DISEASE CONTROL AND PLANT DEFENSE PATHWAYS INDUCED BY *BACILLUS MOJAVENSIS* ISOLATE 203-7 AND B*ACILLUS MYCOIDES* ISOLATE BMJ

by

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DEDICATION

This dissertation is dedicated to the three most important people in my life:

To my mother Ingrid Neher, who nourished and supported my curiosity and my love for nature;

To my wife and friend Dr. Eva Grimme, who encouraged and sometimes pushed me to give my best, and helped me to become the man and researcher I am;

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ABSTRACT

The objective of this study was to investigate the plant defense pathways induced by *Bacillus mojavensis* isolate 203-7 (203-7) and *B. mycoides* isolate BmJ (BmJ) and to test their ability to control fungal pathogens on tomato and cucumber by means of systemic acquired resistance (SAR). An *Arabidopsis thaliana* mutant – *Botrytis cinerea* pathosystem was used to investigate plant defense pathways activated by 203-7 and BmJ. *A. thaliana* wild type (Col-0), *ein2-1*, *jar1-1*, *NahG*, *ndr1-1/npr1-2*, and *npr1-1* mutants were induced by application of bacilli, distilled water, or chemical inducers. Both bacilli reduced disease severity on wild type and *NahG* mutants, but provided no reduction on *jar1-1,* indicating that induction was salicylic acid (SA) independent but jasmonic acid (JA) dependent. 203-7 induced plants had lower disease severity on *npr1-1* and *ein1-2* mutants but were equivalent to buffer controls on *jar1-1* mutants. BmJ did not decrease disease severity on *npr1-1*, *jar1* or *ein2-1* mutants. Enzyme assays confirmed the induction of chitinase, β-1,3-glucanase, and superoxide dismutase by 203-7 and BmJ. These results demonstrate that induction by 203-7 is JA dependent and NPR1 independent. BmJ is SA independent but NPR1 and JA/ethylene dependent. Bacilli were tested for their ability to control *B. cinerea* grey mold on hydroponically grown greenhouse tomatoes and *Glomerella cingulata* var. *orbiculare* on cucumber by means of SAR. Weekly foliar applications of bacilli were able to significantly (α=0.05) reduce the severity of grey mold leaf lesions and to reduce the area under the disease progress curve (AUDPC) calculated for seven Botrytis stem canker ratings. Chitinase, β-1,3-glucanase, and SOD activity of apoplastic fluids were not significantly (α=0.05) increased by the treatments. Bacilli reduced total and live spore production of *G. orbiculare* per mm ^{2} of lesion and increased β-1,3-glucanase activity of cucumber apoplastic fluids. Applications of BmJ compared to fungicides for the control of anthracnose in cucumber (var. 'General Lee') and cantaloupe (var. 'Athena') were evaluated in 2004 and 2005 field experiments. BmJ applied seven days before inoculation provided disease reduction in cucumber and cantaloupe statistically equal to the fungicide treatments.

CHAPERT 1

INTRODUCTION

Biological Control

Overview

The Merriam-Webster Online Dictionary (2008) defines the term "biological control" as "the reduction in numbers or elimination of pest organisms by interference with their ecology (as by the introduction of parasites or diseases)". A more current version of the definition for biological control also includes an environmental component: "Biological control is an environmentally sound and effective means of reducing or mitigating pests and pest effects through the use of natural enemies" (Elsevier, 2008). References to the term biological control can be found as early as 1893, when Dr. Carl Freiherr von Tubeuf published a paper on the biological control of forest insects, followed by a paper (Biological control of fungus diseases of plants) in 1914, where this term was applied to plant diseases for the first time (as cited by Maloy and Lang, 2003). Biological control has since become an interdisciplinary science combining entomology, microbiology, plant pathology, weed science, and virology with the goal to reduce and control pathogens, microorganisms, insects, and plants alike, which can cause damage to crop plants (Eilenberg et al., 2001).

Biological control is widely used in field and greenhouse production, and plays an integral part in integrated pest management (IPM). The IPM approach combines biological control with cultural methods such as planting of less susceptible varieties,

crop rotation with non host plants, control of environmental conditions, sanitation, and low dose applications of pesticides (Anderson, 2008; Bjerre et al., 2006; Daughtrey and Benson, 2005; Hansen et al., 2008; Jacobsen and Backman, 1993). Biological control measures accompanied by adequate IPM strategies can achieve a 95 to 100 % disease control. Without adequate IPM strategies the variability of the biological control agents (BCAs) would be too great (Jacobsen et al., 2004), since they depend strongly on environmental conditions and nutrient availability.

The term biological control agent (BCA) describes the microorganism (bacterial, fungal, viral) or insect used to control destructive pathogens. This includes, but is not limited to, insects such as wasps for the control of aphids or scales; beetles and their larvae to control weeds and aphids; fungi such as *Trichoderma* sp. and *Gliocladium* sp. for the control of soil-borne fungal pathogens; and bacteria like *Pseudomonas* sp. and *Bacillus* sp for the control of soil-borne pathogens, and for foliar diseases caused by fungi and bacteria (Braun-Kiewnick et al., 2000; Fravel, 2005; Jacobsen and Backman, 1993; Jacobsen et al. 2004; Kiewnick et al., 2001; Paulitz and Bélanger, 2001). Pathogens can be controlled by different modes of action such as predation or parasitism, employed by insect BCAs (Strand and Obrycki, 1996; Van Den Bosch, 1971), parasitism by fungal BCAs (mycoparasitism) (Bailey et al., 2008; Sahebani and Hadavi, 2008) or production of antimicrobial compounds by fungal and bacterial BCAs (Bailey et al., 2008; El-Katatny et al., 2006; Iwaki et al., 1972; Jacobsen, 2006; Leifert et al., 1995; Klecan et al., 1990; Vinale et al., 2008).

Since this dissertation is focused on the control of fungal pathogens by means of bacterial and fungal BCAs, their modes of action will be discussed more in detail.

Modes of Action

Most BCAs employ more than one mode of action to control pathogens which can include antibiosis, competition for nutrients and space, parasitism, and induced systemic resistance.

Antibiosis: Antibiosis is the most common mode of action claimed for many fungal and bacterial BCAs and describes the production and excretion of antimicrobial compounds or antibiotics (Bailey et al., 2008; El-Katatny et al., 2006; Iwaki et al., 1972; Jacobsen, 2006; Leifert et al., 1995; Klecan et al., 1990; Vinale et al., 2008). The production of antimicrobial compounds strongly depends on the availability of exogenous nutrients, such as root exudates, leakage of nutrients on the leaf surface, or organic nutrients in the soil, and the overall environmental conditions including root or leaf surface, moisture level, pH range, and the competition by other antagonistic microorganisms (Jacobsen, 2006). Thomashow and Weller (1988) were the first to demonstrate that the control ability of *Pseudomonas fluorescens* 2-79 is based on the production of a phenazine antibiotic by generating phenazine-negative mutants which showed no ability to control *Gaeumannomyces graminis* var. *tritici* (Haas and Keel, 2003). Other known antimicrobial compounds include, but are not limited to, agrocin 85 produced by *Agrobacterium radiobacter* for the control of *A. tumefaciens* (Farrand 1990; Formica, 1990); gramicidin S produced by *Brevibacillus brevis* for the control of *Botrytis*

cinerea (Edwards and Seddon, 2001; Iwaki et al., 1972); peptide and non-peptide antibiotics produced by *Bacillus subtilis* isolate CL 27 for the control of *B. cinerea* (Leifert et al., 1995); flocculosin, an antimicrobial octadecyl cellobiose lipid, produced by *Pseudozyma flocculosa* for the control of *Sphaerotheca fuliginea* (Cheng et al., 2003); and oomycin A produced by *Pseudomonas fluorescens* HV37 for the control of *Pythium ultimum* (damping-off) (Howie and Suslow, 1991).

Competition for Nutrients and Space: BCAs, competing for nutrients with the pathogen, will most likely compete for space in ecological niches such as the surfaces of leaves, flowers or fruits. Examples include rhizosphere colonizing bacteria competing for iron via siderophores (a secreted iron chelating compound)(Van Loon et. al, 1998), and *Pseudomonas syringae* L-59-66 which can control Fusarium dry root of potatoes by colonizing wounds of potato tubers (Kiewnick and Jacobsen, 1997). Also yeasts like *Cryptococcus laurentii* BSR-Y22, *Rhodosporidium toruloides* Y-1091, and *Sporobolomyces roseus* FS-43-238 can provide control of fungal pathogens like *B. cinerea* or *Penicillium* sp. in wounds of different fruits or on geranium leaves by competing for space and nutrients such as fructose and glucose (Buck, 2002; Filonow, 1998; Roberts, 1990; Zhang et al., 2007). The application of *Peniophora gigantea* to control *Fomes annosum* the causal agent of root and butt rot of conifer trees is an example where competition for space is the only mode of action given for the BCA (Agrios, 2005).

Parasitism: Direct parasitism can be distinguished from myco-parasitism, where direct parasitism refers to the infection of hyphae, asexual and sexual structures, and surviving structures of a fungal pathogen by fungal and bacterial BCAs (Inglis and Kawchuk, 2002; Leveau and Preston, 2008). Examples are *Ampelomyces quiqualis* parasitizing powdery mildews such as *Oidium* sp. (the asexual stage of powdery mildews suc as *Erysiphe* sp.), *Erysiphe* sp., and *Sphaerotheca* sp. (Sztejnberg et al., 1989), and *Bacillus amyloliquefaciens* controlling *Sclerotinia sclerotiorum* (Abdullah et al., 2008). Myco-parasitism refers to the lysis of hyphae of fungal pathogens by hydrolytic enzymes excreted by fungal BCAs such as *Trichoderma* species and yeasts like *Tilletiopsis* species (Bailey et al., 2008; Harman, 2006; Inglis and Kawchuk, 2002; Urquhart and Punja, 2002).

Induced Resistance: The term induced resistance describes the activation of certain pathogen-related defense molecules by fungal and bacterial BCAs, chemicals, avirulent races of pathogens, and abiotic factors such as drought or salt stress.

This subject will be explained in more detail because it is critical for the understanding of the following chapters.

Induced Resistance

The phenomenon of induced resistance to pathogens in plants was reported as early as 1933 by Chester and confirmed by Kuć et al. (1959), Loebenstein (1963), and Ross (1961, 1966). An increase in plant resistance to other pathogens was observed when previous exposure to viruses caused localized necrotic lesions. Van Loon et al.

(1998) described this form of resistance as a "state of enhanced defensive capacity", where an induced plant can react faster to a pathogen attack, since its defenses are already activated. In following years, two distinct systems for the induction of plant resistance were discovered: Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR).

Induced Systemic Resistance

ISR is linked to the colonization of plant roots by plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas* spp. (Bakker et al. 2007; Vallad and Goodman, 2004; Van Loon et al., 1998). These PGPR can be applied to the rhizosphere of the plants by soil drenches, soil mixes, root dipping, and seed treatments (Van Loon et al., 1998). ISR induced by PGPR is only effective in certain plant species and genotypes (Yan et al., 2002), and is reported to be successful against fungal and bacterial pathogens, but has shown only limited success in controlling some viral diseases (Raupach and Kloepper, 1998, 2000; Wei et al., 1996; Yan et al., 2002; Zehnder et al., 2001). The induction facilitated by PGPR is linked to the activation of the jasmonate and ethylene (ET) dependent defense pathways and results in the increase of certain defense compounds such as callose, lignin, peroxidase, phenolic compounds, and phytoalexins (Adie et al., 2007; Compant et al., 2005; Dixon et al., 1986). Depending on the type of PGPR pathogenesis-related proteins (PR-proteins) such as chitinases and peroxidases can be expressed (Compant et al., 2005).

Systemic Acquired Resistance

The induction of SAR is the result of the formation of necrotic lesions caused by a hypersensitive response (HR) or as part of disease symptoms caused by a necrotizing pathogen (Durrant and Dong, 2004; Ryals et al., 1996; Sticher et al., 1997). Non-pathogenic microorganisms such as BCAs (Bargabus et al., 2002, 2003, 2004) or avirulent races of pathogens, and chemicals such as phosphate salts or salicylic acid (SA) can induce also SAR in a wide range of monocotyledonous and dicotyledonous plants (Sticher et al., 1997) against bacterial, fungal, viral pathogens, and insect pests. The induction of SAR is accompanied by an increase of SA resulting in the expression of multiple PR-proteins such as chitinase, β-1,3-glucanase, and peroxidase (Conrath et al., 2006; Delaney, 1997), and the formation of callose, lignin, and phytoalexins (Dixon, 1986; Sticher et al., 1997; Vance et al., 1980). Bargabus-Larson and Jacobsen (2007) recently demonstrated a SA independent activation of SAR and the production of PR-proteins by biotic inducers. The effects of the SAR are effectively expressed throughout the plant 3 to 6 days after the application of abiotic and biotic inducers and will last up to 14 to 20 days depending on the plant species and the environmental conditions (Bargabus et al., 2003). Different abiotic and biotic inducers will be discussed in the following sections.

Abiotic Inducers

Abiotic inducer or elicitors include environmental factors such as pH, salt, and water-drought stress, heat shock, partial freezing followed by thawing, wounding, and exposure to ultraviolet (UV) radiation or ozone (Soylu et al., 2002; Walters et al., 2005).

Plant derived organic compounds, such as SA and its derivatives aspirin and 2,6-dihydroxybenzoic acid (Van Loon, 1983), jasmonic acid (JA) and its methyl ester methyl jasmonate (MeJa), ET, systemin, and abscisic acid (ABA) have also been described (Sticher et al., 1997). Chemical elicitors and salts are used as well in the agricultural production to induced SAR. Examples are: aluminum lignosulphonate (sold as BROTOMAX ®), 2,6-dichloroisonicotinic acid (INA), benzo[1,2,3]thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) and its derivative *S*-methyl-benzo[1,2,3]-thiadiazole-7-carbothiate (acibenzolar-*S*-methyl [ASM] sold as BION ® and ACTIGARD ™), probenazole (PBZ, sold as ORYZEMATE ®), 2-chloroethylphosphate (ETHEPHON ®), and DL-3-aminobutyric acid (BABA) (Conrath et al., 2006; Oostendorp et al., 2001; Ryals et al., 1996; Sticher et al., 1997).

Biotic Inducers

Biotic inducers can be necrotizing bacterial, fungal, and viral pathogens, avirulent races of the respective pathogens, foliar applied bacterial and fungal BCAs, and cell wall fractions of bacteria and fungi (Bargabus et al., 2003). Microorganisms involved in the colonization of plant roots such as PGPR and mycorrhizal fungi can also induce the expression of SAR (Conrath et al., 2006; Van Loon et al., 1998). Especially bacterial BCAs such as *Pseudomonas* spp. and *Bacillus* spp., and fungal BCAs like *Trichoderma* spp. play an important role in the induction of SAR (Bargabus et al., 2002, 2003, 2004; Brunner et al., 2005; Emmert and Handelsman, 1999; Hoitink et al., 2006; Harman, 2006; Jacobsen et al., 2004; Jacobsen, 2006; Raupach and Kloepper, 1998, 2000; Wei et al., 1996; Woo et al., 2006). Since the main focus of this dissertation is in the induction of

SAR by foliar applications of *Bacillus mojavensis* and *Bacillus mycoides* both biotic inducers will be described more in detail.

Bacillus mojavensis Isolate 203-7: *B. mojavensis* belongs to the family *Bacilliaceae*, and is characterized by endospore-forming rods. It is closely related to *Bacillus subtilis* (Roberts et al., 1994) and shows the same characteristics such as gram positive, elliptical endospores (0.8 by 1.5-1.8 μm), and rod shaped cells (0.7-0.8 μm by 2-3 μm) which are not in chains and do not show flagella (Bergey's Manual of Determinative Bacteriology, 1994). If grown on nutrient agar, the colonies are round or irregular with a cream-colored surfaces that appears dull and wrinkled. Isolate 203-7 (203-7) was originally isolated from sugar beet seed embryos in 1994 and showed the ability to control fungal diseases in multiple plant species by the induction of SAR (Bargabus et al., 2004; Neher at al., 2007; Neher et al., 2009). 203-7 is able to significantly increase the expression of PR-proteins such as chitinase, β-1,3-glucanase, peroxidase, and superoxide dismutase (Bargabus et al., 2004; Neher et al., 2009). Other *B. mojavensis* isolates show also potential to be used as BCAs (Bacon and Hinton, 2002, 2007; Nair et al., 2002; Thornton et al., 2008).

Bacillus mycoides Isolate BmJ: *B. mycoides* belongs to the family *Bacilliaceae*, and is characterized by endospore-forming rods. Its characteristics include gram positive, elliptical endospores, and rod shaped cells (1.0-1.2 μm by 3-5 μm) which are in chains and not motile (Bergey's Manual of Determinative Bacteriology, 1994). If grown on nutrient agar, the colonies are rhizoid (branching like fungal mycelium) with a dark

cream-colored surfaces that appears dull. Isolate BmJ (BmJ) was originally isolated from sugar beet leaves in 1994 (Bargabus et al., 2002). Foliar applications of BmJ were used to protect sugar beets against *Cercospora beticola*, causal agent of Cercospora leaf spot, and provided a 38-91 % disease control (Jacobsen et al., 2004). This control and the protection of cucumber against anthracnose (*Glomerella cingulata* var. *orbiculare*) (Neher et al., 2009) is facilitated by the induction of SAR which led to an increase of chitinase, β-1,3-glucanase, peroxidase (Bargabus et al., 2002), and superoxide dismutase (Neher et al., 2009) in tested plants. Further studies by Bargabus-Larson and Jacobsen (2007) show that the induction of SAR by BmJ is independent of the SA signaling pathway but dependents on the activation of the NPR-1 gene.

Defense Pathways

Depending on the abiotic or biotic inducer different plant defense pathways are stimulated and include the SA, JA, and ET pathways (Pieterse and Van Loon, 1999; Spoel et al., 2003; Thomma et al., 1998). It is well documented that these pathways are not mutual exclusive, but rather stimulate or inhibit each other (Bostock, 2005; Feys and Parker, 2000; Penninckx et al., 1998; Traw et al., 2003). These interactions are regulated by the regulatory protein Non-expresser of Pathogenesis-Related genes1 (NPR1). In the following sections these pathways, their major signal molecules, and their regulation will be explained in detail.

Salicylic Acid Pathway: SA is not only involved in the expression of localized resistance in response to an attack by a biotrophic pathogen (Delaney et al., 1995), but it is also a 'long distance' messenger molecule within the plant for the induction of SAR and the production of marker PR-proteins like PR-1, PR-2, and PR-5 (Ryals et al., 1996; Sticher et al., 1997). Its significance for the induction of SAR was shown by using *Arabidopsis thaliana* plants transformed with the NahG (naphthalene hydroxylase G) gene which encodes for salicylate hydroxylase degrading SA to inactive catechol (Sticher et al., 1997). Benzoic acid and ortho-coumaric acid, both precursor of SA, are metabolized from phenylalanine via the shikimate-phenylpropanoid pathway (Buchanan et al., 2002; Lee et al., 1995; Sticher et al., 1997). The induction of SAR can be verified by specific SAR marker genes. In the case of *A. thaliana* these genes are PR-1, PR-2 (β-1,3-glucanase), and PR-5 (osmotin-like protein) (Uknes et al., 1992).

Jasmonic Acid / Ethylene Pathway: JA and ET are involved in the induction of induced systemic resistance (ISR) (Pieterse et al., 2000; Ton et al., 2002; Truman et al., 2007) which is associated with the rhizosphere colonization by plant growth promoting rhizobacteria (PGPR) (Van Loon et al., 1998). JA and ET are also important messenger molecules for systemic resistance associated with the attack by necrotrophic pathogens and herbivores (Farmer and Ryan, 1990), and the exposure to stress induced by drought, UV light, or ozone (Shan et al., 2007; Turner et al., 2002). In both cases JA and ET activate genes coding for plant defense-related proteins like thionins and proteinases but in the case of JA not PR-proteins (Pieterse et al., 1998). JA is a precursor of jasmonate, which is a plant hormone regulating the production of viable pollen, fruit ripening, seed

germination, root growth, leaf and fruit abscission, and senescence. JA is metabolized from linolenic acid via the octadecanoid pathway (Buchanan et al., 2002; Shan et al., 2007).

ET has similar function than jasmonate, but in addition it is related to cell elongation and induction of certain PR-proteins such as chitinase and β-1,3-glucanase (Sticher et al., 1997). ET is metabolized from methionine which is converted to 1-aminocyclo-propane-1-carboxylic acid, and followed by ethylene (Bleecker and Kende, 2000; Kende, 1993).

The Role of NPR1: NPR1 plays an important role for the regulation of SAR and ISR (Dong, 2004; Durrant and Dong, 2004; Feys and Parker, 2000). In the case of SAR, NPR1 is essential for the PR-protein gene induction, and also for the SA detoxification and the regulation of its biosynthesis (Kinkema et al., 2000). In addition, NPR1 regulates the cross-talk between SA, JA, and ET pathways (Spoel et al., 2003). Two forms of NPR1 are known: the inactive-multimeric form and the active-monomeric form. The inactive multimeric form of NPR1 is primarily detected in the cytoplasm, whereas after treatments with SAR inducers, NPR1 is translocated into the nucleus, where its active monomeric form is detectable. There NPR1 probably interacts through several members of the transcriptions factors (TGAs) subclass of the basic leucine zipper family of TGAs to regulate PR-protein gene expression (Kinkeman et al., 2000).

