascular tissues (Martínez-Medina et al. 2017).

The fact that *Trichoderma* can inhibit plant growth in axenic co-cultures suggests the possibility that *Trichoderma* can inhibit plant growth in soil under certain conditions. Therefore, to better understand the growth inhibition observed under axenic co-cultures we decided to perform RNA-seq analyses of quinoa roots in the presence of *Trichoderma.* Here, we observed that quinoa roots are activating genes involved in defense response when directly interacting with *Trichoderma*, especially in the cultivar Kurmi (**Paper III**). The results suggest that quinoa is activating both the SA- and the JA-mediated defense signaling cascades. We suggest this to be the defense response signaling that helps *Trichoderma* to activate the ISR and prevent upcoming attacks by pathogens.

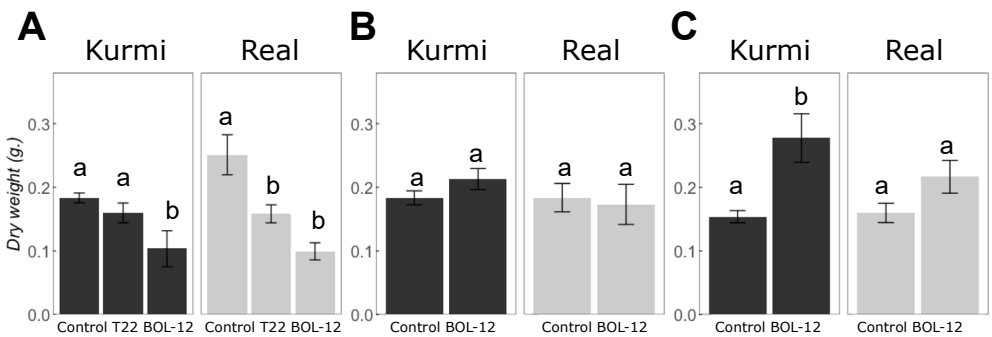

Figure 5 Different outcomes on the interaction between quinoa and *Trichoderma* depending of the growth system and treatment.
The interaction between *Trichoderma* and quinoa was analysed: (A) Quinoa growth inhibition of T-22 and BOL-12 on shoot growth in *C. quinoa* plants at 35 dpi in steamed soil (n = 4). Seeds were surface-sterilized and were coated with a spore suspension [$1*10^6$ sp/ml] and incubated overnight before sowing. (B) Quinoa growth (17 dpi) was not affected by late *Trichoderma* treatment in regular soil. Roots were inoculated with 3 ml of *Trichoderma* spore suspension [$1*10^6$ sp/ml] two weeks after germination (n = 5). (C) Quinoa growth promotion by *Trichoderma* BOL-12 at 35 dpi in regular soil. Quinoa seeds were coated with a spore suspension [$1*10^6$ sp/ml] and incubated for 1 h before sowing (n = 5-9). Data are representative of at least two individual experiments. Data show means ± SE per treatment. Statistically significant differences are denoted with different letters

Finally, in order to better understand the variability of growth effects by *Trichoderma* in quinoa plants we decided to assess the effects of *Trichoderma* on quinoa plants in different soil conditions. Preliminary experiments performed in steamed soil indicated that the quinoa growth was inhibited by our *Trichoderma* strains (Figure 5A). The same *Trichoderma* strains previously promoted growth by VOC exposure and generated growth inhibition in axenic co-cultures (Figure 3 and 4). However, when growing quinoa plants in regular soil with the same *Trichoderma* strains, we instead observed growth promotion (Figure 5C) or no effect (Figure 5B) based on the type of treatment that the plants were exposed to. Thus, we are observing a variable effect by the same plant-*Trichoderma* interaction depending on the co-culture system.

