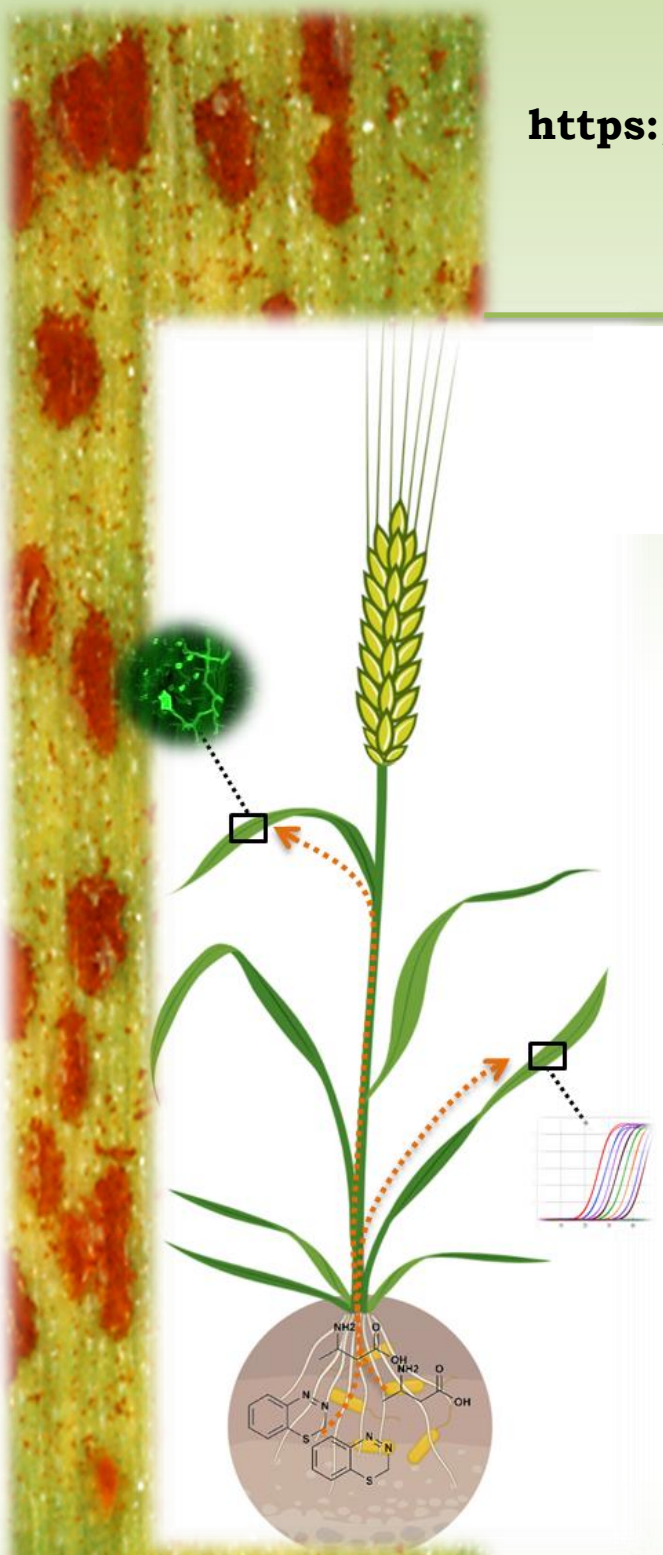
https://doi.org/10.35662/unine-thesis-2819



Induced resistance in wheat

A dissertation submitted to the University of Neuchâtel for the degree of Doctor in Naturel Science by

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"La résistance induite chez le blé"

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Summary

During evolution, plants have developed a variety of chemical and physical defences to protect themselves from stressors. In addition to constitutive defences, plants possess inducible mechanisms that are activated in the presence of the pathogen. Also, plants are capable of enhancing their defensive level once they are properly stimulated with non-pathogenic organisms or chemical stimuli. This phenomenon is called induced resistance (IR) and it was widely reported in studies with dicotyledonous plants. However, mechanisms governing IR in monocots are still poorly investigated. Hence, the aim of this thesis was to study the efficacy of IR to control wheat diseases such leaf rust and Septoria tritici blotch. In this thesis histological and transcriptomic analysis were conducted in order to better understand mechanisms related to IR in monocots and more specifically in wheat plants.

Successful use of beneficial rhizobacteria requires their presence and activity at the appropriate level without any harmful effect to host plant. In a first step, the interaction between *Pseudomonas protegens* CHA0 (CHA0) and wheat was assessed. Our results demonstrated that CHA0 did not affect wheat seed germination and was able to colonize and persist on wheat roots with a beneficial effect on plant growth.

Once we showed the absence of side effects of CHA0 on wheat plants, in the second step, we evaluate efficacy of CHA0 or β-aminobutyric acid (BABA) to induce resistance against leaf rust caused by the biotroph *Puccinia triticina* in wheat. Our results confirmed the capacity of CHA0 to control leaf rust at the seedling stage. BABA showed dose-dependent reduction of leaf rust infection accompanied with plant growth repression at 20 mM. Balancing between protection and growth repression, a concentration of 15 mM was chosen as suitable dose for leaf rust control. Defence reactions such as callose deposition and H_2O_2 regeneration involved in the observed resistance were investigated. Both treatments reduced fungal penetration and haustoria formation of *P. triticina* with differences in timing and amplitude, leading to different levels of resistance to leaf rust. IR in wheat was accompanied with high deposition of callose and the accumulation of H_2O_2 during fungal infection, showing their importance in mechanisms involved in this resistance.

To deeply clarify differences and similarities between CHA0- and BABA-IR at transcriptomic level, in the third step, the expression level of defence-related genes was analysed by RT-qPCR during IR induced by CHA0 and BABA against leaf rust. A correlation between induction of genes and *P. triticina* infection events was observed. A clear difference between the two

induced responses is that BABA target more defence-related genes compared to CHA0 treatment.

The last step was to evaluate the two mentioned elicitors (CHA0 and BABA), *Pseudomonas chlororaphis* PCL1391 and Benzothiadiazole (BTH) for their ability to induce resistance in wheat against the hemibiotrophic fungus *Zymoseptoria tritici*. Only BABA efficiently enhanced plant resistance to *Z. tritici*.

In conclusion, exploiting IR might be a prominent strategy to control wheat disease. However, its effectiveness depends on the combination inducer/pathogen. Arguably, CHA0 bacteria and BABA induce similar defence reactions leading both to enhanced levels of resistance. However, only BABA enhanced defences against the hemibiotroph *Z. tritici*, suggesting that resistance of the plants react to the lifestyle of the pathogen and IR enhances does not involve necessarily all of them. More understanding is needed on both on capacity of the inducer to induce and the plant to become induced.

Key words : induced resistance, wheat, *Pseudomonas* ssp., β-aminobutyric acid, Benzothiadiazole, *Puccinia triticina*, *Zymoseptoria tritici*.

Résumé

Au cours de l'évolution, les plantes ont développé une variété de défenses chimiques et physiques pour se protéger contre les facteurs de stress. En plus de ces défenses constitutives, les plantes possèdent des mécanismes inductibles qui sont activés en présence du pathogène. Les plantes sont aussi capables d'améliorer leur niveau défensif une fois qu'elles sont correctement stimulées par des organismes non pathogènes ou des stimuli chimiques. Ce phénomène est appelé résistance induite (RI) et il a été largement rapporté dans des études avec des plantes dicotylédones. Cependant, les mécanismes dirigeants la RI dans les monocotylédones sont encore peu étudiés. Par conséquent, l'objectif de cette thèse était d'étudier l'efficacité de la RI pour lutter contre les maladies du blé telles que la rouille brune et le septoriose. Dans cette thèse, des analyses histopathologiques et transcriptomiques ont été menées afin de mieux comprendre les mécanismes liés à la RI chez les monocotylédones et plus spécifiquement chez le blé.

L'utilisation réussie de rhizobactéries bénéfiques exige leur présence et leur activité au niveau approprié sans aucun effet nocif pour la plante hôte. Dans un premier temps, l'interaction entre *Pseudomonas protegens* CHA0 (CHA0) et le blé a été évaluée. Nos résultats ont démontré que CHA0 n'affectait pas la germination des graines de blé et pouvait coloniser et persister sur les racines de blé avec un effet bénéfique sur la croissance des plantes.

L'absence d'effets secondaires du CHA0 sur les plantes de blé ayant été établie, dans la deuxième étape, nous avons évalué l'efficacité du CHA0 ou de l'acide β-aminobutyrique (BABA) pour induire la résistance contre la rouille brune causée par le champignon biotrophe *Puccinia triticina* chez le blé. Nos résultats ont confirmé la capacité de CHA0 à contrôler la rouille brune au stade plantules. BABA a montré une réduction dose-dépendante de l'infection par la rouille brune accompagnée d'une répression de la croissance des plantes à une concentration de 20 mM. En équilibrant entre la protection et la répression de la croissance, une concentration de 15 mM a été choisie comme la dose appropriée pour lutter contre la rouille brune. Les réactions de défense telles que le dépôt de callose et la génération de H_2O_2 impliquées dans la résistance observée ont été étudiées. Les deux traitements ont réduit la pénétration fongique et la formation d'haustoria de *P. triticina* avec des différences de temps et d'amplitude, conduisant à différents niveaux de résistance à la rouille brune. La RI chez le blé s'est accompagnée d'un dépôt élevé de callose et de l'accumulation de H_2O_2 lors de l'infection fongique, montrant leur importance dans les mécanismes impliqués dans cette résistance.

Pour clarifier en profondeur les différences et les ressemblances entre la RI par CHA0 et BABA au niveau transcriptomique, dans une troisième étape, le niveau d'expression de gènes liés à la défense a été analysé par RT-qPCR. Une corrélation entre l'induction des gènes et des événements d'infection du champignon *P. triticina* a été observée. La différence entre les deux réponses induites est que le BABA cible plus de gènes liés à la défense que le traitement CHA0.

La dernière étape a consisté à évaluer les deux éliciteurs mentionnés (CHA0 et BABA), *Pseudomonas chlororaphis* PCL1391 et Benzothiadiazole (BTH) pour leur capacité à induire la résistance du blé contre un autre champignon hémibiotrophe, le *Zymoseptoria tritici*. Seul BABA a amélioré efficacement la résistance des plantes à *Z. tritici*.

En conclusion, l'exploitation de la RI pourrait être une stratégie importante pour contrôler les maladies du blé. Cependant, son efficacité dépend de la combinaison inducteur/pathogène. On peut dire que les bactéries CHA0 et BABA induisent des réactions de défense similaires conduisant à la fois à des niveaux accrus de résistance. Cependant, seulement BABA a amélioré les défenses contre l'hémibiotrophe *Z. tritici*, ce qui suggère que la résistance des plantes réagit au mode de vie du pathogène et que la RI ne les implique pas nécessairement toutes. Une meilleure compréhension est nécessaire à la fois sur la capacité de l'inducteur à induire et sur la plante à devenir induite.

Mots clés : résistance induite, blé, *Pseudomonas* ssp., acide β-aminobutyrique, Benzothiadiazole, *Puccinia triticina*, *Zymoseptoria tritici.*

Acknowledgements

I would like to add a few words of appreciation to people who have supported and helped me to accomplish this work.

First of all, I want to thank my thesis directors who were more than generous in giving help. I am especially grateful to Prof. Brigitte Mauch-Mani, for her trust, even before she knows me, when I decided to apply for Swiss Scholarships for Foreign Students; she gave me the opportunity to live this scientific adventure of in her team. Many thanks to her for being always supportive for new ideas, and always open for scientific discussions. Also, I express my special thanks to Dr. Fabio Mascher for all the support, scientific inputs, precious piece of advice in writing the articles and opening the doors to work in Agroscope, not only for that, also for giving me the chance to participate in my first international congress in Italy.

I would also like to acknowledge Prof. Daniel Croll (University of Neuchâtel) and Prof. Victor Flors (Universitat Jaume I, Spain) for taking time to be part of my thesis committee.

I would like to acknowledge all the people I had the chance to work with during this project, namely Chiara Pedrazzini, Mohamed Jasim and Mahnaz Katouzi. Without them; it would not have been possible to accomplish this thesis.

For their technical support advice, I would like to thank Sophie, Guillaume and Ivan for showing me the scientific methods used in the lab. To handle pathogens used in this thesis, I thank Stefan Kellenberger, (Agroscope Changins), Leen Abraham and Nikhil Kumar (University of Neuchâtel). I would also thank Prof. Christophe Keel (University of Lausanne) for providing biological material used in this project.

A very special thank goes to my brother-in-lab Francesco Stefanelli for being kind and supportive during this adventure and for all his scientific, cultural, politic discussions. My thanks go to everybody who supported me through their great friendship, namely Carla, Marie, Andrea, and all the past and present lab members as well as all the kind people from the other labs in Neuchâtel.

Special thanks go to my family, especially to my parents, for being always in my service whenever I need a help. I am grateful to them for supporting me throughout good and bad times. Finally, I have not enough words of “thanks” to the lady of my life who supported me in all the aspects during these years, my biggest thanks to my wife Zina for her patience to be far from her during this life experience. This thesis is dedicated to my little boy Moayed.

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CHAPTER I

General introduction

Chapter I: General introduction

Part I. Biological and chemical plant resistance inducers: novel alternative to control wheat fungal diseases.

I.1. Wheat

Among crop production, cereals are the most cultivated crops and have a prominent role in food and energy requirements of a large part of world's population. Practically half of the cereal production is dependent on rice, maize, and wheat which serve as leading sources of energies and primary food (Shiferaw et al., 2011). In term of global food security, wheat has played an outstanding role in feeding a hungry world. The crop contributes about 20 % of the total dietary calories and proteins worldwide (Shiferaw et al., 2013).

Origin and importance of cultivated wheat

The first cultivation of wheat occurred about 10,000 years ago, as part of the 'Neolithic Revolution', which saw a transition from hunting and gathering of food to settled agriculture (Shewry, 2009). These earliest cultures mainly included diploid and tetraploid wheats and their genetic relationships indicate that they originated from the south-eastern part of Turkey (Dubcovsky & Dvorak, 2007). Around 9000 years ago, hexaploid bread wheat was first seen with spread of cultivation to the Near East (Feldman, 2001). Since then, wheat has become one of the main food sources of humanity (Shewry & Hey, 2015). Today, cultivated wheat belongs to the genus *Triticum*, which groups together several species with varying levels of ploidy. The most cultivated ones are hexaploid (common or bread wheat, *T. aestivum*) and diploid (durum wheat, *T. turgidum* spp *durum*). In 2017, 218 million hectares of agricultural land were devoted to wheat crops in the world, leading to the production of 771 million tonnes of wheat, making it the second largest crop in the world behind maize (http://www.fao.org/faostat, accessed 15 January 2020). Bread wheat accounts for 95% of the world production of cultivated wheat, while the majority of the remaining 5% is used for durum wheat. This predominance can be explained by the great genomic plasticity of bread wheat, which makes it possible to cultivate it in the majority of the agricultural areas in the world, and by its high yield potential (Shewry, 2009). Globally, the average yield was 34 quintals/hectare in 2017 (http://www.fao.org/faostat, accessed 15 January 2020). Its success depends on the gluten protein fraction which confers the viscoelastic properties that allow dough to be processed into bread, pasta, noodles, and other food products. Wheat also contributes essential amino acids, minerals, and vitamins, and

beneficial phytochemicals and dietary fibre components to the human diet, and these are particularly enriched in whole-grain products (Shewry, 2009).

Bread wheat genome

Bread wheat is a hexaploid species (2n = 6× = 42, AABBDD) that has arisen by further hybridization and spontaneous chromosome-doubling events during evolution (Snape & Pánková, 2001) as illustrated in Fig. 1A which also shows examples of spikes and grain. The hexaploid bread wheat is a combination of 3 interrelated diploid genomes originating from the diploid progenitors *T. urartu* (AA), the relative of *Aegilops speltoides* (BB) and *T. tauschii* (DD) (El Baidouri et al., 2017). Less than one million years ago emmer wheat *T. turgidum*, an tetraploid with AABB genomes became into existence. Finally, 10,000 years ago hybridization between *T. turgidum* and *T. tauschii* gave rise to the hexaploid *T. spelta*, which after domestication and selection, resulted in cultivated bread wheat *T. aestivum* (Petersen et al., 2006; Venske et al., 2019).

In comparison to other cereals, the bread wheat genome is considered as one of the largest genomes with a size of ~17000 Mb (Brachypodium 355 Mb, rice 375 Mb, barley 5500 Mb, maize 2365 Mb) (Salse et al., 2009). It is known to be a very complex with high proportion of repetitive sequences (~80%) compared to rice (22%) and maize (~50%), which make wheat genomic research more complicated compared to other cereals or dicots (Borrill, 2019; Linkiewicz et al., 2004; Whitelaw et al., 2003).

Morphology and development of wheat plant

Wheat is an annual herbaceous plant in the *Poacae* (*Gramineae*) family and settles in the *Triticeae* subfamily, with defined growth, having an inflorescence in dense terminal spike and a dry fruit also called caryopsis. Wheat is a grass cereal of 0.5 to 1.5 m tall, grown for its grains. It consists of a vegetative system (aerial system and root system) and a reproductive system (inflorescence) (Fig. 1B). Plant characteristics are the following (Kirby, 2002):

Shoot: This is made up of a series of repeating units or phytomers, each potentially having a node, an elongated internode, a leaf, and a bud in the axil of the leaf.

Tillers: They have the same basic structure as the main shoot; arise from the axils of the basal leaves. Each leave comprises the sheath, wrapping around the subtending leaf, and a lamina (blade). At the junction of the sheath and lamina, there is a membranous structure, the ligule, and a pair of small, hairy projections, the auricles.

Inflorescence: The wheat inflorescence is called a spike or ear, where the grains are born. This is a simply branched structure in which the major axis (rachis) bears two rows of alternating secondary axes (rachilla, the axis of spikelet).

Roots: Two types of roots are found, the seminal roots and the nodal roots (adventitious or crown roots), which arise from the lower nodes of the shoot.

The developmental stages of wheat are well characterized by BBCH scale (Meier, 1997) as described in Fig. 1C. Plant development starts with seed germination and growth of the first leaves as the leaf lamina are unfolded. It is followed by the 2-leaf stage (BBCH 12; Meier, 1997). plants at this stage were mainly used in our experiments. A ramification of the main shoot starts giving different tillers. Later, the stem elongates, the upper internodes extend and grow, and the ear grows and finally pushes through the flag-leaf sheath until complete emergence. Finally, flowering starts and is followed by the grain filling phase. The vegetative growth phase from a single seed results in a plant composed of several ear-bearing shoots at anthesis.

I.2. Wheat disease caused by fungal pathogens

Many biotic and abiotic factors are responsible for agricultural losses. Among abiotic factors, limited soil resources, lack or excess of water, non-optimal temperatures for growth, or problems related with soil salinity are found. But, 20 to 40% of yield losses are currently attributed to biotic factors (Savary et al., 2012). Seven to ten percent of losses are attributed to invasive weeds, 8 to 15% are attributed to pests (insects, nematodes, birds …) and 11 to 13% are attributed to pathogens (fungi, bacteria, viruses) (Oerke, 2006). Among the three categories of pathogens, fungi are the most important in terms of crop damage. A study conducted with 495 plant pathologists from the Molecular Plant Pathology network identified a list of 10 most damaging pathogenic fungi, based on their scientific and economic importance. Among these 10 fungi, six are cereal pathogens (Dean et al., 2012). Four of these six are known to be particularly virulent on wheat: *Puccinia* ssp., *Blumeria graminis*, *Zymoseptoria tritici* and *Fusarium graminearum* (Singh et al., 2016). Due to the distribution, frequency of appearance and levels of epidemic development that they can reach, it is considered that those with economic importance are fungal diseases that affect leaf tissues such as leaf rust caused by *Puccinia triticina* (Herrera-Foessel et al., 2011; Piasecka et al., 2015), and *Septoria* tritici blotch caused by *Zymoseptoria tritici* (Eyal, 1987; Fones & Gurr, 2015). Their importance can vary according to the year, the region of cultivation and according to other factors such as climate, environmental conditions and the susceptibility of the cultivars.

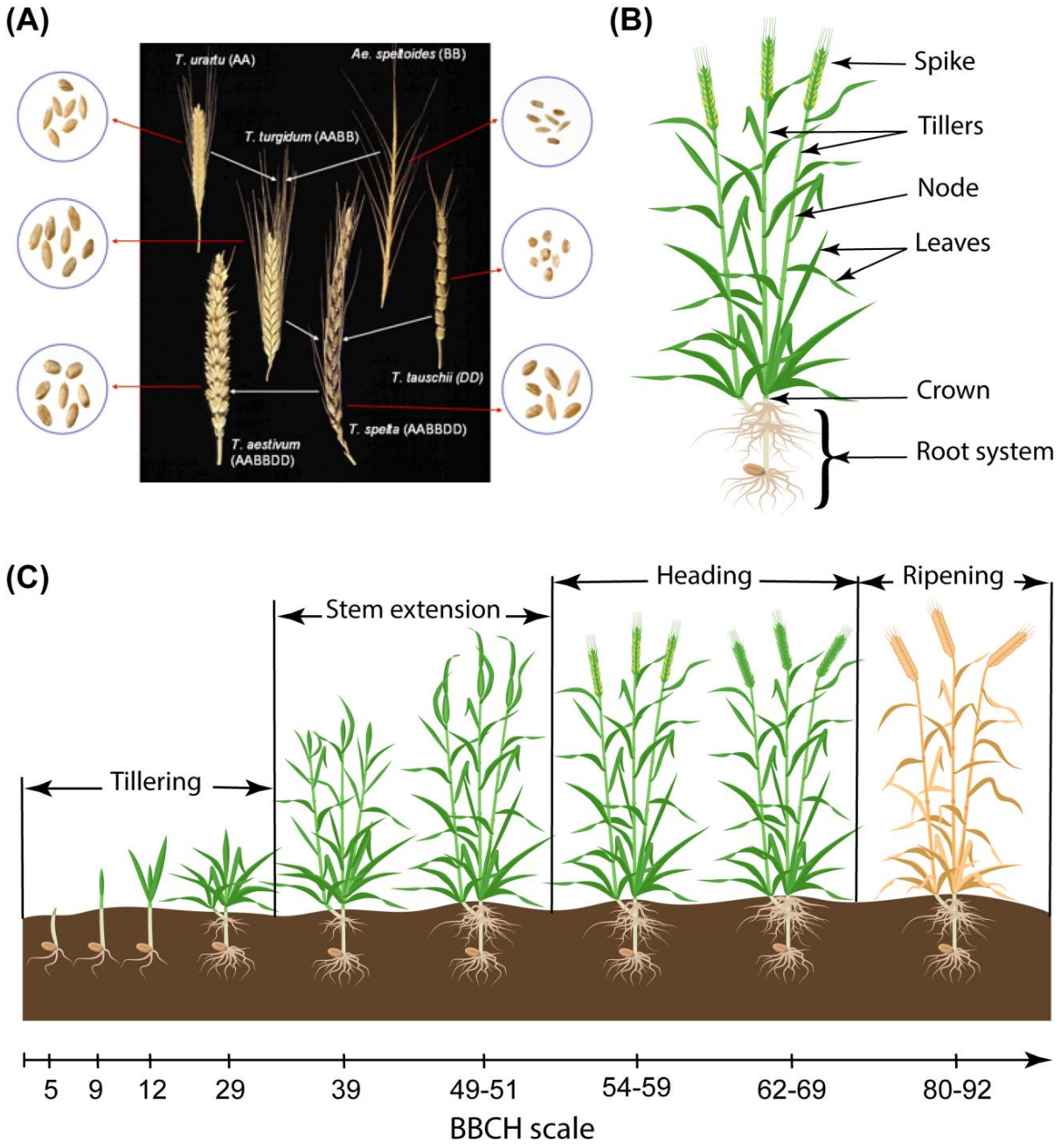

Figure 1: Genome evolution, morphology and development of wheat plant. **A.** The evolutionary and genome relationships between cultivated wheats and related wild diploid grasses, showing examples of spikes and grain (Shewry, 2009). **B.** Wheat plant with its different parts. **C.** Developmental sates of wheat according to BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) scale (Meier, 1997).

In this report, we present in details two examples of wheat fungal diseases that are economically important in Europe.

Leaf (brown) rust of wheat

Symptoms and importance

Brown rust is the product of the interaction between the biotrophic basidiomycete fungus *Pucinia triticina* and wheat. Being an obligate biotrophic pathogen, *P. triticina* requires a living host to grow and complete its life cycle. Plant responses to leaf rust infection depend on the resistance level of the host plant (Fig. 2A). Symptoms on susceptible varieties consist of large uredinia without chlorosis or necrosis in the host tissues. However, resistant cultivars are characterized by various responses from small hypersensitive flecks to small to moderate size uredinia that may be surrounded by chlorotic and/or necrotic zones (Bolton et al., 2008). During host invasion the rust fungi form highly specialized infection structures that are involved in spore attachment, host recognition, penetration, proliferation and nutrition (Mendgen and Hahn, 2002), that affect the plant by exporting assimilates to produce fungal tissues and spores (Robert et al., 2002, 2004) reducing the photosynthetic surface, and accelerating leaf senescence (Robert et al., 2005). This make leaf rust as one of the most widespread of three types of rusts causing significant yield losses over large geographical areas (Dadrezaie et al., 2013; Kolmer, 2013; Roelfs, 1992; Singh et al., 2014). Yield loss due to leaf rust depends on weather conditions, cultivar susceptibility and availability of inoculum (Huerta-Espino et al., 2011). Infection of the flag leaf with rust pathogens is the main cause of grain yield losses, which is thought to be responsible for greater than 70% of grain filling. However, grain losses have been significant and estimated to reach 30-70% or even greater on susceptible varieties (Huerta-Espino et al., 2011; Murray et al., 1994; Ordoñez et al., 2010).

Disease cycle

Rust fungi have without doubt the most complicated life cycles of all fungi with multiple life stages including different types of spore and multiple plant hosts (Ellison et al., 2016). *P. triticina* has five distinct life cycle stages; teliospores, basidiospores, and urediniospores on cereal hosts, and pycniospores and aeciospores on the alternate hosts (Fig. 2B) (Bolton et al., 2008; Kolmer, 2013). At the end of the wheat growing season, urediniospore production is replaced by dikaryotic teliospores (2n). These spores are thick-walled and remain viable during winter. The teliospores germinate and undergo meiosis producing four haploid basidiospores (1n) that are forcibly ejected into the air. Some of these spores land on and successfully parasitize the alternate host (*Thalictrum speciosissimum*). These haploid infections occur on the upper leaf surface generating pycnial structures. The pycnia produce receptive hyphae (female organ), pycniospores (male organ) and a sugary nectar. These elements are required for

fertilization. After fertilization aecia are formed from which wind distributed dikaryotic aeciospores (2n) can infect wheat plants (primary host) to produce urediniospores (2n) in brown-coloured, "rusty" pustules. The urediniospores can re-infect the same or fresh host plants several times during the growing season resulting in exponential increases of inoculum. Wind currents and proper weather conditions can result in heavily infected wheat producing areas, or even epidemics.

The asexual cycle consists of three stages: infection of the leaf, pathogen growth in leaf tissue, and spore production. The asexual phase of leaf rust is most readily observable and it is the rapid multiplication of the urediniospores produced during this stage that is the cause of crop damage. The physiological and molecular processes of infection are well known (Bolton et al., 2008). The infection process (Fig. 2C) starts when infectious rust spores (urediniospores) land on the host plant and start to germinate. Germination of spores leads to the formation of a primary germ tube which is directed towards the stomata of the leaf and results in the formation of an appressorium over the stomatal aperture. After formation of an appressorium, an infection peg grows into the substomatal cavity. From the infection peg, a substomatal vesicle is formed. Subsequently, infection hyphae elongate from this and a haustorium is formed from the haustorial mother cell after hyphae contact leaf mesophyl cells. The haustorium penetrates the plant cell wall of the leaf interior and invaginate the plasma membrane of the infected cell. The haustorium is the most complex infection structure developed by rust fungi while invading the host cell. It serves as a feeding structure to extract nutrients from the host cell towards the pathogen and also leads to the suppression of the plant's triggered defense responses (Staples, 2001). Seven to ten days post-infection, pustules (uredia) erupt through the epidermis on both leaf surfaces. Newly produced asexual urediniospores are released from the pustules and serve as a source for secondary inocula on the same or neighboring plants throughout the growing season (Webb & Fellers, 2006).

The dispersion of spores is mostly carried by the wind. Epidemics result from the succession of four to five cycles of asexual reproduction during the season, when environmental conditions are favourable (Zadoks & Bouwman, 1985).

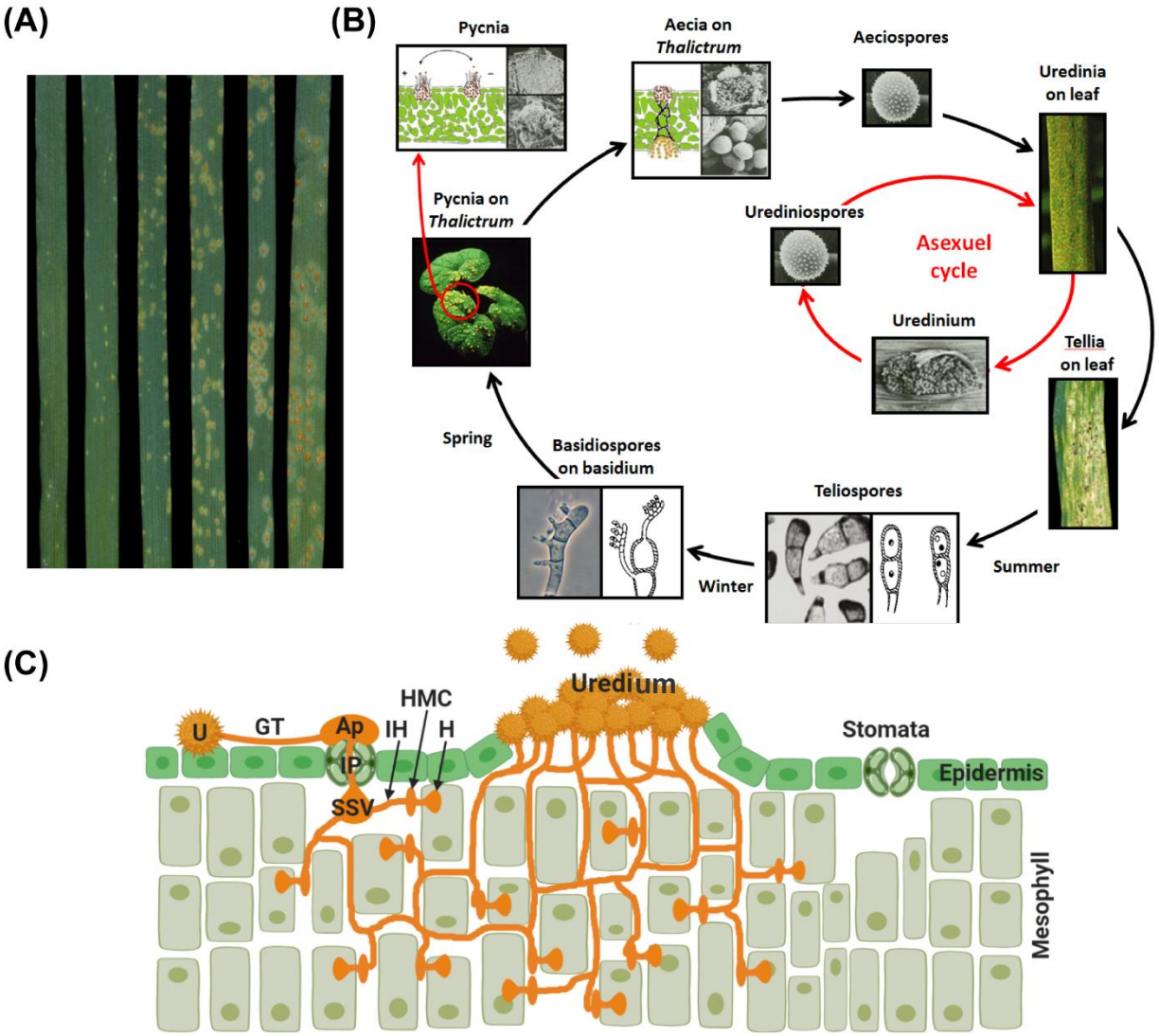

Figure 2: Leaf rust of wheat. **A.** Symptoms of leaf rust on wheat cultivars with different level of resistance (Kolmer, 2013). **B**. Life cycle of *P. triticina* adapted from Bolton et al. (2008) and (Singh et al., 2002). **C.** Infection process of *P. triticina* (leaf rust). **U**: Urediniospore, **GT**: Germination tube, **Ap**: Appressorium, **IP**: Infection peg, **SSV**: Substomatal vesicle, **IH**: Infection hyphae, **HMC**: Haustorial mother cell, **H**: Haustorium.