Activated Defense Compounds

Depending on the type of elicitor, pathogen, or BCA either ISR or SAR is induced in the plant. In the course of this induction, defense compounds such as callose, lignin, peroxidase, phenolic compounds, and phytoalexins (Compant et al., 2005), or PR-proteins are produced and expressed. PR-proteins will be described more in detail, since an understanding is essential for the following chapters.

Pathogenesis-Related Proteins: PR-proteins are classified as proteins "*encoded by the host plant but induced only in pathological or related situations*" (Antoniw and White, 1980). These proteins are characterized by their chemical properties such as low molecular weight or their isoelectric point (pI, acidic or basic), their location either in the apoplast or in the vacuole, and more importantly the fact that they are induced and newly expressed (Van Loon et al., 1994; Van Loon and Van Strien, 1999).

Up to now, 14 PR-protein families are recognized, and only a short summary according to their biological function will be given.

Unknown function (PR-1, PR-4);

β-1,3-glucanase (PR-2);

chitinase (PR-3, PR-8, PR-11);

thaumatin- and osmotin-like protein (PR-5);

proteinase inhibitor (PR-6);

endoproteinase (PR-7);

lignin-forming peroxidase (PR-9);

ribonuclease-like protein (PR-10);

plant defensin (PR-12);

thionin (PR-13), and

lipid-transfer protein (PR-14) (Huang, 2001; Van Loon and Van Strien, 1999). Since different plant families were used to identify PR-protein families, multiple PR-proteins families with the same biological function can occur. For a more in-depth reviewed see Huang (2001).

Model Plant *Arabidopsis thaliana*

Overview

A. thaliana (common English name: wall or mouse-ear cress) belongs to the mustard family (Brassicaceae), which includes species like radish and cabbage. The plant grows 20 to 25 cm tall, with leaves (green to slightly purplish colored) forming a rosette at the base. Leaves have a serrated margin and are covered with trichomes, and can reach 1.5 to 5 cm in length and 2 to 10 mm in with.

A. thaliana is a powerful tool for basic research in genetic and molecular biology (Glazebrook et al., 1997): 1. Its small genome (125 Mb total) is completely sequenced; 2. Multiple well described mutant lines are available from the TAIR stock center (Carnegie Institution of Washington Department of Plant Biology, Stanford, CA); and 3. *A. thaliana* has a rapid life cycle which can be completed within approximately 7 weeks, starting with vernalisation of the dormant seeds at 4 ± 1 °C to mature seeds (Garcia-Hernandez et al., 2002).

Selected Mutants

The following *A. thaliana* mutants were obtained from the TAIR stock center (Carnegie Institution of Washington Department of Plant Biology, Stanford, CA) and had an *A. thaliana* ecotype Columbia backround:

Mutant *ein2-1* (TAIR CS3071) expressing ethylene insensitivity (Vandenbussche et al., 2007; Zipfel et al., 2004).

Mutant *jar1-1* (TAIR CS8072) is jasmonate resistant and reacts with reduced induction of lipoxygenase to exogenous applications of methyljamonate (Zipfel et al., 2004).

Mutant *ndr1-1/npr1-2* (TAIR CS6355) is a nonexpresser of PR-protein genes and is insensitive to salicylic acid (Kirley et al., 2003).

Mutant *npr1-1* (TAIR CS3726) is also a nonexpresser of PR-protein genes and is insensitive to salicylic acid (Spoel et al., 2003).

The *NahG* mutant (obtained from Dr. Bob Dietrich, Syngenta, Greensboro, NC) contains the NahG (naphthalene hydroxylase G) gene which encodes for salicylate hydroxylase. This enzyme degrades SA to the inactive catechol and makes the plant salicylic acid insensitive (Hunt et al., 1997).

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CHAPTER 2

ADAPTATION OF MICRO-PLATE-BASED CHITINASE AND B-1,3-GLUCANASE ASSAYS FOR THE ANALYSIS OF APOPLASTIC FLUIDS EXTRACTED FROM ARABIDOPSIS

Abstract

Based on existing colorimetric methods to quantify chitinase and β-1,3-glucanase activity, new micro-plate-based enzyme activity assays were developed. The micro-plate assays allow up to 28 individual samples (repeated three times, including three standards) to be tested simultaneously, without the restriction of conventional gel diffusion assays (limited sample size and reproducibility). The developed chitinase and β-1,3-glucanase assays can be used interchangeably with either sodium buffer (150 mM NaCl) or standard apoplastic extraction buffer (25 mM MES + 150 mM NaCl) without interference in the colorimetric analysis. The optimal range to test for chitinase or β-1,3-glucanase enzyme activity was determined to be between 9.68 E-04 to 9.68 E-07 units/µl of chitinase and between 1.00 E-03 and 1.56 E-05 units/µl of laminarinase, respectively. In this range the chitinase standards achieved an average R^2 value of 0.938 and the laminarinase standards a R^2 value of 0.9921 regardless of buffer type. When apoplastic fluid samples extracted from *Arabidopsis thaliana* plants were analyzed by either a gel diffusion or colorimetric micro-plate method, similar test results were achieved with either method. However, the micro-plate assay had lower standard deviations and overall better R^2 values (0.9889 chitinase, 0.9925 β-1,3-glucanase) for the enzyme standards than the gel diffusion assay.

The use of a liquid substrate for the enzyme activity and the incubation in a thermocycler also reduces the overall incubation time by 41 % for the chitinase assay and by 77 % for the β-1,3-glucanase assay when compared with the gel diffusion assays.

Introduction

Induction of Systemic Acquired Resistance (SAR) in plants is accompanied by a significant increase of pathogenesis-related proteins (PR-proteins) (Durrant and Dong, 2004; Van Loon et al., 2006). These proteins can also serve as molecular markers for the identification of possible biological control organisms with SAR as their mode of action as described by Bargabus et al. (2004) and include, but are not limited to, β-1,3-glucanase, chitinase, and peroxidase . Gel diffusion assays (Bargabus et al. 2002, 2004; Cote et al, 1989; Velasquez and Hammerschmidt, 2004; Zou et al, 2002) and colorimetric methods (Imoto and Yagishita, 1971) have most commonly been employed to determine specific enzyme activity in plant fluids. Gel assays pose the challenge of achieving reproducible results since multiple factors such as differences in gel thickness which provide an unequal amount of substrate per sample, gel composition, and plate material are sources of experimental variability. Also, differences in sample activity which could lead to a coalescence of the individual activity zones (Image 2.1) and can complicate the analysis. The ability to process multiple samples at the same time can be an advantage of gel assays. Colorimetric assays have the advantage of lower experimental error due to standardized spectrophotometer cuvettes, substrate volume and composition. However, colorimetric assays are more laborious if single cuvettes are used to process multiple

samples. Micro-plate methods have been developed for carboxymethylcellulose based assays to determine endoglucanase and chitinase activity and to overcome the limitations described above (Hung et al., 2002; Xiao et al., 2005). Theoretically, up to 96 individual samples could be analyzed in a single run under conditions that minimize experimental error.

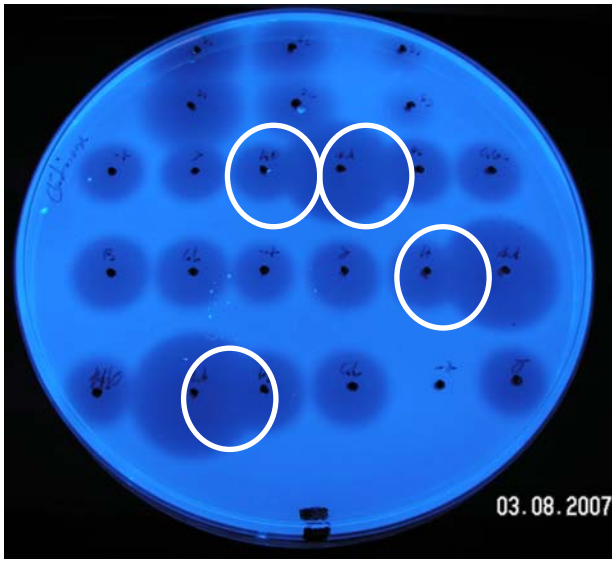


Image 2.1: Chitinase gel diffusion assay under UV light (360 nm). White circles indicate a coalescence of individual activity zones.

The objective of this research was the adaption and modification of existing agarose-based or micro-plate-based assays for the quantification of chitinase and β-1,3-glucanase activity of apoplastic fluids extracted from Arabidopsis plants using a spectrophotometer.

Material & Methods

Chitinase Assay

Based on the chitinase activity assay described by Hung et al. (2002) a 96 well micro-plate assay was developed. Each well of a 96 well PCR plate (VWR International) was loaded with 54 µl of 100 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid), 60 µl of 0.2 % of glycol chitin solution, and 6 µl of chitinase standards (from *Streptomyces griseus*, Sigma). The plate was sealed with a silicone sealing mat (VWR

International) and incubated for 14 h at 37 °C using a thermocycler (GeneAmp PCR System 9600, The Perkin-Elmer Corporation, Norwalk, Connecticut). After this time period 90 µl of the solution were transferred to a new PCR plate and 120 µl of ferri-ferrocyanide reagent (Imoto and Yagishita, 1971) consisting of 0.05 % potassium ferricyanide in 500 mM sodium carbonate was added. The plate was sealed as described before and incubated for 15 min at 99.9 °C. Following incubation 100 µl of the solution were transferred to a 96 well flat-bottom assay plate (Becton Dickinson Labware, Becton Dickinson and Company, Fanklin Lakes, NJ) and the production of reduced sugars (mg of *N*-acetyl-D-glucosamine released/24h/unit of enzyme) was measured as a decrease of absorbance at 420 nm with a SpectraMax plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Comparison of Different Buffers: The previously described chitinase assay was used to measure the chitinase activity in apoplastic fluids extracted from Arabidopsis plants. The effects of different buffers used to extract apoplastic proteins and to prepare the standard enzymes on the production of reducing sugars and their measurement with a spectrophotometer were tested prior to employing this method. Six concentrations (9.68 E-04, 9.68 E-05, 9.68 E-06, 9.68 E-07, 9.68 E-08, and 9.68 E-09 units/µl) of chitinase were suspended either in a MES-sodium chloride buffer (25 mM MES and 150 mM NaCl in double-distilled water at pH 6.2), a sodium chloride buffer (150 mM NaCl in double-distilled water at pH 6.2), or in double-distilled water alone. Plates were prepared as described before with triplicates for each chitinase concentration. Plates were read as described before and results were averaged for each concentration. These values were

plotted on a graph (optical density [OD] at 420 nm versus concentration) and a trend line was calculated. An analysis of variance (Madden et al., 1982) was performed for the different buffers and their interaction with the standards using the ANOVA procedures of the Statistical Analysis System (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The experiments were repeated twice.

Range of Enzymatic Activity: Multiple concentrations (9.68 E-04, 9.68 E-05, 9.68 E-06, 9.68 E-07, 9.68 E-08, and 9.68 E-09 units/µl) of chitinase were tested to establish a range of enzymatic activity measurable with this method. Plates were prepared as described before with triplicates for each chitinase concentration. Plates were read as described before and results were averaged for each concentration. These values were plotted on a graph (OD at 420 nm versus concentration) and a trend line was calculated.

β-1,3-glucanase Assay

The microplate-based carboxymethylcellulose assay described by Xiao et al. (2005) was adapted to be used to determine β-1,3-glucanase activity in apoplastic fluids extracted from Arabidopsis plants. Thirty microliters of 1 % laminaran (from *Eisenia bicyclis*, TCI, Portland, OR) and 1 % CM-Pachyman (Megazyme International Ireland Ltd, Ireland) in 50 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid) and 30 µl of laminarinase standards (from *Trichoderma* sp., Sigma) were combined in a PCR plate well, sealed with a silicone sealing mat and incubated for 4 h at 30 °C using a thermocycler. Following incubation, 60 µl of dinitrosalicylic acid reagent consisting of

30 mM dinitrosalicylic acid (Alfa Aesar, Ward Hill, MA), 14.9 mM phenol, 2.8 mM sodium sulfite, 0.5 M sodium-potassium-tartarate (EMD Chemicals Inc., Darmstadt, Germany), and 0.2 mM NaOH were added to the solution and the PCR plate was incubated again for 5 min at 95 °C as described above. Subsequently 100 µl of solution were transferred to a 96 well flat-bottom assay plate and the absorbance was measured at 540 nm with a SpectraMax plus384 spectrophotometer.

Comparison of Different Buffers: In order to apply this method it was crucial to test the effects of different buffers on the production of reducing sugars and their measurements with a spectrophotometer, so this method can be applied to measure the laminarinase activity in apoplastic fluids extracted from Arabidopsis plants. Six concentrations (1.60 E-02, 4.00 E-03, 1.00 E-03, 2.50 E-04, 6.25 E-05, and 1.56 E-05 units/µl) of laminarinase were suspended either in a MES-sodium chloride buffer (25 mM MES and 150 mM NaCl in double-distilled water at pH 6.2), a sodium chloride buffer (150 mM NaCl in double-distilled water at pH 6.2), or in a double-distilled water alone. Plates were prepared and read as described above with triplicates for each laminarinase concentration. The results were averaged for each concentration, plotted on a graph (OD at 540 nm versus concentration), and a trend line was calculated. An analysis of variance (Madden et al., 1982) was performed for the different buffers, and their interaction with the standards was analyzed using the ANOVA procedures of the Statistical Analysis System (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The experiments were repeated at least once.

Range of Enzymatic Activity: Multiple concentrations of laminarinase (1.60 E-02, 4.00 E-03, 1.00 E-03, 2.50 E-04, 6.25 E-05, and 1.56 E-05 units/µl) were tested to determine the range of enzymatic activity measurable with this method. Plates were prepared as previously described with triplicates for each laminarinase concentration and read as described above. The results were averaged for each concentration, plotted on a graph (OD at 540 nm versus concentration), and a trend line was calculated.

Comparison of Gel Diffusion and Colorimetric Micro-Plate Assays

Newly developed colorimetric micro-plate assays for the quantification of chitinase and β-1,3-glucanase activity were compared to the existing gel diffusion assays described by Bargabus et al. (2002). In addition to chitinase and laminarinase standards, apoplastic fluids extracted from *Arabidopsis thaliana* plants were incorporated when testing the two different methods.

Plant Culture and Treatments: *A. thaliana* ecotype Columbia (Col-0) seeds were sown in 18x13x6 cm flats (T.O. Plastics Inc., Clearwater, MN) containing Sunshine #1 mix (Sun Gro Horticulture Inc., Bellevue, WA) and vernalized for 4 days at 5 ± 2 °C under 80 % relative humidity (RH). Plants were then transferred to a growth chamber, sub-irrigated and kept at 22 ± 2 °C day and 20 ± 2 °C night temperatures with a 10 h photoperiod. Supplemental lighting was provided by Cool white (F96T12/CW/1500) and Gro-Lux (F96T12/Gro/VHO) lamps (3:1 ratio, GTE Products Corporation, Dancers, MA). After 3 weeks, individual plants were transplanted into 10 x 10 x 10 cm plastic pots filled with Sunshine #1 mix supplemented with Osmocote Classic 14-14-14 (The

Scotts Company, Marysville, OH) at a rate of 1.5 kg/m^3 of Sunshine #1 mix. Plants were kept under conditions as described above for 3 weeks.

Apoplastic Fluid Extraction and Protein Quantification: Four 6 week old plants were harvested by cutting the roots 0.5 mm below the crown and submerging the leaf tissue in chilled distilled water on ice. Cuttings were transferred to a filtering flask containing 150 mM NaCl and 25 mM MES in double-distilled water at pH 6.2. A vacuum was applied for 15 min and the buffer was forced into the leaf tissue by releasing the vacuum and swirling the flask content every 3 min. Plants were removed from the flask, blotted dry between paper towels and leaves were cut off 0.2 mm above the crown. Leaves were rolled and inserted into 1.8 ml microcentrifuge tubes (caps and tips removed) with petioles facing the tip. Samples were centrifuged at 2000 rpm for 10 min at 4 °C. Apoplastic fluid was collected in a 2 ml microcentrifuge tube and frozen at -80 °C until analyzed.

The protein content of the apoplastic fluids was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) per manufacturer's instructions using bovine serum albumin (EMD Chemicals Inc., Darmstadt, Germany) as standards.

Chitinase and β-1,3-glucanase Gel Diffusion and Colorimetric Micro-Plate Assays: Chitinase activity was measured using the gel diffusion method described by Bargabus et al. (2002). Two 3-mm wells per sample and three per standard were made in a 14-cm glass Petri dish containing 30 ml of sodium phosphate buffer (100 mM at pH 5.0) with 0.3 ml of 0.1 % glycol chitin and 1 % agarose (OmniPur*, EMD Chemicals

Inc., Darmstadt, Germany). Wells were filled with 4 µl of apoplastic fluid or chitinase standards (9.68 E-04, 9.68 E-05, and 9.68 E-06 units/µl, *S. griseus*, Sigma). Plates were incubated overnight at 37 °C and then flooded with 50 ml of 0.01 % calcofluor white (Fluorescent Brightener 28, Sigma) in 500 mM Tris-HCl (pH 8.9). Following incubation on an orbital shaker at 100 rpm for 10 min, plates were rinsed multiple times with distilled water, flooded and destained overnight. The non-fluorescence area of enzyme activity around each well was assessed under UV light (365 nm) by averaging two perpendicular measurements (in mm) of the diameter. Specific activity of protein samples in apoplastic fluid (mg of *N*-acetyl-D-glucosamine released /24h/ug of apoplastic protein) was estimated by comparison to the non-fluorescence zones produced by the chitinase standards.

β-1,3-glucanase activity was determined using the gel diffusion method described by Bargabus et al. (2004) with the following modifications: Two 3-mm wells per sample and three per standard were made in a 14-cm glass Petri dish containing 40 ml of 0.1 M citrate buffer (pH 4.8) with 1 % agarose, 0.05 % laminaran (from *E. bicyclis*, TCI , Portland, OR), and 0.05% CM-Pachyman (Megazyme International Ireland Ltd, Ireland). Wells were filled with 4 µl of the same apoplastic fluid used for the chitinase assay, or the laminarinase standards (2.5 E-04, 1.25 E-04, 6.25 E-05, and 3.125 E-05 units/µl, from *Trichoderma* sp., Sigma), respectively. Plates were incubated for 24 h at 37 °C, flooded with 0.1 % Congo red (Sigma) solution and incubated overnight on an orbital shaker at 100 rpm. Gels were destained by rinsing with 1 M sodium chloride solution and the area of enzyme activity indicated by a cleared zone around the wells was determined as

described above. The specific activity of apoplastic fluid (mg of glucose released/24h/µg of apoplastic protein) was estimated by comparing the cleared zone diameter to that produced by the laminarinase standards.

The chitinase and β-1,3-glucanase micro-plate assays were performed as described earlier by testing the same apoplastic fluid samples and chitinase and laminarinase concentrations used earlier in the gel diffusion assays.

The experiment was performed twice with four replications per method. All described experiments were tested for homogeneity using the Levene's test. Data of the first experiment were analyzed statistically by calculating the standard deviation for the specific enzyme activity within two subsamples of each apoplastic fluid sample, and conducting an analysis of variance (Madden et al., 1982) using the general linear model procedure of the SAS program.

Results

Chitinase Assay

Three different buffers (standard apoplastic buffer [MES + NaCl], sodium buffer [NaCl], and double-distilled water [dd H_2O]) were evaluated for their suitability as a suspension medium for chitinase enzyme standards and their effects on the measurement with a spectrophotometer. A visual interpretation of the graphs showed a close resemblance of the different buffer curves as indicated by their similar slopes (Figure 2.1). Data were also analyzed using analysis of variance for the individual buffers and the interaction between the buffers and the chitinase standards. The standard apoplastic

buffer, the sodium buffer, and the double-distilled water were not significantly different from each other (P=0.4052), as were their interaction with the chitinase standards (P=0.4601). The R^2 values for all buffers were 0.7832, 0.795, and 0.7789 for dd H_2O, MES+NaCl, and NaCl buffer, respectively.

To improve the R^2 values of the different buffers, individual chitinase standard dilutions (9.68 E-04 to 9.68 E-07, 9.68 E-05 to 9.68 E-08, and 9.68 E-06 to 9.68 E-09 units/µl) were tested for their enzyme activity range (Figure 1.2). For all three buffers, the standard dilutions with 9.68 E-04 to 9.68 E-07 units/µl provided the best R^2 values with 0.9447 for the apoplastic buffer, 0.9243 for the sodium buffer, and 0.9406 for double-distilled water. All other chitinase standard dilutions were in a range of 0.709 to 0.747 irrespective of the type of buffer.