5. Conclusions and future remarks

Plants display a large variation in outcomes when being attacked by pathogens. The progression of these interactions depends on the genotype of the host as well as the pathogen. For example, quinoa plants can have several degrees of resistance or susceptibility to *P. variabilis*, depending on its genotype. The variation in the plant defense responses is mediated by the signaling pathways that each genotype can activate. The genotypes that were assessed in this study were compatible with the isolated *P. variabilis* strain, but not to the same extent. We found that the defense response of quinoa plants during biotrophic compatible interactions may be mediated by the JA signaling pathway (**Paper I**). The literature suggests that the defense response against biotrophic pathogens in compatible interactions might be mediated by both the JA and the SA signaling cascades. Therefore, we suggest performing hormone treatment analysis with JA and SA to verify that the *Arabidopsis*-ortholog genes found to be significantly changed in our experiments are truly induced by JA. Perhaps measuring the hormone levels of SA and JA during compatible and incompatible interactions can further validate the results.

Resistance of quinoa against *P. variabilis* can be better achieved by selecting quinoa genotypes which can activate the SA signaling pathway, as usually observed for certain plant genotypes during incompatible interactions with biotrophic pathogens. Quinoa genotypes that activate the SA pathway have been found in crop fields in the Andes. We suggest selecting quinoa genotypes that can trigger HR against *P. variabilis* through the SA signaling pathway and study its molecular defense response in order to find better markers of resistance against the downy mildew disease. An alternative can be to study the defense responses of genotypes that can trigger HR in the close-related species cañahua (*C. pallidicaule*), given its diploid genome (**Paper IV**).

Similar to plant–pathogen interactions, plant interactions with beneficial microbes also show large variations depending on the genotypes interacting. Such variation in the outcomes is even larger, given that they can oscillate from growth promotion to severe growth inhibition. For example, the beneficial fungi *T. harzianum* BOL-12 can enhance quinoa plant growth and the same *Trichoderma* strain can inhibit quinoa plant growth depending on the plant genotype and the particular growth conditions (**Paper II**). Our results indicate that the activation of plant growth-regulating signaling by fungal metabolites during root-hypha interactions might not be the principal mechanism of *Trichoderma* plant growth promotion. Thus, the results from our axenic studies (**Paper II** and Figure 4) suggest that *Trichoderma* main mechanism of plant growth promotion might be caused by the release of volatile compounds (Figure 3). However, more

research needs to be done to validate these statements. We suggest performing plant-*Trichoderma* co-cultures in soil removing the VOCs from the environment to validate our hypothesis.

Trichoderma, otherwise known to be a beneficial microbe, inhibited quinoa growth in axenic co-cultures (**Paper II**). The molecular signaling behind this interaction shows partial activation of genes involved in the defense against pathogens (**Paper III**). Some of these genes were also induced in quinoa interactions with the pathogen *P. variabilis* (**Paper I**). This indicates that quinoa response to beneficial microbes and pathogens may share its molecular machinery with many similar compounds. The main difference in the response might be in the recognition of microbe signals. To further understand the similarities in quinoa response to beneficial and pathogen microbes we suggest performing a transcriptomic study of the changes of quinoa interacting with *P. variabilis* and compare with our transcriptomic data.

Trichoderma possess the ability to induce systemic resistance in quinoa. Studying the induction of systemic resistance against *P. variabilis* in quinoa might reveal key molecular compounds to understand the beneficial effects of *Trichoderma*. Further, the signals activated during this interaction can reveal key insights of the molecular signaling involved in the resistance of quinoa to *P. variabilis*, especially if *Trichoderma* can induce the SA-mediated defense response and produce HR. From our results presented here, we suggest that induction of SA-mediated defense response by *Trichoderma* could be answered by using cultivars that can and cannot trigger HR against *P. variabilis.* The *Trichoderma* treatment suggested would be seed drench. Finally, we suggest a global gene analysis of quinoa cultivars that can trigger HR pre-treated with *Trichoderma* and infected with *P. variabilis* to recognize a shared response pathway between pathogenic and beneficial microorganisms.

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About the back cover

Molecular structure of Germin 1 from barley (Oxalate oxidase: P45850)[1].
This structure was generated by SWISS-MODEL[2].