Genetic resistance to leaf rust

To control this disease, finding new sources of resistance to leaf rust has always been an objective of plant breeders. Rust resistance is commonly categorized into race-specific seedling resistance, also known as all-stage resistance and race-specific or non-specific adult plant resistance, also known as slow-rusting or partial resistance (Ellis et al., 2014). To date, 79 Lr genes are permanently catalogued in wheat (Qureshi et al., 2018). Race non-specific resistance is usually effective at the adult plant stage (Marone et al., 2009). It is associated with a longer latent period, lower infection frequency, smaller uredinial size, reduced duration of sporulation and less spore production per infection site (Caldwell, 1968). However, combinations of four

or five such *Lr* genes are needed for near-immunity or at least a high level of resistance (Singh et al., 2011). Unfortunately, most *Lr* genes are race-specific and confer hypersensitive reactions and interact with the pathogen in a gene-for-gene manner. This type of resistance is often rapidly overcome (Hysing et al., 2006; Kolmer, 2013; Serfling et al., 2011) thus making breeding for stable and durable leaf rust resistance in wheat difficult to achieve (Kolmer, 2019). Arina is considered as one of the most cultivated bread wheat cultivars in Switzerland, unfortunately, it carries the gene *Lr13* and is susceptible to leaf rust (Winzeler et al., 2000).

Septoria tritici blotch (STB)

Economic importance and symptoms

Septoria tritici blotch, a major wheat foliar disease, is caused by the filamentous ascomycete *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola*). This disease is one of the most devastating diseases of wheat present in many parts of the world and it is the reason for severe yield reductions and important economic losses (Dean et al., 2012; Eyal, 1987; Fones & Gurr, 2015). In Europe, it is also considered very important, mainly because of the suitable climate conditions (Jørgensen et al., 2014). During severe epidemics, losses reaching 50% have been registered in fields planted with wheat cultivars susceptible to STB (Fones & Gurr, 2015).

The pathogen is hemibiotrophic, *e.g.*, biotrophic in the early infection process and necrotrophic during the later stages (Agrios, 2005). The leaf symptoms (Fig. 3A) generally appear 14-21 days after the infection as light green to yellow chlorotic spots. As they enlarge, the lesions become brown and develop darker coloured fruiting bodies (sexual pseudothecia and asexual pycnidia). Plant cells collapse, and necrotic lesions appear on leaves and stems. Lesions on mature leaves are most often long, narrow and delimited by leaf veins, but they can also be shaped irregularly orelliptical (Agrios, 2005; Ponomarenko et al., 2011).

Disease cycle

Ascomycetes are a group of fungi that produce sexual spores, eight ascospores, within asci (Agrios, 2005). Asci are produced inside the pseudothecia, underneath the host epidermis (Ponomarenko et al., 2011). It has been estimated that each pseudothecia contains on average 26 asci, this gives a potential number of 200 ascospores per pseudothecium (Suffert et al., 2011). STB infection is initiated by air-borne ascospores and splash-dispersed conidia contained in pycnidia produced on residues of the previous season's crop (Fig. 3B) (Eyal, 1987; Ponomarenko et al., 2011).

The infection process (Fig. 3C) begins with the deposition of a spore from sexual or asexual reproduction on the surface of a wheat leaf. Spores initiate their development only in an environment that groups certain conditions necessary for good fungal growth during its life cycle (Lucas et al., 2004). Humidity and temperature are major variables that determine this initiation. The germinative capacity of *Z. tritici* spores is very high, Cohen and Eyal (1993) reported that in humid conditions at 22°C, 85 to 90% of spores on wheat leaves germinate within 24-26 hours after inoculation. The hyphae penetrate leaf tissues mainly through the stomata (Cohen & Eyal, 1993; Duncan & Howard, 2000; Kema et al., 1996; Palmer & Skinner, 2002). Several hyphae can penetrate the leaf through the same stoma (Kema et al., 1996). However, hypha can also penetrate directly through the cuticle of the leaf at the periclinal or anticlinal walls of epidermal cells (El Chartouni et al., 2012; Shetty et al., 2003; Siah et al., 2010). Once the hypha penetrates the leaf, it begins a process of colonization of the sub-stomatal chambers and the mesophyll. During this course of leaf colonization, the infection remains asymptomatic and the leaves appear healthy. This symptom-less "latent phase" (also named biotrophic phase) could be explained by the fact that some effectors are secreted and prevent recognition by the plant and may suppress host defences (Rudd, 2015). The mycelium grows extracellularly in the mesophyll and gets nutrients from the plant apoplast. About two weeks later pycnidia are formed (Palmer & Skinner, 2002). Later on, there is a drastic change to necrotrophic phase. The mechanisms underlying this trophic change remain unclear. *Z. tritici* probably uses several mechanisms to induce necrosis, including the production of protein effectors (Gohari et al., 2015; Rudd et al., 2015). It was also suggested that programmed cell death is triggered by the pathogen at the onset of the necrotrophic phase (Keon et al., 2007). Finally, after pycnidia maturation, multi-cellular pycnidiospores are produced and released by water splash and spread the infection (Ponomarenko et al., 2011).

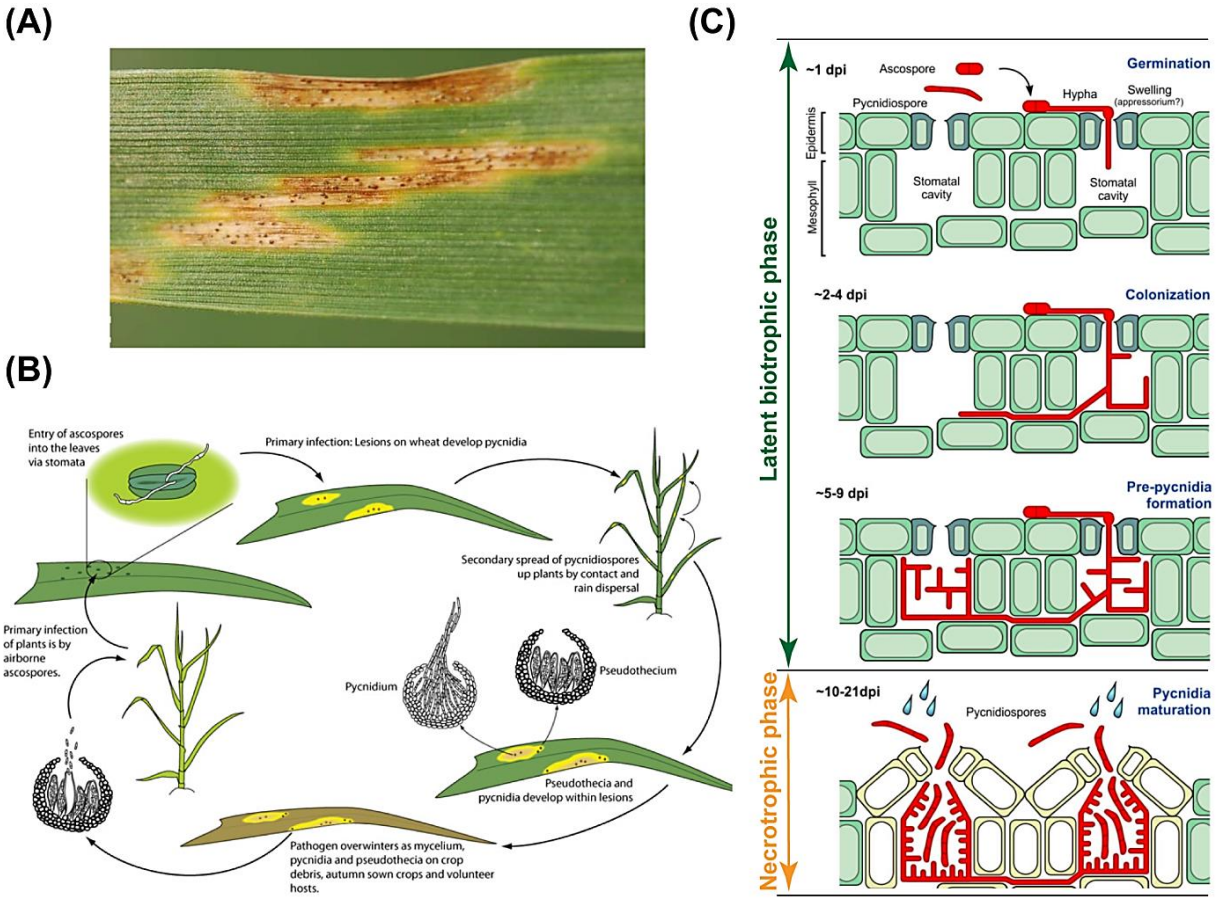

Figure 3: Septoria tritici blotch of wheat. **A.** Symptoms of STB disease on wheat leaves. **B.** Life cycle of *Z. tritici*. The life cycle of the fungal pathogen includes both asexual and sexual reproduction (Ponomarenko et al., 2011). **C.** Infection stages of *Z. tritici*. The ascospores reach the leaf epidermis and form germ tubes that enter leaf tissues throw stomata, colonize the substomatal cavity and form pre-pycnidia. This is the transition to necrotrophic phase, in which the fungus proliferates and forms pycnidia that release pycnidiospores (Steinberg, 2015).

Genetic resistance to STB

An environmentally friendly method against STB is the use of resistant cultivars that reduce the employment of fungicides (Brading et al., 2002). In the last decades, resistant wheat varieties have been selected by crosses between parents that carry the desirable characters. In fact, the aim of breeding is to improve some characters whilst maintaining other characters no lower than in previous cultivars (Johnson, 1992). Therefore, 13 major genes for resistance to STB have been identified and some of them have been found to interact in a gene-for-gene manner with the pathogen (Ponomarenko et al., 2011). However, the process of breeding for disease resistance is subject to several limitations, as the variability of pathogens in relation to host resistance (Johnson, 1992). Gene-for-gene interaction implies that the allele conferring resistance to the plant will be overcome if the pathogen acquires the corresponding virulence

by losing or altering the avirulence allele, by deletion or by genetic change (Brading et al., 2002; Johnson, 1992). Since gene flow can occur between fungal strains the high genetic variation and large effective population sizes found in this fungal pathogen represent ideal conditions for evolution of fungicide resistance and new virulent strains of the fungus (Zhan & McDonald, 2004). For an ideal disease management practice, it will be important to continue breeding wheat plants for resistance (Fones & Gurr, 2015).

I.3. Control management of wheat fungal diseases

In general, cultivated wheat varieties do not have an adequate level of resistance against fungal diseases (Courvoisier et al., 2015; Schaad et al., 2019). In addition to the use of a certain resistance level, management practices provide another method for at least partial control of fungal disease. In case of wheat rust disease, eradication of the alternate host programs showed their efficacy to both eliminate the source of local inoculum and reduce diversity in biotypes in order to provide for stable genetic resistance (Peterson, 2018; Roelfs, 1982). Also, removing the green-bridge from one crop to the next with tillage or herbicides is an effective practical management for epidemics that would result from endogenous inoculum (Singh et al., 2002). Avoidance or escape of pathogen-host contact by modifying cultural dates, or using high wheat cultivars could also help to minimize disease severity. Simón et al. (2005) showed a clear association between STB disease, plant height, and heading date in wheat. A positive correlation between the number of days between sowing and heading stage and STB severity was demonstrated, the late sowing date was therefore likely to decrease STB epidemics (Murray et al., 1990). In wheat disease management, the canopy density is also an important factor, since a high density or modification of canopy architecture increases fungal disease severity (Baccar et al., 2011).

In view of disease pressure and the lack of efficacy of varietal resistance and cultural practices in the field, the fight against wheat fungal diseases is essentially based on the use of chemical fungicides (Fones & Gurr, 2015; Gooding, 2007). Triazoles and strobilurins are the most commonly used fungicides to control fungal diseases. Triazoles are inhibitors of ergosterol biosynthesis, which is the essential part of the fungal cell membrane (Kwok & Loeffler, 1993). These fungicides were effective to control Fusarium head blight (Paul et al., 2008) and leaf rust (Barro et al., 2017). However, its combination with other fungicide was needed to control STB disease (Shah et al., 2014). Strobilurins are mesostemic, which means they possess strong adsorption and cuticle-waxes penetration on leaves. They inhibit mitochondrial respiration (Bartlett et al., 2002). Strobilurins are able to control a large spectrum of fungal disease

including ascomycetes and basidiomycetes, the major groups of plant pathogenic fungi in wheat crops (Bartlett et al., 2002).

Nevertheless, sometimes abusive treatment of plants with the fungicides leads to the emergence of resistant strains of these pathogens (Ponomarenko et al., 2011; Schuerch et al., 2009). Additionally, the use of synthetic fungicides for controlling these diseases may have negative effects on human and animal health (Aktar et al., 2009). To face such issues, research for sustainable alternatives of wheat disease management has become necessary.

I.4. Inducers of plant resistance to control wheat fungal diseases

The use of plant inducers has proven to be a complementary control strategy potentially interesting for protecting wheat from fungal diseases while respecting the environment.

Biological inducers

Microorganisms like fungi and bacteria have frequently been reported to have protective activity against plant pathogens and pests (Köhl et al., 2019; Tahat et al., 2010; Xavier & Boyetchko, 2004). Some fungi belonging to *Trichoderma* spp. naturally present in agricultural soils have been used as biocontrol agents to protect wheat plants (Perelló et al., 2009). The efficiency of the arbuscular mycorrhizal fungus *Funneliformis mosseae* to protect wheat against the foliar biotrophic pathogen *Blumeria graminis* f. sp. *tritici* was shown under controlled conditions. *B. graminis* infection on wheat leaves was reduced in mycorrhizal plants (Mustafa et al., 2016, 2017).

The bacteria *Bacillus megaterium* directly extracted from the wheat rhizosphere and leaves have been studied for STB control (Kildea et al., 2008). *B. subtilis* has also been assessed as biological control agent against *Z. tritici* for the production of cyclic lipopeptides, such as mycosubtilin, surfactin and fengycin (Mejri et al., 2017).

Pseudomonas spp. and wheat disease control

Bacteria of the genus *Pseudomonas* are commonly found among the predominant genera in the rhizosphere, and on and in the below-ground parts of wheat plants (Yoshida et al., 2012).

The genus *Pseudomonas* comprises mostly fluorescent as well as a few non-fluorescent species in two groups: *P. aeruginosa and P. fluorescens*. The latter is divided into six groups, with that of *P. fluorescens* being the most complex and includes nine subgroups (Mulet et al., 2010). *Pseudomonas* has been tested as potential biocontrol agents by controlling STB on adult wheat

plants (Kildea et al., 2008). When wheat seedlings are inoculated with *Pseudomonas* prior to infection, symptoms are less present (Ponomarenko et al., 2011).

Root-colonizing *P. protegens* CHA0 (formerly *P. fluorescens* CHA0) was mostly reported as a potential bacterial antagonist to control plant diseases (Hase et al., 2000; Ramette et al., 2011). In fact, it was isolated for the first time from the roots of tobacco grown in a soil near Payerne, Switzerland, that is naturally suppressive to black root rot in tobacco (Stutz et al., 1986), and their capacity to induce systemic resistance in monocots has been shown (Henkes et al., 2011; Sari et al., 2008)

Chemical inducers

Several chemical inducers have been tested on wheat and have shown good efficacy for controlling various leaf diseases such as powdery mildew, rust and Septoria. Rémus-Borel et al. (2005) have shown that silicon application induces the production of antifungal compounds in wheat infected with powdery mildew. Also, foliar application of potassium chloride induced an osmotic mechanism involved in the control of wheat powdery mildew (Kettlewell et al., 2000). Similarly, Deliopoulos et al. (2010) revealed that bicarbonates ($KHCO_3$ and $NaHCO_3$) are able to protect wheat against rust and silicon against *Septoria nodorum*. Benzothiadiazole (BTH) can also induce resistance against powdery mildew and Septoria (Görlach et al., 1996), but not against Fusarium head blight (Yu & Muehlbauer, 2001). In addition, dos Santos et al. (2011) confirmed the protection conferred by acibenzolar-s-methyl on powdery mildew and rust. Vechet et al. (2005) demonstrated that BTH was effective in reducing wheat powdery mildew. Renard-Merlier et al. (2007) and Tayeh et al. (2013) showed efficacy of SA and HAS (heptanoyl salicylic acid) to control wheat powdery mildew. This work revealed that HAS increases H_2O_2 accumulation and lipid peroxidation. In addition, a recent study has shown that the exogenous application of oxalic acid increases the resistance of wheat to Septoria in the field (Zhuk et al., 2014).

Natural extracts

Several studies demonstrated the efficacy of some resistance inducers based on natural extracts. To control powdery mildew, (Renard-Merlier et al., 2007) used a polysaccharide derived from a brown algae (*Laminaria digitata*) and treated plants showed a decrease in the level of peroxidation. Randoux et al. (2010) also tested the efficacy of acetylated and non-acetylated oligogalacturonides in the protection of wheat against this disease. In case of the wheat-Septoria pathosystem, Joubert et al. (1998) showed the effectiveness of β-1,3-glucan oligomers extracted

from an *L. digitata* against STB. Shetty et al. (2009) showed in their study that the application of glucans extracted from *Z. tritici* walls induces wheat defense mechanisms in both the susceptible and the resistant wheat cultivar. This study demonstrated the overexpression of *PR2* associated with callose deposition. Surfactin extracted from the strain *Bacillus amyloliquefaciens* S499 protected wheat against *Z. tritici* and activated both salicylic acid- and jasmonic acid-dependent defense responses (Le Mire et al., 2018).

β-aminobutyric acid and wheat fungal disease

Additionally to plant resistance inducers mentioned before, β-aminobutyric acid (BABA) is considered as a potential chemical inducer of disease resistance and has been investigated for many years (Cohen et al., 2016; Mauch-Mani et al., 2017; Thevenet et al., 2017). Previous reports demonstrated that the application of BABA induced local or systemic resistance against various plant pathogens (Cohen et al., 2016; Justyna & Ewa, 2013). However, its application to induce resistance to wheat fungal disease is poorly reviewed. Zhang et al. (2007) studied the effect of potential resistance inducers to reduce *Fusarium* head blight (FHB) under greenhouse conditions. They found that BABA at 1 mM significantly reduced FHB severity through induction of systemic resistance. In another study, the efficacy of BABA to reduce FHB development, especially when it was applied in combination with other potential resistance inducers, was confirmed (Hofgaard et al., 2010).

Part II. Plant resistance to pathogens: from innate immunity to induced resistance

II.1. Plant innate immune response

In the last years, revolutionary conceptual advances have been made in understanding the evolution and development of the immune system of the plant (Jones & Dangl, 2006). The establishment of the disease depends on three components described in the conceptual model "disease triangle": plant, pathogen, and the environment. The ideal characteristics for the development of the disease are a sensitive host, a virulent pathogen, and a favourable environment (Agrios, 2005). However, genetic background of each plant gives a level of 'immunity' to pathogens even if environmental conditions are optimal. For establishment of such a disease, pathogens generally have to overcome three types of defense lines usually described in plants as constitutive resistance, total or qualitative resistance, and partial or quantitative resistance.

First line - constitutive immunity

During evolution, plants have acquired a variety of constitutive defences giving them a certain level of passive resistance. This type of resistance includes constitutive structural and chemical barriers.

(i) The constitutive structural barriers

The structural barriers represent the first obstacle that the pathogen encounters (Fig. 4 *Constitutive barriers*), even before being in contact with the walls of the epidermal cells of the plant. Thus, hairs, trichomes, spines and prickles are structural barriers that prevent pathogen attachment to the plant surface (Freeman & Beattie, 2008). However, the cuticle remains the most effective protective structure. Indeed, composed of cutin (Jeffree, 1996), it forms a barrier difficult to cross by pathogens that do not have the necessary enzymes for its degradation (van den Brink & de Vries, 2011).

(ii) The constitutive chemical barriers

Chemical substances called phytoanticipins are present constitutively in the plant. They group together different chemical families: alkaloids, lactones, saponins, glycosides, phenolic compounds (Piasecka et al., 2015). They have a strong antifungal and antibacterial potential that results in a deleterious effect on the life cycle traits of pathogens such as spore germination, mycelial growth and also on the production of hydrolytic enzymes, the synthesis and biological activity of fungal toxins (Freeman & Beattie, 2008).

Second line - quantitative resistance

Plant cells are also capable of perceiving pathogens through the recognition of highly conserved molecular motifs of the pathogen called 'Pathogen Associated Molecular Patterns (PAMPs)', via extracellular membrane receptors, or 'Pattern Recognition Receptors (PRRs)'. This recognition leads to the induction of general defences of the plant, characteristic of innate plant immunity (Fig. 4 *Innate immunity*) called 'PAMP-Triggered Immunity (PTI)' (Jones & Dangl, 2006). The accumulation of antimicrobial compounds during PTI can slow the development of the pathogen and reduce symptoms without avoiding them; this interaction is known to be compatible. However, successful pathogens deploy effectors that contribute to pathogen virulence (Fig. 4 *Innate immunity*). Effectors can interfere with PTI this result in effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). PTI is associated with partial or quantitative resistance (Boller & Felix, 2009), under polygenic control (Fig. 4 *Plant response*). Polygenic characters are generally expressed in a quantitative form in a segregating population, where the responsible loci are defined as 'Quantitative Trait Loci (QTL)' (Corwin & Kliebenstein, 2017).

Third line - qualitative resistance

Total or qualitative resistance is based on gene-for-gene theory established by Flor (1971). It corresponds to the interaction between the products (or their derivatives) of a major Resistance (*R*) gene of the plant and an Avirulence (*Avr*) gene, also called effector of the pathogen (Fig. 4 *Innate immunity*). This type of interaction is described as incompatible between the plant and the pathogen and is under monogenic control (Fig.4 *Plant response*). The highly specific R/Avr recognition via cytoplasmic receptors of the 'Nucleotide-Binding Leucine Rich Repeat' type (NB-LRR) leads to (i) rapid and intense activation of plant defense mechanisms and (ii) to programmed cell death of plant cells at the site of infection causing a hypersensitivity reaction (HR). This results in necrotic lesions that confine the pathogen and stop its progression in the host tissues. The very strong increase in defense responses of the plant corresponds to a mobilization of the specific immune system of the plant known as Effector-Triggered Immunity (ETI) (Jones & Dangl, 2006).

II.2. Systemic resistance

As reviewed in the first part, pathogen recognition can induce the enhancement of plant defences. But, also exogenous biological and chemical stimuli can enhance plant resistance (Mauch-Mani et al., 2017). This defense mechanism implemented by the plants is not limited

only to local responses. The plants are also able to deploy systemic defences (Fig. 4 *Systemic resistance*), in other words generalized to the whole of their tissues (Pieterse et al., 2002; Van Loon, 1997).

Systemic acquired resistance

It has been known for long that the defense of a plant is not only restricted to the pathogen-attacked tissues, but also extends to distal tissues which become more resistant to a second challenge by the same or another pathogen. This phenomenon has been termed 'Systemic Acquired Resistance' (SAR; Ross, 1961).

SAR involves the generation of a signal (or signals) in the primary leaves that upon translocation to the distal tissues activates defense responses resulting in broad-spectrum resistance. Production of this phloem-based mobile signal occurs within 6 hours of pathogen infection in the primary leaves (Chanda et al., 2011), and the signal is rapidly transferred to the distal uninfected tissues (Kachroo & Robin, 2013).

Activation of PTI and ETI in locally infected tissues often triggers SAR (Fu & Dong, 2013). While PTI and ETI are activated rapidly and act locally to limit growth of the specific invader at the site of infection, SAR takes more time to develop but confers an enhanced defensive capacity that is typically effective against a broad spectrum of pathogens (Fu & Dong, 2013; Walters et al., 2013).

Generally, SAR is accompanied by accumulation of salicylic acid (SA) and pathogenicity-related proteins (PR). It can be induced after local HR, may be associated with the production of reactive oxygen species (ROS) and sometimes in the synthesis of phytoalexins (Desender et al., 2007; Durrant & Dong, 2004). Other chemicals that contribute to SAR have been identified (Gao et al., 2014; Kachroo & Robin, 2013; Shah & Zeier, 2013), including the methylated SA derivative MeSA, the dicarboxylic acid azelaic acid (AzA), and the phosphorylated sugar glycerol-3-phosphate (G3P). In addition, SAR is also dependent on the lipid-transfer-like proteins (LTPs; Champigny et al., 2011; Chanda et al., 2011; Yu et al., 2013).

Induced systemic resistance

Plant health is enhanced by useful microbes in the microbiome of plant roots. These microbes contribute to an important mechanism called 'Induced Systemic Resistance (ISR; Smart et al., 1986). ISR permits selected plant growth promoting rhizobacteria (PGPR) and fungi in the rhizosphere to prime the entire plant for enhanced defense against a broad range of pathogens and insect herbivores (Pieterse et al., 2014; Zamioudis & Pieterse, 2012). Colonization of plant

roots by diverse PGPR strains were shown to reinforce the plant immune system in above-ground plant parts, causing a broad-spectrum disease resistance (Song et al., 2011; Weller et al., 2012; Xiang et al., 2014). Numerous studies in dicots and monocots have reported on the ability of PGPR to promote plant health via ISR (Pieterse et al., 2014).

ISR is phenotypically similar to SAR. However, it seems that the induction pathways of SAR and ISR are different, as will be explained below, even if both are based on the transmission of a signal leading to the activation of different defense mechanisms.

ISR has been reported to be efficient against a wide range of attackers: viral (Harish et al., 2009), bacterial (Chithrashree et al., 2011) and fungal (De Vleesschauwer et al., 2009) pathogens, insect herbivores (Saravanakumar et al., 2007a) and parasitic nematodes (Elsen et al., 2008). These studies mainly involved *Bacillus*, *Pseudomonas*, and *Serratia* PGPR strains. In addition, non-pathogenic plant growth-promoting fungi (PGPF) strains from species like *Fusarium oxysporum*, *Trichoderma* spp., and *Piriformospora indica* strains, but also symbiotic arbuscular mycorrhizal fungi have been shown to trigger ISR (Pieterse et al., 2014).

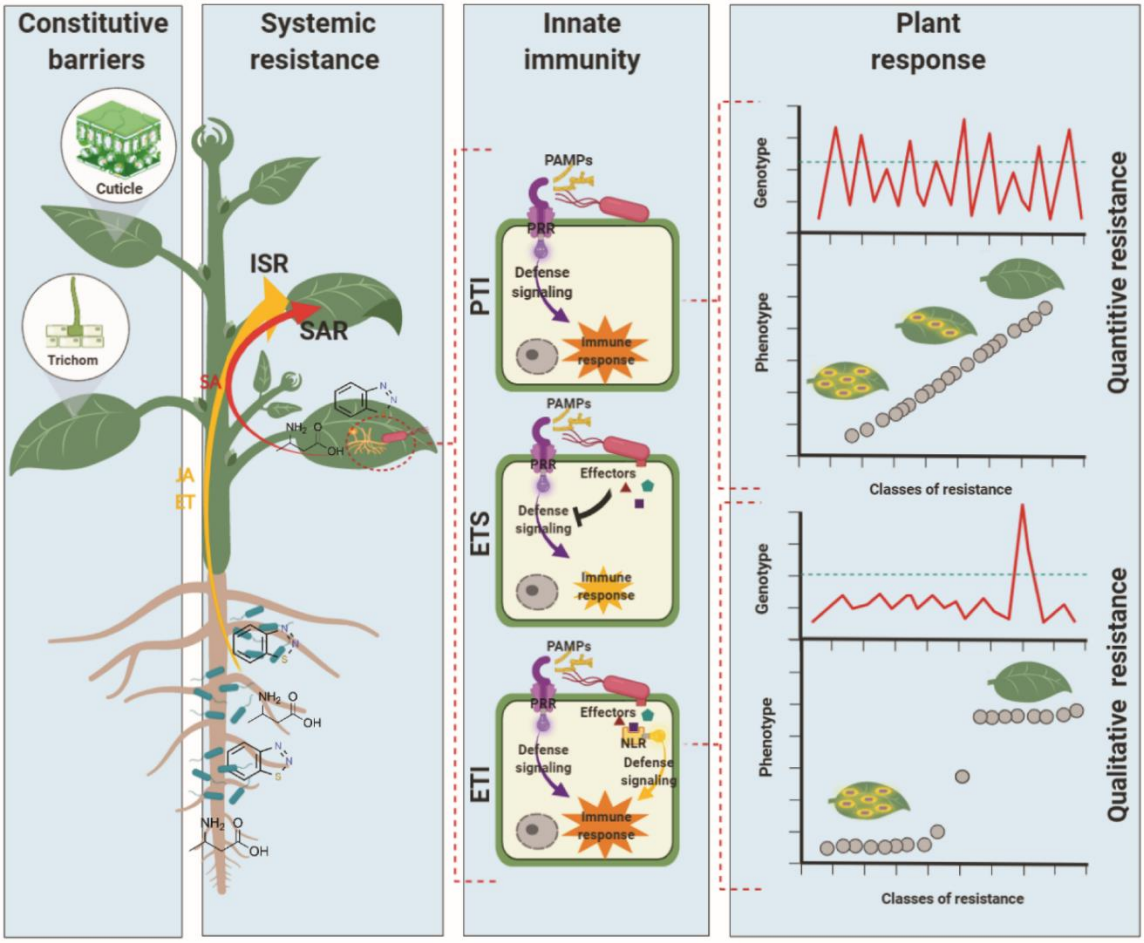

Figure 4: Illustration showing simplified model of plant resistance to pathogen infection: mechanisms and responses of innate and induced resistance. **Constitutive barriers** such as waxy epidermal cuticle and trichromes are considered the first layer of resistance. Plants often wait until pathogens are detected to trigger the **innate immunity.** First, a pattern recognition receptor (PRR) on the plant cell's surface recognizes pathogen-associated molecular patterns (PAMPs) released by invaders, the flagellar proteins from pathogenic bacteria or chitin from fungi, this activates signaling pathways inside the cell leading to an immune response to combat the pathogen. But pathogens can interfere with PAMP-triggered immunity (PTI) by injecting effector molecules into the plant cell which lead to effector-triggered susceptibility (ETS). Intracellular plant protein complexes called nucleotide-binding domain, leucine-rich repeat receptors (NLRs) bind effectors and set off secondary immune cascades under the name effectors-triggered immunity (ETI) of that boost the PTI for a stronger responses. Roots as well as leaves also after a perception of biotic (Bacteria, fungi) and chemical stimuli (BABA and BTH) can trigger a **systemic resistance** throw long-distance signals. Systemic acquired resistance (SAR) starts with a local infection and can induce resistance in yet not affected distant tissues. Salicylic acid (SA) pathway is essential for this response. Induced systemic resistance (ISR) can result from root colonization by non-pathogenic microorganisms and, by long-distance signaling, induces resistance in the shoot. Ethylene (ET) and jasmonic acid (JA) are involved in the regulation of the respective pathways. **Plant response** to pathogen attacks can be qualitative or quantitative. Phenotypically, classes of resistance follows a binary 'susceptible or resistance' distribution for qualitative resistance, but a continuous distribution from susceptibility towards resistance for quantitative resistance. Genetic mapping of qualitative resistance (red lines) results in a single genetic locus (NLR proteins). Quantitative resistance result in large number of genomic loci. Illustration is inspired from previous reviews (Corwin & Kliebenstein, 2017; Pieterse et al., 2012; Roux et al., 2014).

II.2.1. SAR/ISR elicitors

SAR

Inorganic compounds

The inorganic salt potassium phosphite) (K_3PO_3) induces resistance against against the foliar pathogens *Venturia inaequalis* and *Venturia pirina* which cause apple and pear scab respectively (Percival et al., 2009). Another inorganic compound, barium chloride ($BaCl_2$), has been reported to induce resistance against pathogens when applied to plants (Tripathi et al., 2019).

Synthetic compounds

Certain chemical compounds such as SA, dichloroisonicotinic acid (INA) and S-methyl ester of benzo-1,2,3-thiadiazole-7-carbothioic acid are capable of triggering SAR in different plants against various pathogens (Wang & Zhou, 2018).

Since the discovery of White (1979) that treatment of tobacco leaves with SA or acetyl salicylic acid (commonly known as Aspirin) induces resistance to tobacco mosaic virus, there have been many studies of SA effectiveness to stimulate SAR. The activation of SA-SAR provides a broad-spectrum resistance against a wide range of related or unrelated pathogens (Tripathi et al., 2019).

INA is a functional analogue of SA and was reported as SAR inducer in many cases (Durner & Klessig, 1995; Van Kan et al., 1995), since it is capable to induce a SAR response similar to the one induced by pathogens (Tripathi et al., 2019). BTH is another functional analogue of SA and is a powerful inducer of plant immune responses by triggering resistance against a wide range of pathogens. BTH treatment induces the accumulation of many transcripts that also accumulate during pathogen infection (Bektas & Eulgem, 2015; Görlach et al., 1996).

Microbial elicitors

The direct or indirect interaction of gene resistance with an avirulence gene product stimulates a signaling pathway leading to HR, then to SAR (Durrant & Dong, 2004). In general, microbial pathogen compounds, which have eliciting properties of SAR, are not specific for a strain of the pathogen and depend on their chemical nature. They can be surface compounds like lipopolysaccharides (LPS) of gram-negative bacteria (Gerber et al., 2004; Silipo et al., 2010) the peptidoglycan of gram positive bacteria (Silipo et al., 2010; Underhill & Ozinsky, 2002), β-glucans, oligomers derived from chitin, pectin fragments (released from the pathogenic fungus), but also flagellins, glycine-rich proteins and peptides such as syringoline isolated from

pathogenic bacteria (Felix et al., 1999; Imler & Hoffmann, 2001; Klarzynski et al., 2003; Kuć, 2001; Nürnberger & Scheel, 2001; Tanaka et al., 2003; Underhill & Ozinsky, 2002; Wäspi et al., 1998). Some species of *Phytophthora* can secrete small peptides called "elicitins" and can stimulate SAR in tobacco against *Pernospora parastica* (Yu, 1995).