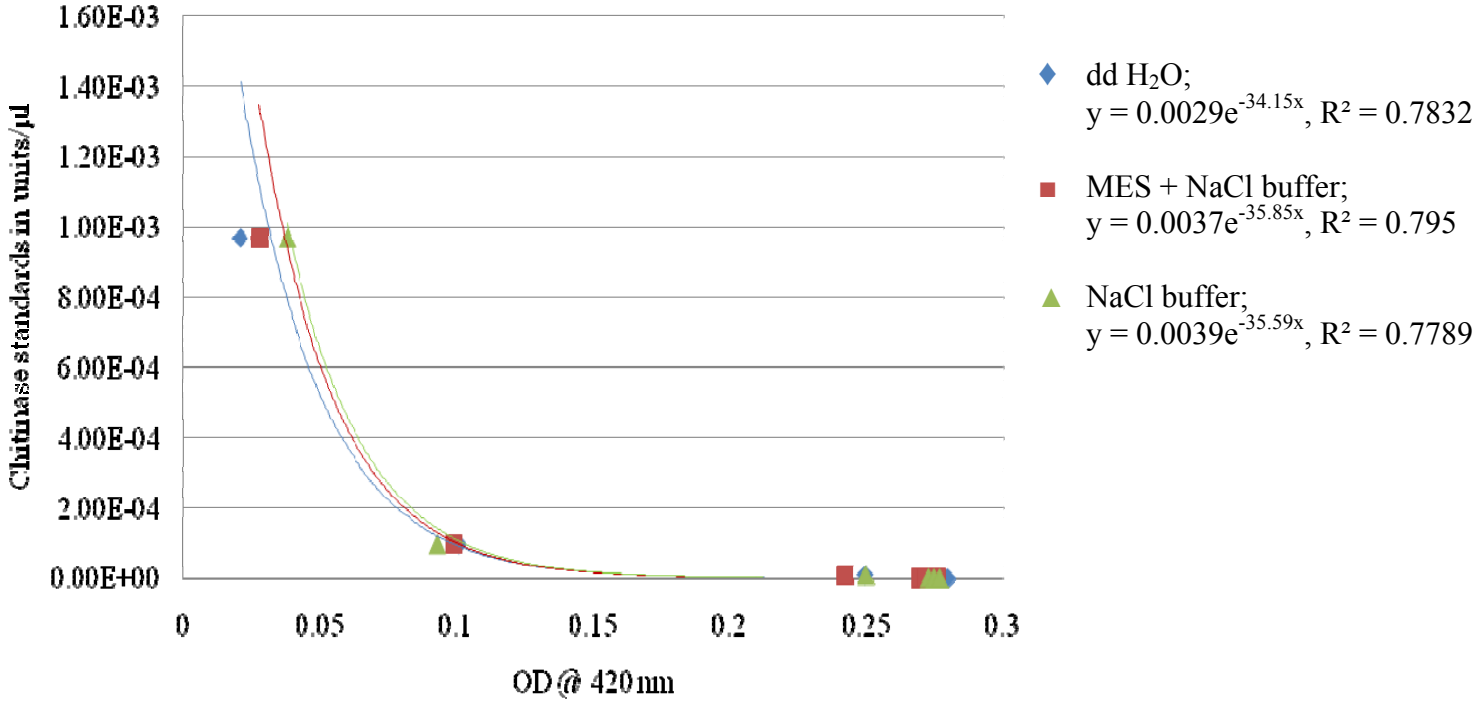


Figure 2.1: Comparison of standard apoplastic buffer (MES + NaCl), sodium buffer (NaCl), and double-distilled water (dd H_2O) as a suspension medium for chitinase enzyme standards (9.68 E-04, 9.68 E-05, 9.68 E-06, 9.68 E-07, 9.68 E-08, and 9.68 E-09 units/µl) and their effects on the production of reducing sugars and their measurement with a spectrophotometer.

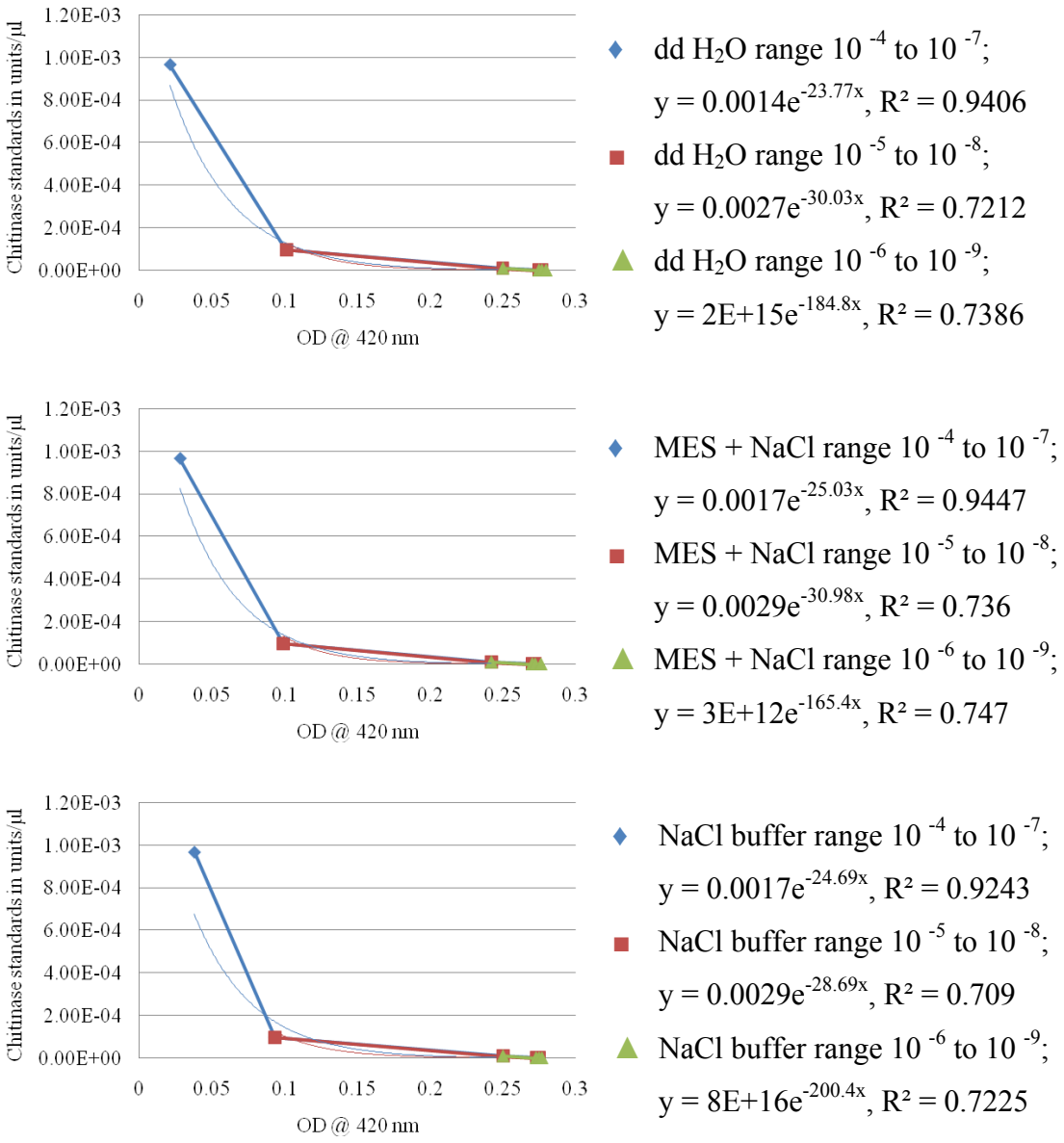


Figure 2.2: Estimation of the enzyme activity range of different chitinase standard dilutions (9.68 E-04 to 9.68 E-07, 9.68 E-05 to 9.68 E-08, 9.68 E-06 to 9.68 E-09 units/µl) and their suspension buffers (standard apoplastic buffer [MES + NaCl], sodium buffer [NaCl], and double-distilled water [dd H_2O]).

β-1,3-glucanase Assay

The standard apoplastic buffer [MES + NaCl], sodium buffer [NaCl], and double-distilled water [dd H_2O] were tested for their effects on laminarinase enzyme activity and on the ability to be read with a spectrophotometer. Buffers tested over the full range of enzyme standards (1.60 E-02, 4.00 E-03, 1.00 E-03, 2.50 E-04, 6.25 E-05, and 1.56 E-05 units/µl) with an OD of 2.000 or higher could not be read since light was not able to penetrate the sample (Figure 2.3). This applied to laminarinase standards of > 4.00 E-03 units/µl, irrespectively of buffer types. The R^2 values of all buffers for laminarinase standards from 1.60 E-02 to 1.56 E-05 were 0.6845 (dd H_2O), 0.719 (NaCl buffer), to 0.7558 (MES + NaCl buffer).

R^2 values were improved on average by 38% for all buffers by restricting the data to samples within the readable range (0.000 to 2.000 OD). For laminarinase standards from 1.00 E-03 to 1.56 E-05 units/µl, the R^2 value for dd H_2O was 0.9863, for MES+NaCl 0.9975, and 0.9924 for NaCl buffer (Figure 2.4).

A visual analysis of the graphs showed a close resemblance, expressed by a similar slope, between the curves of the standard apoplastic buffer and the sodium buffer (Figure 2.4). This observation was supported by the analysis of variance for the two buffers ($P=0.7125$) and the interaction between the buffers and the laminarinase standards ($P=0.0012$). The visual examination and the ANOVA for all three buffers revealed a significant difference ($P<0.0001$) between the standard apoplastic buffer, the sodium buffer, and the double-distilled water.

To improve the R^2 values of the different buffers, individual laminarinase standard dilutions (1.60 E-02 to 2.50 E-04, 4.00 E-03 to 6.25 E-05, and 1.00 E-03 to 1.56 E-05 units/µl) were tested for their enzyme activity range (Figure 2.5). For all three buffers, the standard dilutions with 1.00 E-03 to 1.56 E-05 units/µl provided the best R^2 values with 0.9975 for the apoplastic buffer, 0.9924 for the sodium buffer, and 0.9863 for double-distilled water. This was followed by the standard dilutions with 4.00 E-03 to 6.25 E-05 units/µl, resulting in R^2 values of 0.8965 for the apoplastic buffer, 0.942 for the sodium buffer, and 0.9211 for double-distilled water.

In addition to the chitinase and laminarinase standards, a set of apoplastic fluid samples, extracted from *A. thaliana* Col-0 plants treated with double-distilled water were included for each run to estimate the optimum range of the standards (data not shown). The apoplastic fluid samples were to be found in the 9.68 E-04 to 9.68 E-07 units of chitinase/µl range, but had to be diluted 1:10 to fit within the 1.00 E-03 to 1.56 E-05 units of laminarinase/µl range.

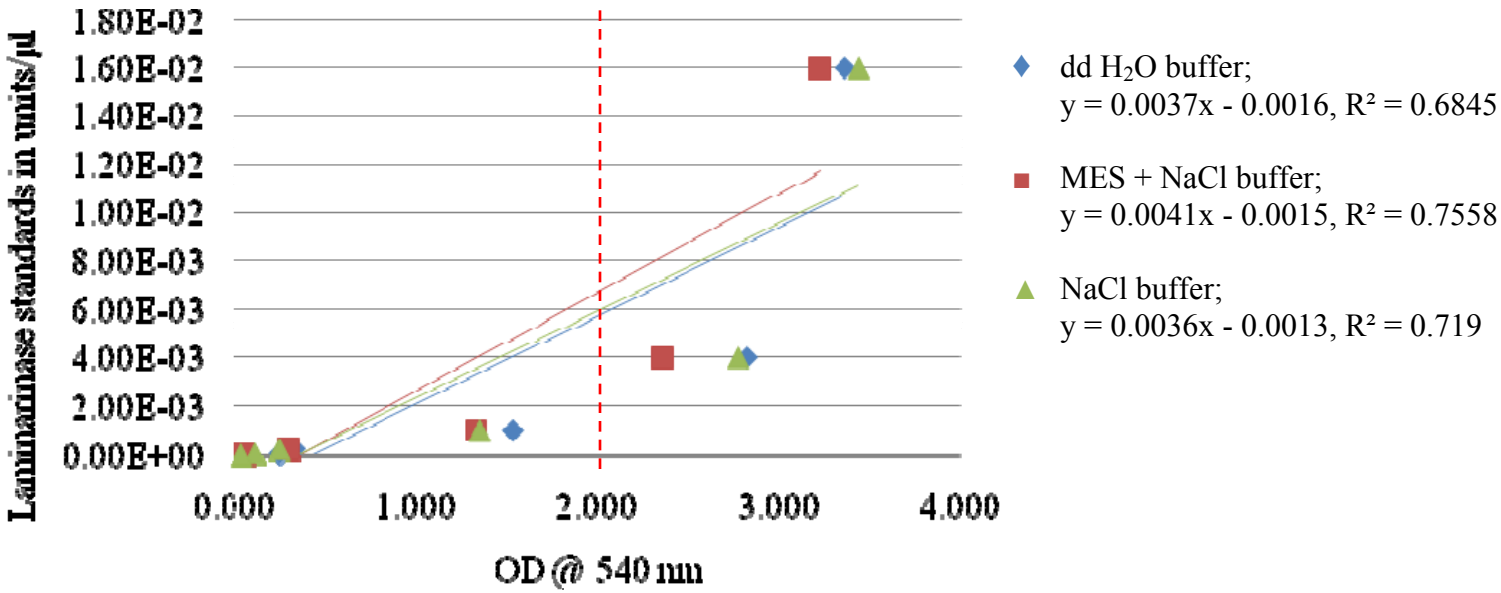


Figure 2.3: Comparison of standard apoplastic buffer (MES + NaCl), sodium buffer (NaCl), and double-distilled water (dd H_2O) as a suspension medium for laminarinase enzyme standards (1.60 E-02, 4.00 E-03, 1.00 E-03, 2.50 E-04, 6.25 E-05, and 1.56 E-05 units/µl) and their measurement with a spectrophotometer.

Red line Starting at an OD of 2.000 samples could not be read accurately because light was not able to penetrate sample.

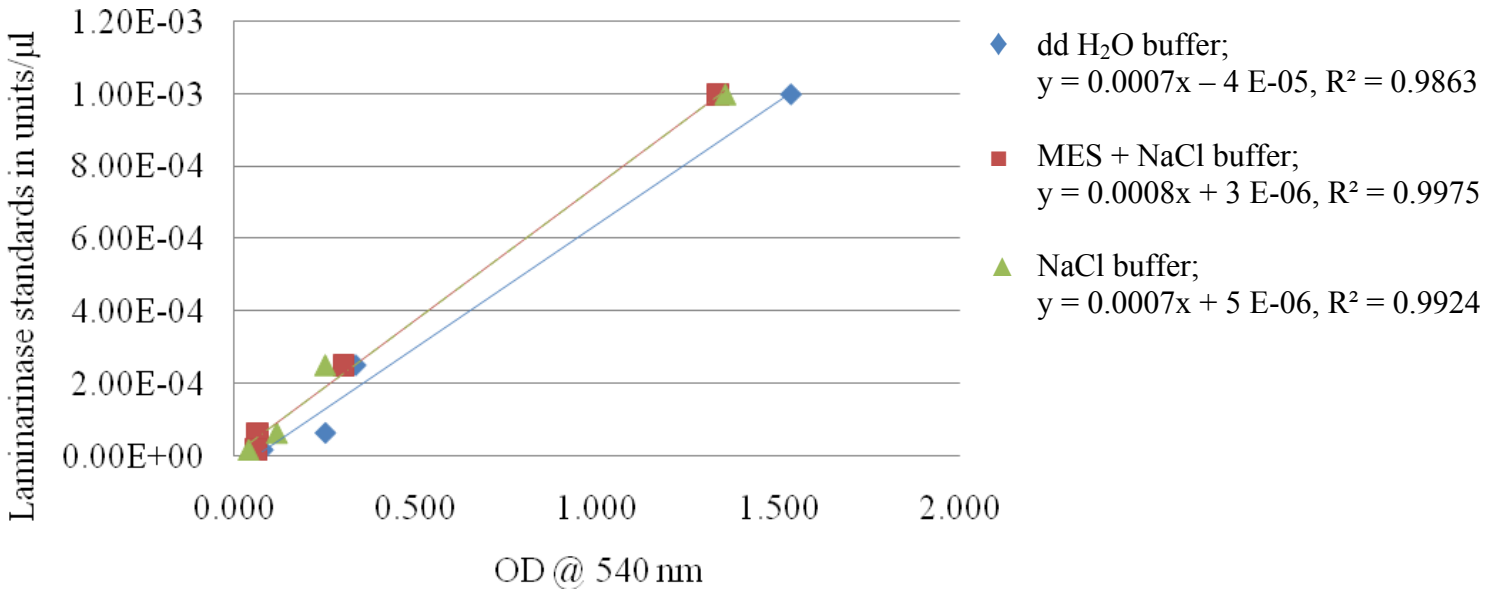


Figure 2.4: Comparison of standard apoplastic buffer (MES + NaCl), sodium buffer (NaCl), and double-distilled water (dd H_2O) as a suspension medium for laminarinase enzyme standards (1.00 E-03, 2.50 E-04, 6.25 E-05, and 1.56 E-05 units/µl) and their measurement with a spectrophotometer – restricted to data points with an OD range of 0.000 to 2.000.

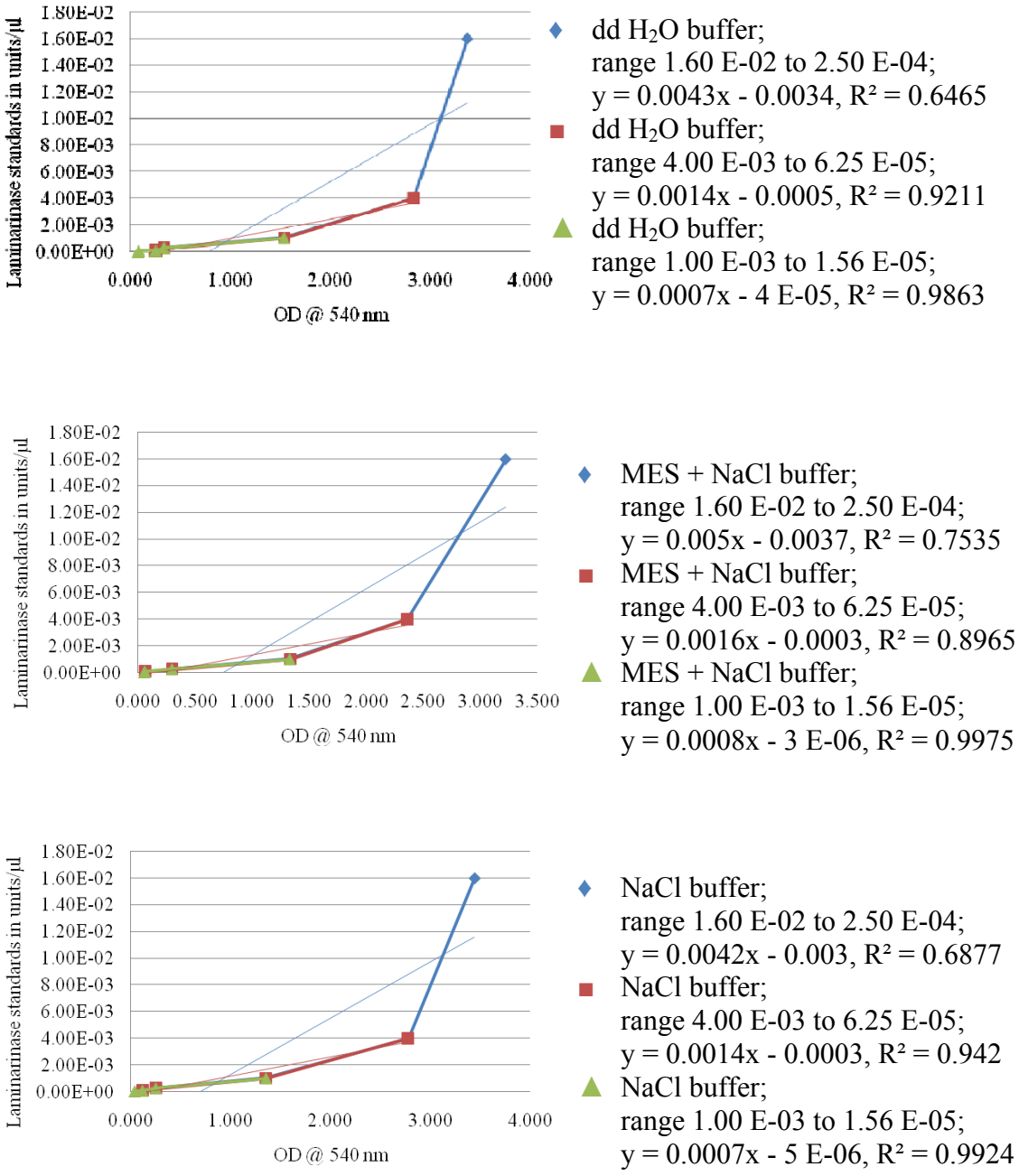


Figure 2.5: Estimation of the enzyme activity range of different laminarinase standard dilutions (1.60 E-02 to 2.50 E-04, 4.00 E-03 to 6.25 E-05, 1.00 E-03 to 1.56 E-05 units/µl) and their suspension buffers (standard apoplastic buffer [MES + NaCl], sodium buffer [NaCl], and double-distilled water [dd H_2O]).