[1]Woo, E.-J., Dunwell, J. M., Goodenough, P. W., Marvier, A. C., and Pickersgill, R. W. 2000. Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nature Structural & Molecular Biology* 7:1036.

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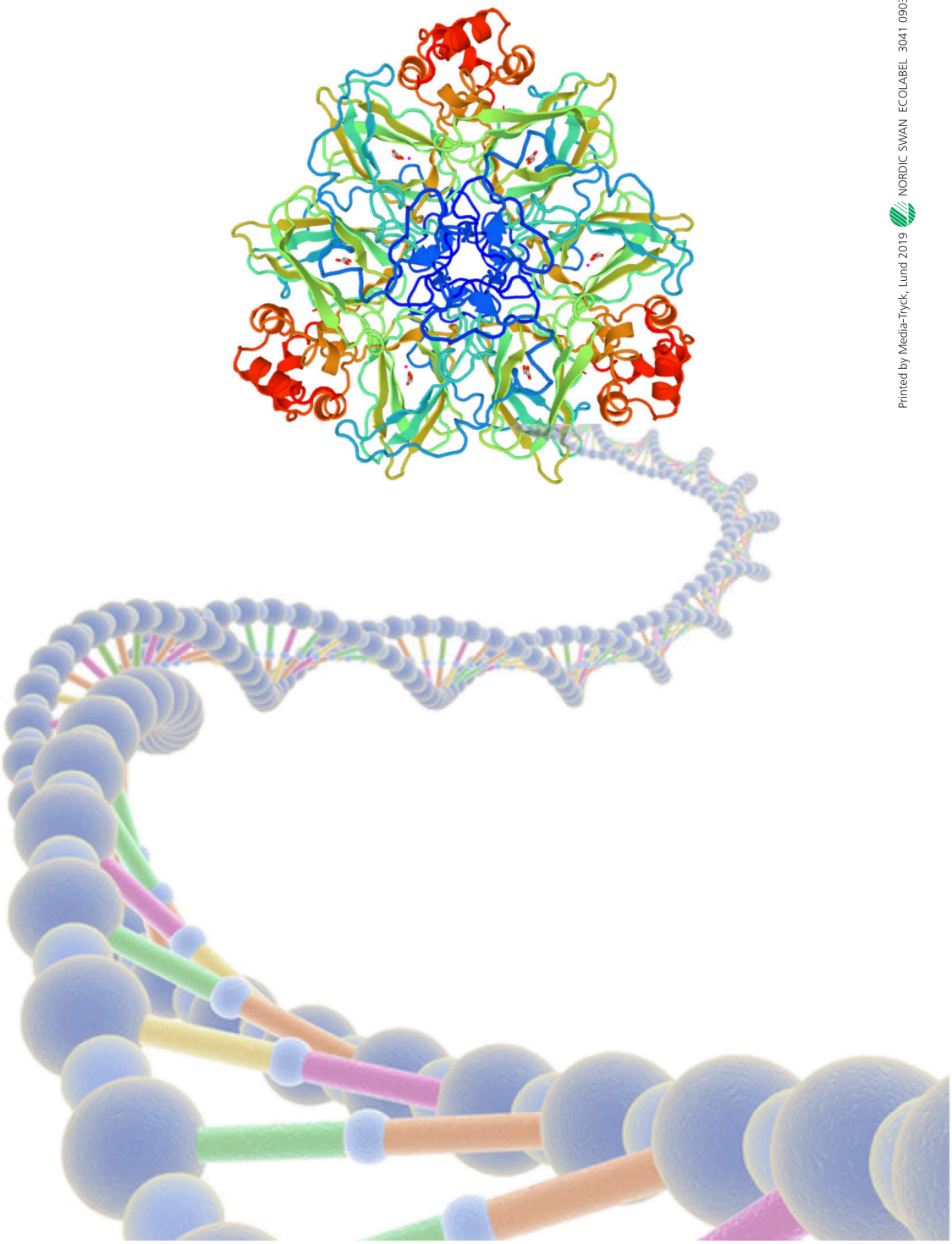

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