ISR

Over the past decade many studies aimed to isolate an increasing amount of rhizobacteria strains that can stimulate ISR in various hosts. However, the mechanism and the biological activity of those non-pathogenic microbes are not well known. In order to stimulate ISR, beneficial microbes must produce elicitors that are responsible for the systemic immunity. Some elicitors are classified into three categories: cell surface components, metabolites regulated by iron, and antibiotics (Ongena et al., 2006)**.**

Cell surface components

Motility, adhesion and growth rate of bacteria are important elements for effective colonization of the host. These properties depend partly on LPS and flagella present on the surface of bacterial membranes (Persello-Cartieaux et al., 2003). Flagellin from *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta*) acts as a major inducer of ISR in non-host plants by inducing HR cell death in its non-host tomato plant but not in the host tobacco plant (Taguchi et al., 2003a). A flagellin-defective mutant of *Pta*, *ΔfliC*, lost its motility and the ability to induce HR cell death in the tomato plant (Taguchi et al., 2003b), and the recombinant flagellin polypeptide including the N-terminal domain showed elicitor activity (Naito et al., 2007). On the other hand, the use of cell envelope extracts, mutants or purified compounds also demonstrated the role of LPS in inducing resistance. This is particularly the case when ISR was induced by some strains of *P. fluorescens, P. putida, Burkholderia cepacia* and *Rhizobium elti,* which LPS were tested with positive results on pathosystems as diverse a*s Arabidopsis / F. oxysporum,* and potato / cyst nematode (Reitz et al., 2002; VanWees et al., 1997).

Metabolites regulated by iron

Among the metabolites regulated by iron, pyoverdines produced by *Pseudomonas fluorescens* strains are chelating molecules of iron (siderophores) having a high affinity for Fe^{3+} ions. Their production allows microorganisms to grow in an iron-poor environment, some research on siderosphores showed that pyoverdines produced by *P. fluorescens* WCS358 can induce ISR in Arabidopsis, beans, tomato and eucalyptus (Höfte & Bakker, 2007). Also in limiting conditions of iron, some microorganisms can produce SA, whose eliciting activity was shown by *P.*

aeruginosa 7NSK2 and KMPCH (De Meyer & Höfte, 1997) and *P. fluorescens* P3 (Maurhofer et al., 1998).

Antibiotics

Elicitors belonging to the class of antibiotics are mainly produced by bacteria of the genus *Pseudomonas*. 2,4-diacetylphoroglucinol (DAPG) synthesized by *P. fluorescens* CHAO is notably one of the first that was highlighted for his action against *Hyaloperonospora arabidopsidis* via stimulation of plant defense in Arabidopsis and tomato (Iavicoli et al., 2003; Siddiqui & Shaukat, 2003).

Among other ISR-elicitors, specific volatile organic compounds produced by beneficial microbes were demonstrated to elicit ISR (Lee et al., 2012; Ryu et al., 2004). Several ISR elicitors were shown to act redundantly, indicating that multiple microbial elicitors can trigger common signaling pathways leading to systemic immunity (Bakker et al., 2003).

II.2.2. SAR/ISR signaling

Accumulation of salicylic acid

SAR, induced by an incompatible pathogen, is notably characterized by an accumulation of SA (Gaffney et al., 1993; Kusajima et al., 2017; Van Loon & Van Strien, 1999). This accumulation of SA occurs locally and systemically. Plants expressing the *NahG* gene (coding for salicylate hydroxylase that converts SA to catechol) are no longer capable of developing SAR, indicating that SA is a molecule essential for the development of this resistance (Lawton et al., 1995). Although SA can be synthesized from L-phenylalanine by phenylalanine ammonia-lyase (PAL), the predominant pathway for SA biosynthesis during infection is from chorismate via isochorismate synthase and isochorismate pyruvate lyase (Shah, 2003). Overexpression of these two enzymes in transgenic plants increases the accumulation of SA (Mauch et al., 2001). In Arabidopsis, *SID2* gene expression (SA Induction Deficient2), which codes for isochorismate synthase, is activated in tissues infected with an incompatible pathogen that induces SAR (Wildermuth et al., 2001).

Contrary to SAR, the induction of ISR in plants by PGPRs is often independent of SA (Ryu et al., 2003; Yan et al., 2002). Transgenic SA-non accumulating Arabidopsis NahG plants mounted wild-type levels of ISR upon colonization of the roots by *P. fluorescens* WCS417r, providing genetic evidence that ISR can be mediated via an SA-independent signaling pathway (Pieterse et al., 1996). However, certain strains of beneficial microbes have been reported to

trigger ISR in an SA-dependent fashion, which resembles pathogen-induced SAR (De Vleesschauwer & Höfte, 2009; van de Mortel et al., 2012)

Response related to jasmonic acid and ethylene

As mentioned above, induction of SAR is clearly SA-dependent. However, ethylene (ET) and jasmonic acid (JA) can modulate SAR, since increasing evidence indicates that the SA- and ET/JA-mediated defense response pathways are mutually antagonistic (Li et al., 2019; van Loon et al., 2006).

In contrast to SAR, the ISR-signaling pathway is generally not associated with the accumulation of SA (van Wees et al., 2000), but rather with the plant hormones JA and ET emerged as important regulators of plant immunity (Pieterse et al., 2012). In *A. thaliana*, ISR-dependence on JA and ET is based on enhanced sensitivity to these hormones, instead of increasing their production (van Wees et al., 2000)**.** Studies with JA-response mutants (*jar1-1*) or ET response mutants (*etr1-1*) showed that the perception of JA and ET is essential to trigger ISR (van Loon et al., 2006; van Wees et al., 2000). For example, the application of ET precursor ACC (1-aminocyclopropane-1-carboxylic acid) showed the stimulation of ISR in *A. Thaliana* plants expressing the gene ETR-1 (which are not able to produce the ET) in the same way as the plants treated with PGPR (Van Loon & Bakker, 2005), On the other hand, it has been demonstrated that the ability to convert ACC to ET is a general response in *A. thaliana* treated with *P. fluorescens* WCS417r. But treating plants with *P. fluorescens* WCS417r primed them by greatly increasing ET production during pathogen attack. This increase in ET may participate in the improvement of the defense capacity against pathogens (Haas & Keel, 2003). Another study with the strain BG03 of *B. subtilis* showed that signaling pathway is not associated to JA but rather to ET, suggesting that the signal due to ET is essential in Arabidopsis treated with this PGPR (Ryu et al., 2004). For many other PGPR genetic evidence pointed to a role for JA and/or ET in the regulation of ISR (Pieterse et al., 2014)

SA-induced SAR is effective against biotrophic pathogens, while JA/ET-related signaling confers resistance against necrotrophic fungal pathogens (reviewed in Backer et al. (2019)). Apparently, an effective defense against biotrophic pathogens usually involves programmed cell death (PCD) related to the HR and the SA-dependent signaling pathway. On the other hand, necrotrophic pathogens can actually benefit from dead host cells and thus are not controlled by PCD and the defense pathways are rather related to signaling via the JA/ET system (Glazebrook, 2005).

Lipid signaling

Signaling by phospholipids is an important component in the signaling pathways in the Eukaryotes. It plays a major role in plant growth and development as well as in systemic response to environmental stresses, including the attack by pathogens (Song & Goodman, 2002)**.** To investigate the involvement of both phospholipase C and D in early responses, *Brassica napus* plants were treated with the chemical inducers of SAR: SA, BTH, and with the inducer mediating the ISR pathway, methyl jasmonate (MeJA). The results show that phospholipases are involved in very early processes leading to systemic responses in plants and that they are most probably initially activated at the post translational level (Profotová et al., 2006).

II.2.3. SAR/ISR regulators

The first regulatory protein identified as being essential for rhizobacteria-ISR was NPR1 (Pieterse et al., 1998). While in SAR, NPR1 functions as a transcriptional co-activator of SA-responsive *PR* genes, JA/ET-dependent ISR typically functions without *PR* gene activation. Hence, the role of NPR1 in ISR seems to be different from that in SAR. In SA signaling, NPR1 is clearly connected to a nuclear function (Fu & Dong, 2013), while in JA/ET signaling and ISR, evidence is accumulating for a cytosolic function of NPR1 (Pieterse et al., 2012; Stein et al., 2008). Interestingly, simultaneous activation of SAR and ISR leads to an additively enhanced defensive capacity (van Wees et al., 2000). Whether this is based on the notion that SAR and ISR do not seem to compete for the same subcellular pool of NPR1 is unknown, as the exact molecular mechanism by which NPR1 functions in JA/ET-dependent ISR remains to be investigated.

Although ISR involves long-distance root-to-shoot signaling, only few studies have investigated the signaling components of the plant root that are involved in the onset of ISR. Analysis of the transcriptome of WCS417-colonized Arabidopsis roots revealed the R2R3 type MYB transcription factor gene *MYB72* as one of the significantly induced genes (Verhagen et al., 2004). Knockout *myb72* mutants are impaired in their ability to express ISR, indicating that this root-specific transcription factor is essential for the onset of ISR (Van der Ent et al., 2008). *MYB72* is also induced in *Trichoderma*-colonized Arabidopsis roots and shown to be crucial for *Trichoderma*-ISR (Segarra et al., 2009), suggesting that MYB72 is a node of convergence in the ISR signaling pathway triggered by different beneficial microbes. Being a transcriptional regulator, it was postulated that MYB72 plays an important role in the generation and/or translocation of a long-distance ISR signal.

II.2.4. Defense mechanisms of systemic resistance

The defense responses described above lead to a new physiological, biochemical and molecular state, which gives the plant the ability to resist against pathogenic agents. This resistance requires often the implementation of complex cascades of events, from the initial mechanism of perception of pathogen to its actual establishment. These molecular and biochemical cascades constitute a favoured objective of research carried out on the mechanisms of resistance. Indeed, their deep understanding is the keystone to develop more efficient and/or more sustainable procedures for crop protection based on this phenomenon.

Accumulation of pathogenesis related proteins

Plant responses to attack by microorganisms are complex, and include the expression of a large number of genes encoding various defense- and pathogenesis-related proteins. As mentioned above, SAR is characterized by the accumulation of SA and PR proteins that serve as markers of the phenomenon (Ali et al., 2018). These PR proteins are currently classified into several groups according to their biochemical and molecular properties and are defined as proteins encoded by the host plant, but specifically induced in pathological or relative situations. They do not accumulate only locally in the infected area but are also induced systematically (Van Loon & Van Strien, 1999). The induction of SAR has been associated with the increase of hydrolytic enzymes such as β-1,3-glucanases (PR2) and chitinases (PR3, PR4, PR8 and PR11) that are capable of damaging fungal or bacterial cell walls (Andreu et al., 2006; Chandrashekar & Satyanarayana, 2006). PR proteins have been originally defined as proteins accumulating inside or outside plant cells following interaction with the pathogen (Hammond-Kosack & Jones, 1996). ISR is not generally associated with PR proteins (Pieterse et al., 1996). However, some non-pathogenic rhizobacteria can sometimes induce defense responses associated with the accumulation of hydrolytic enzymes (Ramamoorthy et al., 2001). For example, ISR induced by *P. fluorescens* strain Pf1 in rice and tea was associated with the accumulation of certain defense enzymes such as peroxidase, polyphenol oxidase, chitinase and β-1,3-glucanases which can inhibit the growth of pathogenic fungi (Nandakumar et al., 2001; Saravanakumar et al., 2007b).

Plant cell wall strengthening

In SAR, and more specifically in the local response linked to HR, it has become evident that the response of plants to the pathogenic organism or to injury is correlated with biochemical and physiological changes of cells which are accompanied by structural changes including thickening of the cell wall, and deposition of newly formed barriers such as callose, lignin or

phenolic compounds at the sites of pathogen entry (Hückelhoven, 2007; Kohler et al., 2002; Schenk et al., 2014; Soylu, 2006).

In the case of ISR as well, some studies have shown some defense mechanisms that are closely related to the establishment of chemical or physical barriers to pathogen entry. Thus, a large amount of callose and phenolic compounds (lignification) are rapidly accumulated at the site of infection in plants treated with PGPR and in certain pathosystems such as Arabidopsis/*Pseudomonas syringae* pv. *tomato* DC3000 by treatment with *Bacillus cereus* AR156 (Niu et al., 2011), cucumber/*Colletotrichum orbiculare* by treatment with *Serratia marcescens* (Jeun et al., 2004), Chilli/ *Pythium aphanidermatum* by treatment with *Pseudomonas fluorescens* EBL 20-PF (Muthukumar et al., 2011). These rapid reactions at the site of infection delay the spread of the pathogen and give the host plant the time necessary to put in place other defense mechanisms to restrict the growth of the pathogen. Furthermore, precursors of lignin and free radicals produced during the polymerization reactions can affect the plasticity of the membrane or inactivate pathogens enzymes. *P. fluorescens* and *B. amyloliquefaciens* for example reinforce cucumber defences against both *P. aphanidermatum* and *Colletotrichum orbiculare* (Chen et al., 2000; Jeun et al., 2004).

Accumulation of phytoalexins

The accumulation of antimicrobial phytoalexins has been widely described in the case of SAR in many plant species. They are defined as low molecular weight antifungal and/or antibiotic compounds, synthesized in plants during biotic or abiotic stress (Ahuja et al., 2011). These compounds accumulate in the plant tissues at the site of penetration of a pathogen, thus making it possible to reduce or stop its progression by disorganizing and killing its cells. In some cases, the speed of phytoalexin accumulation is associated with the plant's resistance to disease caused by a bacteria or fungus (Hammerschmidt, 1999; Kuc, 1995)

In ISR-defense mechanism, phytoalexin accumulation in plants treated with PGPR is still unclear. However, some examples of accumulation of phytoalexins or hydrolytic enzymes are occasionally reported in different plants, *e.g.*, treatment of carnation plants with *Pseudomonas fluorescens* WCS417r stimulated phytoalexin synthesis against wilt disease caused by *Fusarium oxysprum* (Van Peer et al., 1991). In another study on cucumber treated with *Pseudomonas putida* BTP1 phytoalexins accumulation was observed locally and systemically in the plants (Ongena et al., 2000; Ongena et al., 1999).

Involvement of the lipoxygenase pathway

Many previous studies have shown that in some pathosystems ISR is associated with the stimulation of the key enzyme of the lipoxygenase (LOX) pathway. This defense pathway synthesizes antibiotic and signal compounds called oxylipins from poly unsaturated fatty acids. For example, the increase in LOX activity in tomato treated with some PGPR was related to ISR against the pathogen *P. syringae* pv. *tomato* (Silva et al., 2004). A previous study has shown the ability of the PGPR *Pseudomonas putida* BTP1 to stimulate LOX and the synthesis of one of its products, the 13-hydropreoxyoctadecatrienoic acid (13-HPOT), after inoculation with the fungal pathogen *Botrytis cinerea* (Mariutto et al., 2011)

II.3. Priming for enhanced plant defense

Priming is the induction of a physiological state that allows a plant to deploy a more rapid and stronger defense response compared with a non-primed plant (Balmer et al., 2015)**.** In many cases, colonization of plant roots by beneficial microbes does not lead to major changes in defense-related gene expression in the above-ground plant parts. Instead, pathogen infection or insect herbivory on ISR expressing plants often leads to an accelerated expression of defense-related genes in comparison to similarly attacked control plants (Van Oosten et al., 2008; van Wees et al., 1999). This preparation of the whole plant to better combat pathogen or insect attack is characterized by a faster and/or stronger activation of cellular defences upon invasion, resulting in an enhanced level of resistance (Conrath, 2011).

The phenomenon "priming" is not only limited to SAR, but it has been also shown in ISR stimulated by PGPR. This phenomenon has been reported for the first time in carnation treated with *Pseudomonas fluorescens* WCS417r in which the accumulation of phytoalexins was expressed after the infection by *Fusarium oxysprum f.* sp. *danthi* (Van Peer et al., 1991)**.** In addition, treatment of Arabidopsis roots with *P. fluorescens WCS417r* primed the plants to produce more ET immediately after infection with the pathogen *P. syringae* pv. *tomato* (Hase et al., 2003). To date, a large number of studies with PGPR have supported the notion that ISR by beneficial microbes is commonly based on defense priming (Pieterse et al., 2014).

Some studies have investigated the long-term effects of priming. Priming can be inherited epigenetically from disease-exposed plants, and descendants of primed plants exhibit next-generation systemic resistance (Luna et al., 2012; Slaughter et al., 2012), as shown in recent studies comparing the reactions of *Arabidopsis thaliana* plants that had been either primed with an avirulent isolate of *Pseudomonas syringae* pv. *tomato* or primed with BABA. The descendants of primed plants showed a faster and higher accumulation of transcripts of defense-

related genes in the SA-signaling pathway and enhanced disease resistance upon challenge inoculation with a virulent isolate of *P. syringae*. The results suggest that plants can inherit a sensitization for the priming phenomenon and the primed state of plants is transferred to their progeny (Luna et al., 2012; Slaughter et al., 2012).

Thesis outline

Induced resistance in plants against pathogens has been known since the late 19th century. The first publications on this subject described investigations carried out separately by Ray (1901) and Beauverie (1901), which were followed by Chester's observations (1933) on "acquired physiological immunity". The scientific community ignored these investigations for a long period. Until, 1993, works reviewed by Professor Kuć (1995) demonstrated both in the greenhouse and in the field, how some elicitors of resistance could operate efficiently, in order to perform an alternative control of diseases caused by fungi, bacteria and viruses. Undoubtedly, these works succeeded to introduce the topic and to highlight the importance of this strategy, arousing great interest from big companies, which went from simple observation to research of natural or synthetic elicitors that could be an excellent candidates to induce resistance.

Since then the road has progressively cleared. In the last century, studies of induced resistance in many plant species were concluded against a broad spectrum of plant invaders. Therefore, researchers in plant protection opened an immense range of possibilities or alternatives that resistance induction can offer for the management of diseases and pests in crops of economic importance. To date, a good number of these elicitors of defense in plants have been incorporated into commercial-scale agricultural programs.

Currently, the use of induced resistance is emerging, taking into account the enormous amount of literature generated in the last years and the growing interest in this subject. However, studying histological, transcriptomic and biochemical aspect of induced resistance during plant-pathogen interaction gives more insights to accelerate a strategic choice for an efficient plant resistance activator.

Inoculation of plant roots by beneficial microbes does not only inhibit the growth of pathogens, but also induces systemic resistance in the plant, hence confers protection on leaves. Furthermore, the application of the non-protein amino acid, BABA can induce resistance against a broad spectrum of plant pathogens (Baccelli & Mauch-Mani, 2016). Only little is known about rhizobacteria and BABA-induction of resistance in wheat, in particular about the genetic basis governing this phenomenon.

The goal of the study consists in a better understanding of the mechanisms that lead to defences induced by the beneficial bacteria *P. protegens* and BABA in wheat against *P. triticina*, the causal agent of brown (leaf) rust. This PhD project can be divided into different steps:

- The first step of the project consisted on the study of *P. protegens* CHA0 effects on wheat plants. For this purpose, different aspects have been tested: effect on seed germination, bacterial colonization in wheat roots using tagged bacteria (GFP-tagged CHA0), their capacity of plant growth promotion and impact on root architecture.
- The second step was to determine and compare the efficacy of 2 resistance-inducing methods against *P. triticina* in wheat. We investigated whether application of different concentrations of BABA can induce resistance to *P. triticina* in wheat, and we examined the effect of different concentrations of BABA on vegetative growth of wheat seedlings with the aim to find the best-suited concentration to induce resistance with the smallest impact on growth. In this step, the capacity of *P. protegens* CHA0 to induce resistance against leaf rust on wheat seedlings was also investigated. Furthermore, to better understand the mechanisms implicated in the CHA0 and BABA-induced resistance in wheat, a histopathological study was performed.
- The third step of the project was the analysis of the transcriptomic changes occurring in such protected plants compared to un-induced control plants in order to postulate the defense pathway induced by *P. protegens* CHA0 and BABA.
- The last step was to test the efficiency of the two mentioned inducers (*P. protegens* CHA0 and BABA) in addition to *P. chlororaphis* PCL1391 and BTH for their ability to enhance resistance in wheat seedlings against the hemibiotrophic fungus *Zymoseptoria tritici*.
- A general conclusion of the thesis and suggested perspectives for the project are addressed in last chapter.

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CHAPTER II

Wheat inoculation with *Pseudomonas protegens* CHA0: root colonization and growth promotion capacity

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Chapter II: Wheat inoculation with *Pseudomonas protegens* CHA0: root colonization and growth promotion capacity

Abstract

Several rhizobacteria are considered as potential biocontrol and plant growth-promoting agents. Successful use of these beneficial bacteria requires their presence and activity at the appropriate level without any harmful effect to host plant and their survival in critical conditions. Among these rhizobacteria the strain *Pseudomonas protegens* CHA0 (CHA0) is known to be a good colonizer of plant roots and displays a beneficial effect on plant health and productivity. Here we report evidence that seed inoculation with CHA0 did not affect bread wheat germination. CHA0 is also able to colonize and persist on the root of four different wheat cultivars. More than 10^5 CFU/g of root were collected. The level of colonization was not affected 8 days prior a challenge with leaf rust infection. Additionally, CHA0 cells were unregularly distributed along wheat root and were more frequently found in old parts of root. Wheat plants germinated from CHA0-treated seeds showed enhanced vegetative growth (shoot length and shoot biomass) and more developed root system. Moreover, this strain showed its capacity to colonize wheat root in saline condition and its growth *in vitro* was not affected by 240 mM of NaCl.

Introduction

In 1904, Lorenz Hiltner was recognized as the first scientist observed that microorganisms were more abundant in the soil surrounding the plant roots and called this area the rhizosphere (Hartmann et al., 2008). The plant rhizosphere hosts a large and diverse community of microbes; their interactions with roots can influence plant health and productivity (Lambers et al., 2009). Within the rhizo-microbiome, some bacteria are categorized as plant growth promoting rhizobacteria (PGPR) (Lugtenberg & Kamilova, 2009a), which they can promote plant growth and provide better plant health through several indirect or direct mechanisms (Couillerot et al., 2009; Richardson et al., 2009)

Bacteria of the genus *Pseudomonas* are commonly found among the predominant genera in the rhizosphere of many plants (Couillerot et al., 2009; Haney et al., 2015) included wheat plants (Yoshida et al., 2012). They have a wide range of plant-beneficial functions with different modes of action (Ahmad et al., 2008; Loper et al., 2012; Naik et al., 2008) These rhizobacteria can affect (i) plant development; they alter the root architecture by producing plant hormones or modulating hormone production in the plant (García de Salamone et al., 2001; Picard &

Bosco, 2005; Shaharoona et al., 2006), (ii) on its nutrition; they make available to the plant certain essential minerals such as phosphorus (de Werra et al., 2009) and (iii) on its health; they produce secondary metabolites that can harm the development of plant pathogens and/or activate the plant defense mechanisms (Bakker et al., 2002; Gross & Loper, 2009).

Efficient root colonization by a given PGPR is a prerequisite to exert a successful biocontrol effect on the host plant, either by direct or indirect ways (Beneduzi et al., 2012; Lugtenberg & Kamilova, 2009b). In case of pseudomonads, approximately 10^5 CFU/g of root is known to be as threshold required to achieve beneficial effects on the plant (Haas & Défago, 2005). However, the amount and composition of rhizobium was found to be influenced by the host plant especially after a given stress (Neal et al., 2012; Rudrappa et al., 2008). To reach this high density on plant root, different methods of inoculation were used (Malusá et al., 2012). Seeds inoculation by beneficial microorganisms is an ideal way for delivery of high densities of beneficial microorganisms to soil, where they can colonize emerging plant roots (O'Callaghan, 2016). This method of inoculation is widely used for research purposes included for wheat studies (Abaid-Ullah et al., 2015; Akbar et al., 2019; Rosas et al., 2009). However, some *Pseudomonas* can inhibit seed germination (Lee et al., 2013; McPhail et al., 2010). In wheat, some indole-3-acetic acid (IAA) producing *Pseudomonas* isolates dramatically decreased seed germination and was found to be correlated with IAA production (Tabatabaei et al., 2016).

Root-colonizing *P. protegens* CHA0 (CHA0) was more reported as a potential bacterial antagonist to control plant diseases (Hase et al., 2000; Henkes et al., 2011; Ramette et al., 2011). In fact, it was isolated for the first time from the roots of tobacco grown in a soil near Payerne, Switzerland, that is naturally suppressive to black root rot in tobacco (Stutz et al., 1986). It has been also studied as a model for the biological control of fungal diseases as take-all of wheat (Sari et al., 2008).

Within this context, investigating the safe use of seed-inoculation with CHA0 on wheat plants is crucial step in this thesis. For that the objectives of this part were to assess whether (i) seed inoculation with CHA0 affects germination in quantity and quality. (ii) CHA0 is able to colonize and persist on the surface of wheat roots in four Swiss varieties. (iii) Aboveground parts of plant under biotic stress (leaf rust) can generate recruitment of more CHA0 cells in belowground parts. (iv) Effective colonization of wheat roots influences its growth at seedling stage. (vi) Potential changes in the root architecture of wheat can take place upon root colonization by this strain. We investigated also (v) colonization pattern of CHA0 by

quantification and microscopic observations using GFP-tagged bacteria. (vi) Behavior of CHA0 in saline condition.

Materials and methods

Plant material and growth conditions

Wheat seeds cv. Arina, Spluga, Cimetta and Forno (Triticum aestivum L.) were obtained from Agroscope, DSP, Delley. Surface sterilized seeds were used throughout the experiments. For this, the seeds were rinsed in 70% ethanol and incubated for 5 minutes in 5% bleach (sodium hypochlorite solution, Fisher Chemical, U.K.). Subsequently, the seeds were washed three times in sterile distilled water. They were then pre-germinated for 3 to 4 days on humid filter paper (Filterkrepp Papier braun, E. Weber & Cie AG, 8157 Dielsdorf, Switzerland). We selected the seedlings with similar growth state and morphology to plant in 120 mL polypropylene tubes (Semadeni, 3072 Ostermundigen, Switzerland) filled with a standard potting mixture (peat/sand, 3:1, vol/vol). The plants grew in a growth chamber with the following conditions: 16 hours day at 22°C, 8 hours night at 18°C and an irradiance of 300 μmol m^{-2} s^{-1}. The plants were watered as needed.

Bacterial inoculum

The bacterial inoculum consisted of the biocontrol agent *P. protegens* strain CHA0-Rif (Natsch et al., 1994), for inoculum preparation, the strain was grown on solid King's medium B (Pseudomonas agar F, Merck KGaA, 64271 Darmstadt, Germany) supplemented with 50 μg mL^{-1} rifampicin at 25°C in the dark for 3 days. A single colony, of a freshly grown culture, was transferred to 100 mL of King's liquid medium B supplemented with 50 μg mL^{-1} rifampicin. After an overnight incubation at 28°C with continuous shaking at 150 rpm, the bacterial culture was centrifuged at 3700 rpm and washed twice with sterile 10mM $MgSO_4$ solution. The final pellet was re-suspended in 20 mL sterile distilled water and adjusted to 10^6 cfu/mL and used for seed inoculation. For this, the sterilized wheat seeds were immerged into the bacterial suspension for 6 hours with shaking at 35-40 rpm at room temperature. Inoculated seeds underwent the pre-germination procedure as described previously. Control seeds were soaked in distilled water for the same time period before pre-germination.

Inoculation with *P. triticina*

Inoculations with leaf rust (*P. triticina*) were done using freshly harvested urediniospores of isolate Pr2271 (Agroscope, Changins, Switzerland). The urediniospores were generated on leaves of the susceptible cv. Arina. For infections, fresh urediniospores were mixed with talcum

powder in a 1:9 w/w ratio and rubbed gently on the leaf surface. Inoculated plants were placed in a dew box in the dark at 18 to 22°C for 24h to promote infection. Subsequently, the plants were placed in the growth chamber as described above.

Effect of CHA0 strains on wheat seed germination traits

To evaluate the effect of *P. protegens* on germination and emergence traits of wheat seeds, sterilized seeds cv. Arina, Spluga, Cimetta and Forno were germinated in dark at room temperature for 4 days on humid filter paper in plastic bags to maintain the humidity; furthermore, the germinated seeds were counted 4 days post-inoculation with bacteria. A seed was considered as germinated when its radicle emerged by about 2 mm in length. Final germination percentage was measured at day 4 after incubation. The root and shoot lengths were measured and seedling vigour index was determined as described by Tabatabaei et al. (2016): Vigour index = (Mean root length + Mean shoot length) × Germination percentage

Root colonization by CHA0 on wheat plants challenged with leaf rust

In order to assess CHA0 root colonization capacity in wheat and to investigated whether the infection with leaf rust can change bacteria colonization behaviour or not, seeds of four Swiss varieties (Arina, Spluga, Cimetta and Forno) were inoculated with bacterial suspension (10^6 CFU/mL) or with distilled water as previously elucidated. At 2-leaf stage, the plants were then either inoculated with leaf rust spores or talcum as control. Bacterial root quantification was performed 0, 4 and 8 days post challenge with leaf rust, for that, roots from bacteria-inoculated and mock-inoculated plants were cut and washed gently in tap water, subsequently, roots were dried on filter paper and transferred to 50 mL plastic tubes filled with 10 mL of sterile distilled water. Bacteria were extracted from roots by 1 min of agitation using a vortex mixer, followed by 1 min of sonication. Each extract was serially diluted and plated on King's medium B supplemented with 100 µg/mL of rifampicin and were incubated at 28°C in the dark for 24 to 36 hours. The colony-forming units (CFU) per gram of fresh material were then determined.

Plant growth promotion capacity of CHA0

To investigate possible effects of CHA0 treatment on vegetative plant growth, shoot length and shoot dry mass of seedlings was measured in two weeks-old plants inoculated with bacteria or sterile distilled water as control. Shoot length was defined as the upper part of the plant cut at the residue of the seed. The shoots were placed on coffee filter paper and dried separately in an oven at 65° until sample weight remained constant (dry weight).

Effect of CHA0 on wheat root architecture

In order to characterize root architecture, wheat seeds cv. Forno were either mock-inoculated with sterile distilled water or with a fresh overnight bacterial suspension (10^6 CFU/mL) and they were then transferred to soil-free system (Planchamp et al., 2013). 7 day after inoculation, photos of the whole root systems from six replicates were taken. Root images were analyzed by EZ- Rhizo 2 (Laboratory of plant physiology and biophysics, University of Glasgow, UK) (Maurhofer et al., 1994) and morphometric parameters such as total length root and number of lateral roots were evaluated.

Colonization of wheat roots by CHA0

Colonization of CHA0 were monitored on roots of wheat (cv. Forno) cultivated for 7 days in soil-free system and pre-inoculated with GFP-tagged CHA0 (Baehler et al., 2005) kindly provided by Prof. Christophe Keel (University of Lausanne, Switzerland). For microscopy, 1cm long root pieces were cut from the three different zones of root: (i) the old primary roots (zone A), (ii) the lateral roots (zone B) and (iii) the new primary roots (zone C). Samples were immediately mounted in sterile water on glass slides and were then examined with a confocal microscope (Leica SP5 white laser, Leica, Germany). Also, bacteria in each part of root were extracted and quantified as described above.

Bacterial growth in NaCl *in vitro*

Bacterial growth was observed in liquid King's B supplemented with NaCl at 240 mM. CHA0 were incubated overnight (150 rpm, 28°C) in 100 mL King's B medium. Then, 1 mL of bacterial solution was used to inoculate 100 mL of 240 mM NaCl King's B medium. Bacterial concentration was measured at 600 nm each hour until the achievement of the stationary phase of bacteria growth.

Statistical analyses

In all experiments, except for bacterial growth in NaCl, the data were analysed by a *t*-test or by one-way ANOVA followed by Tukey HSD for multiple comparisons. Analyses of bacterial growth in NaCl were made by Weibull-curve. All data analyses were performed in in R (R Core Team, 2017). Significant differences were considered at $P < 0.05$. Experiments were repeated at least twice in two independent assays.

Results

Effect of bacteria on seed germination

Some PGPR can affect the germination process of wheat seeds. To test a potential effect of CHA0 on germination of 4 Swiss varieties of bread wheat, after 4 days in humid filter paper, percentage of germinated seeds and vigor index was calculated in a total of 30 seeds for each treatment with three replicates. The results presented in Fig. 1 show that inoculation with bacteria did not affect germination in the 4 varieties tested in this study. In fact, T test ($p = <0.05$) didn't show any significant difference between bacteria treated plant and control in both parameters (Fig. 1 A and B).