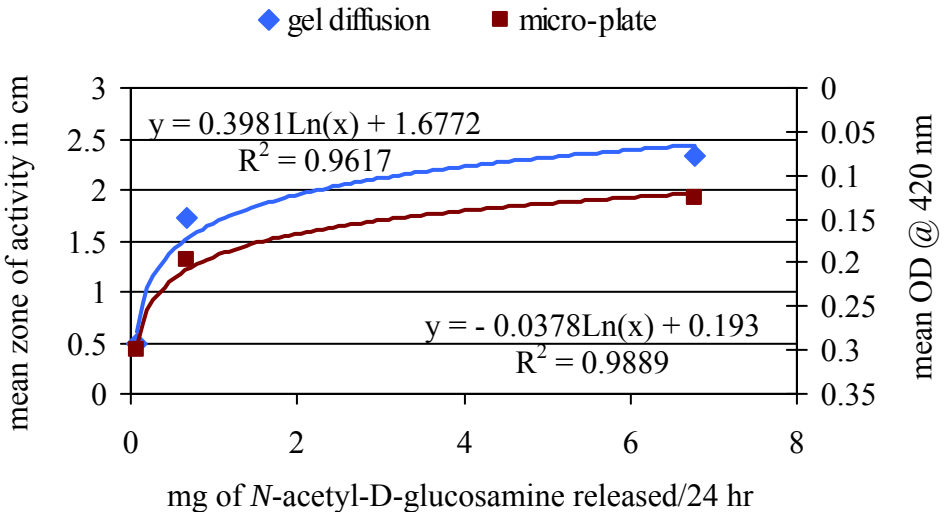
Comparison of Gel Diffusion and
Colorimetric Micro-Plate Assays

The tested gel diffusion and colorimetric micro-plate assays to quantify chitinase or β-1,3-glucanase activity were homogeneous over two experiments (Levene's test for chitinase assays = 0.9361, Levene's test for β-1,3-glucanase assays = 0.1751), consequently only data of the first experiment are presented. There was also no significant difference between the gel diffusion and the micro-plate method for quantifying either chitinase (P=0.2195) or β-1,3-glucanase (P=0.1588) activity.

Within the chitinase or β-1,3-glucanase assays, both methods showed similar slopes for their standard curves (Figure 2.6.A and Figure 2.7.A). The gel diffusion assay had a R^2 for the regression of the chitinase standards versus the non-fluorescence zones of 0.9617. In comparison the micro-plate assay produced a R^2 of 0.9889 for the same standard concentrations versus OD at 420 nm (Figure 2.6.A). The laminarinase standards had a R^2 of 0.934 in the gel diffusion assay and a R^2 of 0.9925 in the micro-plate assay (Figure 2.7.A).

The apoplastic fluid samples tested for chitinase and β-1,3-glucanase activity with either the gel diffusion or colorimetric micro-plate method were never significantly different (α=0.05) from each other (Figure 2.6.B and Figure 2.7.B). The standard deviations of these samples were also not significantly different (α=0.05) from each other. The gel diffusion method had a mean standard deviation of 2.409 versus 0.083 for the micro-plate method in the chitinase assay, and a mean standard deviation of 0.935 versus 0.0325 in the β-1,3-glucanase assay.

A.



B.

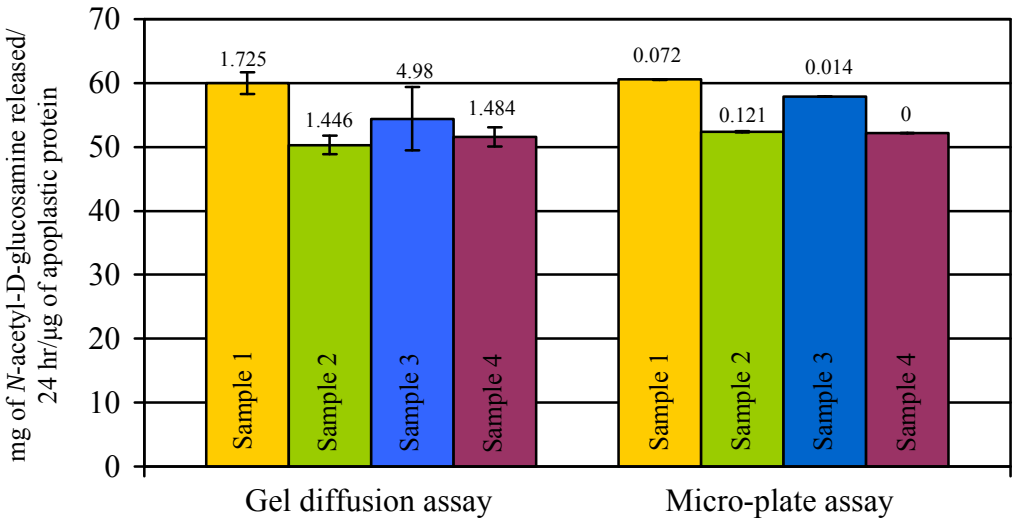
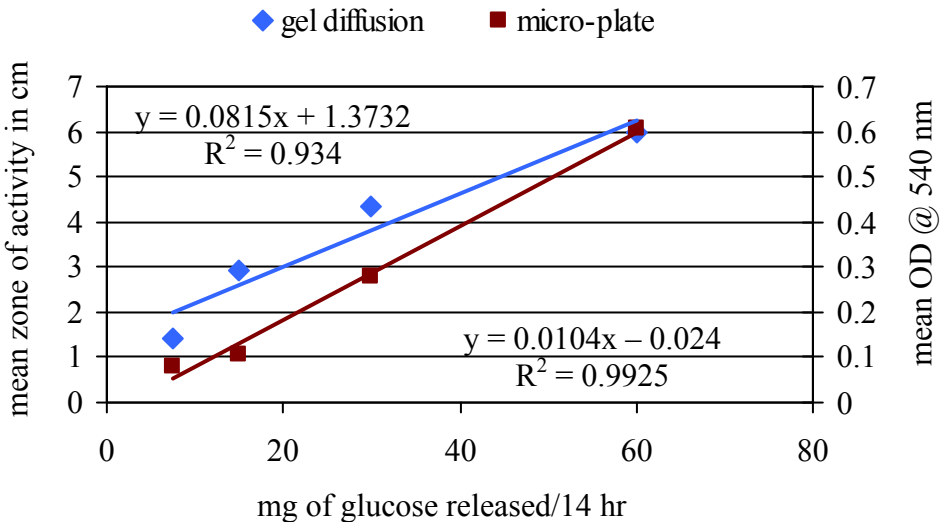


Figure 2.6: Comparison of gel diffusion and colorimetric micro-plate assay for the quantification of chitinase activity. **A.** Chitinase standards at a concentration of 9.68 E-04, 9.68 E-05, and 9.68 E-06 units/µl analyzed either by gel diffusion or colorimetric micro-plate method. **B.** Apoplastic fluid analysis using gel diffusion or colorimetric micro-plate method to determine chitinase activity. Standard deviations were calculated for two subsamples per replication.

A.



B.

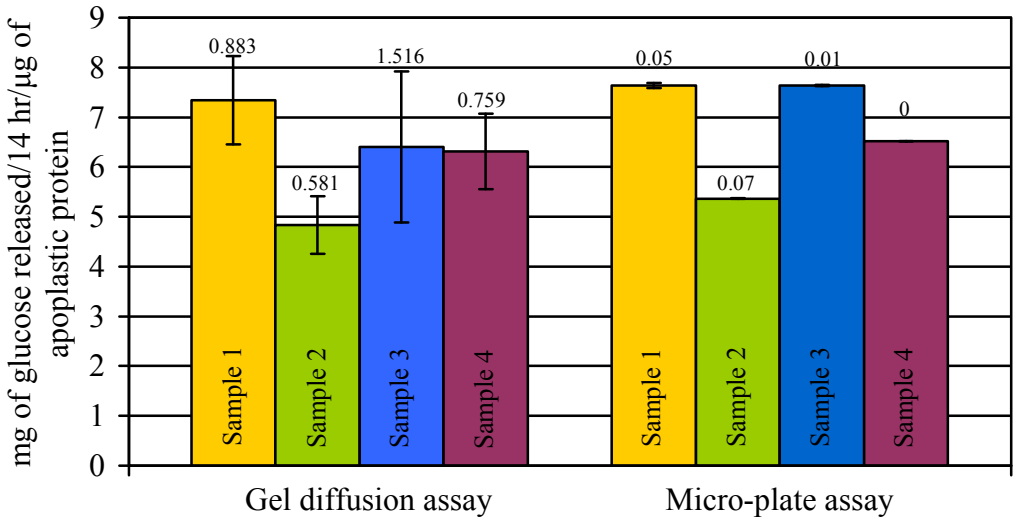


Figure 2.7: Comparison of gel diffusion and colorimetric micro-plate assay for the quantification of β-1,3-glucanse activity. **A.** Laminarinase standards at a concentration of 2.5 E-04, 1.25 E-04, 6.25 E-05, and 3.125 E-05 units/µl analyzed either by gel diffusion or colorimetric micro-plate method. **B.** Apoplastic fluid analysis using gel diffusion or colorimetric micro-plate method to determine β-1,3-glucanse activity. Standard deviations were calculated for two subsamples per replication.

Discussion

The developed micro-plate based chitinase assay was not affected by the buffer type (standard apoplastic buffer, sodium buffer, or double-distilled water) used to suspend the chitinase enzymes. There was no significant difference between the individual buffers, indicating that it would be possible to use individual buffers for specific tasks (standard apoplastic buffer for the extraction of apoplastic proteins or double-distilled water to suspend chitinase enzymes) at the same time without interfering with the enzyme quantification.

The optimal range to test for chitinase enzyme activity was determined to be between 9.68 E-04 to 9.68 E-07 units/µl. All tested buffers had an average R^2 value of 0.938 within this range.

The β-1,3-glucanase assay was not as tolerant to different buffers as the chitinase assay. This was supported by the observation that only the standard apoplastic buffer and the sodium buffer were similar ($P=0.7125$) to each other, but both were significantly different from the double-distilled water ($P<0.0001$). In comparison to the chitinase assay, the β-1,3-glucanase assay only allows the use of the standard apoplastic buffer and the sodium buffer at the same time, and not the double-distilled water. The determined optimal range of the laminarinase enzyme activity for all buffers is situated between 1.00 E-03 and 1.56 E-05 units/µl with an overall R^2 value of 0.9921.

The comparison of both methods, gel diffusion and colorimetric micro-plate assay, resulted in non-significant differences ($\alpha=0.05$) for the tested apoplastic samples, indicating a similar effectivity and accuracy for both methods. However, the micro-plate

assay had overall better R^2 values for the enzyme standards and lower standard deviations for the individual apoplastic fluid samples when compared to the gel diffusion assay. This could reduce the probability for errors within a single run, but also between multiple experiments.

As discussed above, colorimetric assays based on a 96 well multi-plate have the ability to test multiple samples at the same time without the liabilities associated with gel diffusion assays (Bargabus et al., 2002). The amount of processed samples is also more limited with a gel diffusion assay than it is with a 96 well multi-plate. The chitinase assay in Image 1.1 shows a set-up with 3 standards and 6 different samples, all are replicated 3 times. The same set-up (3 standards, 3 replications, plus a blank well) used in a 96 well multi-plate assay would allow 28 individual samples to be tested at the same time, equivalent to a 366 % increase in productivity. Also the adaptation of the chitinase assay described by Hung et al. (2002) to a micro-plate based assay reduced the total volume from 2 ml to 0.12 ml, and the sample size (apoplastic fluids or standard solutions) from 0.1 ml to 0.006 ml.

Furthermore, the use of a liquid substrate for the enzyme activity and the incubation in a thermocycler reduces the overall incubation time by 41 % for the chitinase assay and by 77 % for the β-1,3-glucanase assay when compared with the gel diffusion assays described by Bargabus et al. (2002).

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CHAPTER 3

EFFECTS OF WHOLE PLANT VS. 3 LEAVES INDUCATION BY *BACILLUS MOJAVENSIS* ISOLATE 203-7, *BACILLUS MYCOIDES* ISOLATE BMJ, AND THEIR COMBINED APPLICATION ON *BOTRYTIS CINEREA* DISEASE RESISTANCE AND PR-PROTEINS

Abstract

Foliar applications of *Bacillus mojavensis* isolate 203-7 (203-7) and *Bacillus mycoides* isolate BmJ (BmJ) are able to protect multiple plant species against bacterial and fungal pathogens, by means of systemic acquired resistance (SAR), which leads to an increase of the pathogenesis related (PR) proteins chitinase, β-1,3-glucanase and superoxide dismutase (SOD) without affecting plant growth or yield. The induction of these PR-proteins enables the plant to react faster to a pathogen attack (Priming), but it depends strongly on the applied dose of the biological or chemical inducers. 203-7 or BmJ applied to 3 leaves of *Arabidopsis thaliana* plants had the highest (P=0.05) disease reduction of *Botrytis cinerea* leaf spot of all treatments with 40.2 % and 40.5 % respectively when compared to the water control. When applied to the whole plant 203-7 and BmJ only reduced the disease by 28.9 % and 29.3 % respectively. Plants treated entirely with Actigard (ASM, 50 μg/ml) showed a disease reduction by 31.8 % compared to the control plants. However, when ASM was applied to 3 leaves only, the disease was reduced by 13.4 %. The activity of tested PR-proteins was never significantly different for the two application methods, but 203-7 or BmJ applied to 3 leaves always had a

significantly higher chitinase, β-1,3-glucanase (not BmJ), and SOD activity when compared to the water control. They also showed an increase in these PR-proteins when compared to the whole plant application. ASM showed a significant increase for chitinase and β-1,3-glucanase for both application methods compared to the controls, with non-significant higher levels in the whole plant applications.

Introduction

Plants have the ability to defend themselves against bacterial, fungal, and viral pathogens, and insect pests, through several different mechanisms (Agrios, 2005) such as preexisting structural (waxy layer or hairs on leaf surface, stomata structure) and chemical defense mechanisms (phenolic compounds). Also constitutively expressed or inducible defense enzymes are of major importantance (Herms and Mattson, 1992; Rausher, 2001). Constitutive expression of pathogenesis-related proteins (PR-proteins) may be accompanied by increased fitness costs such as reduced plant growth and seed production (Brown, 2002; Durrant and Dong, 2004; Heil et al., 2000 Heil, 2002; Heil and Baldwin, 2002). In comparison, induced defense enzymes which are only formed after a pathogen attack or a treatment with chemical or biological inducers (e.g. biological control agents [BCAs]) (Bargabus et al., 2002; Friedrich et al., 1996; Görlach et al., 1996; Heil et al., 2000; Kuć, 1982; Ryals et al., 1994) may have no negative effects on the overall plant fitness as measured by growth and yield (Friedrich et al., 1996; Tuzun and Kuć, 1985). However, Cipollini et al. (2003), Durrant and Dong (2004), and Bergelson and Purrington (1996) argue that there may be a significant cost to pay under the special

conditions of systemic acquired resistance (SAR), a form of induced resistance (Kuć, 1982; Ryals et al., 1996; Sticher et al., 1997). The synthesis of PR-proteins associated with SAR and their distribution within the plant might lead to a drain of resources, mostly nitrogen, normally used for growth and seed production (Bergelson and Purrington, 1996; Cipollini, 2002; Heil et al., 2000; Heil, 2002; Heil and Baldwin, 2002). This observation depends on the induced crop, fertility levels, environmental conditions, and the competition within the crop stand (Cipollini, 2002; Dietrich et al., 2005; Heil et al., 2000; Kuć, 1982). The concentration of the chemical or biological inducers may influence the fitness costs. For example, high doses of benzothiadiazole (BTH, Actigard, BION) reduce the growth rate and the seed production, but also increase the disease control (Faessel et al., 2008; Friedrich et al., 1996; Van Hulten et al., 2006), whereas suitable concentrations of BTH will provide disease control without phytotoxicity or negative effects on the plant fitness (Friedrich et al., 1996). Overall, these negative effects on the plant fitness are related to a direct induction of defense mechanisms by high doses of chemical inducer like BTH (Beckers and Conrath, 2007; Conrath et al., 2001; Conrath et al., 2002; Conrath et al., 2006; Van Hulten et al., 2006). They also are more pronounced under low fertility levels and can be reversed with adequate fertilization. But Van Hulten et al. (2006) showed that low doses of BTH put the plant in a primed state. This priming allows the plant to react faster to a pathogen attack (Goellner and Conrath, 2008; Walters and Boyle, 2005) without expressing major reductions in fitness (Van Hulten et al., 2006). The minor fitness costs are being compensated by the fact that under stress situations like pathogen attacks, primed plants express an increase in fitness when

compared to non-primed plants (Goellner and Conrath, 2008; Van Hulten et al., 2006). Also, Hammerschmidt (2005) pointed out that especially in agricultural production, a loss in non-induced plants caused by high disease pressure will be more devastating than the yield loss related to the induction of resistance.

Priming by foliar applications of *Bacillus mycoides* isolate BmJ (BmJ) was used to protect sugar beets against *Cercospora beticola*, causal agent of Cercospora leaf spot (Jacobsen et al., 2004), and to protect cucumber against anthracnose (*Glomerella cingulata* var. *orbiculare*) (Neher et al., 2008). This protection was facilitated by SAR which led to an increase of the PR-proteins chitinase, β-1,3-glucanase, peroxidase (Bargabus et al., 2002), and superoxide dismutase (Neher et al., 2008). BmJ induces SAR in sugar beets via a salicylic acid independent and NPR1 dependent pathway (Bargabus-Larson and Jacobsen, 2007) without affecting plant growth or yield. Jacobsen et al. (2002) showed that sugar beets treated with BmJ always had the highest yield of all treatments when infection with *C. beticola* was either low or absent, indicating that BmJ has no negative effects on the overall plant fitness.

The objective of this research was to determine if the leaf surface covered (whole plant versus partial or 3 leaves) by foliar applications of *Bacillus mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, and BTH would have an effect on disease control and expression of chitinase and β-1,3-glucanase.

Material & Methods

Bacterial Cultures

B. mycoides isolate BmJ (BmJ) was originally isolated from sugar beet leaves (Bargabus et al., 2002). *B. mojavensis* isolate 203-7 (203-7) originally isolated from sugar beet seed embryos was also used in the greenhouse experiments since it demonstrated good induction of SAR in several plant species in previous experiments (Bargabus et al., 2002; Neher et al., 2008). Both isolates were stored at -80 °C in 10 % glycerol and 1 % tryptic soy broth (TSB, EMD Chemicals Inc., Darmstadt, Germany). Bacteria were cultured in 3 % TSB for 24 h at room temperature (22 °C) on an orbital shaker (Model OS-500, VWR International) at 250 rpm. Fresh cells were harvested by centrifugation for 20 min at 5,000 rpm at 4 °C. The pellet was re-suspended in sterile-distilled water and pelleted twice by centrifugation for 20 min at 5,000 rpm at 4 °C to assure that all fermentation beer was separated from the cells. The inoculum density was adjusted to 10^8 colony forming units (CFU)/ml with distilled water and was applied using a Crown aerosol sprayer (Aervoe Industries Inc., Gardnerville, NV) with applications made to run-off.

Fungal Culture

Botrytis cinerea isolate Bot-1 was originally isolated from infected tomato fruits and conidia were stored at -80 °C as described above. For experimental use, conidia were streaked onto 50 % potato dextrose agar (PDA, EMD Chemicals Inc., Darmstadt, Germany) using a sterile loop and incubated at 24 °C for at least 2 weeks or until

mycelium showed signs of sporulation. Plates were flooded with a solution consisting of 6.2 mM KH_2PO_4 and 5.5 mM glucose in sterile-distilled water and conidia were loosened with a sterile glass rod. The conidia suspension was decanted and filtered through two layers of cheesecloth to remove mycelium and agar pieces. The inoculum density was adjusted to 10^5 conidia/ml.

Chemical Inducer

Acibenzolar-s-methyl (ASM) (Actigard 50WG Fungicide, Syngenta, Greensboro, NC) was used as the chemical inducer at a rate of 50 µg/ml and was applied as described above.

Plant Culture, Treatments and Inoculation

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were sown in 18x13x6 cm flats (T.O. Plastics Inc., Clearwater, MN) containing Sunshine #1 mix (Sun Gro Horticulture Inc., Bellevue, WA) and vernalized for 4 days at 5 ± 2 °C under 80 % relative humidity (RH). Plants were then transferred to a growth chamber, sub-irrigated and kept at 22 ± 2 °C day and 20 ± 2 °C night temperatures with a 10 h photoperiod. Supplemental lighting was provided by Cool white (F96T12/CW/1500) and Gro-Lux (F96T12/Gro/VHO) lamps (3:1 ratio, GTE Products Corporation, Dancers, MA). After 3 weeks, individual plants were transplanted into 10 x 10 x 10 cm plastic pots filled with Sunshine #1 mix supplemented with Osmocote Classic 14-14-14 (The Scotts Company, Marysville, OH) at a rate of 1.5 kg/m^3 of Sunshine #1 mix. Plants were kept under conditions as described above for 3 weeks.

Twenty plants per treatment were induced either with distilled water, 203-7, BmJ, or ASM by spraying the whole plant or by applying the treatments to only 3 individual leaves while covering the rest of the plant with plastic wrap. Further, a combined 203-7 and BmJ treatment was included by spraying the bacilli spatially separated from each other to 3 individual leaves. Induced plants were kept under greenhouse conditions as described above for 6 days.

After this time period, 20 plants per treatment were challenge inoculated with Bot-1 conidia solution by placing one 5 µl droplet on 3 individual leaves (ranging from oldest to newest leaves) per plant. For plants of which only 3 leaves were induced, spatially separated and non-treated leaves were chosen for the challenge inoculation. Following inoculation, plants were placed at conditions as described above and under 90 % RH for 7 days to allow disease development. Disease severity was rated on a 0 to 5 scale with 0 = no visible lesion to 5 = lesion expanding into non-inoculated tissue.

The experimental design was a randomized complete block with 20 replications per treatment for the *B. cinerea*-bioassay and 8 replications per treatment for the PR-protein assays. Experiments were performed three times. All described experiments were tested for homogeneity using the Levene's test and collected data were pooled and analyzed statistically by conducting an analysis of variance (Madden et al., 1982) using the general linear model procedure of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at α=0.05. In addition, data for the individual BCAs (203-7 and BmJ alone) were analyzed by performing a pairwise analysis of variance for the

two different application methods (whole plant and 3 leaves). Data from the *B.3 cinerea*-bioassay were also analyzed by performing a non-parametric analysis (Shah and Madden, 2004), to obtain a ranking of the different treatments with regard to the rating scale (0 to 5) which was used to estimate disease development.

Apoplastic Fluid Extraction
and Protein Quantification

Six weeks old plants were induced, in replicates of 8, either with distilled water, 203-7, BmJ, or ASM by spraying the whole plant or by applying the treatments to only 3 individual leaves while covering the rest of the plant with plastic wrap. Further, a combined 203-7 and BmJ treatment was included by applying the bacilli spatially separated from each other to 3 individual leaves. Induced plants were kept under greenhouse conditions as described above for 6 days.

Six days post induction the plants were cut 0.5 mm below the crown and submerged in chilled distilled water on ice. Cuttings were transferred to a filtering flask containing 150 mM NaCl and 25 mM MES in double-distilled water at pH 6.2. A vacuum was applied for 15 min and the buffer was forced into the leaf tissue by releasing the vacuum and swirling the flask content every 3 min. Plants were removed from the flask, blotted dry between paper towels and leaves were cut off 0.2 mm above crown. Leaves were rolled and inserted into 1.8 ml microcentrifuge tubes (caps and tips removed) with petioles facing the tip. Samples were centrifuged at 2000 rpm for 10 min at 4 °C. Apoplastic fluid was collected in a 2 ml microcentrifuge tube and frozen at -80 °C until analyzed.

The protein amount of the apoplastic fluids was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) per manufacturer's instructions using bovine serum albumin (EMD Chemicals Inc., Darmstadt, Germany) as standards.

Chitinase Assay

Based on the chitinase activity assay described by Hung et al (2002) a 96 well micro-plate assay was developed (see chapter 2). For each standard or sample 3 wells of a 96 well PCR plate (VWR International) were filled with 54 µl of 100 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid), 60 µl of 0.2 % of glycol chitin solution, and 6 µl of chitinase standards (0.1 units/ml, 0.01 units/ml, and 0.001 units/ml from *Streptomyces griseus*, Sigma) or apoplastic fluid samples. The plate was sealed with a silicone sealing mat (VWR International) and incubated for 14 h at 37 °C using a thermocycler (GeneAmp PCR System 9600, The Perkin-Elmer Corporation, Norwalk, Connecticut). After this time period 90 µl of the solution were transferred to a new PCR plate and 120 µl of ferri-ferrocyanide reagent (Imoto and Yagishita, 1971) consisting of 0.05 % potassium ferricyanide in 500 mM sodium carbonate was added. The plate was sealed as described before and incubated for 15 min at 99.9 °C. Following incubation 100 µl of the solution were transferred to a 96 well flat-bottom assay plate (Becton Dickinson Labware, Becton Dickinson and Company, Fanklin Lakes, NJ) and decrease of absorbance was measured at 420 nm with a SpectraMax plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The specific enzyme activity (in mg of *N*-acetyl-D-glucosamine released/24h/µg apoplastic proteins) of the apoplastic fluid samples was determined by comparison to chitinase standards.

β-1,3-glucanase Assay

The microplate-based carboxymethylcellulose assay described by Xiao et al. (2005) was adapted to be used to determine β-1,3-glucanase activity in apoplastic fluids extracted from Arabidopsis plants (see chapter 2). Thirty microliters of 1 % laminaran (from *Eisenia bicyclis*, TCI, Portland, OR) and 1 % CM-Pachyman (Megazyme International Ireland Ltd, Ireland) in 50 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid) and 30 µl of laminarinase standards (0.25, 0.125, 0.0625, and 0.03125 units/ml from *Trichoderma* sp., Sigma) or apoplastic fluid samples were combined in a PCR plate well with 3 wells per standard or sample. The plate was sealed with a silicone sealing mat and incubated for 4 h at 30 °C using a thermocycler. Following incubation, 60 µl of dinitrosalicylic acid reagent consisting of 30 mM dinitrosalicylic acid (Alfa Aesar, Ward Hill, MA), 14.9 mM phenol, 2.8 mM sodium sulfite, 0.5 M sodium-potassium-tartarate (EMD Chemicals Inc., Darmstadt, Germany), and 0.2 mM NaOH were added to the solution and the PCR plate was incubated again for 5 min at 95 °C as described above. Subsequently, 100 µl of solution were transferred to a 96 well flat-bottom assay plate and the change of absorbance was measured at 540 nm with a SpectraMax plus384 spectrophotometer. The specific enzyme activity (in mg of glucose released/4h/µg of apoplastic proteins) of the apoplastic fluid samples was determined by comparison to laminarinase standards.

Superoxide Dismutase Assay

Quantification of superoxide dismutase (SOD) activity in the apoplastic fluid samples was performed according to the method described by Ewing and Janero (1995).

Under subdued room light, 200 µl of 0.1 mM EDTA, 62 µM nitro blue tetrazolium (TCI America, Portland, OR), and 98 µM NADH in 50 mM phosphate-buffered saline (pH 7.4) were combined in a 96 well flat-bottom assay plate with 25 µl of SOD standards (4, 8, 12, and 16 ng/µl from bovine liver, CALBIOCHEM, San Diego, CA) or apoplastic fluid samples. Standards and samples were run in triplicate. Five minutes after the reaction was started by adding 25 µl of 33 µM phenazine methosulfate (TCI America, Portland, OR) and 0.1 mM in 50 mM phosphate-buffered saline (pH 7.4), the endpoint absorbance was measured at 560 nm with a SpectraMax plus384 spectrophotometer. The specific enzyme activity (in ng of SOD/µg of apoplastic proteins) of the apoplastic fluid samples was determined by comparison to SOD standards.

Results

The non-parametric analysis performed for the *B. cinerea*-bioassays showed significant ($P<0.0001$) differences between treatments and a similar ranking of the treatments in regard to disease development when compared to Fisher's protected LSD.

Under all application methods (whole plant and 3 leaves), all tested treatments for the control of *B. cinerea* had always significantly lower ratings ($\alpha=0.05$) when compared to the double-distilled water control (Table 3.1). 203-7 and BmJ applied to 3 leaves had the highest ($\alpha=0.05$) disease reduction of all treatments with 40.2 and 40.5 % respectively when compared to the double-distilled water control. They were followed by the combined application of 203-7 and BmJ on 3 leaves each (32.7 %), ASM (31.8 %), BmJ (29.3 %), and 203-7 (28.9 %) applied to the whole plant. These treatments were always

significantly (α=0.05) different to 203-7 and BmJ applied to 3 leaves and the double-distilled water control. ASM applied to 3 leaves only had the lowest (α=0.05) disease reduction of all treatments with 13.4 %. The pairwise analysis of variance showed a significant difference between the application methods (3 leaves or the whole plant) when compared at the individual treatment level (203-7: P=0.0008; BmJ: P=0.0005), with the highest disease reduction for treatments applied to 3 leaves.

Table 3.1: Disease reduction of *Botrytis cinerea* leaf spot on *Arabidopsis thaliana* by means of induced SAR resulting from foliar applications of *Bacillus mojavensis* isolate 203-7 and *B. mycoides* isolate BmJ to either the whole plant or to three leaves per plant only.

		% disease reduction in comparison to distilled water control
whole plant	203-7	28.9 c [zy]
	BmJ	29.3 c
	Actigard	31.8 c
3 leaves	203-7	40.2 d
	BmJ	40.5 d
	Actigard	13.4 b
	203-7 & BmJ	32.7 c

[z] Means followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).
[y] All means were significant different from the distilled water control according to Fisher's protected LSD (α=0.05).

The SOD activity of the bacilli treatments under all application methods was significantly (α=0.05) higher than to the double-distilled water control (Table 3.2). ASM applied to the whole plant or to 3 leaves only was neither significant different to the water control nor to the other treatments with the exception of BmJ applied to 3 leaves only which had the highest (α=0.05) increase of SOD activity by 1434.3 % when compared to the control.

ASM treatments, applied to either the whole plant or 3 leaves only, had the highest (P=0.05) β-1,3-glucanase activity (371.2 and 215.8 % compared to the double-distilled water control) of all treatments (Table 3.2). Applications with 203-7 or its combination with BmJ, to either the whole plant or to 3 leaves only, resulted in a significant (α=0.05) increase in enzyme activity (whole plant: 83.1 %, 3 leaves: 114 %, and 3 leaves, 203-7 & BmJ: 94.3 %) when compared to the water control. 203-7 and 203-7 & BmJ were never significantly different from each other or from BmJ treatments. BmJ had the lowest increase of β-1,3-glucanase activity with 44.6 % for the whole plant and 70.7 % for the 3 leaves application and was not significant different to the double-distilled water control.

The highest (α=0.05) increase of chitinase activity, could be observed for Actigard applied to the whole plant (Table 3.2) with an increase of enzyme activity by 270.6 % compared to the double-distilled water control. Actigard, BmJ and 203-7 applied to 3 leaves each, followed with a significant (α=0.05) increase by 163, 91.8 and 90.8 % respectively when compared to the double-distilled water control. These treatments were significantly different from Actigard applied to the whole plant, but they were not significantly different from 203-7 or BmJ applied to the whole plant, or the combination of both. The later treatments were also not significantly different from the double-distilled water control. The pairwise analysis of variance for the application methods (3 leaves or the whole plant) showed no significant differences for SOD, chitinase, and β-1,3-glucanase activity when compared at the individual treatment level (203-7 or BmJ).

Table 3.2: Superoxide dismutase (SOD), β-1,3-glucanase and chitinase activity assays for apoplastic fluids of *Arabidopsis thaliana*, extracted after treating either the whole plant or only three leaves per plant with *Bacillus mojavensis* isolate 203-7 or *B. mycoides* isolate BmJ.

		SOD activity [z]		β-1,3-glucanase activity [y]		chitinase activity [x]	
whole plant	203-7	2.35	abc [w]	50.55	c	5.58	cd
	BmJ	2.23	abc	39.93	cd	6.35	cd
	Actigard	0.58	cd	130.1	a	13.04	a
	distilled water	0.20	d	27.61	d	3.52	d
3 leaves	203-7	2.72	ab	58.43	c	6.98	bc
	BmJ	3.42	a	46.59	cd	7.02	bc
	Actigard	1.20	bcd	86.22	b	9.63	bc
	203-7 & BmJ	2.43	ab	53.03	c	6.41	cd
	distilled water	0.22	d	27.30	d	3.66	d

[z] SOD activity measured in ng of SOD/μg of apoplastic proteins [v].
[y] β-1,3-glucanase activity measured in mg of glucose released/4h/μg of apoplastic proteins [v].
[x] Chitinase activity measured in mg of *N*-acetyl-D-glucosamine released/24h/μg apoplastic proteins [v].
[w] Means followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).
[v] Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

Discussion

Priming of plants is widely used to protect crops against multiple fungal, bacterial, and viral pathogens (Conrath et al., 2006). The protective effect is based on the systemic induction of plants with a low dose of chemical inducers (e.g. BTH) or BCAs. This enables the plant to react faster to a pathogen attack, since the systemic induction of the plant increases certain PR-proteins, including chitinase and β-1,3-glucanase known to be associated with reduction in disease (Kuć, 1982; Ryals et al., 1996). It is known that high doses of the inducers lead to a direct induction of defense mechanisms, sometimes accompanied by fitness costs like reduced growth or seed production (Faessel et al., 2008; Friedrich et al., 1996). Nevertheless, very little is known about the relationship between leaf coverage by chemical inducers or BCAs and their efficacy in inducing systemic resistance in the plant. Will they behave like a chemical pesticide and express reduced disease control when coverage is poor?

Contrary to the results presented by Van Hulten et al. (2006), the chemical inducer ASM showed a significant increase in disease severity and a significant decrease in chitinase and β-1,3-glucanase activity when applied to 3 leaves only, indicating that the applied concentration of ASM was probably too low to achieve a priming effect in the plant. ASM induced a non-significant ($\alpha=0.05$) increase in SOD activity, while significant increases of chitinase and β-1,3-glucanase activity were noted for whole plant treatments compared to 3 leaves treatments.

Both BCAs showed a significant increase in disease control, on average by 38.5 % when applied to 3 leaves only, indicating a priming effect of the plant. This effect was

not significant for the tested PR-proteins and SOD, but still provided on average an increase in chitinase activity by 34.5 %, in β-1,3-glucanase activity by 48 %, and in SOD activity by 20 %, when compared to whole plant applications. The differences between disease control and enzyme activity could be explained by the activation of additional or different PR-proteins than the previous tested. It is shown in chapter 4 that 203-7 and BmJ activate different defense pathways in *A. thaliana* wild type and mutants plants. These pathways are salicylic acid (SA) independent, but in the case of 203-7 jasmonic acid (JA) and of BmJ ethylene and NPR1 dependent. JA and ethylene dependent pathways can lead to the induction of defensive compounds and the plant defensin PDF1.2 (Pieterse et al., 1998).

It is debatable if an experimental set-up with a whole plant versus 3 leaves application is similar to a high dose versus low dose application of BCAs. But it seems that a reduction in leaf surface, exposed to a biological or chemical inducer, and therefore a reduction in colony forming units applied to the plant has beneficial effects on disease control and PR-protein expression, which are comparable the effects of low BTH concentrations (Van Hulten et al., 2006). In addition, if the fitness costs related to high dose applications are not only expressed as reduced physiological activity (Faessel et al., 2008; Friedrich et al., 1996; Van Hulten et al., 2006), but also as a decrease in disease resistance and PR-protein expression which is contradictory to the above mentioned references, than the whole plant application would be equal to high dose treatments. Since no data on growth rate and seed production were collected and all treatments applied to the whole plant still showed increased disease control and PR-protein activity

when compared to the distilled water control, another explanation for the significant differences in disease control between the whole plant and 3 leaves applications needs to be found.

A minor objective of this research was to determine if it is possible to "stack" enzyme activity. BmJ and 203-7, both induce chitinase and β-1,3-glucanase, but plants treated with BmJ express a higher level of chitinase activity and a lower level of β-1,3-glucanase activity when compared to 203-7 treated plants. By applying both BCAs to the plant (spatially separated from each other, on 3 individual leaves), it might be possible to "stack" the enzyme activity. "Stacking" should have resulted in an equally elevated chitinase and β-1,3-glucanase activity above the thresholds of BmJ and 203-7. The results of these experiments disprove this hypothesis. The combination of 203-7 and BmJ was neither significantly different (α=0.05) for the control of disease caused by Bot-1, nor for the induction of chitinase, β-1,3-glucanase, or SOD when compared to the whole plant applications of 203-7 or BmJ. One reason for the unsuccessful "stacking" might be the high dose response described earlier. A simultaneous application of 203-7 and BmJ to 3 leaves each, equals an overall plant coverage of approximately 30 % which might be enough to increase the fitness costs to a point where a further increase of enzyme activity is not feasible. As discussed earlier, 203-7 and BmJ induce different plant defense pathways. The simultaneous activation of the JA and ethylene dependent pathway may lead to an inhibition of one or the other. This phenomenon of cross talk or inhibition of different defense pathways can be observed for SA and JA pathways induced by pathogens and insects respectively. Thaler et al. (1999) showed an increased

susceptibility to bacterial pathogen when plants were sprayed with JA, resulting in a reduction of PR-protein expression induced by benzothiadiazole, which induces the SA dependent pathway.

In conclusion: The induction of PR-proteins and the resulting disease control appears to depend strongly on the applied dose of the biological or chemical inducers. The bacilli based BCAs applied at lower concentrations (similar to the 3 leaves application) had the highest disease reduction of *B. cinerea* leaf spot of all treatments when compared to the water control. However, the chemical inducer ASM when applied at lower concentrations similar to 3 leaves only showed less disease reduction when compared to the higher concentration or whole plant application. Also, the non-significantly differences between the induced and tested PR-proteins of plants treated with the BCAs suggest that other, not tested PR-proteins are expressed and might be involved in the disease reduction.

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CHAPTER 4

ARABIDOPSIS DEFENSE PATHWAYS ACTIVATED BY *BACILLUS MOJAVENSIS* ISOLATE 203-7 *AND BACILLUS MYCOIDES* ISOLATE BMJ

Abstract

Foliar applications of *Bacillus mojavensis* isolate 203-7 (203-7) and *Bacillus mycoides* isolate BmJ (BmJ) are able to induce systemic acquired resistance (SAR) in multiple plant species, expressed by elevated levels of pathogenesis related proteins (PR-proteins) like chitinase, β-1,3-glucanase, and superoxide dismutase (SOD). Differences in enzyme activity and expressed disease resistance were noticeable between biological control agents (BCAs) and induced plant species. An *Arabidopsis thaliana* mutant – *Botrytis cinerea* pathosystem was used to investigate the plant defense pathways activated by 203-7 and BmJ. *A. thaliana* wild type (Col-0), *ein2-1*, *jar1-1*, *NahG*, *ndr1-1/npr1-2*, and *npr1-5* mutants were induced by application of washed cells of the BCAs, sterile dH_2O, or the chemical inducers: acibenzolar-S-methyl, methyl jasmonate, ethephon or probenazole. Both BCAs reduced disease severity on wild type and *NahG* mutants but provided no disease reduction on *jar1-1*, indicating that induction was salicylic acid independent but jasmonic acid dependent. BmJ did not decrease disease severity on *npr1-5*, *jar1* or *ein2-1* mutants. 203-7 induced plants had lower disease severity on *npr1-5* and *ein1-2* mutants but were equivalent to buffer controls on *jar1-1* mutants. Enzyme assays confirmed induction of chitinase, β-1,3-glucanase and superoxide dismutase by 203-7 and BmJ. These results demonstrate that induction by

BmJ is salicylic acid independent, and both NPR1 and jasmonic acid/ethylene dependent. 203-7 is jasmonic acid dependent and NPR1 independent.

Introduction

Plants have an innate ability to defend themselves against biotic agents such as bacterial, fungal, or viral pathogens, insects causing diseases and damage, and abiotic factors including but not limited to heat and water stress. Plants rely either on pre-existing defense systems like waxy layers, hairs, lignin depositions, and other structural features, or on the induction of localized and/or systemic defense responses resulting in the accumulation of certain defense molecules (pathogenesis related proteins [PR-proteins]) and defense related secondary antimicrobial metabolites (Agrios, 2005).

Three major systemic signals: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are involved in the induction of systemic resistance. SA is not only involved in the expression of localized resistance in response to an attack by a biotrophic pathogen (Delaney et al., 1995), but it is also a 'long distance' messenger molecule within the plant for the induction of systemic acquired resistance (SAR) and the production of PR-proteins like chitinase, β-1,3-glucanase, or peroxidase (Ryals et al., 1996; Sticher et al., 1997). SA applied to the plant surface can also serve as an exogenous trigger for the induction of SAR, whereas JA and ET are involved in the induction of induced systemic resistance (ISR) (Pieterse et al., 2000; Ton et al., 2002) which is associated with the rhizosphere colonization by plant growth promoting rhizobacteria (PGPR) (Van Loon et al., 1998). JA and ET are also important messenger

molecules for systemic resistance associated with the attack by necrotrophic pathogens and herbivores (Farmer and Ryan, 1990). In both cases JA and ET activate genes coding for plant defense-related proteins like thionins and proteinases but not PR-proteins (Pieterse et al., 1998).

Depending on the damaging agent, different defense pathways can be activated (Thomma et al., 1998). *Peronospora parasitica* induces the SA dependent pathway in *Arabidopsis thaliana* (Rairdan and Delaney, 2002) by causing necrotic lesions which will lead to an activation of the PR-protein genes PR-1 (unknown function), PR-2 (β-1,3-glucanase), and a defense protein PR-5 (thaumatin-like protein). In comparison *Alternaria brassicicola* and *Botrytis cinerea* will stimulate only the JA dependent pathway in *A. thaliana*, but not the SA pathway, activating the PR-3 and PR-4 genes (both chitinases) (Thomma et al., 1998).

Unlike these pathogens, two Bacillus based biological control agents (BCA) *Bacillus mojavensis* isolate 203-7 (203-7) and *Bacillus mycoides* isolate BmJ (BmJ) are able to induce SAR following foliar applications without causing visible damage to the plant surface (Bargabus et al., 2003). Bargabus et al. (2002, 2004), showed that BmJ and 203-7 are able to protect sugar beets by inducing SAR and as a result to elevate PR-proteins like β-1,3-glucanase and chitinase. Later work revealed that the induction facilitated by BmJ is SA independent but NPR1 (non-expresser PR-proteins) dependent (Bargabus-Larson and Jacobsen, 2007).

Other isolates of *B. mojavensis* are reported to be BCAs as well. Examples are *B. mojavensis* isolate RRC101 (Bacon and Hinton, 2002) and isolate AB1 (Nair et al. 2002).

Their activity, however, was linked to the production of antifungal metabolites and not associated with the induction of SAR.

Foliar application of BmJ is effective against a wide range of pathogens including *Alternaria solani*, *B. cinerea, Cercospora beticola*, *Erwinia carotovora* subsp. *betavasculorum, Glomerella cingulata* var. *orbiculare, Pseudomonas syringae* pv. *lachrymans,* and cucumber mosaic virus (CMV) on many different hosts including: cantaloupe, cucumbers, peppers, potatoes, sugar beets, and tomatoes (Bargabus et al., 2002, 2003, 2004; Jacobsen et al., 2004; Neher et al., 2007 abc; Neher et al., 2008).