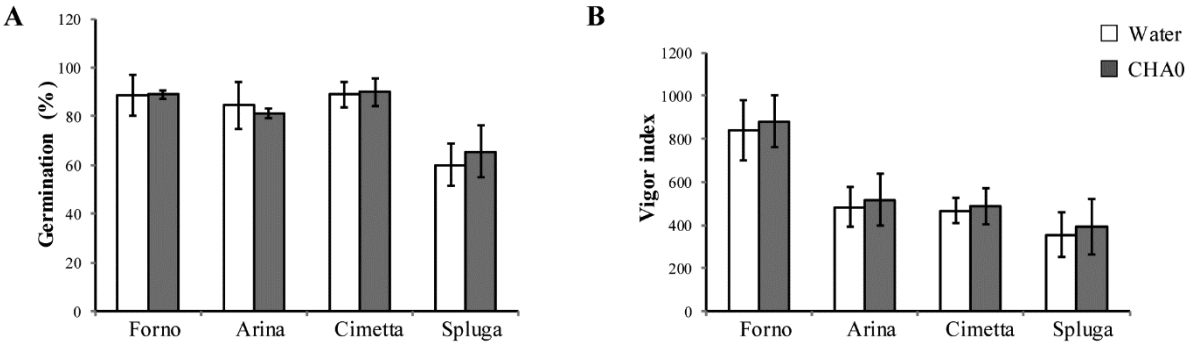

Figure 1: Response of bread wheat seeds to inoculation with CHA0: changes in germination (A) and vigor index (B) developed in humid filter paper containing 30 seeds. Error bars indicate the standard error of the mean (n = 3).

Root colonization capacity of CHA0

The aim of this experiment was to evaluate the capacity of CHA0 to colonize the roots of bread wheat. Therefore, four Swiss wheat varieties Forno, Arina, Cimetta and Spluga were seed-inoculated with distilled water or with a suspension of CHA0 (10^6 CFU/mL) and root colonization was assessed in 0, 4 and 8 days post challenge with leaf rust. The results (Fig. 2) showed that the strain CHA0 equally colonized roots of 4 Swiss varieties and no significant differences were observed, moreover, the level of root colonization remained stable over all time points tested in this study and the number of bacteria recovered was more than 10^5 CFU/g of root fresh weight in all varieties at different time points. The infection of wheat seedlings with leaf rust did not affect colonization of roots by CHA0; in fact, bacteria quantity in roots of infected and non-infected plant was not statistically different.

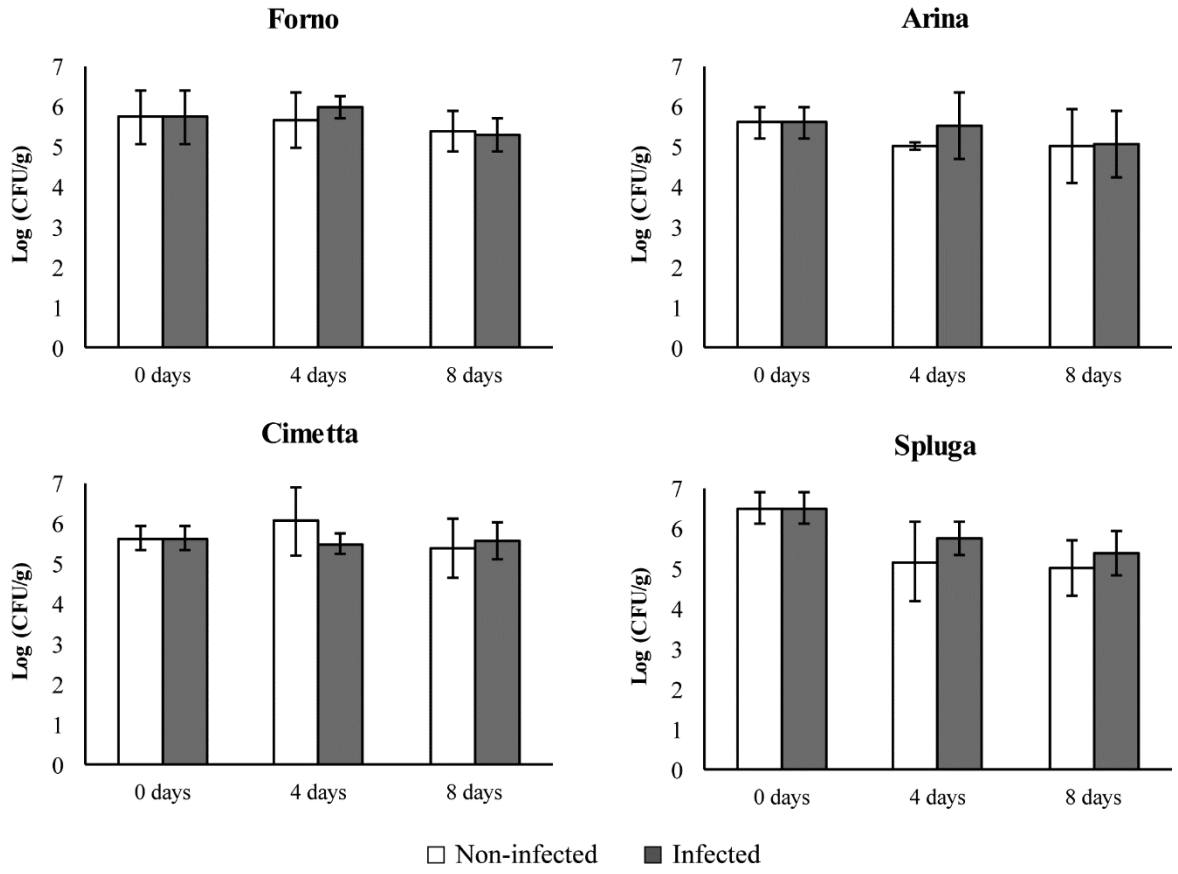

Figure 2: Root colonization capacity of CHA0 of wheat seedlings challenged with leaf rust. Plants were seed-inoculated with bacterial suspension (10^6 CFU/mL) and either inoculated with leaf rust spores or mock-inoculated with talcum at two-leaf age plants. Error bars indicate the standard error of the mean (n = 5).

Plant growth promoting capacity of CHA0

To evaluate the effect of inoculation with CHA0 on growth of wheat plants (cv. Forno, Arina Cimetta and Spluga) in comparison to mock-inoculated plants, we assessed the parameters linked to plant fitness; shoot length and shoot dry weight (Fig. 3). We observed a clear increase on length shoot after inoculation with bacteria in all varieties tested in this study (Fig. 3-A), in fact, bacteria treated plants were significantly higher than control ones. Same pattern was observed with the dry biomass of shoot and bacterized plants showed significantly more biomass compared with non-bacterized plants (Fig. 3B).

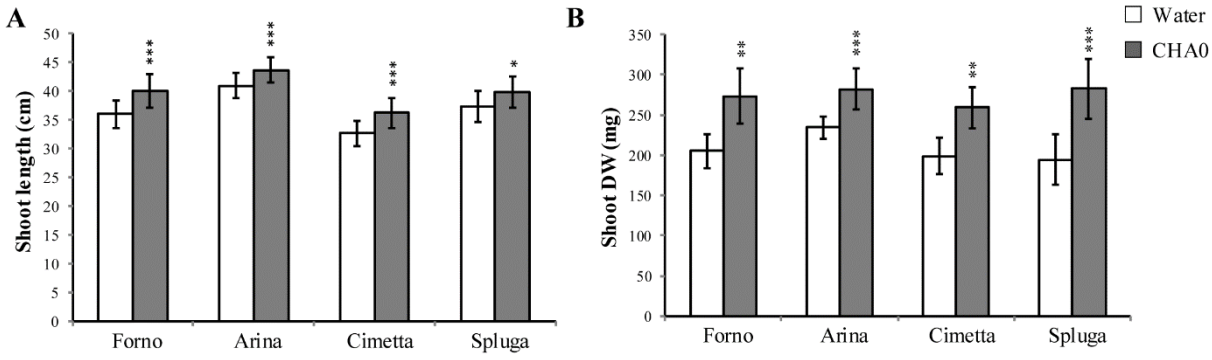

Figure 3: Growth and biomass production of 2 weeks-old plants treated or not with CHA0. **A:** shoot length, measured from the seed to the top of the longest leaf. **B:** shoots dry weight obtained with drying at 65° until the weight remained constant. Error bars indicate the standard error of the mean (n =18). Asterisks indicate statistically significant differences in response to CHA0 treatment (Student's t-test; *P<0.05; **P<0.01; ***P<0.001)

Effect of CHA0 on wheat root architecture

To assess the impact CHA0 on some root architecture parameters, images of root from bacterized and non-bacterized plants were analysed by the software EZ- Rhizo 2 and the results are presented in Fig. 4. Clearly, bacteria inoculation improved root development and enhanced root branching (Fig. 4A). Results showed significantly a higher number of lateral roots in bacteria treated plants comparing to control and total length root were higher in plant inoculated with bacteria (10^6 CFU/mL) compared to non-treated ones (Fig. 4B).

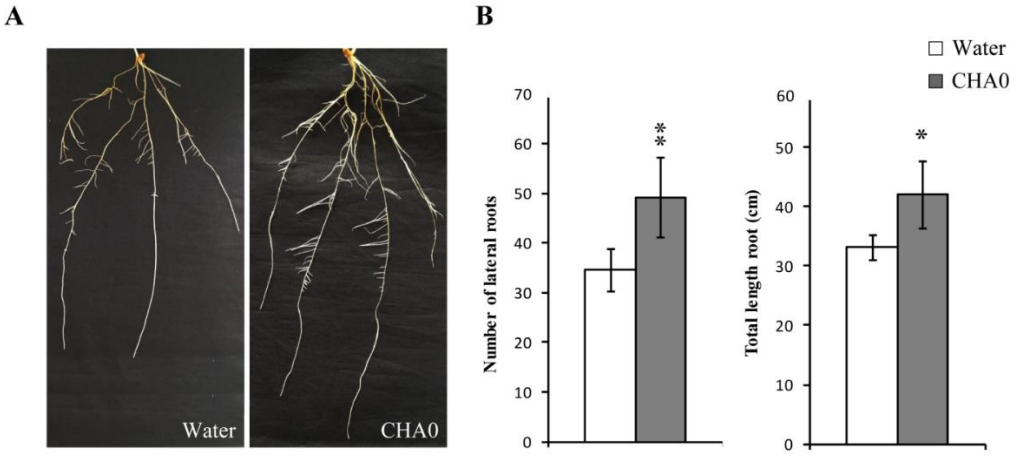

Figure 4: response on root architecture of 7 days-old wheat seedlings to inoculation with *P. protegens* CHA0. **A:** Roots of bacterized (CHA0 10^6 CFU/mL) and non-bacterized (Water) wheat plants grown in soil-free system. **B:** Root architecture parameters of 7 days-old wheat plants inoculated or not with *P. protegens* CHA0, Error bars indicate the standard error of the mean (n =6). Asterisks indicate statistically significant differences in response to CHA0 treatment (Student's t-test; *P<0.05; **P<0.01; ***P<0.001)

Colonization pattern of wheat roots by CHA0

A combination of microscopic observations (Fig. 5A) and bacterial quantification by dilution plating (Fig. 5B) allowed to characterize distribution and colonization patterns of bacterial cells according to different zones of roots of 7-day-old plants. The results showed that bacteria are unregularly distributed along wheat root. Stronger colonization was observed in old primary roots (zone A) in comparison to new formed parts; lateral roots (zone B) and new primary roots (zone C). The old primary roots were mostly covered by cells localized between epidermal root cells and organized in strings (biofilms) (Fig. 5A, Zone A).Whereas, in the two root zones B and C, only few cells were observed and bacteria were mostly present as single cells (Fig. 5A, Zone B and C).

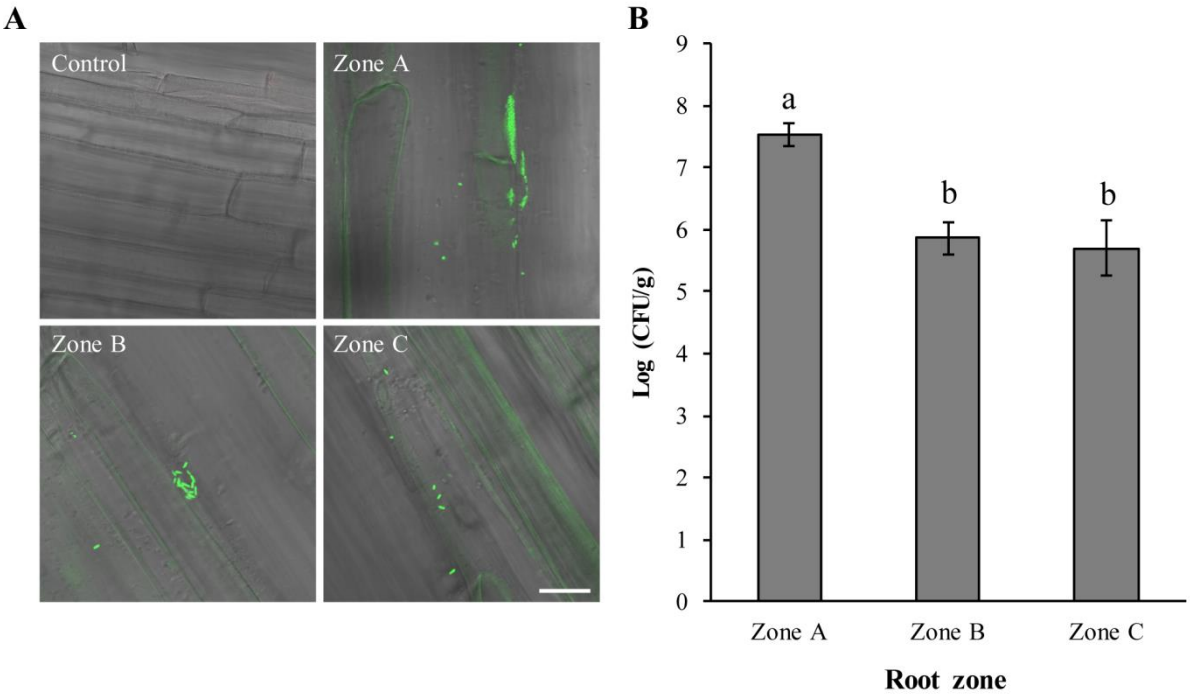

Figure 5: Root colonization pattern of CHA0 in 7 day-old wheat plants. **A.** Confocal images of 3 different parts of wheat root colonized by GFP-tagged of CHA0. **B.** Comparison of bacterial densities after inoculation in the different root zones. Bacterial densities were assessed by dilution plating. For a given root zone, bars with the same letter are not significantly different according to Tukey HSD (P < 0:05) for multiple comparisons. Zone A: old primary roots, Zone B: lateral roots, Zone C: new primary roots. Bars = 10 µm

Bacterial growth in NaCl

For possible application of CHA0 in saline condition, growth and root colonization of this bacterium was assessed after NaCl treatment (Fig. 6). Bacterial growth was observed in King's B medium supplemented with NaCl (240 mM) (Fig. 6A). CHA0 was able to survive in saline

condition, no significant differences were observed between bacterial growth in NaCl (240 mM) and in control condition. Also, CHA0 colonization of wheat root cv. Arina was assessed one week after salt treatment (Fig. 6B). No significant differences were observed between salt-treated plant and untreated control.

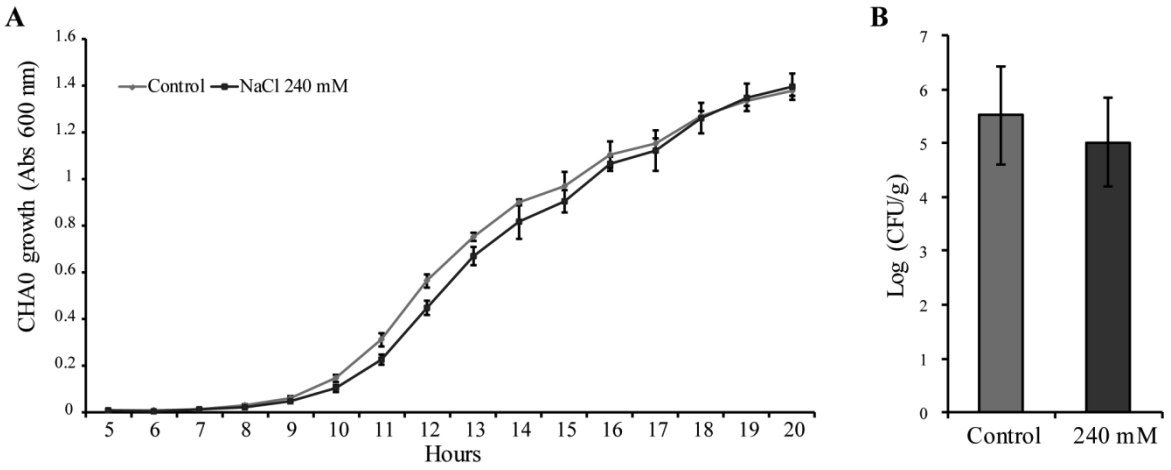

Figure 6: Effect of saline conditions on CHA0 survival and its root wheat colonization. **A.** Bacterial growth in King'B medium supplemented or not with NaCl (240 mM). No significant differences were observed by semi-parametric curves and Weibull test. **B.** Bacteria amount in wheat root one week after salt stress; bacteria were extracted and quantified by dilution plating. Bars show averages of 3 replicates.

Discussion

The application of PGPR requires certain conditions for an efficient beneficial effect (Lugtenberg & Kamilova, 2009a). This study provides an initial assessment of CHA0-wheat interaction in order to evaluate whether this strain could be safely used in future works as biocontrol. Our results demonstrated that CHA0 is a potential PGPR on the four tested Swiss varieties of bread wheat with a beneficial effect.

Seed inoculation with CHA0 did not affect wheat germination

Seed inoculation with PGPR is an ideal tool to supply the soil with a high density of beneficial microorganisms. However, it was reported that some *Pseudomonads* could exert inhibitory effects on seed germination (Lee et al., 2013; McPhail et al., 2010). In this work, tow parameters linked to seed germinated was investigated. Neither percentage of germination nor vigor index was affected by CHA0 inoculation in four tested cultivars of bread wheat. Contrary to our results, Tabatabaei et al. (2016) found that some indole-3-acetic acid (IAA) producing *Pseudomonas* isolates inhibit seed germination durum wheat and was correlated with IAA production. However, CHA0 was reported to produce small amounts of IAA (Beyeler et al.,

1999) which could explain its neutral effect on wheat seeds germination. Positive effect of PGPR on wheat seed germination was also reported, Selvakumar et al. (2009) showed that soaking seeds in a culture suspension of *P. fragi* significantly increased the percent germination. Concluding that rhizobacteria affects germination differently and is related to the specie of pseudomonad used for seed-inoculation.

CHA0 successfully colonized bread wheat

The amount of rhizobacteria on root surface after inoculation is a prerequisite to exert effective growth promotion or disease protection in the host plant (Haas & Défago, 2005). In this study, the capacity of CHA0 to colonize the roots of four Swiss wheat varieties was assessed by dilution plating. CHA0 was successfully able to colonize four Swiss wheat varieties and more than 10^5 CFU/g of root fresh weight were found on all tested varieties, which is known to be the threshold required to provoke beneficial plant effects with plant growth-promoting pseudomonads (Haas & Défago, 2005; Raaijmakers et al., 1995). In support of our results, CHA0 root colonization capacity and its beneficial effects on wheat were reported in several works (Henkes et al., 2011; Imperiali et al., 2017; Sari et al., 2008). This strain was widely reported to be a good colonizer of other species. Indeed, it was isolated from tobacco roots in Switzerland (Stutz et al., 1986). In addition, it was found to colonize roots of cucumber (Hase et al., 2000), Arabidopsis (Iavicoli et al., 2003), barley (Henkes et al., 2011), maize (Chiriboga et al., 2018) and sorghum (Deepika et al., 2019).

The emerging “cry for help” hypothesis posits that plants recruit microbes that are able to alleviate plant stress in a given situation (López-Ráez et al., 2011; Neal et al., 2012; Rudrappa et al., 2008). The hypothesis that amount of CHA0 on roots can change after leaves attack was investigated in this works. For that, CHA0-inoculated plants at the two-leaf stage were challenged by leaf rust disease. Our results did not confirm the hypothesis; in fact the amount of bacteria remained stable during all time points with or without rust infection, which could be explain by reaching the required amount of CHA0 to exert a beneficial effect (Haas & Défago, 2005; Raaijmakers et al., 1995), plants did not need additional recruitment of more bacteria in wheat roots. In Contrast, aboveground white-fly feeding significantly increased the population density of beneficial belowground microflora including beneficial bacteria (Kloepper & Ryu, 2006).

Colonization pattern of CHA0 in wheat roots

A combination of bacterial quantification and microscopic observations using GFP-tagged bacteria gave us more insight on root colonization pattern of this strain. This study was conducted with cv. Forno that presented a good growth in soil-free system (data no shown). Plant-bacteria interaction was studied in gnotobiotic system (Chin-A-Woeng et al., 1997; Planchamp et al., 2013; Simons et al., 1996) included the strain CHA0 (Keel et al., 1989). Here, we have provided evidence that wheat root colonization by the strain CHA0 was not uniform. CHA0 cells were more frequently found in old parts of root. This was also observed in CHA0-maize root colonization study conducted in a microcosm system, confirming that older and/or decaying maize roots represent a favourable niche for the inoculant (Troxler et al., 1997). Moreover, distribution of CHA0 along the root of tomato varied from 10^6 CFU per cm near the root base to 10^2 CFU per cm near the root tip. In the same study, Similar colonization patterns were found for the *P. protegens* biocontrol strains WCS365 and F113(Chin-A-Woeng et al., 1997). Our results observed in soil-free system were similar with those reported by Planchamp et al. (2013) where they found more cells of *P. putida* KT2440 present in the upper parts than in the lower parts of roots in both conditions; soil-pot standard system and soil-free system. This common observation of CHA0-root colonization pattern could be explained either due to abundance of nutrients and/or bacterial chemo-attractants, or simply as a consequence of being a more protective microenvironment for the bacteria. However, other species of *Pseudomonas* were found to be more abundant near root hairs (Mercado-Blanco et al., 2016; Prieto et al., 2011)

CHA0 promoted wheat growth and affected root architecture

Once effective colonization of wheat roots by CHA0 was demonstrated we aimed to assess whether this bacterium was able to promote plant growth in wheat after seed treatment. Our results showed the capacity of CHA0 to enhance wheat growth at seedling level in four Swiss cultivars. This strain was more reported as a potential bacterial antagonist to control plant diseases (Hase et al., 2000; Henkes et al., 2011; Iavicoli et al., 2003; Keel et al., 1989; Stutz et al., 1986). However, in field experiment, a significant positive effect of the application of beneficial soil organisms, including CHA0, on performance of wheat crop was observed specially when plants were under biotic stress (Imperiali et al., 2017). The observed plant growth promotion of CHA0 could be explained by production of phytohormones and the increase of nutrient availability to plants, in particular phosphate. CHA0 can solubilize mineral phosphate and improve plant growth in phosphate-limiting conditions (de Werra et al., 2009).

Moreover, we provide evidence that CHA0 inoculation improves root development and enhances root branching. This could also explain the observed plant growth promotion exerted by this stain, since enhancement in root surface and expansion of plant root systems increase the plant's ability to acquire nutrients as was observed under effect PGPRs (Rosier et al., 2018).

As was reported before, CHA0 is able to produce low concentrations of IAA (Beyeler et al., 1999) which may stimulate primary root elongation of wheat as was proved in other studies of PGPR-root development (Patten & Glick, 2002; Remans et al., 2008). Moreover, it was found that CHA0 produces DAPG (Notz et al., 2001). DAPG produced by PGPR can modify root system architecture by enhancing root branching (Brazelton et al., 2008; Couillerot et al., 2011; Walker et al., 2011) Indeed, applications of exogenous DAPG, at a concentration around 10 μM, stimulated lateral root production in tomato seedlings (Brazelton et al., 2008) as was observed in our study where we found more lateral root in CHA0-treated plants.

CHA0 survived in saline conditions

Finally, survival of this strain in saline conditions and its capacity to colonize wheat root was investigated. Neither *In vitro* growth nor root colonization capacity of CHA0 was affected by NaCl (240 mM), providing evidence that CHA0 could be used as biocontrol of plant disease in saline soils as was reported in case of other *Pseudomonas* (Egamberdieva, 2012; Paul & Nair, 2008; Rangarajan et al., 2003; Walker et al., 2011). Recently, studies have been demonstrated the capacity of *Pseudomonas* species to induce tolerance in plants (Azadikhah et al., 2019; Chu et al., 2019; Egamberdieva et al., 2015). However, the stain CHA0 was not yet tested for its capacity to induce salt tolerance in wheat. Our findings open the door to this strain for possible induction of salt tolerance studies in wheat.

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CHAPTER III

Histopathological aspects of induced resistance by *Pseudomonas protegens* CHA0 and β-aminobutyric acid in wheat against *Puccinia triticina*

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Manuscript submitted.

Chapter III: Histopathological aspects of induced resistance by *Pseudomonas protegens* CHA0 and β-aminobutyric acid in wheat against *Puccinia triticina*

Abstract

After perception of specific biotic or abiotic stimuli, such as root colonization by rhizobacteria or selected chemicals, plants are able to enhance their basal resistance against pathogens. Due to its sustainability, such induced resistance is highly valuable for disease management in agriculture. Here we study an example of resistance against wheat-leaf rust induced by *Pseudomonas protegens* CHA0 (CHA0) and β-aminobutyric acid (BABA), respectively. Seed dressing with CHA0 reduced the number of sporulating pustules on the leaves and the expression of resistance was visible as necrotic or chlorotic flecks. Moreover, a beneficial effect of CHA0 on growth was observed in wheat seedlings challenged or not with leaf rust. BABA was tested at 10, 15 and 20 mM and a dose-dependent reduction of leaf rust infection was observed with the highest level of protection at 20 mM. However, BABA treatment repressed plant growth at 20 mM. Balancing between BABA-impact on plant growth and its protective capacity, we selected 15 mM as suitable concentration to protect wheat seedlings against leaf rust with the least impact on vegetative growth. To understand the mechanisms behind the observed resistance, we have studied the histological aspects of the fungal infection process. Our results showed that the pre-entry process was not affected by the two resistance inducers. However, both treatments reduced fungal penetration and haustoria formation. The timing and the amplitude of the resistance reactions was different after bacterial or chemical induction, leading to different levels of resistance to leaf rust. During fungal colonization of the tissues, a high deposition of callose and the accumulation of H_2O_2 in both CHA0- and BABA-treated plants pointed to an important contribution to resistance.

Key words:

Leaf rust, callose deposition, hydrogen peroxide (H_2O_2), plant resistance inducers

INTRODUCTION

Plants dispose of several layers of sophisticated defense mechanisms to defend themselves against pathogen attack. The first layer is given by preformed physical and chemical barriers that impede the pathogen to penetrate into the plant and to initiate infection (Ferreira *et al.* 2006). Once the presence of the pathogen has been detected, the plant activates further chemical and physical barriers that block or at least delay the attack (second layer; Jones and Dangl

(2006)). Defense success depends on the readiness of the plant to detect the pathogen. In the case of the interaction between wheat and the leaf rust pathogen (*Puccinia triticina*), the plant can detect specific fungal avirulence factors (elicitors) with leaf rust resistance genes (Lr). This gene-by-gene interaction is a very rapid recognition-reaction event leading to an elevated degree of resistance against the disease. However, the avirulence patterns can change and the pathogen may become undetectable by the plant. This resistance breakdown happened recently with yellow rust (Hovmøller *et al.* 2010) and stem rust (Singh *et al.* 2011).

In the case of unspecific recognition of the pathogen, the plant is still able to contain the development of the pathogen but with a reduced and variable degree of severity of infection (Jones and Dangl 2006). The degree of this quantitative resistance is linked to the readiness of the plant defences and depends on a series of genetic and environmental factors. Besides the pathogen itself, biological and abiotic stimuli as well as certain chemicals can enhance plant resistance (Mauch-Mani *et al.* 2017). Such induced resistance can be limited to the site of the inducing treatment but it can also be systemic and thereby effective in parts of the plant distant from the site of induction (Van Loon 1997). For instance, certain root-associated bacteria such as the biocontrol strain *Pseudomonas protegens* CHA0 (formerly *P. fluorescens* CHA0) induce systemic resistance against viral and fungal diseases in various dicots (Maurhofer *et al.* 1994; Haas and Keel 2003; Iavicoli *et al.* 2003) and monocots (Sari *et al.* 2008; Henkes *et al.* 2011). Certain chemical compounds can also induce disease resistance in plants, *e.g.* the non-protein amino-acid β-amino-n-butyric acid (BABA). Root colonizing bacteria and BABA root treatment reduce significantly the severity of infection caused by the oomycete *Hyaloperonospora arabidopsis* on *Arabidopsis thaliana*, and the induced state is regulated by different defense signaling pathways, depending on the inducing agent and the challenging pathogen (Van der Ent *et al.* 2009).

In the present work, we aimed to study the mechanisms underlying induced resistance by CHA0 and BABA in wheat against leaf rust. A previous study has shown that root colonization by *Pseudomonas protegens* strain CHA0 reduces the number of leaf rust uredia on susceptible wheat seedlings in wheat (Sharifi-Tehrani *et al.* 2009). The enhanced resistance is very likely due to a resistance priming event by induction of systemic resistance (ISR). This priming enables the plant to cope with the pathogen at an early stage of infection. To study this, in the work presented here, we followed the interaction between the plant and the pathogen at the microscopic level (De Vleesschauwer *et al.* 2008).

The infection process of leaf rust is well known (Bolton *et al.* 2008). After adhesion of a urediniospore on the leaf surface, germination, directed growth of the germ tube on the plant surface towards a stoma, and recognition of the guard cell lips take place. A small appressorium is formed over the stomatal opening and then, a penetration hypha is entering through the stomatal pore. Following penetration, a substomatal vesicle, and haustorium develop (Bolton *et al.* 2008).

Primed plants recognize the pathogen and produce reactive oxygen species (ROS) and deposit callose at the infection sites (Balmer *et al.* 2015). This rapid local oxydative burst generates, among other, hydrogen peroxide (H_2O_2) during pre-haustorial resistance against wheat leaf rust caused by *P. triticina* (Wesp-Guterres *et al.* 2013; Serfling *et al.* 2016). Callose is an effective barrier that is induced at the sites of attack during the early stages of pathogen invasion (Luna *et al.* 2011). A strong deposition of callose has been reported for the wheat Thatcher near-isogenic lines carrying leaf rust resistance genes (Wang *et al.* 2013).

Few studies have investigated rhizobacteria- and BABA-induced resistance against wheat leaf rust. In this study, we aimed to compare mechanisms involved CHA0-ISR and BABA-IR during interaction between leaf rust and wheat. To this end, we evaluated microscopically the development of fungal structures, the occurrence of callose deposition and hydrogen peroxide accumulation in leaf tissues.

MATERIALS AND METHODS

Induced resistance assay

Plant material and growth conditions

Experiments were done with the leaf rust-susceptible bread wheat cultivar Arina (Agroscope/DSP). Surface sterilized seeds were used in all experiments. Wheat seeds were rinsed with 70% ethanol, incubated for 5 minutes in 5 % bleach (sodium hypochlorite solution, Fisher Chemical, U.K.) and washed three times in sterile distilled water. The sterilized seeds were germinated on humid filter paper (Filterkrepp Papier braun, E. Weber & Cie AG, 8157 Dielsdorf, Switzerland) in plastic bags maintained in the dark at room temperature. Three to 4 days later, seedlings at similar growth state and morphology were selected and planted in 120 mL polypropylene tubes (Semadeni, 3072 Ostermundingen, Switzerland) filled with a standard potting mixture (peat/sand, 3:1, vol/vol). The tubes were placed in a growth chamber with the 16 hours day at 22°C and 8 hours night at 18°C and with 300 μmol m^{-2} s^{-1} light. The plants were watered regularly keeping the potting soil wet yet avoiding its saturation.

Bacterial inoculum

The bacterial inoculum consisted of the biocontrol agent *P. protegens* strain CHA0-Rif (Natsch *et al.* 1994) (in the following called CHA0), a spontaneous rifampicin resistant strain of *P. protegens* strain CHA0 (Stutz *et al.* 1986; Ramette *et al.* 2011). Both strains are similar in terms of growth rates, production of antimicrobial compounds (Natsch *et al.* 1994) and their capacity to induce resistance in wheat (Sharifi-Tehrani *et al.* 2009). Routinely, the strain was grown on solid King's medium B (Pseudomonas agar F, Merck KGaA, 64271 Darmstadt, Germany) supplemented with 50 μg mL^{-1} rifampicin at 25°C in the dark for 3 days. For long-term storage, 1mL of a freshly grown bacterial suspension in King's liquid medium B (30g proteose-peptone, 1.5g K_2HPO_4, 2.46 g $MgSO_4$, 1.5g glycerol in 1 L distilled water) was mixed with 1mL glycerol (87%) and conserved at -80°C. For inoculum production, a single colony of a freshly grown culture was transferred to a 300 mL Erlenmeyer flask filled with 100 mL of King's liquid medium B supplemented with 50 μg mL^{-1} rifampicin. After 12 h incubation at 28°C with continuous shaking at 150 rpm, the bacterial culture was centrifuged at 3700 rpm and washed twice with sterile 10mM $MgSO_4$ solution. The final pellet was re-suspended in 20 mL sterile distilled water and adjusted to an OD_{600} of 0.1 corresponding to approximately 10^6 CFU/mL and used for seed inoculation. For this, the sterilized wheat seeds were immersed in the bacterial suspension for 6 hours with shaking at 35-40 rpm at room temperature. Inoculated seeds underwent the pre-germination procedure as described above. Control seeds were soaked in distilled water for the same time period before pre-germination.

Treatment with β-aminobutyric acid

The resistance inducer BABA was purchased at Sigma-Aldrich (Buchs SG, Switzerland). Dilutions of 10, 15 and 20 mM of BABA in distilled water were used as a soil drench. For this, 10 ml of BABA solution were added to the soil to plants were at the 2 leaf stage, 48 hours before infection with leaf rust. Control plants were treated with the same amount of distilled water.

Effect of CHA0 and BABA on plant development

In a first step, the impact of the inoculation of CHA0 and BABA treatment on the plant was assessed. To measure root colonization by CHA0, 0.1g each of inoculated or control roots were shaken each in 10 mL sterilized distilled water during 1 min on a benchtop vortex mixer, followed by 1 min of sonication. The root extract was serially diluted and plated on solid King's

medium B supplemented with 100 μg mL^{-1} of rifampicin. The plates were incubated at 28°C in the dark and the number of CFUs was determined after 24h to 36h.

To investigate possible effects of CHA0 and BABA treatments on plant growth, the dry mass of the shoot of pre-treated seedlings was measured at 12 days after inoculation with leaf rust. Shoot length was defined as the upper part of the plant cut at the residue of the seed. The shoots were weighed (fresh weight), placed on coffee filter paper and dried separately in an oven at 65° until sample weight remained constant (dry weight).

Inoculation with P. triticina

Inoculations with leaf rust (*P. triticina*) were done at the 2-leaf stage (BBCH 12 (Meier 1997)) using freshly harvested urediniospores of isolate Pr2271 (Agroscope, Changins, Switzerland). The urediniospores were generated on leaves of cv. Arina. For infections, fresh urediniospores were mixed with talcum powder in a 1:9 w/w ratio and rubbed gently on the leaf surface. Inoculated plants were placed in a dew box in the dark at 18 to 22°C for 24h to promote infection. Subsequently, the plants were placed in the growth chamber as described above. After 12 days or when the symptoms were sufficiently developed on the control plants, the infection type was assessed using the 0–4 scoring system (Table S1) described by Roelfs (1992).

Histochemical assessment of leaf rust infection in presence of CHA0 and BABA

Assessment of fungal growth and development

Leaf rust growth was observed on 2 cm leaf segments from the centre of the second leaves at 0, 6, 12, 24, 48, 72 and 96 hai (hours after inoculation). The leaf segments were immerged in 96% ethanol for 2-3 days to remove chlorophyll. The distained leaf segments were washed in an ethanol/ water (1:2 v/v) solution and then incubated in 0.5 M sodium hydroxide for 15 min with slight shaking. The leaf segments were incubated for 15 min in distilled water and before soaking for 2 h in 0.1 M Tris–HCl buffer (pH 8.5). Fungal structures were then stained with a 0.2% Calcofluor White solution in water (Sigma-Aldrich, Germany) for 5 min. After four washings in distilled water, the samples were stored in 50 % (*v*/v*)* glycerol for microscopic observation.

The preparations were examined with an epifluorescence microscope (Model E800; Nikon Instruments Europe, Badhoevedorp, The Netherlands) using excitation at 365 nm in combination with a 450 nm barrier filter and a dichroic mirror at 400 nm. This installation allowed the determination of position and number of all fungal organs on and in the leaf,

namely germinated and non-germinated spores, appressoria, sub-stomatal vescicles and haustoria.

Identification and quantification of callose deposition

Assessment of callose deposition was done on segments from the centre of the second leaf at 0, 24, 48 and 72 hai with leaf rust according to Scalschi *et al.* (2015). The leaf tissue was distained for 48h in 96% ethanol until transparent. Subsequently, the leaf tissue was rehydrated in 0.07 M phosphate buffer (pH =9) for 30 min and incubated for 15 min 0.05% aniline-blue (Sigma, St. Louis) prepared in 0.07 M phosphate buffer and were finally stained overnight in 0.5% aniline-blue microscopic observations were performed with the epifluorescence microscope using a UV filter as described above.

The presence and the quantity of deposited callose was determined from digital photographs by counting the number of white pixels (representing callose deposits) in 20 infection sites for each replicate, using the GNU Image Manipulation Program (GIMP 2.10.10) software. Contrast settings of the photographs were adjusted to obtain an optimal separation of the callose signal from the background signal. Callose was automatically identified using the "Color Range" tool and callose-corresponding pixels were recorded as the area covered by the total number of selected pixels (Scalschi *et al.* 2015).

Accumulation of H_2O_2 at the infection sites

Detection of H_2O_2 was carried out using DAB (3,3-diaminobenzidine, Sigma-Aldrich, Switzerland) staining as described (Thordal-Christensen *et al.* 1997). The second fully expanded leaves were cut at 0, 24, 48 and 72 hai and immediately immersed in a solution containing 1mg mL^{-1} DAB dissolved in HCl acidified distilled water (pH 3.8). Leaves were incubated in the dark for 8 h to allow DAB uptake and reaction with H_2O_2. Subsequently, leaves were cleared in saturated chloral hydrate and scanned at 1.200 dpi (Epson perfection, V370 PHOTO).

In presence of H_2O_2, DAB is reduced to a dark-brown deposit that can be easily visualized in the leaves. The H_2O_2 content of the leaves was quantified by counting the number of dark-brown DAB pixels using GIMP 2.10.10 software and the percentage of DAB stain was calculated corresponding to total leaf area (Luna *et al.* 2011). The dark-brown DAB pixels were selected using "Color selection" and the total area of leaves was using the "Free Selection" tool.

Experimental set up and statistical analyses.

All experiments were repeated at least twice. The induced resistance assay consisted of seven biological replicates. The fungal growth and the callose deposition assessments were done with three independent replicates and the H_2O_2 quantification on ten biological replicates.

Fungal development structures were identified and counted at 50 sites in each replicate. The percentage of germinated spores = (germinated spores/observed spores) ×100, the percentage of stomatal appressoria = (stomatal appressoria/germinated spores)×100, percentage of sub-stomatal vesicles = (sub-stomatal vesicles/stomatal appressoria) x 100 and percentage of haustoria = (haustoria/ sub-stomatal vesicles ×100) were determined.

Data were collected and stored in spreadsheets (Microsoft® Excel 2010, Redmond USA). Statistical analysis was conducted in R (R Core Team, 2017).

In all experiments, statistically significant differences in response to CHA0 and BABA treatment compered to control were tested with Student's T-test, Except, the experiment data of the effect of CHA0 and BABA on plant development were analyzed by two-way ANOVA with the factors; treatment (CHA0 and BABA) and rust inoculation (infected or not). Tukey Honest Significant Differences (HSD) test was used for multiple comparisons. Significant differences were considered at $P < 0.05$.

RESULTS

Plant growth and biomass production in presence of CHA0, BABA and following inoculation with leaf rust

Twelve days after planting of the seedlings, 5×10^5 CFU/g on average of CHA0 were recovered on the fresh roots, showing the capacity of the bacterium to successfully colonize the roots. In preliminary experiment, the initial concentration of bacteria (10^4, 10^6 and 10^8 CFU/mL) used for seed inoculation did not alter the final number of bacteria on the roots (data not shown).

The effect of CHA0 and BABA on plant length and biomass production is presented in Fig. 1. The results indicate that the plants treated with CHA0 grew significantly longer and produced significantly more biomass. The growth and the biomass were not influenced by the presence of the pathogen (Fig. 1A and B). In contrast, plants treated with BABA at 20 mM were significantly shorter and produced significantly less biomass than the untreated control. Meanwhile, the plants treated with 10 or 15mM of BABA were not different to the untreated control. The infections with leaf rust did not affect plant growth or biomass, exception made, for the treatment with BABA at 20 mM (Fig. 1C and D).

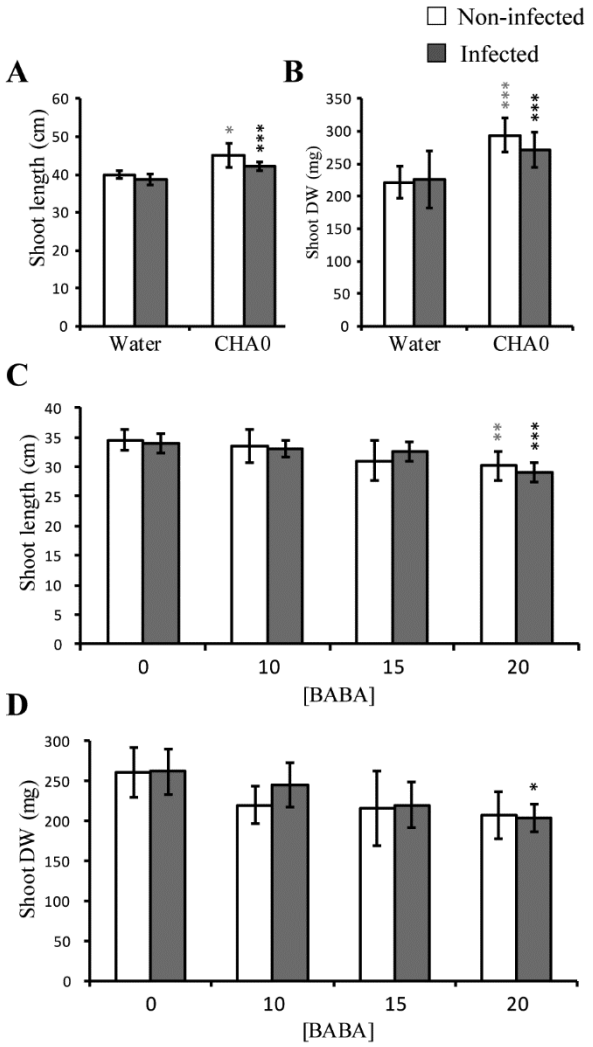

Figure 1: Growth and biomass production of plants treated with CHA0 (**A** and **B**) and BABA (10, 15 and 20 mM) (**C** and **D**) at 12 dpi with *P .triticina.* Shoot length was measured from the seed to the top of the longest leaf. Shoots dry weight was obtained after drying samples at 65° until the weight remained constant. Error bars indicate the standard errors for the average values of 7 replicates, grey and dark stars indicate significant differences compared to non-infected and infected control respectively (Tukey's test; *P<0.05; **P<0.01; ***P<0.001).

Phenotypic reaction to leaf rust of seedling pre-treated with CHA0 or BABA

Twelve days after inoculation with *P. triticina*, control plants were totally healthy (Fig. 2a) while the rust inoculated plants presented uniformly uredia with chlorosis corresponding to score 3 (high infection type (HI) (Roelfs 1992)) (Fig. 2b). When treated with CHA0 (Fig. 2c), leaves showed overall a lower number of uredia compared to the infected control. The symptoms are heterogeneous, namely chlorotic flecks (score ";", low infection type (LI)) but also uredia without sporulation (score "2", LI) and with sporulation and a chlorotic halo (score "3", HI). Similarly, BABA treatments also resulted in a mix of chlorotic flecks (score ";") and small to medium pustules with and without low sporulation scored as "1" and "2". Generally, all BABA treatments lead to low infection type symptoms. Yet, the scores were clearly dose-dependent, since the higher the BABA concentration, the lower the scores (Fig 2d, e, f).

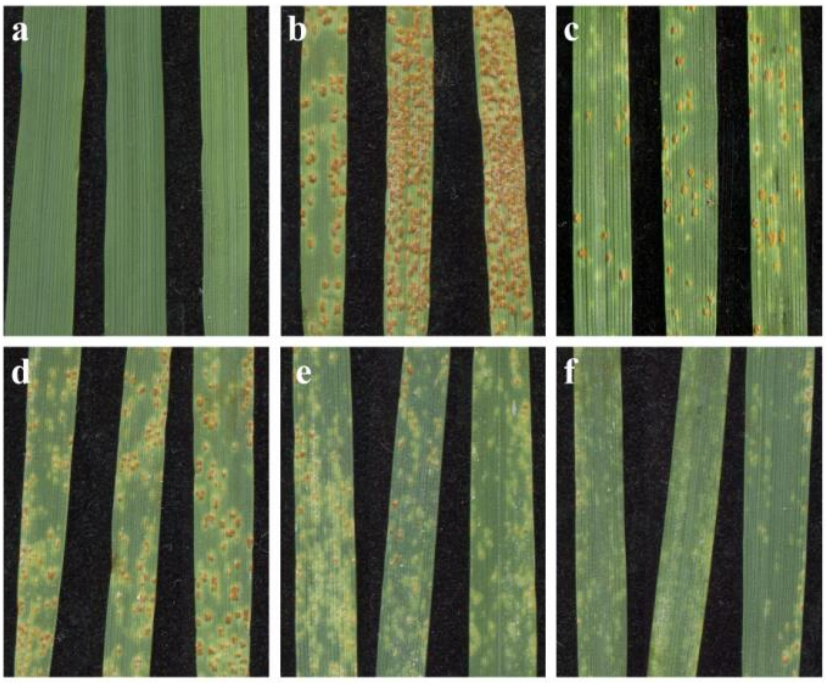

Figure 2: Leaf rust infection on seedling leaves of cultivar Arina at 12 dpi. **a,** control plant non-infected, **b,** infected plants non-treated, **c,** infected plants pre-treated with CHA0, from **d** to **f** infected plant treated with different concentration of BABA 10, 15 and 20 mM, respectively. Images were obtained by scanning at 1.200 dpi a segment of 3 to 4 cm from the centre part of the second leaf.

Fungal infection structures

Calcofluor white staining was used to track the pathogen structures during the first 96 hours after infection (hai) with leaf rust in non-treated control and on the plants pre-treated with CHA0 and BABA at 15mM. Within 6 hai, germ tubes started to elongate (Fig. 3A, 1). Independent of the pre-treatment, about 90% of urediniospores had germinated within 6 hai, in all treatments (Fig. 3B, 1). In the following, the proportion of germinated spores remained constant.

Once germinated, the fungus forms appressoria at the stomatal regions (Fig. 3A, 2). The formation of appressoria started at 6 hai (data not shown). Yet, at 24 hai, 85-88 % of the germinated spores had formed appressoria (Fig. 3B, 2). This proportion hardly varied between the control and the bacterial and BABA treatments.

Through the appressoria, the fungus penetrated into the cavities below the stomata, forming infectious vesicles in the substomatal cavity (Fig. 3A, 3). Our observations indicate that the formation of those vesicles started at 12 hai (data not shown). On the leaves of non-treated control plants, about 37% of appressoria had formed vesicles after 24 hai, with the proportion increasing to 50% after 48 hai. In plants inoculated with CHA0, about 29% of the appressoria formed. Yet, after 48 hai, the proportion of formed vesicles was about 23%. In BABA (15mM)

treated plants, the proportion of formed vesicles was 10% and increased to 30% at 48 hai (Fig. 3B, 3).

At 48h we noticed the formation of haustoria out of the vesicles (Fig. 3A, 4). At 72 hai, more than 80% of the sub-stomatal vesicles had formed haustoria in the untreated control plants. This proportion did not change at the last time point, at 96 hai. In the CHA0 treated plants, the proportion of formed haustoria was not different to the control plants, at both time points (i.e. 72 and 96 hai). However, the absolute number of haustoria was significantly lower in the CHA0 treated plants compared to the control. With BABA treatment, only about 50% of the vesicles formed haustoria. This is significantly less haustoria formed than in the control. At 96 hai, haustoria formation increased in the BABA treatment to 70% and there was no significant difference with the other treatments. Also, in the BABA treatment, the absolute number of haustoria was lower compared to the control plants (Fig. 3B, 4).

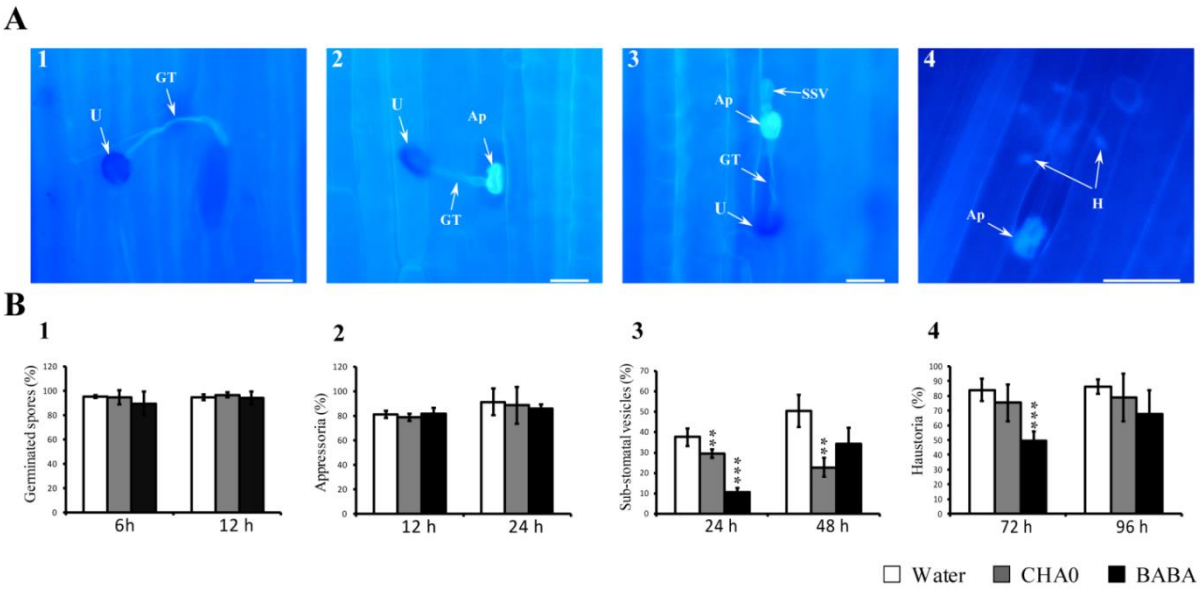

Figure 3: Microscopic observations and quantification of fungal structures *P. triticina* in wheat seedlings. **A**, fungal structures stained with calcofluor white and visualized under the epifluorescence microscope. In **B,** Percentages of fungal infection structures during infection of wheat with *P. triticina*: (1) spores germination, (2) appressoria, (3) sub-stomatal vesicles and (4) haustoria. Treatments: **CHA0**, plants obtained from seeds inoculated with CHA0 (10^6 CFU/ml), **BABA**, plants soil-drenched with BABA (15 mM) 48h before rust infection, **Water**, plants mock-treated with sterile distilled water. Fungal structures: **U**, urediniospore, **GT**, germ tube, **Ap**, appressorium, **H**, Haustoria. Error bars indicate the standard error of the average values of 3 replicates at 50 infection sites for each replicate. Asterisks indicate statistically significant differences in response to CHA0 or BABA treatment (Student's *t*-test; *P<0.05; **P<0.01; ***P<0.001). Bar 20 µm.

Callose deposition after leaf rust inoculation

Callose was quantified at 24, 48 and 72 hai after inoculation with leaf rust in the control, the CHA0 and the BABA 15mM treatment (Fig. 4). Callose deposition at the infection sites was made visible by aniline bleu. The amount of deposited callose was measured by counting pixels of stain around the infection sites (suppl. Fig. 1S). Overall, callose deposition occurred in all treatments within the first 24 hai (Fig.4). However, in plants pre-treated with CHA0 and BABA, a significantly higher quantity of callose was observed compared to the control. With CHA0, callose accumulated at the guard cells and was highest at 72h hai. In plants treated with BABA, we measured the highest callose deposition after 48 hai. Here, callose was not only observed at the guard cells (stomata) but also in mesophyll cells, at 72h.

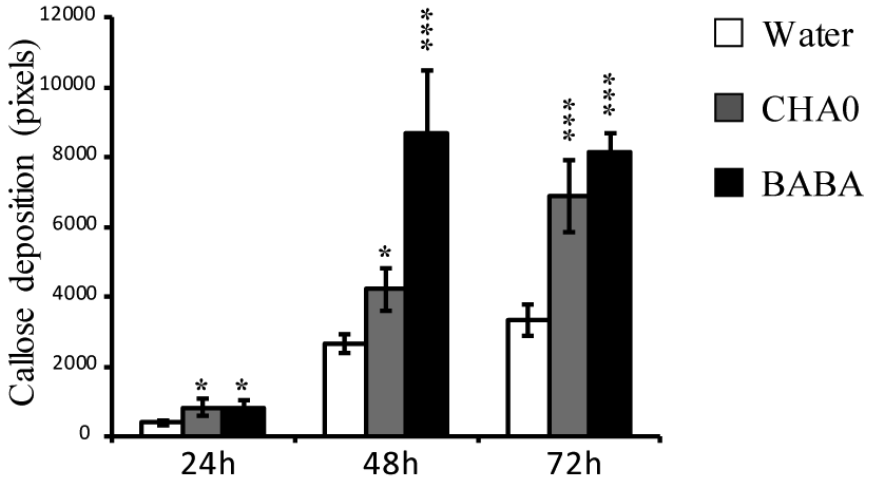

Figure 4: Callose deposition in wheat leaves in response to *P. triticina* infection in treated and control plants at 24, 48 and 72 hai. Callose was identified as white spots around the infection sites and quantified from digital photographs by measuring the number of pixels of the white surface at 20 infection sites for each of the three replicates. Treatments: **CHA0**, plants obtained from seeds inoculated with CHA0 (10^6 CFU/ml), **BABA**, plants soil-drenched with BABA (15 mM) 48h before rust infection, **Water**, plants mock-treated with sterile distilled water. Error bars indicate the standard error of the average values in 20 infection sites for each of three replicates. Asterisks indicate statistically significant differences in response to CHA0 or BABA treatments (Student's t-test; *P<0.05; **P<0.01; ***P<0.001).

Accumulation of H_2O_2 following the inoculation with leaf rust

The hydrogen peroxide released by plant tissue was measured between 0 and 72 hai with leaf rust in the control, the CHA0 and the BABA 15mM treatment. We monitored hydrogen peroxide with the DAB staining that produces dark-brownish dots (suppl. Fig. 2S). The surface

of the dots was measured as the proportion of dark-brown pixels on the total leaf surface (Fig. 5). Differences within the treatment became evident at 24 hai with higher H_2O_2 concentrations in the CHA0 and the BABA treated plants than in the control. Similarly, at 48 hai, in CHA0 and BABA treatments, the concentration of released H_2O_2 was significantly higher than in the control. At 72 hai, the accumulation of H_2O_2 in the CHA0 treatment dropped to the level of the control, while the BABA treatment increased to 12 % of the surface.

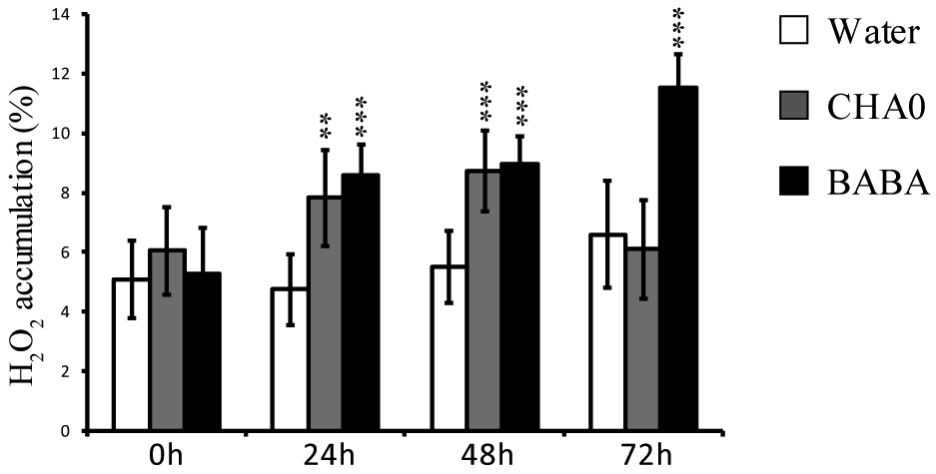

Figure 5: Hydrogen peroxide (H_2O_2) accumulation in wheat leaves in response to *P. triticina* infection. The generation of H_2O_2 was visible as dark-brown dots after DAB staining. The quantification based on the measurement of the dark-brown pixels on the photograph in proportion to the total leaf area. Treatments: **CHA0**, plants obtained from seeds inoculated with CHA0 (10^6 CFU/ml), **BABA**, plants soil-drenched with BABA (15 mM) 48h before rust infection, **Water**, plants mock-treated with sterile distilled water. Error bars indicate the standard error for the average values of 10 replicates. Asterisks indicate statistically significant differences in response to CHA0 or BABA treatment (Student's t-test; *P<0.05; **P<0.01; ***P<0.001).

DISCUSSION

Induced resistance has proven to be a complementary control strategy potentially interesting for protecting wheat from foliar diseases (Görlach *et al.* 1996; Sharifi-Tehrani *et al.* 2009). Here, we confirmed the efficacy of both beneficial bacteria CHA0 and BABA.to induce resistance in wheat against leaf rust

We first assessed the physiological effect of both resistance inducers on wheat growth. Efficient root colonization by a given plant growth promoting bacterium is a prerequisite to exert a successful biocontrol effect on the host plant, either directly (*e.g.* disease suppression) or indirectly (*e.g.* ISR) (Lugtenberg and Kamilova 2009; Beneduzi *et al.* 2012). In our study, after seed inoculation, CHA0 was able to colonize wheat roots and more than 10^5 CFU were recovered from 1g of root fresh weight. Our preliminary results showed that the initial

concentrations used for seed inoculum (10^4, 10^6 and 10^8 CFU/mL) did not affect final root colonization. The bacterial titer in wheat root was high enough for an effective plant protection as shown for soils suppressive to take-all of wheat and barley caused by *Gaeumannomyces graminis* var. *tritici* (Weller *et al.* 2007), Fusarium wilt of pea mediated by *Fusarium oxysporum* f. sp. *pisi* (Landa *et al.* 2002) and black root rot of tobacco (Stutz *et al.* 1986). Additionally, the growth promotion capacity of this stain was apparent with or without presence of leaf rust infections. In field experiments, a significant positive effect of beneficial soil organism application, including CHA0, on performance of wheat crop was observed especially when plants were under biotic stress (Imperiali *et al.* 2017). The observed plant growth promotion of CHA0 could be explained by production of phytohormones and the increase of nutrient availability to plants, in particular phosphate. CHA0 can solubilize mineral phosphate and improve plant growth in phosphate-limiting conditions (de Werra *et al.* 2009).

Recently, Thevenet *et al.* (2017) showed that BABA is a natural product in plants including wheat, but unfortunately, application of BABA can reduce plant growth in some plants (Cohen *et al.* 2016), At the relatively high concentration of 20 mM, BABA induced resistance but reduced the growth of the plant. The costs of induced resistance have also previously been linked to the reduction in plant growth (van Hulten *et al.* 2006; Heil 2007). Nevertheless, soil drenching with relatively low concentrations of BABA (15 mM) did not affect plant growth and reduced infection types in wheat seedlings infected with leaf rust, suggesting the possibility to optimize the BABA dose for an effective wheat protection against *P. triticina* with smallest impact on plant growth. Similarly, Luna *et al.* (2016) successfully identified feasible application methods of BABA by decreasing the concentration, which induced resistance in tomato against *Botrytis cinerea* without concurrent impacts on plant growth.

BABA is a well-recognized inducer of resistance against a broad spectrum of pathogens such as fungi, bacteria, virus and nematodes (Baccelli and Mauch-Mani 2016; Cohen *et al.* 2016). It is often applied as a soil drench (Hodge *et al.* 2005; Luna *et al.* 2016). Several studies demonstrate that BABA was effective when applied 1-3 days post-infection against a large spectrum of pathogens (Justyna and Ewa 2013). In the present, BABA was applied as a soil drench. 2 days before inoculation with leaf rust. The treatment significantly reduced leaf rust in wheat similar to results obtained with other rust species on wheat (Amzalek and Cohen 2007; Barilli *et al.* 2012). Inoculation with *P. protegens* strain CHA0 led to a specific reaction to infection with *P. triticina*: while mock-inoculated plants displayed many sporulating uredia (high infection type), plants with the bacterial treatment on the seeds had a mix of sporulating

uredia and chlorotic and necrotic flecks, suggesting that *P. protegens* strain CHA0 partially reduced infection with *P. triticina* in the wheat seedlings.

Histopathological studies after induction of resistance were performed to identify the events that occur during pathogenesis, and ultimately lead to better understanding of the resistance mechanism. The infection process of *P. triticina* in wheat plants has been well described (Bolton *et al.* 2008). During this infection process, the growth of the fungus can be interrupted at different phases. In principle, each of these phases can be affected by the action of resistance inducers. Our results regarding the infection events indicated that the pre-entry processes between plants treated with the two resistance inducers and control plants did not differ. In support of our results, reports show that the first steps of wheat rust infection (spore germination and appressoria formation) were not affected during the resistance implicated by the host plant (Wang *et al.* 2007; Orczyk *et al.* 2010). However, after penetration, distinct differences in fungal spread and host responses between CHA0- and BABA-treated plants were observed. In BABA-treated plants, fungal penetration was strongly aborted 24 hai, based on the reduced percentage of substomatal vesicles. Moreover, the percentage of haustoria formed in BABA-treated plant was significantly reduced at 72 hai. This was also observed in durable resistance to leaf rust in the Brazilian wheat variety Toropi, where the number of haustoria formed was significantly reduced (Wesp-Guterres *et al.* 2013). Being effective at two important stages of fungal development could explain the infection type observed in BABA-treated plants, showing no to very small uredia formation surrounded by chlorotic flecks.

While, in CHA0-treated plants, small to medium uredia with no or low sporulation were apparent at the leaf surface and the number of pustules were reduced compared to infected control, this infection type could be explained by the fact that the CHA0 treatment was partially effective before haustorium formation and reduction in fungal penetration was observed at 24 and 48 hai. The successful penetrations generated a lower number of haustoria, giving later rise to small uredia.

To investigate the observed effect on fungal spread exerted by BABA and CHA0 treatment, assessment of callose deposition and hydrogen peroxide (H_2O_2) were performed. During fungal infection, callose can be deposited at infection sites, which provides a physical barrier preventing the penetration of a pathogen (Voigt 2016). Our results showed that callose depositions were mainly detected in guard cells. In support of our observations, Wang *et al.* (2015) demonstrated that the resistance response to *Puccinia graminis* f. sp. tritici is associated with callose deposition in the wheat guard cells. The increase of callose in BABA and CHA0-

treated plants might restrict penetration and development of *P. triticina*, correlating with the increase of resistance in wheat seedlings against leaf rust. This defense mechanism of the plants is enhanced at the post-challenge primed state after perception of a stimulus from beneficial bacteria and BABA (Mauch-Mani *et al.* 2017). In addition to guard cells, callose was observed in the mesophyll cells of BABA-treated plants. This could also explain the high resistance observed compared to plants inoculated with bacteria. Same pattern was observed in defence mechanisms induced by *P. fluorescens* WCS417r and BABA against *Hyaloperonospora arabidopsis*. Both WCS417r and BABA prime for enhanced deposition of callose. However, more callose accumulated in BABA-treated plants (Van der Ent *et al.* 2009).

Reactive oxygen species (ROS) and especially H_2O_2 constitute a further important plant defence mechanism in interactions between plants and pathogens. We investigate H_2O_2 accumulation after infection with leaf rust in plants treated with BABA and CHA0. H_2O_2 accumulation was mostly detected in guard cells. At this site of penetration, an appressorium over the stomatal opening is generated. It seems likely that during the recognition or the formation of an appressorium, the generation of H_2O_2 in guard cells is induced possibly following secretion of rust elicitors. It might also be that mechanical forces during adhesion of the appressorium over the stoma elicit H_2O_2 generation in guard cells. In Arabidopsis, it was reported that H_2O_2 accumulation in guard cells is involved in the signal transduction during ABA-mediated stomatal closing (Sun *et al.* 2017). This could explain that accumulation of H_2O_2 in guard cells following recognition of leaf rust structures might be involved in stomatal closure, this being supported by the fact that we observed more accumulation of ABA in plants infected with leaf rust (data no shown), It had been reported that appressoria formation of *P. triticina* also caused stomata closure in wheat leaves (Bolton *et al.* 2008). Further studies shoe a correlation between H_2O_2 generation and hypersensitive reaction (HR) in resistance mechanism against wheat rust species (Wang *et al.* 2007; Orczyk *et al.* 2010; Serfling *et al.* 2016). In the current study, the accumulation of H_2O_2 due to both resistance inducers was observed 24 hai which corresponds to the beginning of haustorium generation. This suggests that H_2O_2 might initiate HR-defence mechanism. Our results are in line with the observation of Serfling *et al.* (2016) where HR was observed in mesophyll cells that were in contact with fungal haustorial mother cells at 24 hai, and the observed pre-haustorial resistance in the resistant accession PI272560 is due to an early HR of the first infected mesophyll cells. An HR accompanied by H_2O_2 accumulation also occurs in other interactions of plants with fungal parasites and causes non-host resistance to wheat stripe rust in broad bean (Cheng *et al.* 2012).