Comparing the efficacy of 203-7 and BmJ to control certain plant pathogens and their ability to induce β-1,3-glucanase and chitinase in certain plant species revealed an obvious difference between these bacilli. 203-7 was able to provide better control of *G. cingulata* var. *orbiculare* on cucumber when compared to BmJ (Neher et al., 2008), while it was inferior to BmJ for the control of *B. cinerea* on tomato (Neher et al., 2007 c). Induction of β-1,3-glucanase and chitinase by 203-7 was higher on tomato (see chapter 5), but lower on cucumber when compared to BmJ (Neher et al., 2008).

These results indicated a difference in defense pathway activation by which 203-7 or BmJ are inducing certain PR-proteins and managing to control certain plant pathogens.

The objective of this research was to determine the individual defense pathways activated by foliar applications of 203-7 and BmJ.

The *A. thaliana – B. cinerea* patho-system was chosen since different lines/mutants deficient or resistant to the individual systemic signal molecules (SA, JA, and ET) as well as knock-out mutations related to important genes (*npr1-1*, *ndr1-1/npr1-*

2) are available (Glazebrook et al., 1997; Thomma et al., 1998, 2001). The mutants (*NahG*, *ein2-1*, *jar1-1*, *ndr1-1/npr1-2*, and *npr1-1*) used in this experiment are well defined and referenced in their mutation and are commonly used in this type of research (for a summary see Table 4.1).

Material & Methods

Bacterial Cultures

B. mycoides isolate BmJ (BmJ) was originally isolated from sugar beet leaves. *B. mojavensis* isolate 203-7 (203-7) originally isolated from sugar beet seed embryos was included in the greenhouse experiments since it demonstrated good induction of SAR in several plant species in previous experiments (Bargabus et al., 2004; Neher et al., 2008). Both isolates were stored at -80 °C in 10 % glycerol and 1 % tryptic soy broth (TSB, EMD Chemicals Inc., Darmstadt, Germany). Bacteria were cultured in 3 % TSB for 24 h at room temperature (22 °C) on an orbital shaker (Model OS-500, VWR International) at 250 rpm. Fresh cells were harvested by centrifugation for 20 min at 5,000 rpm at 4 °C. The pellet was re-suspended in sterile-distilled water and pelleted twice by centrifugation for 20 min at 5,000 rpm at 4 °C to assure that all fermentation beer was separated from the cells. The inoculum density was adjusted to 10^8 colony forming units (CFU)/ml with distilled water, and was applied using a Crown aerosol sprayer (Aervoe Industries Inc., Gardnerville, NV) with applications made to run-off.

Fungal Culture

B. cinerea isolate Bot-1 was originally isolated from infected tomato fruits. Conidia were stored at -80 °C as described above. For experimental use, conidia were streaked onto 50 % potato dextrose agar (PDA, EMD Chemicals Inc., Darmstadt, Germany) using a sterile loop and incubated at 24 °C for at least 2 weeks or until mycelium showed signs of sporulation. Plates were flooded with a solution consisting of 6.2 mM KH_2PO_4 and 5.5 mM glucose in sterile-distilled water and conidia were loosened with a sterile glass rod. Conidia suspension was decanted and filtered through two layers of cheesecloth to remove mycelium and agar pieces. The inoculum density was adjusted to 10^5 conidia/ml.

Arabidopsis thaliana Wild Type and Mutants

A. thaliana ecotype Columbia (*Col-0*) was supplied by Dr. Robert Sharrock, Montana State University. The following *A. thaliana* mutants were obtained from the TAIR stock center (Carnegie Institution of Washington Department of Plant Biology, Stanford, CA) and had a Col-0 backround: *ein2-1* (TAIR CS3071, ethylene insensitive), *jar1-1* (TAIR CS8072, jasmonate resistance), *ndr1-1/npr1-2* (TAIR CS6355, nonexpresser of PR genes and salicylic acid insensitive), and *npr1-1* (TAIR CS3726, nonexpresser of PR genes and salicylic acid insensitive). The *NahG* mutant (encoding salicylate hydroxylase and salicylic acid insensitive) was obtained from Dr. Bob Dietrich, Syngenta, Greensboro, NC (for a summary see Table 4.1).

Chemical Inducers

Acibenzolar-s-methyl (ASM) (Actigard 50WG Fungicide, Syngenta, Greensboro, NC) was used as the chemical inducer for *Col*-0 and *NahG* plants at a rate of 50 µg/ml of sterile-distilled water. Probenazole (PBZ) (Chem Service, West Chester, PA) was used for *ndr1-1/npr1-2* and *npr1-1* mutants at a rate of 2 mM PBZ suspended in 0.1 M potassium phosphate buffer with 0.01 % trition X-100 (Bargabus-Larson and Jacobsen, 2007). *Jar1-1* plants were treated with methyl jasmonate (MeJa) (TCI America, Portland, OR) at a rate of 7.5 mM in 0.8 % ethanol. 1mM of 2-Chloroethylphosphonic acid (Ethephon, Acros Organics, NJ) dissolved in sterile-distilled water was the chemical inducer for *ein2-1*. All chemical inducer were applied using a Crown aerosol sprayer with applications made to run-off (for a summary see Table 4.1).

Table 4.1: *Arabidopsis thaliana* wild type and mutants and their specific chemical inducer.

Arabidopsis lines	**Mutation**	**Chemical inducer**			**References**
Col-0…….	wild type	acibenzolar-s-methyl	ASM	50 µg/ml	
ein2-1……	ethylene insensitive	2-chloroethylphosphonic acid	Ethephon	1 mM	Zipfel et al., 2004 Vandenbussche et al., 2007
jar1-1……	jasmonate resistance	methyl jasmonate	MeJa	7.5 mM	Zipfel et al., 2004
ndr1-1/npr1-2	nonexpresser of PR genes and salicylic acid insensitive	probenazole	PBZ	2 mM	Kirley et al., 2003
npr1-1……	nonexpresser of PR genes and salicylic acid insensitive	probenazole	PBZ	2 mM	Spoel et al., 2003
NahG……	encoding salicylate hydroxylase and salicylic acid insensitive	acibenzolar-s-methyl	ASM	50 µg/ml	Hunt et al., 1997

Plant Culture, Treatments and Inoculation

All Arabidopsis seeds were sown in 18x13x6 cm flats (T.O. Plastics Inc., Clearwater, MN) containing Sunshine #1 mix (Sun Gro Horticulture Inc., Bellevue, WA) and vernalized for 4 days at 5 ± 2 °C under 80 % relative humidity (RH). Plants were transferred to a growth chamber, sub-irrigated and kept at 22 ± 2 °C day and 20 ± 2 °C night temperatures with a 10 h photoperiod. Supplemental lighting was provided by Cool white (F96T12/CW/1500) and Gro-Lux (F96T12/Gro/VHO) lamps (3:1 ratio, GTE Products Corporation, Dancers, MA). After 3 weeks, individual plants were transplanted into 10 x 10 x 10 cm plastic pots filled with Sunshine #1 mix supplemented with Osmocote Classic 14-14-14 (The Scotts Company, Marysville, OH) at a rate of 1.5 kg/m^3 of Sunshine #1 mix. Plants were kept under conditions as described above for 3 weeks.

Twenty plants per treatment were induced either with distilled water, buffer, 203-7, BmJ, or mutant specific chemical inducer by spraying the whole plant. Induced plants were kept under greenhouse conditions as described above for 6 days.

After this time period, 20 plants per treatment were challenge inoculated with Bot-1 conidia solution by placing one 5 µl droplet on 3 individual leaves (ranging from oldest to newest leaves) per plant. Following inoculation, plants were placed at conditions as described above with RH adjusted to 90 % RH for 7 days to allow disease development. Disease development was rated on a 0 to 5 scale with 0 = no visible lesion to 5 = lesion expanding into non-inoculated tissue.

Apoplastic Fluid Extraction
and Protein Quantification

Six weeks old plants were induced, in replicates of 8, either with distilled water, buffer, 203-7, BmJ, or mutant-specific chemical inducer by spraying the whole plant. Induced plants were kept under greenhouse conditions as described above for 6 days.

Six days post induction the plants were cut 0.5 mm below crown and submerged in chilled distilled water on ice. Cuttings were transferred to a filtering flask containing 150 mM NaCl and 25 mM MES in double-distilled water at pH 6.2. A vacuum was applied for 15 min and the buffer was forced into the leaf tissue by releasing the vacuum and swirling the flask content every 3 min. Plants were removed from the flask, blotted dry between paper towels and leaves were cut off 0.2 mm above crown. Leaves were rolled and inserted into 1.8 ml microcentrifuge tubes (caps and tips removed) with petioles facing the tip. Samples were centrifuged at 2000 rpm for 10 min at 4 °C. Apoplastic fluid was collected in a 2 ml microcentrifuge tube and frozen at -80 °C until analyzed.

The protein amount of the apoplastic fluids was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) per manufacturer's instructions using bovine serum albumin (EMD Chemicals Inc., Darmstadt, Germany) as a standard.

Chitinase Assay

Based on the chitinase activity assay described by Hung et al (2002) a 96 well micro-plate assay was developed (see chapter 2). For each standard or sample, 3 wells of

a 96 well PCR plate (VWR International) were filled with 54 µl of 100 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid), 60 µl of 0.2 % of glycol chitin solution, and 6 µl of chitinase standards (0.1 units/ml, 0.01 units/ml, and 0.001 units/ml from *Streptomyces griseus*, Sigma) or apoplastic fluid samples. The plate was sealed with a silicone sealing mat (VWR International) and incubated for 14 h at 37 °C using a thermocycler (GeneAmp PCR System 9600, The Perkin-Elmer Corporation, Norwalk, Connecticut). After this time period 90 µl of the solution were transferred to a new PCR plate and 120 µl of ferri-ferrocyanide reagent (Imoto and Yagishita, 1971) consisting of 0.05 % potassium ferricyanide in 500 mM sodium carbonate was added. The plate was sealed as described before and incubated for 15 min at 99.9 °C. Following incubation 100 µl of the solution were transferred to a 96 well flat-bottom assay plate (Becton Dickinson Labware, Becton Dickinson and Company, Fanklin Lakes, NJ) and decrease of absorbance was measured at 420 nm with a SpectraMax plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The specific enzyme activity (in mg of *N*-acetyl-D-glucosamine released/24h/µg apoplastic proteins) of the apoplastic fluid samples was determined by comparison to chitinase standards.

β-1,3-glucanase Assay

The microplate-based carboxymethylcellulose assay described by Xiao et al. (2005) was adapted to be used to determine β-1,3-glucanase activity in apoplastic fluids extracted from Arabidopsis plants (see chapter 2). Thirty microliters of 1 % laminaran (from *Eisenia bicyclis*, TCI, Portland, OR) and 1 % CM-Pachyman (Megazyme International Ireland Ltd, Ireland) in 50 mM sodium acetate buffer (adjusted to pH 4.0

with glacial acetic acid) and 30 µl of laminarinase standards (0.25, 0.125, 0.0625, and 0.03125 units/ml from *Trichoderma* sp., Sigma) or apoplastic fluid samples were combined in a PCR plate well with 3 wells per standard or sample. The plate was sealed with a silicone sealing mat and incubated for 4 h at 30 °C using a thermocycler. Following incubation, 60 µl of dinitrosalicylic acid reagent consisting of 30 mM dinitrosalicylic acid (Alfa Aesar, Ward Hill, MA), 14.9 mM phenol, 2.8 mM sodium sulfite, 0.5 M sodium-potassium-tartarate (EMD Chemicals Inc., Darmstadt, Germany), and 0.2 mM NaOH were added to the solution and the PCR plate was incubated again for 5 min at 95 °C as described above. Subsequently 100 µl of solution were transferred to a 96 well flat-bottom assay plate and the change of absorbance was measured at 540 nm with a SpectraMax plus384 spectrophotometer. The specific enzyme activity (in mg of glucose released/4h/µg of apoplastic proteins) of the apoplastic fluid samples was determined by comparison to laminarinase standards.

Superoxide Dismutase Assay

Quantification of superoxide dismutase (SOD) activity in the apoplastic fluid samples was performed according to the method described by Ewing and Janero (1995). Under subdued room light, 200 µl of 0.1 mM EDTA, 62 µM nitro blue tetrazolium (TCI America, Portland, OR), and 98 µM NADH in 50 mM phosphate-buffered saline (pH 7.4) were combined in a 96 well flat-bottom assay plate with 25 µl of SOD standards (4, 8, 12, and 16 ng/µl from bovine liver, CALBIOCHEM, San Diego, CA) or apoplastic fluid samples. Standards and samples were run in triplicate. Five minutes after the reaction was started by adding 25 µl of 33 µM phenazine methosulfate (TCI America,

Portland, OR) and 0.1 mM in 50 mM phosphate-buffered saline (pH 7.4), the endpoint absorbance was measured at 560 nm with a SpectraMax plus384 spectrophotometer. The specific enzyme activity (in ng of SOD/μg of apoplastic proteins) of the apoplastic fluid samples was determined by comparison to SOD standards.

Statistical Analysis

The experimental design was a randomized complete block with 20 replications per treatment for the *B. cinerea*-bioassay and 8 replications per treatment for the PR-protein assays and all experiments were performed three times. All described experiments were tested for homogeneity using the Levene's test and collected data were pooled and analyzed statistically by conducting an analysis of variance (Madden et al., 1982) using the general linear model procedure of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at α=0.05. Data from the *B. cinerea*-bioassay were also analyzed by performing a non-parametric analysis (Shah and Madden, 2004), to obtain a ranking of the different treatments in regard to the rating scale (0 to 5) which was used to estimate disease development.

Results

Arabidopsis – *B. cinerea* – Inoculation

For all tested *A. thaliana* lines (Col-0 and mutants), the non-parametric analysis performed for the *B. cinerea*-bioassays showed significant ($P<0.0001$) differences between treatments and a similar ranking of the treatments in regard to disease development when compared to Fishers protected LSD.

The bacilli treatments and the chemical inducers had always significantly ($\alpha=0.05$) lower disease ratings when compared to the control plants of all tested lines treated with distilled water except for BmJ in *ein2-1* mutants (Table 4.2). Plants treated with the buffer controls alone were also significantly different from the control plants, but had overall the lowest disease reduction when compared to the other treatments. *Col-0* plants treated with BmJ were not significantly different ($\alpha=0.05$) from plants treated with Actigard or 203-7 which showed the least ($\alpha=0.05$) disease reduction. Plants treated with 203-7 showed a significant ($\alpha=0.05$) disease reduction in *npr1-1*, *NahG*, and *ein2-1* mutants when compared to the specific chemical inducers, but were not significantly different from PBZ in the *ndr1-1/npr1-2* mutant or from BmJ treated *NahG* mutants. *Jar1-1* mutants treated with either 203-7 or BmJ showed the lowest ($\alpha=0.05$) disease reduction when compared to methyl jasmonate and were never significantly different ($\alpha=0.05$) from each other or from plants treated with buffer. Applications with BmJ resulted in the lowest ($\alpha=0.05$) disease reduction in *ein2-1* mutants and in disease reduction similar ($\alpha=0.05$) to buffer treatment in *npr1-1* mutants.

Table 4.2: Percent disease reduction of *Botrytis cinerea* leaf spot on *Arabidopsis thaliana* Col-0 and mutants by means of induced SAR resulting from foliar applications of *Bacillus mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, chemical inducers, buffers, or distilled water.

	% disease reduction in comparison to distilled water control					
Arabidopsis lines / **treatment**	***Col-0***	***npr1-1***	***ndr1-1/npr1-2***	***jar1-1***	***NahG***	***ein2-1***
203-7	38.4 b [z]	52.4 d	46.7 d	13.0 bc	49.5 c	36.8 c
BmJ	41.3 bc	9.9 b	23.3 c	15.0 c	49.5 c	4.2 a
chemical inducer	45.9 c (Actigard)	41.9 c (PBZ)	48.9 d (PBZ)	49.9 d (methyl jasmonate)	32.6 b (Actigard)	28.1 b (ethephon)
buffer	na	7.9 b	17.5 b	10.2 bc	na	na
distilled water	0 a	0 a	0 a	0 a	0 a	0 a

[z] Means in the same column followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).

Chitinase, β-1,3-glucanase and
Superoxide Dismutase Activity Assays

Chitinase activity in apoplastic fluids extracted from *Col-0* plants, *npr1-1*, *ndr1-1/npr1-2*, *NahG*, and *ein2-1* mutants treated with 203-7 was always significantly different (α=0.05) from plants treated with distilled water (Table 4.3). In *jar1-1* mutants treated with 203-7 chitinase activity was equivalent to plants treated with buffer or distilled water. Plants induced with 203-7 showed an increase (α=0.05) of apoplastic chitinase activity in *Col-0* plants and the *ndr1-1/npr1-2* mutant, similar to the ASM and PDZ inducers and greater than the distilled water and buffer controls. Treatments with 203-7 significantly increase apoplastic chitinase activity in the *npr1-1* mutant when compared to the ASM and PBZ inducers and the distilled water and buffer controls. BmJ treated *Col-0* plants, *jar1-1*, and *NahG* mutants expressed elevated levels of apoplastic chitinase activity which was significantly different (α=0.05) from distilled water treated plants (Table 4.3). No significant increase of apoplastic chitinase activity over distilled water and buffer controls could be observed in BmJ treated *npr1-1*, *ndr1-1/npr1-2*, and *ein2-1* mutants. Chitinase activity of all Arabidopsis lines with the exception of *npr1-1* mutants was significantly different (α=0.05) from the distilled water control when treated with the specific chemical inducer, but never when treated with the inducer specific buffer (Table 4.3).

Table 4.3: Chitinase activity assays for apoplastic fluids of *Arabidopsis thaliana*, extracted after treating plants with *Bacillus mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, chemical inducers, buffers, or distilled water.

	chitinase activity [z]					
Arabidopsis lines / **treatment**	***Col-0***	***npr1-1***	***ndr1-1/npr1-2***	***jar1-1***	***NahG***	***ein2-1***
203-7	7.00 c [y]	20.28 c	10.90 b	4.05 a	5.88 b	8.30 b
BmJ	3.95 b	8.16 ba	6.30 ab	7.44 bc	4.76 b	6.17 a
chemical inducer	8.24 c (Actigard)	11.85 ba (PBZ)	8.76 b (PBZ)	8.40 bc (methyl jasmonate)	9.81 c (Actigard)	13.06 c (ethephon)
buffer	na	9.15 ba	6.43 ab	5.38 ab	na	na
distilled water	1.95 a	4.77 a	3.60 a	4.12 a	2.11 a	4.80 a

[z] Chitinase activity measured in mg of *N*-acetyl-D-glucosamine released/24h/µg apoplastic proteins [x].

[y] Means in the same column followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).

[x] Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

203-7 induced *Col-0*, *npr1-1, ndr1-1/npr1-2, NahG* and *ein2-1* mutants had a significantly increased (α=0.05) β-1,3-glucanase activity when compared to plants treated with distilled water (Table 4.4). The highest (α=0.05) β-1,3-glucanase activity was observed in 203-7 induced *npr1-1* and *ndr1-1/npr1-2* mutants and was similar to the specific chemical inducers. Induction by 203-7 resulted in a significant increase of enzyme activity in all lines except the *NahG* mutant when compared to BmJ. BmJ was only able to increase β-1,3-glucanase activity significantly in apoplastic extracts of *jar1-1* and *NahG* mutants and showed no significant increase in *Col-0* plants, *npr1-1*, *ndr1-1/npr1-2*, or *ein2-1* mutants when compared to distilled water or buffer control. With the exception of *npr1-1* and *ndr1-1/npr1-2* mutants, Arabidopsis lines treated with the specific chemical inducer were always significantly different to the water control or buffer.

Table 4.4: β-1,3-glucanase activity assays for apoplastic fluids of *Arabidopsis thaliana*, extracted after treating plants with *Bacillus mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, chemical inducers, buffers, or distilled water.

	β-1,3-glucanase activity [z]					
Arabidopsis lines / **treatment**	***Col-0***	***npr1-1***	***ndr1-1/npr1-2***	***jar1-1***	***NahG***	***ein2-1***
203-7	73.22 b [y]	59.56 c	17.46 b	24.76 a	70.13 b	58.30 b
BmJ	43.66 a	20.83 ab	11.01 a	55.53 c	48.33 b	29.91 a
chemical inducer	105.85 c (Actigard)	43.30 bc (PBZ)	15.88 b (PBZ)	58.20 c (methyl jasmonate)	120.9 c (Actigard)	67.71 c (ethephon)
buffer	na	29.80 ab	14.75 b	38.82 b	na	na
distilled water	37.17 a	19.18 a	11.50 a	29.36 ab	21.44 a	21.51 a

[z] β-1,3-glucanase activity measured in mg of glucose released/4h/μg of apoplastic proteins [x].

[y] Means in the same column followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).

[x] Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

The SOD activity assay showed elevated of SOD levels in apoplastic extracts of *Col-0* plants, *npr1-1*, *NahG*, and *ein2-1* mutants when treated with 203-7 and were significantly different (α=0.05) from distilled water treatments (Table 4.5). Plants induced by 203-7 showed no significant increase in SOD activity in *jar1-1* mutants and were not different from either the distilled water or buffer controls. *Jar1-1*, *NahG*, and *ein2-1* mutants induced by 203-7 or the specific chemical inducer were not significantly different (α=0.05) from each other. BmJ showed significantly elevated (α=0.05) levels of SOD activity in *Col-0* plants, *jar1-1*, and *ein2-1* mutants when compared to buffer or distilled water controls. BmJ was neither significantly different from 203-7 in *Col-0* plants and *NahG* mutants, nor from the specific chemical inducer in *npr1-1* and *jar1-1* mutants. The specific chemical inducer resulted in a significant increase (α=0.05) of SOD activity in *Col-0* plants, *NahG*, and *ein2-1* mutants, but not in *npr1-1*, or *jar1-1* mutants where the chemical inducers were never significantly different from the distilled water or buffer control. *Ndr1-1/npr1-2* mutants showed a reduction of SOD activity for all treatments which resulted in values below the distilled water control and therefore in negative numbers.

Table 4.5: Superoxide dismutase (SOD) activity assays for apoplastic fluids of *Arabidopsis thaliana*, extracted after treating plants with *Bacillus mojavensis* isolate 203-7. *B. mycoides* isolate BmJ, chemical inducers, buffers, or distilled water.

	SOD activity [z]					
Arabidopsis lines / **treatment**	***Col-0***	***npr1-1***	***ndr1-1/npr1-2***	***jar1-1***	***NahG***	***ein2-1***
203-7	11.20 b [y]	70.86 b	4.74 ab	0.30 a	25.13 bc	7.91 b
BmJ	11.17 b	25.69 a	2.96 a	1.35 b	24.16 b	3.02 a
chemical inducer	14.70 c (Actigard)	16.97 a (PBZ)	2.48 a (PBZ)	0.67 ab (methyl jasmonate)	26.34 c (Actigard)	10.17 b (ethephon)
buffer	na	21.62 a	4.96 ab	0.46 a	na	na
distilled water	6.95 a	1.81 a	6.28 b	0.14 a	8.71 a	1.19 a

[z] SOD activity measured in ng of SOD/μg of apoplastic proteins [x].
[y] Means followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).
[x] Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

Discussion

The objective of this research was to determine the individual defense pathways activated by foliar applications of *B. mojavensis* isolate 203-7 and *B. mycoides* isolate BmJ. Different *A. thaliana* mutants (*ein2-1*, *jar1-1*, *ndr1-1/npr1-2*, *npr1-1*, and *NahG*) were tested for their ability to be systemically induced by these bacilli. The induction was verified by performing bioassays using *B. cinerea* isolate Bot-1 and quantifying the disease reduction resulting from induced systemic resistance, and by measuring the enzyme activity of SOD and two PR-proteins: chitinase and β-glucanase, associated with induced systemic resistance (Ryals et al., 1996; Sticher et al., 1997).

Induction of systemic resistance facilitated by 203-7 is independent of the SA and ET defense pathways and the PR-protein expression is not regulated by the NPR1 gene.

This conclusion was supported by the significant disease reduction (on average by 46.3 %) and the increase in enzyme activity observed in Arabidopsis *npr1-1*, *ndr1-1/npr1-2*, *NahG*, and *ein2-1* mutants. The defense pathway stimulated by 203-7 appeared to be JA dependent (Image 4.1) as evidenced by reduced disease control and decreased enzyme activity in *jar1-1* mutants when compared to the chemical inducer methyl jasmonate. A similar induction in Arabidopsis via the JA dependent, SA- / NPR1-independent pathway was observed for the gram-negative plant growth-promoting rhizobacteria *Serratia marcescens* strain 90-166 which required, in contrast to 203-7, responsiveness to JA and ET (Pieterse et al., 1998).

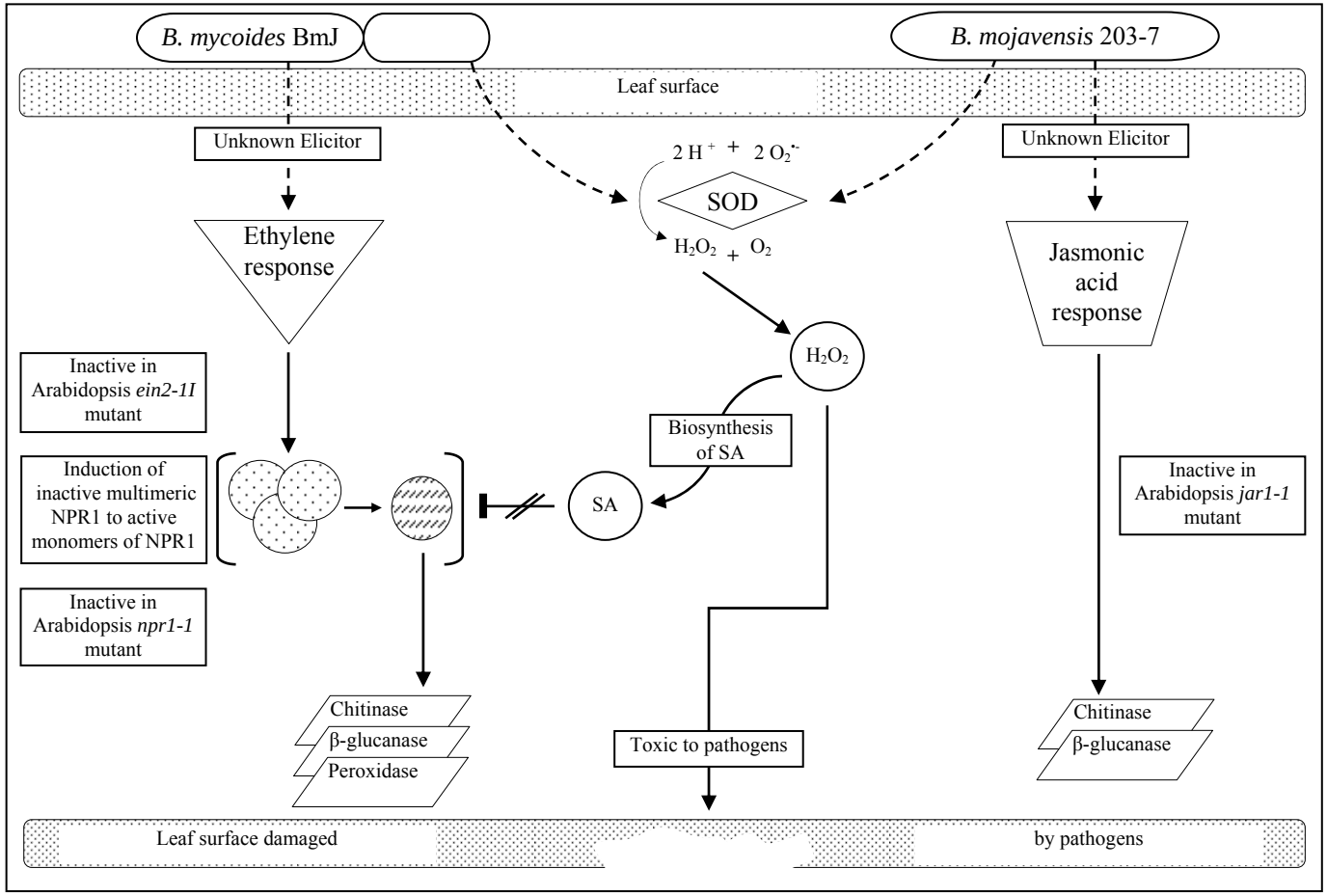

Image 4.1: Model for proposed induction of defense pathways induced by *Bacillus mojavensis* isolate 203-7 and *Bacillus mycoides* isolate BmJ. ═ indicates pathway not activated by 203-7 or BmJ, SA = salicylic acid, SOD = superoxide dismutase.

As reported by Bargabus-Larson and Jacobsen (2007), the induction of systemic resistance in sugar beets via foliar applications of BmJ is SA independent, but is regulated by NPR1 genes. The same observation could be made for the Arabidopsis system, where BmJ was able to significantly increase the disease control (on average by 32 %) and the measured enzyme activity of chitinase, β-1,3-glucanase, and SOD in *jar1-1* and *NahG* mutants. Arabidopsis *npr1-1* and *ndr1-1/npr1-2* mutants induced with BmJ showed a reduced ability to control the infection facilitated by Bot-1 and also expressed significantly lower levels of enzyme activity, indicating a regulation of the tested PR-proteins by the NPR1 genes. Surprisingly, the Arabidopsis *ein2-1* mutant also showed a decrease in disease control and enzyme activity, supporting the conclusion that the pathway induced by BmJ is ET dependent (Image 4.1). Similar results were observed by Pieterse et al. (1998) for the induction of induced systemic resistance (ISR) by *Pseudomonas fluorescens* WCS417r via a JA/ET pathway regulated by NPR1 genes. This induction leads to defense responses similar to the ones observed for SAR.

In the past, SAR was associated with biotrophic pathogens, SA, or SA analogues like 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH) as biological or chemical inducers. These proved to be important for the induction of systemic resistance and the associated PR-proteins, including PR-1, chitinase (PR-3), or β-1,3-glucanase (PR-2) (Durrant and Dong, 2004; Ryals et al, 1996; Sticher et al., 1997). However, recent research indicated an activation of SAR and the induction of associated PR-proteins without the accumulation of SA and consequently independent of the SA pathway (Larson and Jacobsen, 2007; Vernooij et al., 1995; Yasuda et al., 2003). Yasuda

et al. (2003) were able to show that *N*-cyanomethyl-2-chloroisonicotinamide induces SAR without the activation of the SA dependent pathway and by elevating the levels of resistance towards *Pseudomonas syringae* pv. *tomato* DC3000 and increasing the activity of PR-proteins in Arabidopsis. This form of resistance was neither induced by ET nor JA, but by a signal located between the SA accumulation and the NPR1 genes. Similar observations were made by Vernooij et al. (1995) for INA inducing SAR without the accumulation of SA and activation of a component downstream of this molecule. Expressions of PR-3 and PR-2 proteins are not only induced by SA, but also by exogenous applications of JA or ET which trigger the corresponding pathways (Mauch and Staehelin, 1989; Van Loon and Van Strien, 1999). The JA or ET pathways are normally associated with ISR, resulting from an attack by necrothrophic pathogens or insects, and the expression of its PDF1.2 marker gene (Heil and Bostock, 2002; Pieterse et al., 2000). It is not yet clear, how JA and ET co-regulate the expression of PR-3 and PR-2 (Thomma et al., 1998; Van Loon and Van Strien, 1999), and the function of NPR1 also is still controversial. Thomma et al. (1998) saw indications for a NPR1 independent activation, others (Spoel et al., 2003; Truman et al., 2007) support the NPR1 dependency.

Nevertheless, Thomma et al. (1998), Truman et al. (2007), and Schenk et al. (2000) propose an interaction between the SA- and JA/ET-dependent pathways, with a co-regulatory effect as described above and/or a side-by-side action of both pathways (Truman et al., 2007).

In conclusion: Systemic resistance induced by 203-7 and measured by disease control and induction of PR-proteins is controlled by SA and NPR1 independent pathways, but JA dependent pathways without the involvement of ET.

BmJ activated defense responses are SA independent, but dependent on ET and NPR1 regulated pathways. These findings make it obvious that the differentiation of induced resistance into ISR and SAR needs to be reconsidered. We suggest that perhaps "induced resistance" should be used since the old definitions for SAR and ISR are no longer appropriate as the underlying mechanisms are revealed using Arabidopsis mutants.

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CHAPTER 5

COMPARISON OF 3 *BACILLUS* SSP. BASED BIOCONTROL AGENTS FOR THEIR ABILITY TO CONTROL BOTRYTIS GREY MOLD IN GREENHOUSE TOMATOES

Abstract

Three bacillus based biological control agents (BCAs), *Bacillus mojavensis* isolate 203-7 (203-7), *Bacillus mycoides* isolate BmJ (BmJ), and *Bacillus subtilis* strain QST 713 (Serenade Max™ WP), and *Trichoderma harzianum* strain T-22 (T-22, PlantShield® HC) were tested for their ability to control *Botrytis cinerea* grey mold and to induce superoxide dismutase (SOD), chitinase, and β-1,3-glucanase on hydroponically grown greenhouse tomatoes (*Lycopersicon esculentum* var. *esculentum*). Weekly foliar applications of bacillus based BCAs and T-22 significantly (α=0.05) reduced the severity of grey mold leaf lesions when compared to the distilled water control. BmJ had the lowest rating with a reduction of 60 %, followed by Serenade and T-22 (both 45 %), and 203-7 (41 %). Only the bacilli treatments were able to significantly (α=0.05) reduce the Area Under the Disease Progress Curve (AUDPC) calculated for seven Botrytis stem canker ratings when compared to control plants. Serenade reduced the AUDPC by 85 % when compared to the control and had the lowest AUDPC of all treatments, but was not significantly different from 203-7 or BmJ which were able to reduce the AUDPC by 63 and 64 % respectively. No significant (α=0.05) effects on total yield could be observed for the tested treatments. Neither β-1,3-glucanase, chitinase, nor SOD activities were

significantly (α=0.05) increased by the treatments. Only 203-7 showed a significant increase (α=0.05) of chitinase activity by 44 % when compared to the control.

Introduction

Greenhouse production of tomatoes involved 1726 ha within North America and resulted in an estimated 528,078 metric tons of production. Canada produced 220,114 metric tons under 446 ha of glass and plastic structures, followed by the U.S. with an estimated 159,664 metric tons on 330 ha, and Mexico with 148,300 metric tons on 950 ha (Cook and Calvin, 2005). Greenhouse tomato producers are confronted with multiple disease problems promoted by the unique greenhouse environment. Foliar pathogens include *Botrytis cinerea*, *Colletotrichum coccodes*, *Phytophthora infestans*, and *Oidium neolycopersici*. Soilborne pathogens include *Pythium ultimum* and *Rhizoctonia solani* (UC IPM, 2005). *B. cinerea*, the causal agent of grey mold and stem canker on tomato, is commonly a serious pathogen under the unique environmental conditions, characteristic of greenhouse production. Components of this favorable environment include high relative humidity, warm temperatures, and high-density planting. Specialized cultural practices like pruning provide sites favorable to infection (Dik and Wubben, 2004; Paulitz and Bélanger, 2001). Grey mold can cause significant losses of up to 72 % despite numerous cultural control methods and the common use of fungicides (Dik and Wubben, 2004; Shtienberg et al., 1998). The increased occurrence of fungicide resistant strains of *B. cinerea*, consumer prejudice against pesticide residues, and the restricted reentry period interfering with cultural practices have led to a reduction of fungicide use.

Integrated pest management approaches (Morgan, 1984; Hoffland et al., 1999; Elad and Volpin, 1993) and biological control agents (BCAs) have become widely used alternatives to fungicides. BCAs are very successful under the unique conditions of greenhouse production, since the uniform environmental conditions are favorable to most BCAs. Other factors favorable promoting the use of BCAs are the acceptance by consumers; their short re-entry periods and short harvest interval, all of which are important for the tomato production with its continuous harvest (Paulitz and Bélanger, 2001). Multiple fungal and bacterial BCAs have been tested for their efficacy to control *B. cinerea*. These include: *Trichoderma hamatum*, *T. harzianum* strain T-39, *T. viride*, *Aureobasidium pullulans*, *Cryptococcus albidus*, *C. luteus*, *C. laurentii*, *Gliocladium catenulatum*, *G. roseum*, *Pichia guilliermondii*, and *Bacillus licheniformis*, *B. pumilus*, *B. subtilis* strain QST713, *Bacillus* sp., *Brevibacillus brevis*, *Pseudomonas* sp., (Audenaert et al., 2002; Daggas, 2002; Dik et al., 1999; Eden et al., 1996; Elad and Stewart, 2004; Lee et al., 2006; Paulitz and Bélanger, 2001; Utkhede and Mathur, 2002). Bacillus based BCA have provided good results and have been widely researched (Ingram and Meister, 2006; Lee et al., 2006; Mari et al., 1996; Zhao et al., 2003). An advantage of bacillus based BCAs is the formation of spores as a resting structure which makes them less susceptible to unfavorable environmental conditions than gram-negative bacteria such as *Pseudomonas* ssp. (Emmert and Handelsman, 1999). Individual BCAs employ different modes of action to control *B. cinerea* such as: competition for nutrients and space, the production of antimicrobial compounds and cell wall-degrading enzymes, parasitism, or induced systemic resistance/systemic acquired resistance (ISR/SAR) (for an in-depth

review see Elad and Stewart, 2004; Jacobsen, 2006; Paulitz and Bélanger, 2001). ISR/SAR is potentially of great importance since this form of resistance is able to protect the plant not only against fungal but also against bacterial and viral pathogens at the same time (Fontanilla et al., 2005; Sticher et al., 1997; Van Loon, 1997; Van Loon et al., 1998). Other modes of action like parasitism and the production of antimicrobial compounds have limitations, like pathogen specificity or the need for direct contact between the BCAs and the pathogen. ISR/SAR and the related defense mechanisms, like the induction of pathogenesis-related proteins (PR-poteins; Van Loon, 1997), do not have these limitations because the plant itself produces the PR-proteins and distributes them to even distal plant parts. This phenomenon is used to protect tomato plants against a wide range of bacterial and fungal pathogens by using either chemical inducers (Małolepsza, 2006; Spletzer and Enyedi, 1999; Varina et al., 2004) or BCAs including multiple *Bacillus* ssp. (Jetiyanon, 2007; Ongena et al., 2007; Silva et al., 2004; Yan et al., 2002). Research on *B. mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 has been focused on the biological control of Cercospora leaf spot (*Cercospora beticola*) of sugar beets and Anthracnose (*Glomerella cingulata* var. *orbicular*) of cucumber by means of SAR induced foliar applications (Bargabus et al., 2002, 2004; Neher et al., 2009).

The objective of this research was to test and compare the ability of BmJ and 203-7 to control Botrytis grey mold and stem canker in greenhouse tomatoes (*Lycopersicon esculentum* var. *esculentum*) with *Bacillus subtilis* strain QST 713 (Serenade Max™ WP) and *Trichoderma harzianum* strain T-22 (PlantShield® HC). In addition to the preceding treatments, Acibenzolar-s-methyl (Actigard 50WG Fungicide) was used to determine the

involvement of SAR/ISR and the possible induction of chitinase, β-1,3-glucanase, and superoxide dismutase.

Material & Methods

Biological Control Organisms

B. mycoides isolate BmJ (BmJ) was originally isolated from sugar beet leaves. *B. mojavensis* isolate 203-7 (203-7), originally isolated from sugar beet seed embryos, was included in the greenhouse experiments since it demonstrated good induction of SAR in several plant species in previous experiments (Bargabus et al., 2002; Neher et al., 2009). 203-7 was stored at -80 °C in 10 % glycerol and 1 % tryptic soy broth (TSB, EMD Chemicals Inc., Darmstadt, Germany) and cultured in 3 % TSB for 24 h at room temperature (22 °C) on an orbital shaker (Model OS-500, VWR International) at 250 rpm. Fresh cells were harvested by centrifugation for 20 min at 5,000 rpm at 4 °C. The pellet was re-suspended in sterile-distilled water and pelleted twice by centrifugation for 20 min at 5,000 rpm at 4 °C to assure that all fermentation beer was separated from the cells.

B. mycoides isolate BmJ was stored as a freeze-dried spore preparation and the final inoculum density for both bacilli, 203-7 and BmJ, was adjusted to 10^8 colony forming units (CFU)/ml by adding distilled water.

B. subtilis strain QST 713 formulated as Serenade Max™ WP (Serenade) was obtained from AgraQuest, Inc. (Davis, CA) and was applied at a concentration of 2.5 %

(oral recommendation by Denise C. Manker, AgrarQuest, Davis, CA) suspended in distilled water.

T. harzianum strain T-22 (T-22) sold as PlantShield® HC (BioWorks, Ltd., Fairport, NY) was applied at a rate of 3.8 g/l distilled water.

The distilled water control and all above mentioned biological control organisms were applied using a CO_2 pressurized sprayer with applications made to run-off.

Fungal Culture

B. cinerea isolate Bot-1 the causal agent of grey mold and Botrytis stem canker of tomato was originally isolated from infected tomato fruits. Conidia were stored at -80 °C as described above. Conidia were streaked onto 50 % potato dextrose agar (19.5 g/l PDA, EMD Chemicals Inc., Darmstadt, Germany) using a sterile loop and incubated at 24 °C for at least 2 weeks or until mycelium showed signs of sporulation. Plates were flooded with a solution consisting of 6.2 mM KH_2PO_4 and 5.5 mM glucose in sterile-distilled water, and conidia were loosened with a sterile glass rod. Conidia suspension was decanted and filtered through two layers of cheesecloth to remove mycelium and agar pieces. The inoculum density was adjusted to 10^5 conidia/ml and was applied using a Crown aerosol sprayer (Aervoe Industries Inc., Gardnerville, NV).

Chemical Inducer

Acibenzolar-s-methyl (ASM) (Actigard 50WG Fungicide, Syngenta, Greensboro, NC) was used as the chemical inducer in the greenhouse experiments at a rate of 50 µg/ml and was applied using a Crown aerosol sprayer with applications made to run-off.