Plant-pathogen interaction can be modulated after induced resistance. Here we present a model for wheat-rust interaction (Fig. 6) where the infection of a host plant and growth of fungal structures have been interrupted at different phases in response to BABA or rhizobacteria-induced resistance. In control conditions, urediniospores of leaf rust were able to accomplish the infection cycle giving finally rise to an uredium with a normal size (Fig. 6A, left and right). In this case, callose accumulation in guard cells was not enough to prevent fungus penetration. Moreover, low generation of H_2O_2 was not able to initiate the required mechanisms to stop rust infection. While, in BABA and CHA0-treated plants fungal spread was differently affected (Fig. 6) with the exception of the pre-entry process where the spores germinated normally and appressoria were formed over the stomatal opening in both cases. In CHA0-treated plants, callose deposition in guard cells was highly elevated leading to an abortion of fungal penetration (Fig. 6B, left). However, when the fungus overcame the first barrier, callose deposition was not effective anymore. Here, we suggest that H_2O_2 accumulation can be accompanied by the activation of HR in some haustorium penetration sites which could partially stop fungal spread leading to the formation of small uredia (Fig. 6B, right), In the case of BABA, in addition to what we observed in CHA0 treatment, an accumulation of callose was noted in mesophyll cells. This could explain the high resistance observed after BABA treatment (Fig. 6C, left). Moreover, the high accumulation of H_2O_2 initiated HR in cells penetrated by rust haustoria and fungal spread totally stopped without any uredia formation (Fig. 6B, right).

The present study provides new insights into histological basis of BABA- and rhizobacteria-induced resistance against leaf rust of wheat showing the important role of callose deposition and H_2O_2 generation to prevent penetration and spread of leaf rust. Future studies will focus on expression analysis of some defense-related genes during the infection process of the fungus in order to underline differences and similarities in defence mechanisms induced by CHA0 and BABA.

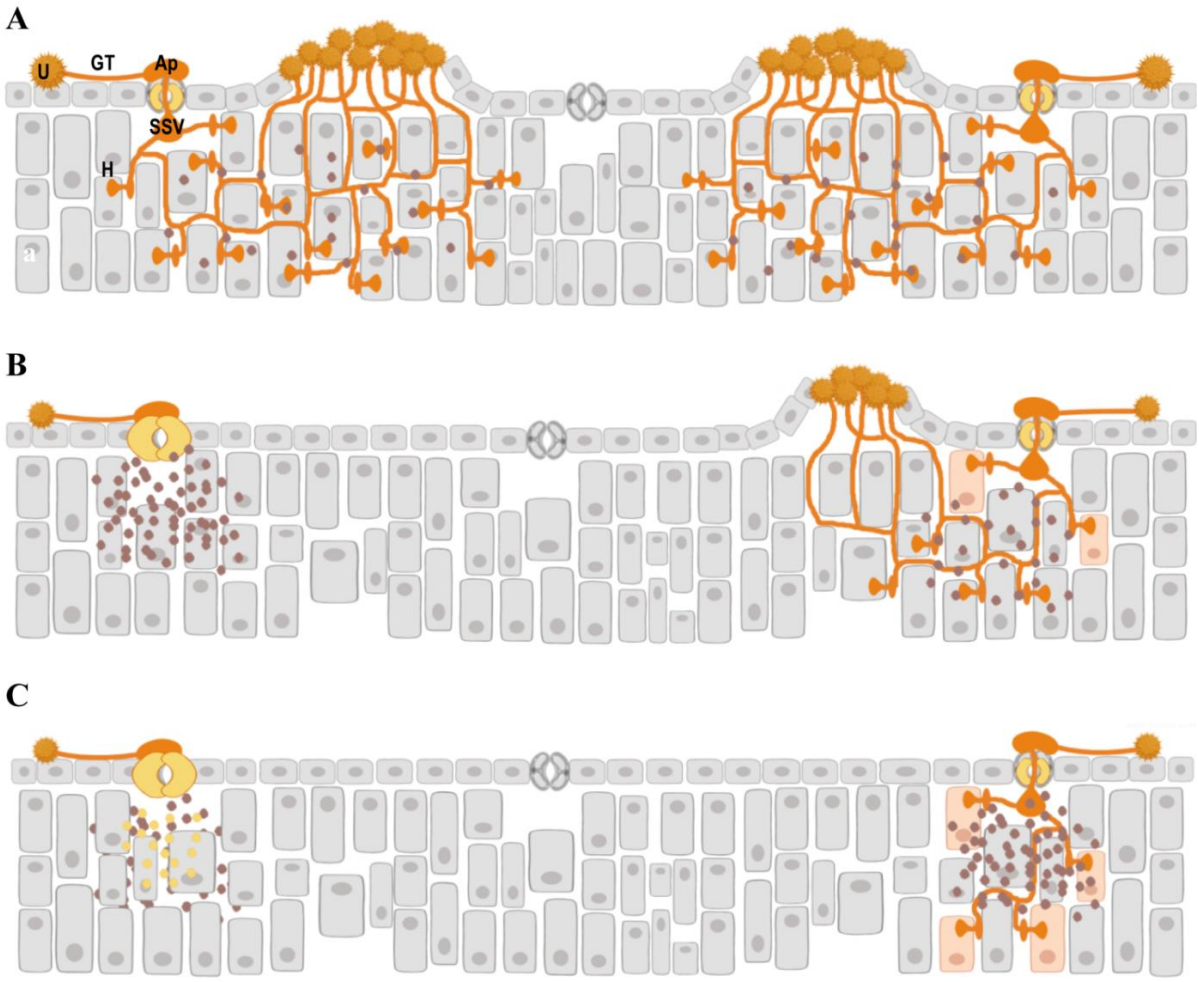

Figure 6: Diagram showing an overview of the fungal development and determined defense reactions of wheat to leaf rust infection under the effect of resistance inducers. **A**, compatible interaction between host and pathogen. In the un-treated plant, *P. triticina* overcomes the resistance mechanisms and is able to complete the infection cycle producing urediniospores (left and right). **B**, enhanced defense reactions in plants treated with CHA0; on the left, fungus penetration aborted after callose deposition in the guard cells, on the right, fungus spread partially but stopped after H_2O_2 accumulation and activation of HR in some haustorium penetration sites. Formation of small uredia without or with low spore production. **C**, enhanced defense reactions to leaf rust infection in BABA-treated plants; on the left, fungus penetration aborted after callose deposition in guard and mesophyll cells, on the right, fungus growth is totally blocked after accumulation of elevated quantities of H_2O_2 and HR activation in cells penetrated by rust haustoria. Fungal structures: **U**, urediniospore. **GT**, germ tube. **SSV**, substomatal vesicle. **Ap**, appressorium. **H**, Haustorium. Yellow dots are callose depositions. Brown spots present H_2O_2 generation.

Acknowledgements

We thank Stefan Kellenberger, Agroscope Changins, Nyon, for technical support advice to handle the leaf rust pathogens. FB gratefully acknowledges the financial support by the Swiss Federal Commission for Scholarships for Foreign Students and BMM the financial support of the Swiss National Science Foundation, Grant No. 312 310030_160162.

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Supplementary material

Tables:

Table S1: Simplified scheme of infection types of wheat leaf rust caused by *P. triticina* according to (Roelfs 1992)

Response (class)	Infection type	Disease symptoms
Immune	0	No uredia or other macroscopic sign of infection
Nearly immune	;	No uredia but hypersensitive necrotic or chlorotic flecks present
Very resistant	1	Small uredia surrounded by necrosis
Moderately resistant	2	Small to medium uredia surrounded by chlorosis or necrosis
Moderately susceptible	3	Medium-sized uredia that may be associated with chlorosis

Figures:

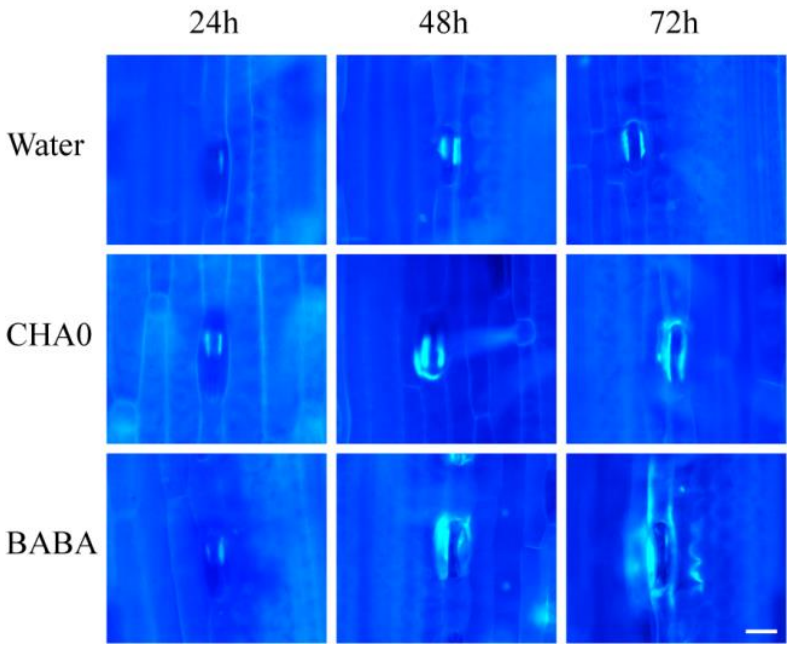

Figure 1S: Localization of callose at 24, 48 and 72 hai by *P. triticina* in wheat leaves treated with CHA0 or BABA. Photographs show stained leaves (Aniline-blue) exposed to UV light. Treatments: **CHA0**, plants obtained from seeds inoculated with CHA0 (10^6 CFU/ml), **BABA**, plants soil-drenched with BABA (15 mM) 48h before rust infection, **Water**, plants mock-treated with sterile distilled water. Bar 20 µm.

Figure 2S: *In situ* detection of hydrogen peroxide (H_2O_2) using DAB staining at 0, 24, 48 and 72 hai by *P. triticina* in wheat leaves treated with CHA0 or BABA. Images were obtained by scanning at 1.200 dpi the stained second leaf. Treatments: **CHA0**, plants obtained from seeds inoculated with CHA0 (10^6 CFU/ml), **BABA**, plants soil-drenched with BABA (15 mM) 48h before rust infection, **Water**, plants mock-treated with sterile distilled water.

CHAPTER IV

RT-qPCR analysis of host defense-related genes during induction of resistance by *Pseudomonas protegens* CHA0 and β-aminobutyric acid in wheat against *Puccinia triticina*

Fares Bellameche, Mahnaz Katuzi, Brigitte Mauch-Mani, Fabio Mascher

Chapter IV: RT-qPCR analysis of host defense-related genes during induction of resistance by *Pseudomonas protegens* CHA0 and β-aminobutyric acid in wheat against *Puccinia triticina*

Abstract

We have previously shown the efficacy of *Pseudomonas protegens* CHA0 (CHA0) and β-aminobutyric acid (BABA) in inducing resistance in wheat against *Puccinia triticina.* To understand the molecular mechanisms underlying this induced resistance we used quantitative real-time PCR to investigate the transcript levels of defence-related genes during the infection process of *P. triticina* in wheat plants pre-treated with CHA0 or BABA. Our results show that the timing of defense response gene induction is correlated with *P. triticina* infection events, where the upregulation of most of the studied genes was affected during the initial attempts of fungal penetration (12 hai). According to a comparative analysis, an additional effect on the regulation of some genes was observed in both treatments with more altered genes in BABA-treated plants. Genes such as *PAL* and *TaERF* showed their role in induced resistance with both activators. While, WCI*2*, *COI1* and *LTP2* were only involved in BABA induced resistance.

Introduction

Among the foliar diseases of wheat, brown (leaf) rust caused by *Puccinia triticina* is the most important factor diminishing wheat crops and wheat quality all over the world (Herrera-Foessel et al., 2011). Therefore, developing cultivars resistant to leaf rust has always been an objective of plant breeding programs but the high degree of virulence variation in *P. triticina* has made breeding for stable and durable leaf rust resistance in wheat difficult to achieve (García-Andrade et al., 2011; Kolmer, 2019; Kolmer & Hughes, 2013). In the bread wheat market, most cultivars are susceptible to fungal diseases including leaf rust (Courvoisier et al., 2015; Schaad et al., 2019). In recent years, novel sustainable approaches to induce resistance in such susceptible cultivars have increased and concurrently the use of biological (Chaudhary & Shukla, 2019; Dimkpa et al., 2009; Vurukonda et al., 2016) and chemical inducers (Görlach et al., 1996; Jakab et al., 2001; Justyna & Ewa, 2013; Karthikeyan & Gnanamanickam, 2011). In a previous study we have demonstrated the capacity of two types of induced resistance, namely β-aminobutyric acid-induced resistance (BABA-IR) and *Pseudomonas protegens* CHA0-induced systemic resistance (CHA0-ISR) to enhance resistance against leaf rust (see chapter 2 of this thesis, unpublished data and Fig. 1).

The molecular mechanisms involved in disease resistance of plants have been studied in some detail during the last thirty years (Jones & Dangl, 2006). The interaction of plants with their pathogens initiates one or more signaling pathways involving a large number of defence genes. Signaling pathways such as salicylic acid (SA), jasmonic acid (JA) and ethylene activate a series of defence responses that restrict or eliminate the pathogen (Denancé et al., 2013; Navarro et al., 2008). These responses include the hypersensitive response (HR), upregulation of phenylalanine ammonium lyase (PAL), a key enzyme in plant defence, deposition of cell wall reinforcing materials such as callose, and the synthesis of a wide range of antimicrobial compounds including pathogenesis related (PR)-proteins (e.g. chitinases and b-1,3-glucanases), phytoalexins and peroxidases (POX) (Hammond-Kosack & Jones, 1996; Veronese et al., 2003). Regulator genes such as the nonexpressor of pathogenesis-related *NPR1* are also important in both local and systemic resistance by controlling the expression of many stress-related genes, including those that encode PR proteins and proteins involved in the secretory pathway (Dong, 2004; Wang et al., 2005)

Recently, genes that are either upregulated or downregulated during the wheat–rust interaction have been identified using a variety of approaches (Casassola et al., 2015; Farrakh et al., 2018; Herrera-Foessel et al., 2011; Lu et al., 2005; Sharma et al., 2018). Moreover, the gene expression profile after treatment with plant resistance inducers in wheat was also studied. Molina et al. (1999) reported that *PR-1.1* and *PR-1.2*, considered as indicators of systemic acquired resistance (SAR) in dicotyledonous plants, were unresponsive to SA and other SAR activators in wheat, a monocotyledonous species. In wheat, Benzothiadiazole (BTH) application induces a set of genes referred to as the wheat chemically-induced (WCI) genes and provides resistance to powdery mildew (Görlach et al., 1996). During interactions with *Pseudomonas fluorescens* with wheat roots, transcripts encoding pathogenesis-related proteins and hypersensitive response protein were induced in coleoptiles (Okubara et al., 2010).

Such defence genes can be studied through transcriptome analysis of the resistant and the susceptible hosts after inoculation with the virulent pathotype (Manickavelu et al., 2010; Sharma et al., 2018). This approach would help in the identification of genes involved in one or more resistance signaling pathways. In our laboratory, we showed that during CHA0- and BABA-induced resistance, H_2O_2 accumulation and callose deposition played a fundamental role to enhance resistance of wheat cv. Arina against leaf rust (Bellameche et al., chapter 2 of present thesis). However, deciphering the molecular vocabulary of this observed resistance could help to formulate the exact mechanisms underlying CHA0-ISR and BABA-ISR. To this

end, genes of wheat were selected based on their potential roles in plant defense reported in wheat and their expression profile was investigated using RT-qPCR in CHA0 or BABA pre-treated wheat seedlings infected with leaf rust during infection process of the pathogen.

Material and methods

Plant material and growth conditions

Experiments were done with the leaf rust-susceptible bread wheat cultivar Arina (Agroscope/DSP). Surface sterilized seeds were used in all experiments. Wheat seeds were rinsed with 70% ethanol, incubated for 5 minutes in 5 % bleach (sodium hypochlorite solution, Fisher Chemical, U.K.) and washed three times in sterile distilled water. The sterilized seeds were germinated on humid filter paper (Filterkrepp Papier braun, E. Weber & Cie AG, 8157 Dielsdorf, Switzerland) in plastic bags maintained in dark at room temperature. Three to 4 days later, the seedlings with similar growth state and morphology were selected and planted in 120 mL polypropylene tubes (Semadeni, 3072 Ostermundingen, Switzerland) filled with a standard potting mixture (peat/sand, 3:1, vol/vol). The tubes were placed in a growth chamber with the 16 hours day at 22°C and 8 hours night at 18°C and with 300 µmol m^{-2} s^{-1} light. The plants were watered as needed.

Bacterial inoculum

The bacterial inoculum consisted of the biocontrol agent *P. protegens* strain CHA0-Rif (Stutz et al., 1986) (in the following called CHA0). The strain was grown on solid King's medium B (Pseudomonas agar F, Merck KGaA, 64271 Darmstadt, Germany) supplemented with 50 µg mL^{-1} rifampicin at 25°C in the dark for 3 days. For long-term storage, 1mL of a freshly grown bacterial suspension in King's liquid medium B (30g proteose-peptone, 1.5g K_2HPO_4, 2.46 g $MgSO_4$, 1.5g glycerol in 1 L distilled water) was mixed with 1mL glycerol (87%) and conserved at -80°C. For inoculum production, a single colony of a freshly grown culture was transferred to a 300 mL Erlenmeyer flask filled with 100 mL of King's liquid medium B supplemented with 50 µg mL^{-1} rifampicin. After 12 h incubation at 28°C with continuous shaking at 150 rpm, the bacterial culture was centrifuged at 3700 rpm and washed twice with sterile 10mM $MgSO_4$ solution. The final pellet was re-suspended in 20 mL sterile distilled water and adjusted to an OD_{600} of 0.1 corresponding to approximately 10^6 CFU/mL and used for seed inoculation. For this, the sterilized wheat seeds were immersed in the bacterial suspension for 6 hours with shaking at 35-40 rpm at room temperature. Inoculated seeds underwent the pre-germination

procedure as described above. Control seeds were soaked in distilled water for the same time period before pre-germination.

Treatment with β-aminobutyric acid

BABA was purchased at Sigma-Aldrich (Buchs SG, Switzerland). BABA (15mM) solution was dissolved in distilled water and 10 mL were used as soil-drench 2 days before *P. triticina* inoculation. Control plants were just watered with distilled water.

Inoculation with *P. triticina* and sampling

Inoculations with leaf rust (*P. triticina*) were done at the 2-leaf stage using freshly harvested urediniospores of isolate Pr2271 (Agroscope, Changins, Switzerland). The urediniospores were generated on leaves of cv. Arina. For infections, fresh urediniospores were mixed with talcum powder in a 1:9 w/w ratio and rubbed gently on the leaf surface. Inoculated plants were placed in a dew box in the dark at 18 to 22°C for 24h to promote infection. Subsequently, the plants were placed in the growth chamber as described above.

P. triticina inoculated leaves and mock-inoculated leaves were collected from 3 independent plants pre-treated with BABA or CHA0 or H_2O as control. Leaves were sampled at each of the following time points; 0, 6, 12, 24, 48 and 72 hours after inoculation (hai). Samples were immediately frozen in liquid nitrogen and stored at -80°C. Leaf rust infections were verified 12 days after inoculation (Fig. 1)

RNA extraction and gene expression analysis

Total RNA was extracted from the frozen wheat leaf tissues. Plant RNA isolation was performed according to manufacturer's instructions using the NucleoSpin® RNA Plant kit (MACHEREY-NAGEL, Germany). RNA was treated with DNase (MACHEREY-NAGEL) and reverse-transcribed into cDNA using SuperScript III RT (Invitrogen, http://www.invitrogen.com).

Sixteen defense-related genes with different molecular functions were selected, and their expression levels were studied. The gene names, primer sequence, and accession number are given in Table 1. The coding-sequence of each gene from databases (https://www.ncbi.nlm.nih.gov/genbank/) was blasted on the Arina genome using an online blast tool available on https://webblast.ipk-gatersleben.de/wheat_ten_genomes/viroblast.php. Primers were designed on the corresponding blasted genes in Arina genome using Geneious 8 (available from http://www.geneious.com). Quantitative real-time PCR was performed using the SensiFAST™ SYBR® No-ROX Kit (Bioline, http://www.bioline.com). Runs were performed on a MIC qPCR Cycler (Bio Molecular Systems). The reaction volume was 10 µL,

consisting of 0.2 µL nuclease-free water, 5 µL SensiFast SYBR master mix, 0.4 µL forward and reverse primer (each 10 µM) and 4 µL cDNA (400 ng). Non-template controls (NTS) were included along with each primer master mix. PCRs were performed as a three-step reaction (initial hold step, 95°C for 10 min; 40 cycles of amplification, 95°C for 15 s, 60°C for 20 s, 72°C for 20 s) with a final melting curve analysis (67°C-95°C). Melting curve analysis, cycle threshold (Ct) values and efficiency of each primer was performed on Mic qPCR Cycler software v.2.6.5 (Bio Molecular Systems). The minimal accepted efficiency for the primers was set to 0,8. Ct values were used to quantify gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) relative to control mock-infected plants in regard to the housekeeping gene ARF (Scholtz & Visser, 2013). The gene expression data was further visualized using the software MeV viewer (http:// www.tm4.org).

Statistical analysis

The gene expression experiment in this study was repeated twice. Gene expression was quantified in 3 independent biological replicates per sample in technical duplicates. Significant differences between the expression levels in CHA0- or BABA-treated plants in comparison to H_2O-treated control were analysed by a Student's *t*-test ($P < 0.05$). All statistical analyses were conducted in R (Team, 2018).

Results

Reduction of leaf rust symptoms on plants pre-treated with CHA0 or BABA

Twelve days after inoculation with *P. triticina*, the phenotypic reactions to leaf rust of seedling pre-treated with either CHA0 or BABA were verified (Fig. 1). Rust inoculated plants pre-treated with H_2O as control (Fig. 1 *H_2O*) presented uniformly uredia with corresponding chlorosis, while leaves of plants treated with CHA0 (Fig. 1 *CHA0*) showed a lower number of uredia compared to the infected control. The symptoms were heterogeneous, namely chlorotic flecks but also uredia without sporulation. Treatment of plants with BABA (Fig. 1 *BABA*) at 15 mM also resulted in a mix of chlorotic flecks and small to medium pustules with low or no sporulation.

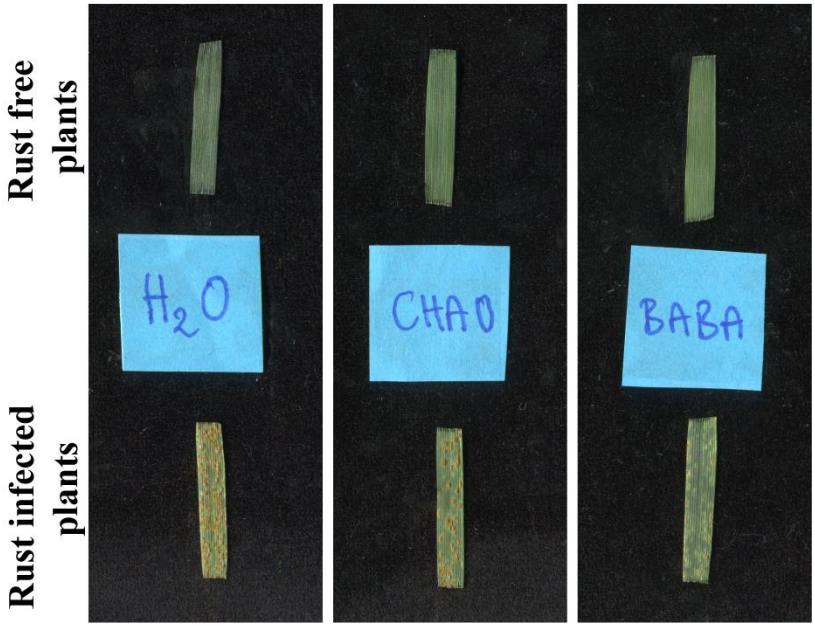

Figure 1: Leaf rust infection on seedling leaves of cultivar Arina at 12 dpi. **Rust free plants** mock-infected with talcum and **rust infected plants** were pre-treated with **H_2O**, **CHA0** (10^6 CFU/mL) or **BABA** (15 mM). Images were obtained by scanning at 1.200 dpi a segment of 3 to 4 cm from the center part of the second leaf.

Expression of defense-related genes in response to leaf rust infection

The expression pattern of various defense-related genes was investigated in CHA0 or BABA pre-treated wheat seedlings infected with leaf rust using RT-qPCR. Primers for 16 defence-related genes were designed (Table 1). Transcriptomic responses presented by fold change in response to infection with *P. triticina* were assessed within each treatment including H_2O as positive control at distinct time points (6, 12, 24, 48 and 72 hai). Primer specificity was checked by melting curve analysis, and single sharp peak with no primer-dimer has been observed in all used primers. Two independent experiments were conducted. Genes presenting a different expression pattern between the two experiments are illustrated in Fig. S2. While genes with a similar expression pattern in both experiments are visualized as a heatmap (Fig. 2) and will be discussed below.

Table 1: DNA primers used to assay the gene expression by real-time PCR

Gene	GenBank Accession	Gene description	Forward primer (5' to 3')	Reverse primer (5' to 3')	T_m (°C)	Ref
ARF	AB050957.1	ADP-ribosylation factor	AGTGAGCTAGTATCAGGTCAGG	TTGGCAACCACAATTCAATCTTG	60;60	(Scholtz & Visser, 2013)
PAL	AY005474.1	Inducible phenylalanine ammonia-lyase	GCCGACGAGACAAGGTAA	CTCCCGCTTCACAGTCTT	60;60	(Casassola et al., 2015)
POX2	X85228.1	Peroxidase	TCACGACGGCCATGATCAAG	TCATTCACGTGTCGTGCTGC	62;62	(Casassola et al., 2015)
PR1.2	AJ007349	(Neutral), pathogenesis-related protein 1	AGCTAGTCATGGCATCTTCC	TTGTGCAGGTTGACGAAGTCC	58;58	(Molina et al., 1999)
PR1.13	HQ541973	Pathogenesis-related protein 1-13	ACTCAAAGGCACCACAGTACG	ACACTATCTGCGTGTAGTGCC	60;60	(Lu et al., 2011)
PR1. RK2	KX673540	Pathogenesis-related protein 1-RK2	TACTACTGCAGTGCGTGGTC	TAGCCTGCCAGGGTGTCATC	60;60	(Lu et al., 2011)
PR2	Y18212	Beta-1,3-endoglucanase	AACCATGCACGCGTTAAC	ATCACAATTCATAACTGCTTCCA	62;62	(Desmond et al., 2005)
CHI4	AF112966	Class 4 acidic chitinase	AAGGGGCGAGCAAGGATTACTG	TGTTGAGCCCGTCGAACC	60;60	(Liu et al., 2016)
Glu3	AY091512	Basic β-1,4-glucanase	ATCCCTCATAACGGTGTCAAG	TCAGGGCCAGCAACCATTG	60;60	(Yang et al., 2013)
LTP1	DQ286560.1	Lipid transfer protein 1	TCTCACCATCTCCAGCTGAG	AGGCTACGAGGAGCATAGC	60;60	(Lu et al., 2005)
LTP2	AF334185.1	Lipid transfer protein 2	ACACCACTACTATTGCTAGCTTG	GAGATTACAGCATCTGTGGCTAT	60;60	(Lu et al., 2005)
COI1	HM447645.1	Coronatine insensitive 1	ACTGGAATAAGCGCATTGGT	GTCACAGTTCACTCGCACAA	62;62	(Okubara et al., 2010)
WCI2	U32428	Wheat chemically induced (WCI) gene 2	TGGCCTTCATCCACACCATCAC	ACGCCTTTTTCGCCTTGACATC	60;60	(Görlach et al., 1996)
WCI3	U32429	Wheat chemically induced (*WCI*) gene 3	ACAAGCCAATACACACACCAA	TTGTCGATGCAAGTTTCTGC	60;60	(Görlach et al., 1996)
TaERF	GU452719	Wheat ethylene-responsive factor-like transcription factor	TCCGAGACAGACCGAGGAG	ACCTCAACGCCGGTTTGATC	60;60	(Zhang et al., 2007)
Lipase	DN551653	Wheat lipase	ATTCACGCCCGACAACATGATTG	ATGGTCAAGGGCACCGATGG	58;58	(Lu et al., 2005)
TaNPR1	AX049430	Wheat nonexpressor of pathogenesis-related Genes 1	TACACAGCACTCCACCTAGC	GCGGCCATCATCTGTCAATT	60;60	(Liu et al., 2016)

T_m values represent melting temperatures for primers.

During the infection process of isolate Pr2271 in cv. Arina plants (Fig. 2A) a clear pattern of upregulated genes (Fig. 2B) was observed at 12 hai during the first attempt of fungal penetration until the formation of haustoria starting at 48 hai.

The candidate defence-related genes responded differentially to CHA0 and BABA treatments among the time course investigated. Three patterns of gene expression (Fig. 2B) were observed: (1) transcripts that increased in response to infection with leaf rust after treatment with plant inducers included *TaERF*, *WCI2*, *Glu3*, *LTP1* and *PAL*; (2) transcripts that mostly decreased in all treatment included *POX2* and *PR2*; (3) transcripts that were not affected by the treatment included *PR1.2* and *COI1*.

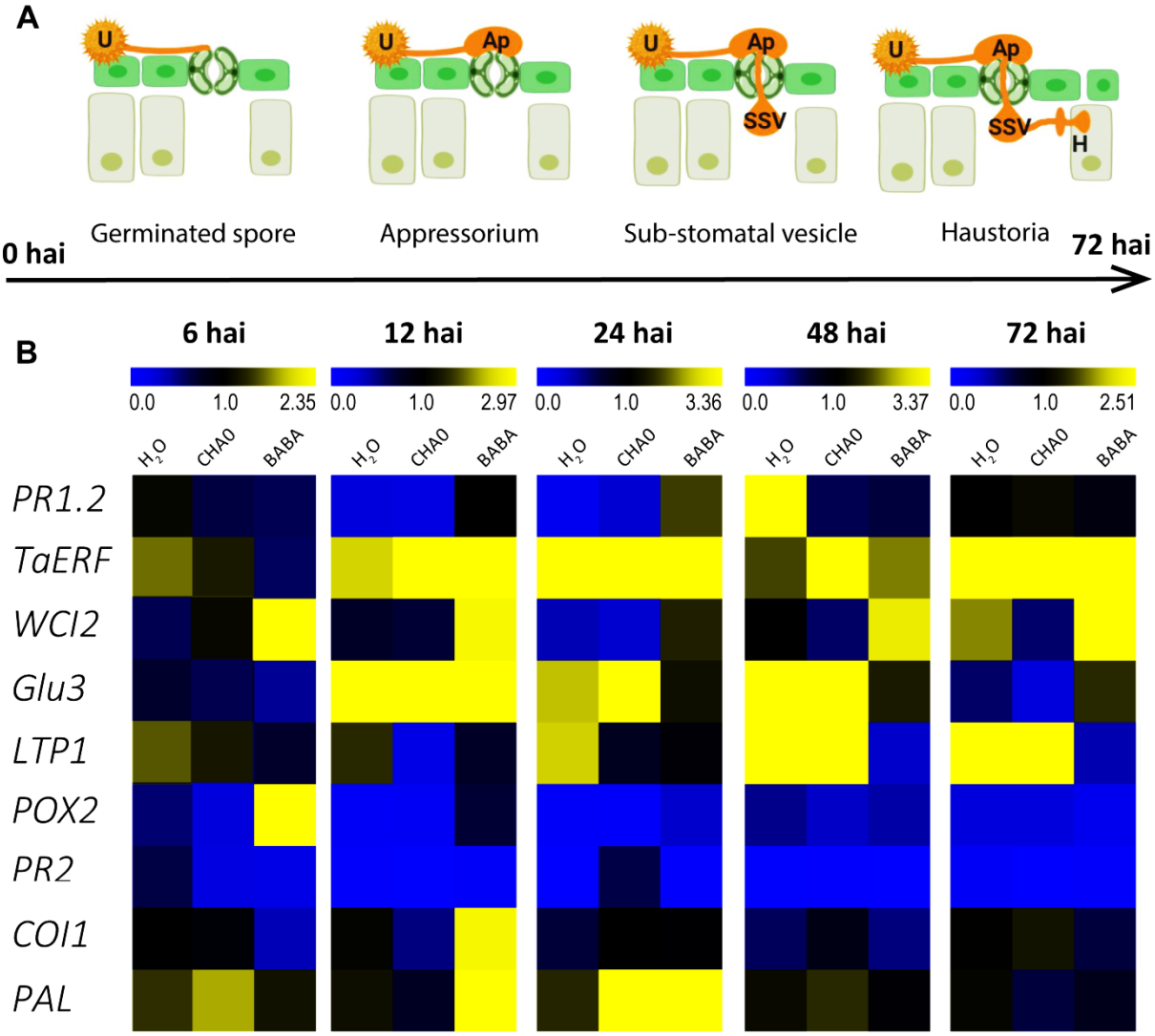

Figure 2: Expression profile of nine defense-related genes during wheat-rust interaction under effect of plant resistance inducers. **A**. Infection process of *P. triticina* isolate Pr2271 in cv. Arina. **U**: Urediniospore, Ap: Appressorium, **SSV**: Substomatal vesicle, **H**: haustorium B. Heat map of gene expression indicated as fold induction compared to mock-inoculated plants previously treated with H_2O, CHA0 or BABA; blue =downregulated genes, yellow = upregulated genes.

A comparative analysis in response to CHA0 or BABA treatments is summarized in Table 2. Genes like *PR2, POX2* and *COI1* were similarly expressed in the different treatment as well as at different time point, with some exceptions: at 6 hai, *POX2* was significantly highly upregulated up to 4.5-fold in BABA-treated plants compared to control treatment (Table 2), and *COI1* was differently expressed only in BABA treatment at 12 hai, but no significant differences were observed. Surprisingly, *PR1.2* was upregulated at later time point (48 hai) only in H_2O treatment. *TaERF* that was highly upregulated starting from 24 to 72 hai and the highest values were observed at 72 hai in BABA treatment with a 9-fold expression. Expression level of *TaERF* in CHA0-treated plant was significantly higher than the control at later time points (48 and 72 hai), while in BABA treatment; *TaERF* was statistically different only at 72 hai. Interestingly, the wheat chemically induced gene *WCI2* was rapidly upregulated at 6 hai in BABA treatment with 3.5-fold. The upregulation of this gene was significantly higher in BABA treatment compared to control in all time points (Table 2). For *Glu3,* the expression was mostly upregulated in the time course investigated in this study in CHA0 and H_2O treatment (Fig. 2). However, in plants treated with BABA a lower transcriptional level of *Glu3* was observed. Similarly to *Glu3*, the *LTP1*transcript was decreased in BABA-treated plant showing a significantly lower level at 72 hai. Finally, *PAL* expression was mainly induced in plants treated with BABA and CHA0, in fact, it was significantly higher in BABA at 12 and 48 hai and in CHA0 at 48h (Table 2).

Discussion

During last decades, gene expression studies have defined molecular pathways and cellular processes that are transcriptionally regulated during biotic interactions, and have expanded our understanding of how plants respond to pathogens (Casassola et al., 2015; Farrakh et al., 2018; Sharma et al., 2018). A transcriptomics analysis of wheat seedlings subjected to leaf rust infection under the effect of resistance inducers revealed more insights into CHA0-ISR and BABA-IR mechanisms. We determined that rust infection deferentially regulated genes involved in defence mechanisms. A significant additional effect on the expression level of some of these genes was observed when plants additionally were treated with plant resistance inducers.

Table 2: Comparative analysis of defence genes expression induced by leaf rust infection in wheat plants pre-treated with plant resistance inducers.

Genes		*PR1.2*	*TaERF*	*WCI2*	*Glu3*	*LTP1*	*POX2*	*PR2*	*COI1*	*PAL*
6h	H_2O	1.04 ± 0.61	1.88 ± 0.54	0.91 ± 0.56	0.69 ± 0.19	1.71 ± 0.36	0.70 ± 0.54	0.84 ± 0.75	1.32 ± 0.66	1.18 ± 0.85
	CHA0	0.85 ± 0.50	1.56 ± 0.39	1.53 ± 0.35	0.66 ± 0.63	1.21 ± 0.26	0.32 ± 0.05	0.30 ± 0.02	1.23 ± 0.62	1.95 ± 1.28
	BABA	0.79 ± 0.64	0.76 ± 0.12	3.49 ± 1.33*	0.60 ± 0.40	0.95 ± 0.77	4.42 ± 1.73**	0.26 ± 0.03	0.49 ± 0.14	1.11 ± 1.02
12h	H_2O	0.24 ± 0.13	1.66 ± 0.97	0.72 ± 0.20	2.46 ± 1.01	1.24 ± 0.73	0.07 ± 0.16	0.04 ± 0.10	1.01 ± 0.74	1.10 ± 0.28
	CHA0	0.21 ± 0.11	2.20 ± 0.99	0.67 ± 0.14	3.37 ± 1.14	0.19 ± 0.32	0.10 ± 0.07	0.02 ± 0.10	0.49 ± 0.27	0.74 ± 0.34
	BABA	0.98 ± 0.32	2.92 ± 0.84	2.86 ± 0.33*	5.48 ± 2.28	0.72 ± 0.51	0.67 ± 0.49	0.06 ± 0.21	2.85 ± 0.92*	3.12 ± 0.96**
24h	H_2O	0.20 ± 0.12	4.69 ± 1.77	0.60 ± 0.32	2.76 ± 0.89	2.85 ± 1.29	0.10 ± 0.17	0.01 ± 0.04	1.07 ± 0.57	1.05 ± 0.90
	CHA0	0.46 ± 0.23	3.89 ± 2.18	0.48 ± 0.14	3.36 ± 0.98	1.22 ± 0.67	0.08 ± 0.15	0.02 ± 0.04	1.59 ± 0.83	4.41 ± 1.74**
	BABA	1.20 ± 1.08	3.46 ± 1.23	1.01 ± 0.72**	1.82 ± 0.61	1.49 ± 1.09	0.49 ± 0.16	0.04 ± 0.01	1.56 ± 0.68	3.56 ± 1.28*
48h	H_2O	2.49 ± 0.85	1.09 ± 0.92	0.93 ± 0.31	3.65 ± 0.86	4.73 ± 2.56	0.44 ± 0.13	0.02 ± 0.12	0.54 ± 0.22	0.97 ± 0.35
	CHA0	0.56 ± 0.89	4.23 ± 1.38*	0.52 ± 0.12	4.37 ± 2.98	3.52 ± 1.08	0.30 ± 0.15	0.03 ± 0.15	0.77 ± 0.37	1.05 ± 0.97
	BABA	0.61 ± 0.29	1.15 ± 1.02	2.30 ± 0.50*	1.02 ± 0.64	0.29 ± 0.19*	0.38 ± 0.05	0.01 ± 0.05	0.47 ± 0.30	0.88 ± 0.55
72h	H_2O	1.16 ± 0.88	5.46 ± 0.28	1.86 ± 0.71	0.64 ± 0.35	4.36 ± 1.55	0.29 ± 0.18	0.09 ± 0.12	1.18 ± 0.50	1.23 ± 0.55
	CHA0	1.29 ± 0.54	7.22 ± 1.81**	0.63 ± 0.23	0.28 ± 0.14	2.51 ± 1.35	0.28 ± 0.15	0.01 ± 0.06	1.38 ± 0.53	0.76 ± 0.48
	BABA	1.01 ± 0.40	9.46 ± 1.01***	3.35 ± 1.81*	1.53 ± 0.49	0.45 ± 0.22**	0.17 ± 0.10	0.05 ± 0.15	0.76 ± 0.25	0.91 ± 0.32

Data indicate mean values ± SE (n=3) of fold change in response to rust infection in plant treated with H_2O, CHA0 or BABA at different time points. Asterisks indicate significant differences in response to BABA or CHA0 treatment determined by Student *t*-test: *P<0.05; **P<0.01; ***P<0.001.

Chemically induced resistance in wheat is associated with the expression of various genes called *WCI* genes (Görlach et al., 1996). Our results confirmed the importance of the *WCI2* gene in chemically induced resistance in wheat, since its level was highly upregulated in plants treated with BABA compared to control. This has also been shown in other cereals species, where a differential expression of barley and rice chemical induced genes was observed after chemical treatment (Beßer et al., 2000; Schaffrath et al., 2000). *WCI2* is a member of a lipoxygenase gene family that putatively synthesizes hydroperoxide fatty acid precursors of JA (Göbel et al., 2002). Leaf rust infection under BABA treatment resulted in higher *WCI2* mRNA levels during the time course of this study, suggesting that in BABA-treated plant, recognition of *P. triticina* may trigger jasmonate synthesis and signaling activity. However, in case of resistance induction with CHA0 this gene seems not to be involved in the induction process.

Generally, *PR1* gene expression is not a reliable indicator for IR in cereals (Molina et al., 1999). In the present gene expression analysis, treatment with both plant resistance inducers did not affect the regulation of *PR1.2* during most of time, showing that *PR1.2* gene is not essential for BABA-IR and CHA0-ISR in wheat. Similar results were observed when wheat plants were treated with inducers of acquired resistance in wheat (Görlach et al., 1996; Schaffrath et al., 1997).

PAL has been proposed as a critical enzyme in the pathway leading to products that are related to plant defense, such as salicylic acid and phytoalexins (Ward et al., 1991). Many studies exhibited that PAL is associated with cereal resistance against pathogens such as *Fusarium graminearum*, *Fusarium culmorum* and *Puccinia hordei* (Beccari et al., 2011; Orzali et al., 2014; Prats et al., 2008). Here, when wheat plants were treated either with CHA0 or with BABA, infection with leaf rust lead to a significant early upregulation of the *PAL* gene 12-24 hai in BABA and 24 hai in CHA0, strongly suggesting a role for this gene in the prevention of leaf rust infection, since this period coincided with the first attempts of cell invasion with haustoria.

Peroxidases (POXs) are considered as pathogenesis related protein 9 subfamily (*PR9*). They catalyze the oxidation of a number of substrates by H_2O_2 (Almagro et al., 2008; van Loon et al., 2006). POX also plays a role in the reinforcement of cell wall physical barriers and lignification (Bowles, 1990; Passardi et al., 2004). Genes participating in the oxidative burst are highly induced at 6–8 hai, which represents the first step in triggering plant defence (Wang et al., 2007). Casassola et al. (2015) reported high expression of *PR9* in wheat plants resistant to leaf rust. Here, the *POX2* gene was only upregulated at 6 hai in BABA-treated plants and not

in CHA0 treatment, which is in contrast with previous results where H_2O_2 was highly accumulated following CHA0 and BABA treatment (Bellameche et al., chapter 2 of present thesis), suggesting that other genes could be involved in the generation of the oxidative burst during CHA0-ISR and BABA-IR against leaf rust, as has been observed in a transcriptome study of wheat seedling resistance (*Lr28)* to leaf rust where genes such as glutathione, transferase and lipoxygenase were involved in the oxidative burst (Sharma et al., 2018).

PR2 is a β-1,3-glucanase, a group of enzymes known to play a major role in plant defence and general stress responses through the regulation of callose deposition (Oide et al., 2013; van Loon et al., 2006). Glucans are also found in fungal cell walls and plant β -1,3-glucanases are able to hydrolyze fungal glucans and callose (van Loon et al., 2006). Downregulation of *PR2* at all time points indicated that wheat used callose as a resistance mechanism against *P. triticina* and probably by *PR2* upregulation, the callose structure could be degraded. This is in line with results obtained in our previous work, where callose deposition was observed in wheat seedlings in response to leaf rust infection and CHA0 and BABA showed an additional deposition of callose compared to control (Bellameche et al., chapter 2 of present thesis),

Lipid transfer proteins (LTPs) have certain effects on plant defense mechanisms and they are classified as PR proteins having antibiotic properties (Wu et al., 2014). The results showed that *LTP1* expression reached a peak at 48 and 72 hai in CHA0, H_2O treatment but not in BABA. It seems *LTP1* is an important signaling response gene in the wheat-rust interaction. However, it was significantly less expressed under BABA treatment, excluding a role in BABA-IR in wheat.

Transcript levels for *Glu3* increased at the beginning of fungal penetration (12 hai) until a total invasion of the tissue (48 hai). A neutral regulation of this gene was observed in BABA-treated plants. Glu3 are homologous to a wheat seedling β -1,4-glucanases (Lai et al., 1993) and they are enzymes that hydrolyze cellulose or (1,4)-β-D-glucans, the major component of plant cell walls (Minic, 2008). In this study, the upregulation *Glu3* gene might have been manipulated by the fungus in order to hydrolyze wheat cell walls and to facilitate its penetration. BABA treatment seems to help the plant overcome this manipulation leading to a high level of resistance.

The pathogen-inducible *ERF* gene (ethylene-response factor) is a member of a transcription factor family that is involved in regulating the defence response (Singh et al., 2002). The identification and characterization of a wheat *ERF* gene revealed its importance to defence responses and its induction kinetics after different pathogen infections and hormone treatments

(Zhang et al., 2007). Here we confirmed the role of this gene in defence mechanism against leaf rust in wheat seedlings, showing also its importance in BABA-IR and CHA0-ISR in wheat, since the *ERF* transcript level was significantly higher at 48 hai in CHA0 treatment and at 72 hai in both treatment suggesting that upregulation of this transcription factor could lead to the regulation of other defence-related gene. Induction of the jasmonate signal pathway component *COI1* by *P. fluorescens* has been observed in a previous study (Okubara et al., 2010). Here, a modest induction of the key pathway regulator *COI1* was observed only in BABA-treated plants at 12 hai. Otherwise, its regulation was not affected by leaf rust infection with or without treatment.

The results of this study extend current knowledge of defence mechanisms in wheat against leaf rust attack treated with plant defence activators. Overall, in BABA-IR, the expression levels of defence-related genes were more altered compared to CHA0-IR. Similarly to our results, Van der Ent et al. (2009) reported differences and similarities in defence mechanisms induced by *P. fluorescens* WCS417r and BABA. A quantitative PCR-based genome-wide screen for putative WCS417r- and BABA-responsive transcription factor genes revealed a higher induction of these genes in BABA-treated plants than in bacteria ones.

Our work has to be completed by validation of expression pattern of genes that differently reacted to our treatments in two independent experiments, which could help to understand better the differences and similarities in defence mechanisms underlying BABA-IR and CHA0-ISR. Since genes such as *PR1.13, PR1.RK2*, class 4 acidic chitinase (*CHI4*), lipid transfer protein 2 (*LTP2*), wheat chemically induced gene 3 (*WCI3*), wheat lipase (Lipase) and the wheat nonexpressor of pathogenesis-related gene 1 *(TaNPR1*) have been shown to be involved in wheat defence against fungal disease (Görlach et al., 1996; Liu et al., 2016; Lu et al., 2011).

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Supplementary material

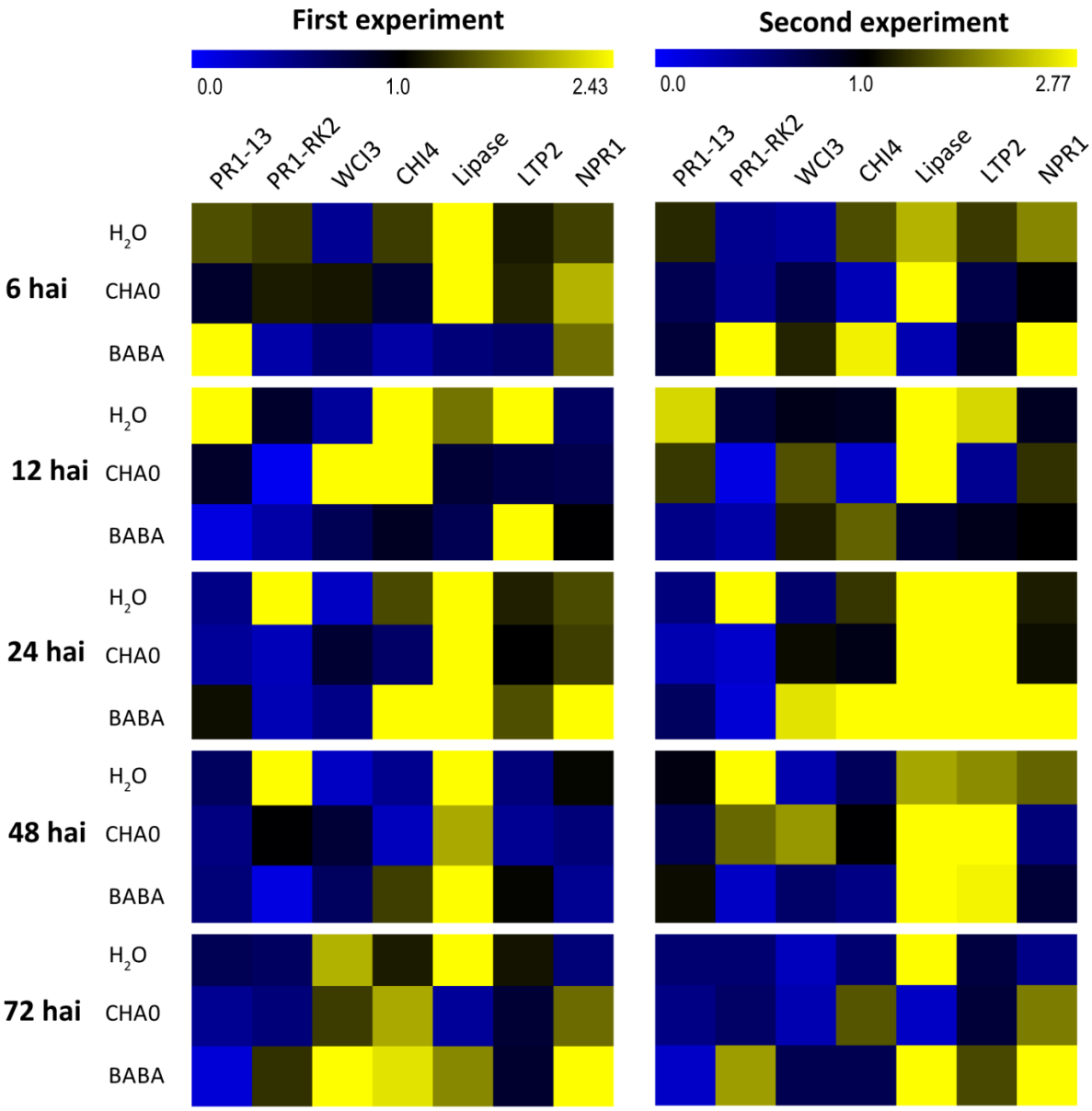

Figure S1: Heat map of seven defense-related genes expression profile during wheat-rust interaction under effect of plant resistance inducers. Gene expression pattern were different in first and second independent experiments. Gene expression is indicated as fold induction compared to mock-inoculated plants previously treated with H_2O, CHA0 or BABA; blue =downregulated genes, yellow = upregulated genes

CHAPTER V

Efficiency of biological and chemical inducers for controlling Septoria tritici leaf blotch (STB) on wheat (*Triticum aestivum L.*)

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Manuscript submitted.

Chapter V: Efficiency of biological and chemical inducers for controlling Septoria tritici leaf blotch (STB) on wheat (*Triticum aestivum* L.)

Abstract

The hemibiotrophic fungus *Zymoseptoria tritici* is the causative agent of Septoria tritici leaf blotch (STB) disease of wheat (*Triticum aestivum* L.), the economically most damaging disease of wheat in Europe. Today, ecofriendly plant protection methods compatible with sustainable agriculture are strongly desirable. Here, we tested two chemical inducers β-aminobutyric acid (BABA) and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) and the two biotic inducers *Pseudomonas protegens* CHA0 (CHA0) and *P. chlororaphis* PCL1391 (PCL) for their ability to induce resistance against STB in wheat seedlings. At 21 days after inoculation, only plants treated with BABA showed a smaller area covered by lesions and less pycnidia compared to the untreated control plants. We evaluated spore germination and fungal development on inoculated wheat leaves at early infection stages using calcofluor white staining. Overall, spores of *Z. tritici* germinated less on plants soil-drenched with BABA and BTH and their hyphal growth was significantly delayed. On the contrary, CHA0 and PCL seed treatments did not affect fungal growth in wheat leaves. In conclusion, BABA efficiently enhanced plant resistance to *Z. tritici*, BTH delayed fungal development at early stages while the two biotic inducers did not enhance resistance against STB disease.

Keywords: Induced resistance, β-aminobutyric acid, Benzothiadiazole, *Pseudomonas protegens* CHA0, *Pseudomonas chlororaphis* PCL1391

Introduction

The fungus *Zymoseptoria tritici* causes Septoria tritici blotch (STB) on wheat. *Z. tritici* is considered the most damaging wheat pathogen in Europe, mainly because of the suitable climatic conditions (Jørgensen et al. 2014). During severe epidemics, up to 50% of losses have been registered in a field planted with wheat cultivars susceptible to STB (Fones and Gurr 2015). Currently, no genetic resistance source in wheat cultivars is known to confer full resistance against STB. For control, farmers rely on the use of cultivars with partial resistance and on conventional fungicides (Schaad et al. 2019; Torriani et al. 2015). Yet, the durability of chemical control may remain ineffective in the field because *Z. tritici* displays a high capacity of adaptation and often succeeds in developing new resistances to fungicides and the overcoming of the host resistance (Cowger et al. 2000; Cheval et al. 2017). As chemical fungicides can have a negative impact on human and animal health and the environment (Aktar

et al. 2009; Berny 2007), we decided to evaluate a more sustainable approach to combat this disease in the form of induced resistance.

One possibility of inducing disease resistance is the use of plant-growth promoting rhizobacteria (PGPR). Numerous cases of a successfully use of PGPR to improve plant nutrition and/or help plants overcome biotic or abiotic stresses have been documented (Chaudhary and Shukla 2019; Vurukonda et al. 2016; Dimkpa et al. 2009). These studies mainly involved the genus *Pseudomonas* commonly found among the predominant genera present in the rhizosphere and also in the root system of wheat plants (Weller et al. 2007; Yoshida et al. 2012). Many of these Pseudomonads are well-characterized PGPR and are able to exert plant-beneficial functions, including the suppression of plant diseases and stimulation of plant defences (Vacheron et al. 2013; Mauchline and Malone 2017). A subgroup including the species *P. protegens* and *P. chlororaphis* has been widely studied (Haas and Défago 2005; Mercado-Blanco and Bakker 2007; Hol et al. 2013; Vacheron et al. 2013). The strain *P. protegens* CHA0 (CHA0) is naturally suppressive to black root rot in tobacco (Stutz et al. 1986). It has been reported as a potential bacterial antagonist to control plant diseases (Hase et al. 2000; Ramette et al. 2011), and also for its capacity to induce resistance in dicotyledonous plants (Maurhofer et al. 1994; Iavicoli et al. 2003), as well as in monocots (Henkes et al. 2011; Sari et al. 2008). The strain *P. chlororaphis* PCL1391 (PCL) was first isolated from the rhizosphere of tomato, showing a high antagonistic activity against *Fusarium oxysporum*, the causal agent of tomato root rot (Chin-A-Woeng et al. 1998). The capacity of PCL to protect plants against different other attackers has also been documented (Imperiali et al. 2017; Bardas et al. 2009; Flury et al. 2017).

Specific chemicals also have the capacity to enhance plant resistance to disease. For instance, synthetic compounds such as benzo[1,2,3]thiadiazole-7-carbothionic acid-*S*-methyl ester (BTH, also called acibenzolar-S-methyl) and β-aminobutyric acid (BABA) have been reported to induce resistance in plants against a wide range of microbial pathogens without possessing direct antimicrobial activity (Görlach et al. 1996; Jakab et al. 2001; Justyna and Ewa 2013; Karthikeyan and Gnanamanickam 2011). The plant defence activator BTH is a functional analogue of salicylic acid and was one of the first chemical compounds shown to enhance activation of several defense responses against major fungal and bacterial pathogens in various crops including wheat (Iriti et al. 2004; Karthikeyan and Gnanamanickam 2011; Soleimani and Kirk 2012; Görlach et al. 1996; Vallad and Goodman 2004). BABA induces resistance in a wide range of economically important crop species and against a broad spectrum of pathogens

including nematodes, virus, bacteria, oomycetes and fungi (Jakab et al. 2001; Barilli et al. 2010; Porat et al. 2003; Amzalek and Cohen 2007). Expression of BABA-induced resistance coincides with a faster and stronger defence response following pathogen attack, a phenomenon that has been termed priming (Cohen et al. 2016; Balmer et al. 2015). Even though BABA, BTH, CHA0 and PCL have been tested in many different pathosystems, to our knowledge, they have never been tested in wheat against STB.

Z. tritici is a filamentous fungal pathogen having the particularity of being hemibiotrophic, with two distinct phases of infection. Following inoculation onto the leaf surface by rain splash, spores germinate (Kema et al. 1996), giving rise to a single hypha. The hypha invades leaf tissues mainly through stomata (Rohel et al. 2001; Palmer and Skinner 2002) growing slowly in the apoplastic space between mesophyll cells, typically during up to 9–11 days (Kema et al. 1996; Shetty et al. 2003). Here the fungus survives by assimilating nutrients in solution in the apoplast (Shetty et al. 2003; Keon et al. 2007; O'Driscoll et al. 2014). This has also been referred to as 'biotrophic'. During this first phase, no symptoms are visible. At a later time point, notably 12 to 20 days after penetration, the plant shows the first symptoms on the leaves. The appearance of symptoms coincides with the passage of the fungus to the 'necrotrophic' phase of the fungus (Palmer and Skinner 2002; Shetty et al. 2003). The infection process ends with the appearance of pycnidia, and leaf symptoms generally appear as light green to yellow chlorotic spots. As they enlarge, the lesions become brown and develop darker colored fruiting bodies (Ponomarenko et al. 2011).

In this study we aimed to assess the efficacy of the two chemical inducers (BABA and BTH) and the two biological inducers (CHA0 and PCL) as a preventive treatment on wheat seedlings against *Z. tritici*. The effect of the plant resistance inducers on fungal development was investigated at an early stage of infection. To exclude any direct inhibitory effect, we also assessed the antifungal activity of the chemical inducers on *Z. tritici* growth by in vitro assays.

Materials and methods

Plant material and growth conditions

Experiments were carried out with the STB susceptible wheat variety Spluga (Agroscope/DSP). Prior to seeding, seeds were surface sterilized by rinsing with 70% ethanol and incubating for 5 minutes in 5 % bleach (sodium hypochlorite solution, Fisher Chemical, U.K.). Subsequently, the seeds were rinsed three times in sterile distilled water. The seeds were then pre-germinated for 3 to 4 days on humid filter paper (Filterkrepp Papier braun, E. Weber & Cie AG, 8157

Dielsdorf, Switzerland). We selected the seedlings with similar growth state and morphology to plant in 120 mL polypropylene tubes (Semadeni, 3072 Ostermundigen, Switzerland) filled with a standard potting mixture (peat:sand, 3:1, vol:vol). The plants grew in a growth chamber with the following conditions: 16 hours day at 22°C, 8 hours night at 18°C and an irradiance of 300 μmol m^{-2} s^{-1}. The plants were watered as needed.

Treatment with biological inducers

The biological inducers used in the following trials were the rifampicin-resistant mutants CHA0-RIF of *Pseudomonas protegens* strain of CHA0 (Natsch et al. 1994), and PCL-RIF of strain *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998). The strains were routinely grown on solid King's Medium B (KMB; *Pseudomonas* agar F, Merck KGaA, 64271 Darmstadt, Germany) supplemented with rifampicin 100 μg/mL at 24°C for 4 days. From this culture, a single colony was transferred to 100 mL of King's liquid medium B (30g protease-peptone, 1.5g K_2HPO_4, 2.46 g $MgSO_4$, 1.5g glycerol in 1 L distilled water) supplemented with 50 μg mL^{-1} rifampicin and incubated overnight at 28°C with continuous shaking at 150 rpm. The resulting bacterial culture was centrifuged at 3700 rpm and washed twice with sterile 10mM $MgSO_4$ solution. The final pellets were re-suspended in sterile distilled water and adjusted to 10^6 cfu/mL (OD_{600} of 0.1) and used for seed inoculation. To this end, the wheat seeds were immerged into the bacterial suspension for 6 hours with shaking at 35-40 rpm at room temperature. Control seeds were soaked in distilled water for the same duration. Inoculated seeds underwent the pre-germination procedure as described above.

Chemical inducer treatment

BTH formulated as BION® 50 WG (50% active ingredient) was obtained from Syngenta (Basel, Switzerland) and BABA from Sigma-Aldrich (Buchs, Switzerland). BTH (2mM) and BABA (15mM), respectively, were dissolved in distilled water and 10 mL per growing tube were used as soil-drench 2 days before *Z. tritici* inoculation. Control plants were just watered with distilled water. The concentration of BTH used in this study was chosen according to Görlach et al. (1996). While, BABA at 15 mM was chosen as ideal concentration to induce resistance against leaf rust without any effect on plant growth (unpublished data).

Fungal cultures and plant inoculation

Z. tritici isolate 3D7 (Zhan et al. 2002) was provided by Prof. Daniel Croll (University of Neuchâtel, Switzerland). The isolate was stored at -80°C in 50% glycerol. Stock cultures were cultivated on Yeast-Sucrose Agar (10 g L^{-1} yeast extract, 10 g L^{-1} sucrose, 1.2% agar)

supplemented with kanamycin (50 μg/mL). For inoculum preparation, the strain was cultured in Yeast-Sucrose Broth (YSB) amended with 50 μg/mL kanamycin and incubated for 8 days at 18°C under continuous shaking at 150 rpm. After incubation, the suspension was filtered through a sterile cheese cloth and rinsed with sterile distilled water. Prior to infection, the spore concentration was adjusted to 10^5 spores/mL in distilled water using a haemocytometer. After adding 0.1% tween 20 to the spore suspension, at 3-leaf stage, each plant was spray-inoculated until runoff. The plants were then maintained at 100% relative humidity for 48 hours. After this, the plants were placed in a growth chamber as described above.

Infection quantification

At 21 days after inoculation (dai), symptoms on wheat plants were quantified as described by Stewart et al. (2016). Briefly, the third leaf of each plant was excised, fixed on a sheet of paper and immediately scanned at 1.200 dpi (Epson perfection, V370 PHOTO). The leaf surface covered with pycnidia, lesions or leaf necrosis was measured using an automated image analyses macro for the software ImageJ version 1.x (Schneider et al. 2012). The disease severity was the expressed as percentage of leaf area covered by lesions (PLACL).

***In planta* fungal growth**

Monitoring of spore germination and hyphal growth of *Z. tritici* on the leaf surface was performed using Calcofluor White (Sigma-Aldrich, Germany) staining according to Siah et al. (2010). Briefly, third leaf segments (4 cm) from three randomly selected replicates of each treatment were harvested at 24, 48 and 120 hours after inoculation (hai) and immersed for 5 minutes in 0.1% (*w/v*) Calcofluor White solution prepared in 0.1 M Tris-HCl buffer pH 8.5. After washing with sterile distilled water, the leaf segments were dried in darkness at room temperature. After covering with a cover slip, the preparations were examined under the epifluorescence microscope (Model E800; Nikon Instruments Europe, Badhoevedorp, The Netherlands) using excitation at 365 nm in combination with a 450 nm barrier filter and a dichroic mirror at 400 nm.

***In vitro* antifungal assay**

A potential antimicrobial effect of BTH and BABA on growth of *Z. tritici* was spectrophotometrically assessed in liquid YSB supplemented with kanamycin 50 μg/mL. Since BION contains additional ingredients that can influence the absorbance measurement, the active molecule Acibenzolar-S-methyl (Sigma-Aldrich, Germany) was used. BABA and BTH were first dissolved in distilled water and filter-sterilized with a 0.22-μm syringe filter (Millex GP,

Millipore). Concentrations of 0, 0.02, 0.2 and 2 mM of BTH and 0, 0.15, 1.5 and 15 mM of BABA were tested. Aliquots of 40 mL culture medium were inoculated with 50 µL of fresh fungal spore suspension (10^5 spores/mL) and placed at 18°C in the dark under continuous shaking at 150 rpm. Fungal growth was assessed daily by measuring the optical density at 405 nm.

Statistical analyses

All experiments were repeated twice. Infection quantification was carried out in eight biological replicates. The germination of conidia *in planta* was observed in at least 50 spores on three independent replicates. For both assessments, PLACL and the germination class of conidia, comparisons between the treatments were carried out with one-way ANOVA. After ascertaining that the residues were normally distributed, significant differences between treatments were tested *post-hoc* using Tukey's HSD test.

For the growth inhibition assay, the area under the growth curve was calculated for each BABA and BTH concentration in three independent replicates. Significant difference in response to dose-treatment were analysed by a Student's *t*-test in comparison to the control (0 mM BABA or BTH).

In all trials, significant differences were considered at $P < 0.05$. All statistical analyses were conducted in R (R Core Team 2018).

Results

Disease severity evaluation after pre-treatment with resistance inducers

Symptoms on leaves were assessed on the third leaf, at 21 days after infection (Fig. 1a). Infected leaf tissue initially became chlorotic and later turned necrotic. In the untreated control, in the bacteria-treated plants and in the BTH treatment a large proportion of the leaves was necrotic and only a small part was alive (green). On the contrary, plants treated with 15 mM BABA presented less symptoms compared to the untreated control and leaves were green. The extension of the lesions (PLACL) was significantly lower in plants treated with BABA in comparison with the untreated control and the other pre-treatments (Fig. 1b). Similarly, the density of pycnidia was significantly reduced in BABA treated leaves but not in the other treatments (Table 1)

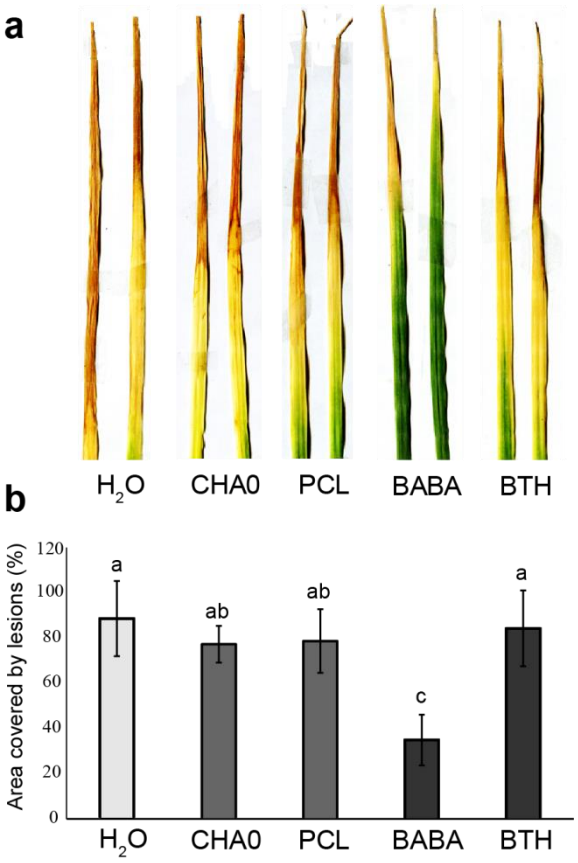

Figure 1: Response of wheat seedlings cv. Spluga to infection with *Z. tritici* after pre-treatment with H_2O (control), CHA0, PCL, BABA and BTH, respectively. Symptoms were observed at 21 dai (A) and percentage of total leaf area covered by lesions (B) was obtained from scanned images analysed with an ImageJ macro. Error bars indicate the standard error for the average values of 8 replicates. Bars with the same letter are significantly not different at $P < 0.05$.

Early effect of plant resistance inducers on *in planta* spore development

Spore germination and hyphal growth of *Z. tritici* on the leaf surface of wheat plants (cv. Spluga) and growth structures were counted at 24, 48 and 120 hai. To quantify the effect of resistance inducers during this observation period, four distinct developmental classes have been determined (Fig. 2a): class 1, spore non-germinated; class 2, geminated spore with a short germ tube; class 3, geminated spore with a well-developed germ tube; class 4, germinated spore with branched hyphae. Fig. 2b shows the percentage of these classes in each treatment.

Table 1: Pycnidia density per leaf (pycnidia/cm^2) in H_2O-treated control plants and CHA0-, PCL-, BABA- or BTH-treated plants. Pycnidia density was assessed on the third leaf of each biological replicate (n = 8).

Treatment	Pycnidia/cm^2
H_2O	97.79 ± 39.78 ^{a}
CHA0	109.94 ± 25.04^{a}
PCL	112.39 ± 13.76^{a}
BABA	14.29 ± 19.81^{b}
BTH	88.48 ± 36.29^{ab}

Values with the same letter are significantly not different at $P < 0.05$.

At 24 hai, fungal development displayed similar proportions of class 1 and class 2. Only in the control and in the CHA0 treatment a small proportion of class 3 structures (about 3%) were present. At 48 hai, the control presented about 45% of class 1, 42% of class 2 and about 13% of class 3 structures. The proportions in the bacterial treatments were similar to the control and statistically not different. Following chemical resistance induction, more spores were in class 1 and 2 compared to the control. While the proportions were statistically not different between BTH treatment and the bacterial treatments, the BABA treatment presented a significantly higher number of class 1 structures compared to the bacterial CHA0 treatment.

At 120 hai, a small proportion of class 4 (hyphae with branching) was present in the control and both bacterial treatments and BTH treatment but not in BABA-treated plants. This was also true for growth class 1. In BABA-treated plants, 70% of spores were in growth class 1. This was 25% higher than in BTH-and CHA0-treated plants. The number of spores in class 1 was not different between the CHA0, PCL, BTH and the control treatment. For class 2, the number of spores that produced a small germ tube did not differ between treatments. Yet, the number of spores with a well-developed germ tube (class 3) was not significantly different between the bacterial treatments and the control and the bacterial treatments and BTH-treated plants. This proportion was very small in BABA-treated and not different between BABA-and BTH-treated plants but highly significantly different between BABA / BTH treatments and the control.

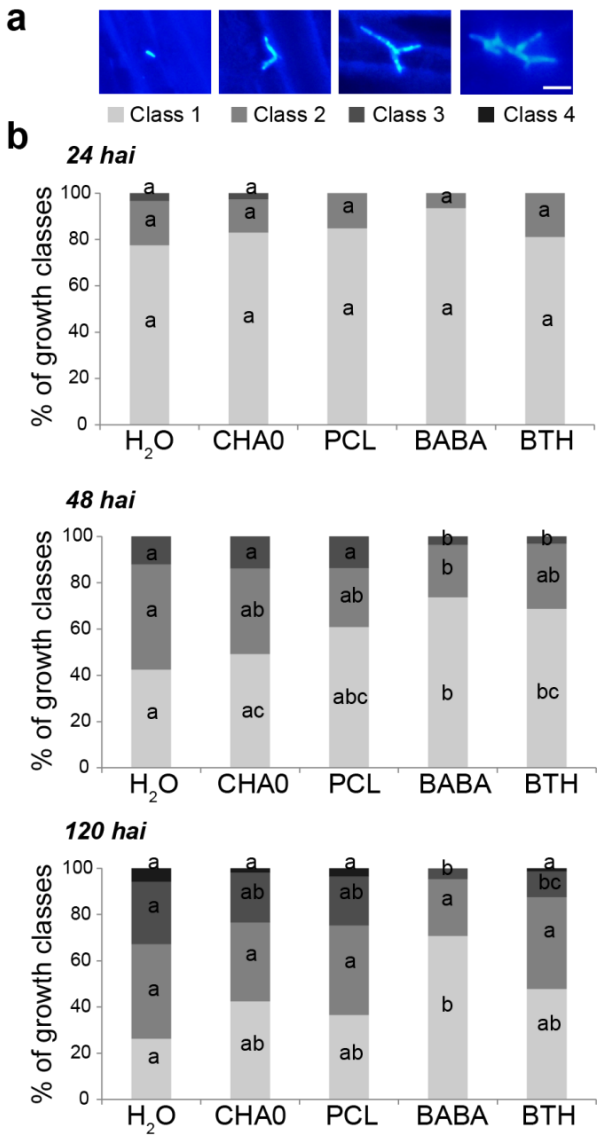

Figure 2: Effect of different plant resistance inducers on spore germination and hyphal growth of *Z. tritici* on leaves of wheat cv. Spluga. Four types of fungal developmental classes were defined (A): class 1, spore non-germinated; class 2, geminated spore with a short germ tube; class 3, geminated spore with a well-developed germ tube; class 4, germinated spore with branched hyphae. Scale bar = 10 µm. On 3 biological replicates, 50 spores were observed. The percentage of each class was assessed in each treatment at 24, 48 and 120 hai (B). Bars with the same letter are significantly not different at $P < 0.05$.

In vitro antifungal activity of BABA and BTH on *Z. tritici* growth

In order to test whether BABA or BTH have a direct inhibitory effect on fungal growth, *Z. tritici* was grown in YSB liquid medium amended with the two inducers (Fig. 3). No antifungal activity was observed for all tested BTH concentrations. At the highest concentration (BTH 2 mM), *Z. tritici* growth was slightly inhibited (Fig. 3a) but no significant differences were observed. When BABA was added to the medium only the highest tested concentration (15 mM) led to a significant delay in fungal growth compared to the control without BABA (Fig. 3b).

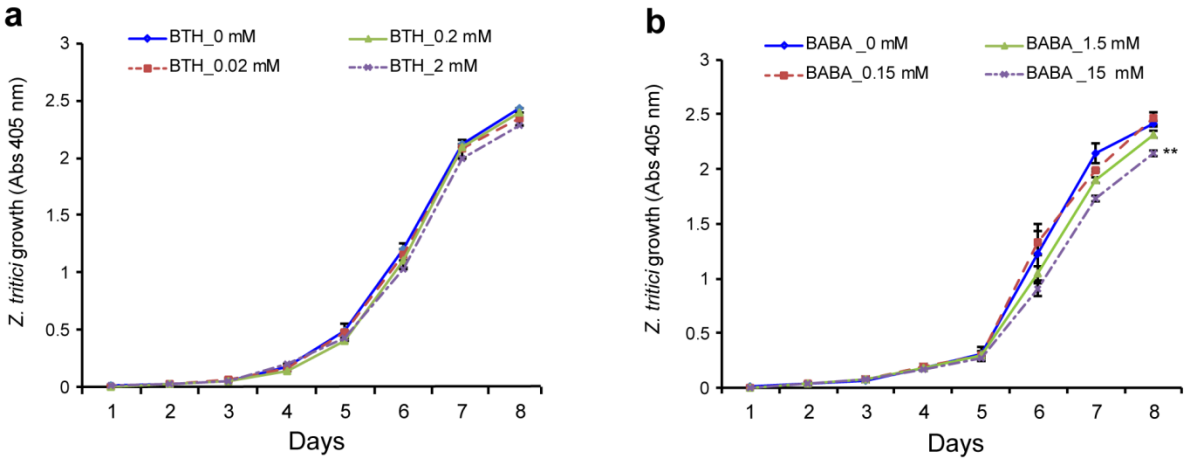

Figure 3: *In vitro* dose–response curves of *Z. tritici* to BABA (A) and BTH (B). Fungal growth was spectrophotometrically measured at 405 nm during 8 days in YSB amended with BABA 0.15, 1.5 and 15 mM or BTH 0.02, 0.2 and 2 mM. Error bars indicate the standard error for the average values of 3 replicates. Asterisks indicate significant differences in area under curves in response to dose-treatment determined by Student's *t*-test: *P<0.05; **P<0.01; ***P<0.001.

Discussion

Plant resistance inducers are a promising alternative to control fungal disease (Wang and Zhou 2018; Chaudhary and Shukla 2019). Here, we report on the possibility to significantly reduce the severity of Septoria leaf blotch, caused by *Z. tritici* when BABA is applied as a preventive treatment in wheat.

General leaf symptoms of Septoria leaf blotch, such as chlorosis and necrosis, were assessed during 21 dai. Severe symptoms were observed in the untreated control as well as in BTH-, PCL- and CHA0-treated plants. Only BABA-treated plants showed a significantly lower percentage of leaf area covered by lesions (PLACL) and a significantly lower number of pycnidia.

To understand the response to infection with *Z. tritici* in wheat treated with resistance inducers, at the early stage, the fungal development was tracked by microscopy at 24, 48 and 120 hours post-infection. As expected, in BABA-treated plants, pathogen growth was significantly delayed. Therefore, BABA as a well-known priming agent, presumably activated a fast and robust response to fungal attack in the host plants (Zimmerli et al. 2000; Ton and Mauch-Mani 2004; van Hulten et al. 2006).

Exogenous application of BABA can inhibit development of disease directly by antimicrobial effect or indirectly via BABA-IR (Cohen et al. 2016). Since BABA is highly systemic, readily taken up by roots, and transported to the leaves (Cohen and Gisi 1994), it was not possible to

conclude whether the observed resistance was direct or not. A potential direct fungicidal action by BABA on the growth of *Z. tritici* was excluded in the *in vitro* growth assay. Only a high concentration of BABA (15 mM) reduced fungal growth. Similar results showing that a high concentration of BABA exhibited a toxic effect on pathogen *in vitro* growth have been reported. Porat et al. (2003) observed that a very high concentration of BABA (100 mM) completely inhibited spore germination and mycelial growth of *Penicillium digitatum.* Similarly, the addition of BABA (50 to 200 mM) to the suspension culture of *Penicillium italicum* inhibited spore germination and germ tube elongation *in vitro* (Torriani et al. 2015). In another study, Fischer et al. (2009) showed that BABA inhibited mycelial growth and germination of *Botrytis cinerea* in a concentration-dependent manner, suggesting that direct antifungal effects of BABA may be associated with its concentration. In our study, low concentrations of BABA (0.15 and 1.5 mM) did not limit *Z. tritici* growth. It is important to note that the concentration of BABA inside wheat leaves at 2 and 6 days post application of 15 mM BABA to the roots were 16 and 6 µM respectively (Table S1), this is far below the *in vitro* inhibition concentration. Therefore, for our *in planta* assays, we postulate that BABA primes resistance mechanisms of the plant that inhibit the germination of *Z. tritici* in the wheat leaves.

As mentioned before, at 21 dai, BABA-treated plants showed less PLCAL and a lower number of pycnidia. This could be explained by results observed in the fungal growth assessment, where BABA treatment significantly limited *Z. tritici* growth. During the transition to the necrotrophic phase, the fungus releases cell wall-degrading enzymes such as β-1,4-endoxylanase, which have been shown to be correlated with symptom and sporulation levels of *Z. tritici* (Siah et al. 2010; Douaiher et al. 2007). Hence, the limitation in fungal growth may decrease the production of cell wall-degrading enzymes resulting in less PLCAL and a lower number of pycnidia.

In the initial infection phase (48 hai), BTH limited fungal development. However, the effect of BTH did not persist during the whole infection process. One hundred and twenty hours after inoculation, hyphal development on BTH-treated plants hardly differed from the non-treated controls. In addition, 21 dai, symptoms on plant leaves were similar to untreated plants. This suggests that BTH did not enhance resistance against Z. *tritici* in wheat seedlings. We suppose that the delay of spore germination observed during the initial infection phase, may be due to an indirect effect since none of the tested concentrations of BTH delayed or inhibited *Z. tritici* growth *in vitro*. Recently, Mejri et al. (2019) studied the protection efficacy of several salicylic acid conjugated derivatives on wheat against *Z. tritici*, and observed no correlation between direct fungicidal activity *in vitro* and protection of wheat plant.

Previous studies have shown that BTH enhances plant resistance to fungal pathogens by activating the systemic acquired resistance signal transduction pathway (Benhamou and Bélanger 1998; Liu et al. 2005; Azami-Sardooei et al. 2013; Abdel-Monaim et al. 2011). BTH treatment induces the accumulation of many transcripts that also accumulate during pathogen infection in Arabidopsis (Görlach et al. 1996; Lawton et al. 1996). In wheat, BTH can induce resistance to powdery mildew (*Blumeria graminis*), leaf rust (*Puccinia triticina*) and Septoria leaf spot and this resistance is accompanied by the induction of a number of wheat chemically induced (WCI) genes (Görlach et al. 1996). However, BTH did not provide resistance to Fusarium head blight in wheat (Yu and Muehlbauer 2001).

Neither CHA0 nor PCL induced plant resistance to *Z. tritici* infection in wheat. Seed treatment by both rhizobacteria did not affect spore germination and hyphal growth in the early infection phase of *Z. tritici*. Moreover, symptoms on bacteria-treated plants were the same as in water-control plants. Following seed inoculation, both bacteria colonized the wheat roots to more than 10^5 CFU/g of root fresh weight (data not shown). This degree of colonization provided effective plant protection in soils suppressive to take-all of wheat and barley caused by *Gaeumannomyces graminis* var. *tritici* (Weller et al. 2007), Fusarium wilt of pea mediated by *Fusarium oxysporum* f. sp. *pisi* (Landa et al. 2002) and black root rot of tobacco (Stutz et al. 1986). Previous work conducted in our laboratory showed the effectiveness of seed treatment with CHA0 to induce resistance against leaf rust caused by *Puccinia triticina* (unpublished). Also, in other studies, *P. fluorescens* species, including CHA0 and PCL, were reported to be efficient suppressive agents of fungal pathogens by inducing systemic resistance (Defago et al. 1990; Howell and Stipanovic 1980; Voisard et al. 1989; Tziros et al. 2007; Bardas et al. 2009). The control of *Z. tritici* by beneficial *P. fluorescens* was attributed to a direct inhibition *in situ* of the fungus by hydrogen cyanide and antimicrobial compounds (Flaishman et al. 1996; Levy et al. 1992). Also other biocontrol organisms such as *Bacillus subtilis* were effective in protecting wheat against STB disease through a direct antifungal activity of their cyclic lipopeptides (Mejri et al. 2018). In this study, inoculation of the STB-susceptible wheat cv. Spluga was performed using *Z. tritici* isolate 3D7, which was collected in a Swiss wheat field in 1999 and was found to be highly aggressive on several wheat cultivars (Zhan et al. 2002; Zhan et al. 2005). This high virulence might be the reason for the observed lack of resistance induction by the tested bacteria.

The present study shows that BABA applied as a soil-drench was effective in protecting wheat seedlings from *Z. tritici* infection, whereas, in plants soil-drenched with BTH, fungal growth was only delayed during the early germination phase. In this case, foliar application may be

more effective. Unexpectedly, wheat seed treatment with CHA0 or PCL did not enhance resistance to STB disease in wheat. Recently, Imperiali et al. (2017) demonstrated the possibility to combine CHA0 and PCL without affecting their capacity to colonize wheat roots. Hence, a combination of these two strains could result in a synergistic effect that may help to control STB disease, as was reported in other case of biological control in wheat (El-Sharkawy et al. 2018; Pierson and Weller 1994).

The results suggest the possibility of developing effective protective measures against *Z. tritici* infection of wheat based on chemical inducer application. However, a histochemical analysis of plant reactions during the infection process should be performed. This will lead to a better understanding of the defence mechanism involved in the observed resistance and providing a conscious choice between the disease resistance inducers.

Acknowledgements

We thank Daniel Croll, Leen Abraham and Nikhil Kumar Singh from the Evolutionary Genetics Laboratory, University of Neuchâtel, for technical support and advice to handle the STB pathogen. FB gratefully acknowledges the financial support by the Swiss Federal Commission for Scholarships for Foreign Students and BMM the financial support of the Swiss National Science Foundation, Grant No. 310030_160162).

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Supplementary material:

Table S1: BABA levels in leaf tissues of wheat plants 2 and 6 days post treatment with BABA as soil-drench. The extraction and quantification of BABA was conducted as previously described by Thevenet et al. (2017).

	BABA concentration (µM)	
Treatment	2 days	6 days
H_2O	0.02 ± 0.009	0.02 ± 0.001
BABA	15.53 ± 4.95	4.58 ± 0.76

The values represent the mean ± SE of 3 biological replicates

CHAPTER VI

General conclusions and perspectives

Chapter VI: General conclusions and perspectives

Conclusions

The results presented in this thesis highlight the capacity of induced resistance to enhance the resistance of wheat against fungal leaf disease and to better understand the underlying resistance mechanisms. In the following section, a synthesis of the main conclusions in different aspects is given.

Physiological aspect: plant growth

The use of plant resistance inducers is a promising approach to control plant disease. However, their inappropriate use may lead to some side effects on plants including phytotoxicity, growth suppression and fitness-cost of such induced resistance in case of chemical stimuli (Cohen et al., 2016; Luna et al., 2016; van Hulten et al., 2006). Inoculation with beneficial bacteria can also lead to some negative effects mainly on seed germination (Lee et al., 2013; McPhail et al., 2010; Tabatabaei et al., 2016). Our results show that *Pseudomonas protegens* CHA0 can safely be used to inoculate wheat seeds in order to perform studies on induced resistance in wheat. On the other hand, optimizing BABA-dose for an efficient use to induce of resistance in wheat was revealed, showing possibility to use 15 mM of BABA to protect wheat seedlings against leaf rust with smallest impact on vegetative growth (Fig. 1).

Phytopatholgical aspect: plant protection

Foliar diseases of wheat are common throughout all wheat-producing areas. If weather conditions favour the development of these diseases, in case control measures are not implemented, losses can exceed 20% of the crop production (Johnson & Townsend, 2009). In this thesis, biological and chemical resistance inducers were tested for their efficiency to control foliar pathogens of wheat; including leaf rust caused by *P. triticina* and Septoria tritici leaf blotch (STB) caused by *Zymoseptoria tritici.* We conclude that plant resistance inducers could be an alternative to protect wheat against foliar disease but they are not always affective and the result depends on the combination inducer/pathogenic agent. As was expected, we confirmed that BABA is a potent inducer of resistance in wheat.

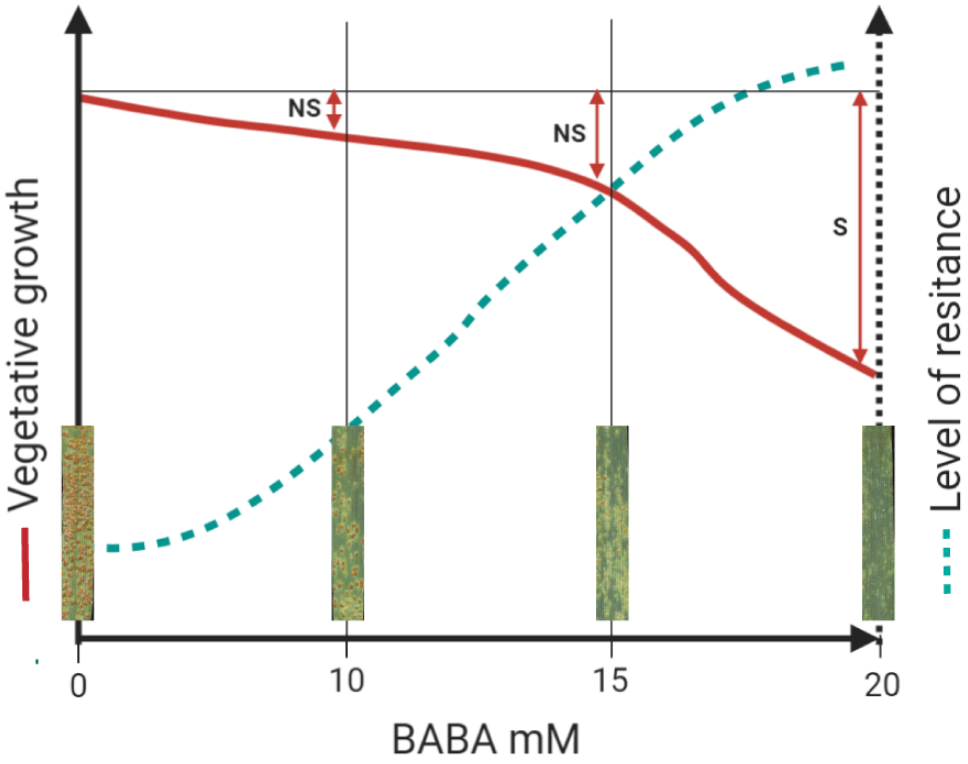

Figure 1: Scheme of the physiological state of wheat plants that have been subjected to a gradient of BABA concentrations challenged with leaf rust. From right-to-left: symptoms of leaf rust infection on plant treated with 0, 10, 15 and 20 mM. A dose-dependent protection of BABA was observed (Red line) accompanied with a dose-reduction in vegetative growth (Dashed blue line), no significant (NS) reduction was observed with 10 and 15 mM. BABA at 20 mM significantly (S) repressed plant growth. Intersection of curves presents a balance point between BABA protectively against leaf rust and plant growth reduction.

Histopathological and molecular aspect: defence mechanisms

Over the past decade, research has focused on elucidating the mechanisms of induced resistance based on physiological changes and signaling pathways in induced plants. Moreover, several works have also studied induced resistance at the cytological level (De Vleesschauwer et al., 2008; Jeun et al., 2001; Jeun et al., 2004; Mahesh & Sharada, 2018). Histopathological and transcriptomic studies of host-pathogen interactions after induction of resistance can help to identify the events that occur during pathogenesis, and ultimately lead to better understanding of the resistance mechanism. Our results concerning mechanisms involved in BABA- and CHA0-induced resistance against wheat leaf rust are summarized in Fig. 2 showing that CHA0 bacteria and BABA induced at least partially similar defence reactions with differences in timing and amplitude. A clear difference between the two induced responses is that they target distinct defence-related genes.

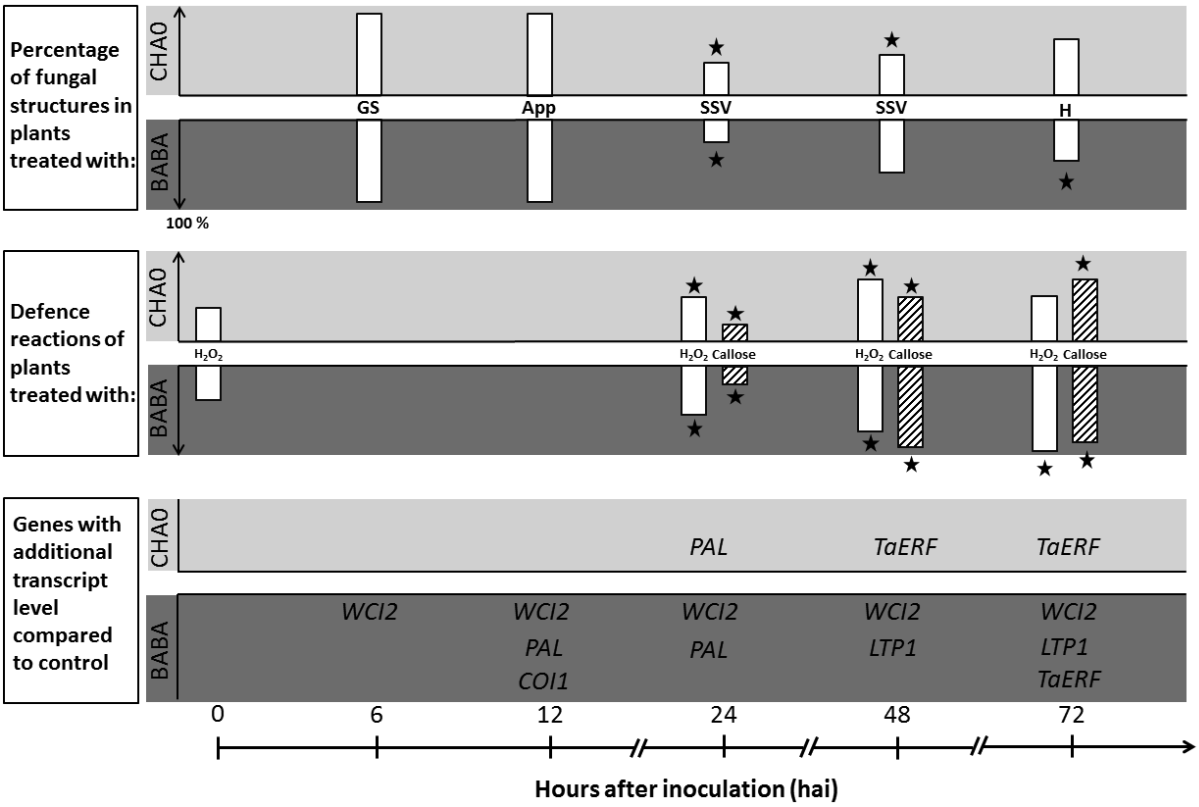

Figure 2: A comparative overview of defense reactions and differentially expressed genes during fungal development in CHA0- or BABA-treated plants. **GS**−Germinated spore, **App** −Appressorium, **SSV**−Substomatal vesicle, **H**−Haustorium. Stars indicate significant differences in comparison with untreated control. ***PAL***−Phenylalanine Ammonia-Lyase, ***WCI2***−Wheat Chemically Induced 2, ***TaERF***−Wheat Ethylene-Responsive Factor, ***COI1***−Coronatine Insensitive 1, ***LTP2***−Lipid Transfer Protein 1.

Perspectives

Several interesting future research perspectives arose from this thesis as presented in the following points:

Is CHA0-induced resistance efficacy dependent on pathogens' lifestyle?

Plant pathogens can be divided into biotrophs and necrotrophs, according to their lifestyles. Biotrophs feed on living host tissue, whereas necrotrophs kill host tissue and feed on the remains (Glazebrook, 2005). Some pathogens exhibit more than one lifestyle. They may initially be biotrophic, but later change to a necrotrophic lifestyle. These can be termed hemibiotrophs (Vleeshouwers & Oliver, 2014). In general, induced systemic resistance by rhizobacteria is mediated by jasmonic acid and ethylene signaling and is effective against necrotrophic pathogens (Nie et al., 2017; Pieterse et al., 2014). However, some beneficial micro-organisms induce the salicylic acid-dependent pathway effective against biotrophic pathogens (Gehring et al., 2005; Molitor et al., 2011). De Vleesschauwer et al. (2006) observed that treatment of rice roots with *P. aeruginosa* 7NSK2 induced resistance against *Magnaporthe grisea*, a hemibiotrophic pathogen. In our study, we showed that induced resistance by CHA0 in wheat is effective against a biotroph (*P. triticina*) via enhancement of callose deposition and H_2O_2 accumulation, and it was not effective against the hemibiotrophic pathogen (*Z. tritici*). In our discussions, we explain ineffectiveness of CHA0 in controlling *Z. tritici* by the fact that the used strain was highly virulent. To clarify this point, first, it would be interesting to test if CHA0-induced resistance gives the same result in case of other *Z. tritici* stains with a different virulence degree. Secondly, to test the efficacy of this induced resistance against necrotrophic pathogens of wheat such as *Parastagonospora nodorum*, causative agent of Septoria nodorum blotch of wheat (Mehra et al., 2019). Comparative cytological and molecular studies of CHA0-induced resistance in case of biotrophic, necrotrophic and hemibiotrophic pathogens may provide a deeper understanding on the relationship between rhizobacteria-induced resistance and fungal lifestyle.

Induced resistance by CHA0 and BABA against other stresses in other parts of the plant?

In addition to our results obtained in this thesis, it would be interesting to test if CHA0- and BABA-induced resistance can be effective against other stresses, such as biotic root attacks by pathogens or nematodes. Direct, as well as indirect, defences could be induced by the presence of both inducers. By using a split-root system (Sari et al., 2008), it would be also possible to

test if the presence of inducers in one part of wheat roots could induce a systemic resistance in non-treated root parts.

Beneficial microbes as well as BABA have been also referred to as potential plant tolerance inducer in the context of abiotic stress conditions in the surrounding plant environment. A master project conducted in our laboratory tested the capacity of CHA0 and BABA to induce wheat tolerance against salt stress. However, repetition of these experiments would be necessary.

Use of CHA0 as a beneficial microbe in the field

CHA0 are well-known rhizobacteria that have an efficient capacity of root colonization for a wide range of plants of agronomic interest (Chiriboga et al., 2018; Deepika et al., 2019; Haas & Défago, 2005; Henkes et al., 2011; Stutz et al., 1986), even in combination with other beneficial organisms (Imperiali et al., 2017). Moreover, our results show that CHA0 are salt-tolerant rhizobacteria. Despite these encouraging facts, only few is known on the effect of CHA0 on wheat plants. Our laboratory studies enhanced the possibility of CHA0 as being interesting rhizobacteria for an agricultural use. Many microbes have been described as beneficial for plant health and an increased demand for sustainable approaches to control crop disease favoured the application of such bioagents in fields. However, exploiting induced resistance elicitors in agriculture is still not common. Inconsistency of induced resistance and a less efficient disease control in comparison with standard pesticide are the main reasons (Walters et al., 2013). Research under laboratory conditions has shown the potential of CHA0-induced resistance as an alternative to fungicides. However, further research to verify the efficacy of induced resistance under field conditions remains to be done. In addition to technical and financial challenges linked to the production of bacterial formulation, there is probably also work to do on the general opinion to gain more acceptance for utilizing induced resistance as a crop protection method.

What could happen at the root level in case of CHA0 or BABA treatment?

Most studies of induced resistance have been essentially focusing on aboveground plant parts (Görlach et al., 1996; Iavicoli et al., 2003; Van der Ent et al., 2009). Roots are not only essential for nutrient and water uptake, they have an important impact on plant capacity to react to stress (Koevoets et al., 2016). In addition, the root system plays a key role in beneficial interactions and perception of chemical stimuli during induced resistance. Our study focused on mechanisms deployed at leaf level. It would be interesting to investigate such as molecular

changes upon treatment with CHA0 and BABA followed by biotic or abiotic challenge at leaf level. In our laboratory, Planchamp et al. (2013) developed a soil-free system for a clean sampling during root-microbe interaction studies. This system was nicely adapted for wheat plants and used to study bacteria localization in roots (see chapter II). Nevertheless, optimization of the system is needed due to some limitations such as the restricted size of the pouches, which does not allow growing the plants for longer than 15 days. Additionally, wheat plants grown in this system presented a normal growth at the beginning, but later on, a chlorosis on leaves appeared (data not shown). This could have negative impact on pathogen infection especially those with biotrophic lifestyle.

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Appendix

a. Submitted manuscripts

- **Histopathological aspects of induced resistance by *Pseudomonas protegens* CHA0 and β-aminobutyric acid in wheat against *Puccinia triticina***

  Bellameche, F., Jasim, M. A., Mauch-Mani, B., & Mascher F.
  Phytopathologia Mediterranea, submission date February 2020.

- **Efficiency of biological and chemical inducers for controlling Septoria tritici leaf blotch (STB) on wheat (*Triticum aestivum* L.)**

  Bellameche, F., Pedrazzini, C, Mauch-Mani, B., & Mascher F.
  European Journal of Plant Pathology, submission date January 2020.

b. Published abstracts for a conference

- **Induction of resistance in wheat against leaf rust by application of biotic and abiotic inducers**

  Bellameche, F., Jasim, M. A., Mauch-Mani, B., & Mascher F.
  IOBC-WPRS Bulletin 135: 101-103, publication 2018.

- **Expression of the antifgungal protein afp from *Aspergillus giganteus* for increasing fungal resistance in strawberry**

  Palomo Ríos, E., Pliego, C., Bellameche, F., Benthami, H., Pliego-Alfaro, F., & Mercado J.A.
  International Strawberry Symposium IX, submission date, 2020

c. Awards

- **Best poster "**Efficiency of biological and chemical resistance inducers for controlling *Septoria tritici* leaf blotch in wheat (*Triticum aestivum* L.)**"**

  Bellameche, F., Pedrazzini, C, Mauch-Mani, B., & Mascher F.
  Journée d'automne 2018 de la Société Suisse de Phytiatrie (SSP)