Plant Culture, Treatments and Inoculation

Tomato (*L. esculentum*) seeds of the variety "Trust" were started in Oasis ® Rootcubes (2.54 x 2.54 x 3.8 cm, Smithers-Oasis North America, Kent, OH) at 22 ± 2 °C day and 18 ± 2 °C night temperatures and were kept in a misting chamber under 95 % relative humidity (RH) with a 16 h photoperiod. Additional lighting was provided by GE Multi-Vapor MVR1000/C/U lights, (GE Lighting, General Electric Company, Cleveland, OH). At the two leaf stage, plants were removed from the misting chamber and watered daily with a fertilizer solution consisting of 0.06 % Chem-Gro 4-18-38 fertilizer (Chemgro Fertilizer CO Inc., East Petersburg, PA), 0.03 % $CaNO_3$ and 0.03 % $MgSO_4$ which was adjusted to pH 6.5. Six week old plants at an approximate height of 15 ± 2 cm were then transplanted into 11.4 l Coconut EZ Gro Bags (Hydro-Gardens, Colorado Springs, CO) and irrigated with a single sprayer (flow rate 16 l/h). The plants were kept in a greenhouse at 24 ± 2 ˚C day and 16 ± 2 ˚C night temperatures under a 16 h photoperiod (GE Multi-Vapor MVR1000/C/U) and fertilized 3 times per day for 1 min (total volume of 800 ml per bag) with a fertilizer solution consisting of 0.06 % Chem-Gro 4-18-38 fertilizer, 0.06 % $CaNO_3$ and 0.03 % $MgSO_4$ which was adjusted to pH 6.2. Bags were placed in five rows 60 cm apart with 12 bags per treatment arranged as a completely randomized experiment.

Starting 4 weeks after transplanting on a weekly interval, one set of lower leaves per plant was removed. Treatments consisting of a distilled water control, 203-7 (10^8 cfu/ml distilled water), BmJ (10^8 cfu/ml distilled water), Serenade (2.5 %), and T-22 (3.8 g/l distilled water) were applied as described earlier.

In week 5, following the routine pruning of the lower leaves and the application of the treatments, plants were inoculated by spraying the lower stems with the Bot-1 spore solution. The inoculation was followed by an overhead mist-irrigation to increase the humidity and to establish grey mold infection. The mist-irrigation was applied every night for 12 h in 30 min intervals for 30 sec.

After symptoms were well developed (4 weeks after inoculation) grey mold disease data were collected by estimating the leaf lesions coverage on 10 random individual leaves per plant using following rating scale: 0 = no lesions, 1 = multiple single lesions (leaf coverage < 10%), 2 = 25% of leaf covered with lesions, 3 = 50% of leaf covered, 4 = 75% of leaf covered, 5 = hole leaf covered (100%). Stem canker data were collected by rating the disease development using the following rating scale: 0 = no stem canker, 1 = stem canker present, 2 = water soaking of lesion, 3 = water soaking extends into stem tissue, 4 = stem infected, 5 = stem girdled by canker. Yield data were collected over several weeks by harvesting and weighing ripe tomatoes.

The entire experiment was performed twice, and all described experiments were tested for homogeneity using the Levene's test and experiments were pooled. By combining seven stem canker ratings, the area under the disease progress curve (AUDPC, Bjarko and Line, 1988) was calculated. All collected data were analyzed statistically by conducting an analysis of variance (Madden et al., 1982) using the general linear model procedure of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at α=0.05. Data from the *B. cinerea*-tomato greenhouse were also analyzed by

performing a non-parametric analysis (Shah and Madden, 2004). A ranking of the different treatments in regard to the estimated disease development (rating scale 0 to 5) was obtained.

Apoplastic Fluid Extraction
and Protein Quantification

For the extraction of apoplastic fluids a separate set of tomato plants grown in 10 x 10 x 10 cm plastic pots filled with equal parts (by volume) of PGC Soil Mix (1/3 loam soil, 1/3 washed concrete sand, 1/3 Canadian Sphagnum peat moss plus AquaGro 2000 G [Aquatrols, Paulsboro, NJ] wetting agent, aerated steam pasteurized at 80 °C for 45 min) and Sunshine Mix #1 (Sun Gro Horticulture Inc., Bellevue, WA) was used. Plants were grown for 10 weeks under greenhouse conditions at 24 ± 2 °C day and 16 ± 2 °C night temperatures with a 16 h photoperiod. Supplemental lighting was provided by SON AGRO 430 WATT high pressure sodium lights (Philips Lighting Company, Somerset, NJ). Vigorous growth was maintained by watering daily and fertilizing twice a week with Peters Professional 20-20-20 General Purpose (The Scotts Company, Marysville, OH) at a rate of 200 µg/ml nitrogen.

The first true leaves of 10-week-old tomato plants were induced, in replicates of five, with distilled water, 203-7, BmJ, Serenade, T-22 and ASM at concentrations as described above. Plants were kept under greenhouse conditions. Six days post induction the terminal leaflet of the second true leaf and the terminal leaflets of the consecutive leaves of each replication were collected and submerged in chilled distilled water on ice. Leaflets were transferred to a filtering flask containing 150 mM NaCl and 25 mM MES

in double-distilled water at pH 6.2. A vacuum was applied for 15 min and the buffer was forced into the leaflet tissue by releasing the vacuum and swirling the flask content every 3 min. Leaflets were removed from the flask, blotted dry between paper towels and rolled into 20 ml syringe barrels with petioles facing the syringe tip. Samples were centrifuged at 2000 rpm for 10 min at 4 °C. Apoplastic fluid was collected in a 2 ml microcentrifuge tube and frozen at -80 °C until analyzed.

The protein amount of the apoplastic fluids was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) per manufacturer's instructions using bovine serum albumin (EMD Chemicals Inc., Darmstadt, Germany) as standards.

Chitinase Assay

Based on the chitinase activity assay described by Hung et al (2002) a 96 well micro-plate assay was developed (see chapter 2). For each standard or sample, 3 wells of a 96 well PCR plate (VWR International) were filled with 54 µl of 100 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid), 60 µl of 0.2 % of glycol chitin solution, and 6 µl of chitinase standards (0.1 units/ml, 0.01 units/ml, and 0.001 units/ml from *Streptomyces griseus*, Sigma) or apoplastic fluid samples. The plate was sealed with a silicone sealing mat (VWR International) and incubated for 14 h at 37 °C using a thermocycler (GeneAmp PCR System 9600, The Perkin-Elmer Corporation, Norwalk, Connecticut). Susequently 90 µl of the solution were transferred to a new PCR plate and 120 µl of ferri-ferrocyanide reagent (Imoto and Yagishita, 1971) consisting of 0.05 % potassium ferricyanide in 500 mM sodium carbonate were added. The plate was sealed as described before and incubated for 15 min at 99.9 °C. Following incubation 100 µl of

the solution were transferred to a 96 well flat-bottom assay plate (Becton Dickinson Labware, Becton Dickinson and Company, Fanklin Lakes, NJ) and decrease of absorbance was measured at 420 nm with a SpectraMax plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The specific enzyme activity (in µg of *N*-acetyl-D-glucosamine released/24h/µg apoplastic proteins) of the apoplastic fluid samples was determined by comparison to chitinase standards.

β-1,3-glucanase Assay

The microplate-based carboxymethylcellulose assay described by Xiao et al. (2005) was adapted to determine β-1,3-glucanase activity in apoplastic fluids extracted from tomato plants (see chapter 2). Thirty microliters of 1 % laminaran (from *Eisenia bicyclis*, TCI, Portland, OR) and 1 % CM-Pachyman (Megazyme International Ireland Ltd, Ireland) in 50 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid) and 30 µl of laminarinase standards (0.25, 0.125, 0.0625, and 0.03125 units/ml from *Trichoderma* sp., Sigma) or apoplastic fluid samples were combined in a PCR plate well with 3 wells per standard or sample. The plate was sealed with a silicone sealing mat and incubated for 4 h at 30 °C using a thermocycler. Following incubation, 60 µl of dinitrosalicylic acid reagent consisting of 30 mM dinitrosalicylic acid (Alfa Aesar, Ward Hill, MA), 14.9 mM phenol, 2.8 mM sodium sulfite, 0.5 M sodium-potassium-tartarate (EMD Chemicals Inc., Darmstadt, Germany), and 0.2 mM NaOH were added to the solution and PCR plate was incubated again for 5 min at 95 °C as described above. Subsequently, 100 µl of the solution were transferred to a 96 well flat-bottom assay plate and the change of absorbance was measured at 540 nm with a SpectraMax plus384

spectrophotometer. The specific enzyme activity (in μg of glucose released/4h/μg of apoplastic proteins) of the apoplastic fluid samples was determined by comparison to laminarinase standards.

Superoxide Dismutase Assay

Quantification of superoxide dismutase (SOD) activity in the apoplastic fluid samples was performed according to the method described by Ewing and Janero (1995). Under subdued room light, 200 μl of 0.1 mM EDTA, 62 μM nitro blue tetrazolium (TCI America, Portland, OR), and 98 μM NADH in 50 mM phosphate-buffered saline (pH 7.4) were combined in a 96 well flat-bottom assay plate with 25 μl of SOD standards (4, 8, 12, and 16 ng/μl from bovine liver, CALBIOCHEM, San Diego, CA) or apoplastic fluid samples. Standards and samples were run in triplicate. The endpoint absorbance was measured at 560 nm with a SpectraMax plus384 spectrophotometer 5 min after the reaction was started by adding 25 μl of 33 μM phenazine methosulfate (TCI America, Portland, OR) and 0.1 mM in 50 mM phosphate-buffered saline (pH 7.4). The specific enzyme activity (in units of SOD/mg of apoplastic proteins) of the apoplastic fluid samples was determined by comparison to SOD standards.

Results

The non-parametric analysis performed for the *B. cinerea*-tomato-greenhouse experiment showed significant ($P<0.0001$) differences between treatments and a similar ranking of the treatments in regard to disease development when compared to Fisher's protected LSD at $\alpha=0.05$.

Weekly applications of BmJ significantly ($P=0.05$) reduced the leaf lesion coverage by 59.7 % when compared to the distilled water control and had the lowest ($\alpha=0.05$) disease rating of all treatments (Table 5.1). Treatments with 203-7, Serenade, and T-22 also resulted in a significant reduction of the leaf lesion coverage (on average 44 %) when compared to the distilled water control. However, the treatments were never significantly different from each other.

All bacilli treatments significantly ($\alpha=0.05$) reduced the development and spread of stem cankers on tomato when compared to the distilled water control, as indicated by individual ratings (Figure 5.1) and the AUDPC (Table 5.1). 203-7, BmJ, and Serenade were never significantly different from each other at any time. At the first and fourth rating, Serenade had significantly lower stem canker ratings than T-22. Ratings of BmJ or 203-7 were similar to T-22 (Figure 5.1).

Table 5.1: Effect of weekly applications of *Bacillus mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, Serenade, and T-22 on development and spread of grey mold leaf lesion and on disease severity of stem canker on tomato variety 'Trust' caused by *Botrytis cinerea*.

Treatments	Leaf lesion coverage [z]	AUDPC [y] for stem canker
distilled water control	1.479 c [x]	32.579 c [c]
B. mojavensis isolate 203-7	0.871 b	12.075 ab
B. mycoides isolate BmJ	0.596 a	11.591 ab
Serenade (*B. subtilis* strain QST 713)	0.817 b	5.017 a
T-22 (*T. harzianum* strain T-22)	0.812 b	24.757 bc

[z] Leaf lesions coverage on 10 random individual leaves per plant estimated using following rating scale: 0 = no lesions, 1 = multiple single lesions (leaf coverage < 10%), 2 = 25% of leaf covered with lesions, 3 = 50% of leaf covered, 4 = 75% of leaf covered, 5 = hole leaf covered (100%).

[y] Area under disease-progress curve (AUDPC) calculated over seven stem canker ratings.

[x] Means followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).

At the last rating, all bacilli were not significantly different from T-22. The distilled water control had the greatest AUDPC (32.579, α=0.05) and all bacilli treatments had significantly lower stem canker ratings. Serenade resulted in an 84.6 % reduction of the AUDPC, while BmJ and 203-7 had a 64.42 and 62.94 % reduction compared to the distilled water control. T-22 had a 24.01 % reduction, which was not significantly different from the distilled water control or to 203-7 or BmJ.

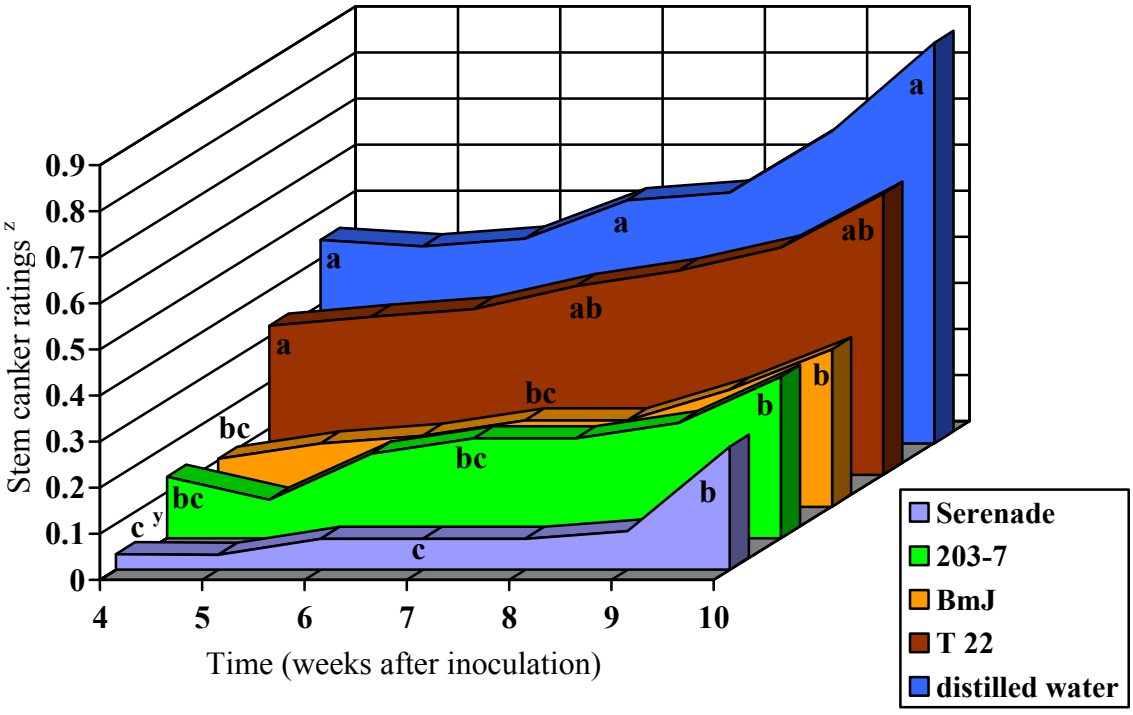

Figure 5.1: Effect of weekly applications of *Bacillus mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, *B. subtilis* strain QST 713, and *Trichoderma harzianum* strain T-22 on disease development and spread of stem cankers on tomato variety 'Trust' caused by *Botrytis cinerea*.

[z] Stem canker data collected using following rating scale: 0 = no stem canker, 1 = stem canker present, 2 = water soaking of lesion, 3 = water soaking extends into stem tissue, 4 = stem infected, 5 = stem girdled by canker.

[y] Means followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).

The yield of plants treated with the biological control organisms was not significantly different (α=0.05) to plants treated with distilled water alone. Apparently the disease pressure was not high enough to cause significant yield losses. None of the treatments had any effect on flower onset or fruit onset (data not presented).

203-7 had the significantly (α=0.05) highest chitinase activity with an increase by 43.96 %, while Serenade and T-22 had a significant reduction in chitinase activity by 30.83 and 19.19 % when compared to the distilled water control (Table 5.2). The results of the β-1,3-glucanase and SOD assays were never significantly different (α=0.05) for the biological control organisms and the distilled water control (Table 5.2). Actigard, the chemical inducer had the lowest β-1,3-glucanase enzyme activity with a reduction by 25 % and the significantly (α=0.05) lowest activity for the SOD and chitinase assays (54.6 and 58 % reduction) when compared to the distilled water control.

Table 5.2: β-glucanase, chitinase, and superoxide dismutase activity assays for apoplastic fluids of tomato variety 'Trust', extracted after foliar treatments with of *Bacillus mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, Serenade, T-22, and Actigard.

Treatments	β-glucanase assay [z]	Chitinase assay [y]	SOD assay [x]
distilled water control	5.6 ab [w]	272.5 dc	15.3 b
B. mojavensis isolate 203-7	6.5 b	392.3 e	15.9 b
B. mycoides isolate BmJ	5.1 ab	290.4 d	17.2 b
Serenade (*B. subtilis* strain QST 713)	5.9 ab	188.4 b	9.1 ab
T-22 (*T. harzianum* strain T-22)	5.0 ab	220.2 b	13.5 ab
Actigard	4.2 a	123.7 e	6.4 a

[z] Specific enzyme activity in μg of glucose released/4h/μg of apoplastic protein [v].
[y] Specific enzyme activity in μg of N-acetyl-D-glucosamine released/24h/μg of apoplastic protein [v].
[x] Specific enzyme activity in units/mg of apoplastic protein [v] compared to the superoxide dismutase standard.
[w] Means followed by the same letter are not significantly different according to Fisher's protected LSD (α=0.05).
[v] Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

Discussion

The objective of this research was to test and compare the ability of three different *Bacillus* species (*B. mojavensis* isolate 203-7, *B. mycoides* isolate BmJ, and *B. subtilis* strain QST713 [Serenade Max ™ WP, AgraQuest, Inc., Davis, CA]) and of *T. harzianum* strain T-22 (PlantShield ® HC) to control Botrytis grey mold and stem canker in greenhouse tomatoes by inducing SAR.

203-7 and BmJ are known inducers for SAR in sugar beets and cucurbits (Bargabus et al., 2002, 2004; Neher et al., 2009). According to the U.S. EPA Biopesticide Registration Action Document (2000) Serenade is supposed to be able to induce resistance against bacterial pathogens in multiple crops including tomatoes. All BCAs were tested for their ability to induce chitinase, β-glucanase, and SOD, in addition to their ability to control *B. cinerea* by means of antibiosis. To test for antibiosis, i*n vitro* discs assays were performed with the supernatants of all BCA spray solutions, where bacterial cells and fungal spores had been removed by filtration with a sterile 0.2 µm syringe filter. No inhibition of the Bot-1 mycelium could be observed for the tested spray solutions (data not shown). Individual isolates of the bacilli based BCAs and T-22 were also tested *in vitro* for the production of antimicrobial compounds. 203-7 and Serenade showed a strong inhibition of Bot-1 mycelium growth. Only a slight inhibition by BmJ, and the formation of a barrage zone between T-22 and Bot-1 could be observed (data not shown).

As discussed by Jacobsen (2006), *in vitro* experiments for the detection of antibiosis sometimes provide false positive results since abundant nutrients in the

medium can lead to a production of antimicrobial compounds. Most of the time, these results cannot be reproduced *in vivo* simply because there are not enough nutrients available on the leaf or plant surface to support the production of antimicrobial compounds, if not supplied with the spray solution. 203-7 and BmJ were applied as a spore solutions suspended in distilled water without additional nutrients. Since the composition of T-22 and Serenade is unknown, it must be assumed that there could be some additional nutrients available to T-22 and Serenade when applied to the leaf surface. Nevertheless, as reported earlier, there was no indication for antimicrobial compounds associated with the spray solutions of T-22 and Serenade used in these experiments.

It is debatable if BmJ is able to control *B. cinerea* by means of induced systemic acquired resistance in tomato, because the tested PR-proteins showed only an insignificant increase of chitinase (6.6 %) and SOD activity (12.4 %) when compared to the water control. Nevertheless, BmJ showed a significant increase in disease reduction for leaf lesions (59.7 %) and stem cankers caused by Bot-1 without producing abundant antimicrobial compounds. Since only chitinase, β-1,3-glucanase, and SOD activity and no other PR-proteins such as defensins were tested, an induction of systemic resistance cannot be ruled out. As previously described in chapters 3 and 4, BmJ is able to control *B. cinerea* by inducing the ethylene dependent pathway resulting in SAR in *Arabidopsis thaliana* plants. Also Díaz et al. (2002) showed that the ethylene dependent pathway is important for the resistance of tomato plants to *B. cinerea*, since this pathway may induce PR-proteins and phytoalexins (Fan et al., 2000; Rodrigo et al., 1993).

203-7 produced some antimicrobial compound and resulted in a significant increase in disease reduction as well (41.1 % for leaf lesions). However the increase in chitinase (by 44 %), β-1,3-glucanase (by 16.1 %), and SOD (by 3.9 %) suggest a disease control by means of SAR.

While the supernatant of the Serenade spray solution was only tested for antimicrobial compounds, it cannot be ruled out that the insignificant increase in β-1,3-glucanase activity by 5.4 % and of other untested defense compounds is related to some of its components. Nevertheless, Serenade offered significant disease reduction for leaf lesions by 44.8 % and had the lowest AUDPC for the stem canker ratings of all treatments, but it is debatable if this effect is related to SAR or to antimicrobial compounds produced by *B. subtilis* strain QST713. There are no scientific data available (Paulitz and Bélanger, 2001) to support or reject the presented findings for Serenade.

T. harzianum Strain T-39 (sold as TRICHODEX 20P) was specifically developed for the control of *B. cinerea* (Paulitz and Bélanger, 2001). Since this product is no longer available in the US, T-22 was included in this experiment to provide another *T. harzianum* species. Bailey and Lumsden (1998) and Harman et al. (2004) showed that T-22 has the ability to induced systemic resistance when applied to the rhizosphere of plants. We hoped to be able to protect tomato plants against *B. cinerea* by means of systemic resistance induced by foliar applications with T-22. T-22 was only effective in reducing leaf lesions (45.1 %), but showed no effects on stem cankers and was not able to increase the activity of the tested PR-proteins. Seaman (2002) reported a failed control of *Alternaria solani* (early blight) on tomatoes when T-22 was applied as a foliar

application, but observed significant reduction in disease for T-22 applied as a soil drench. This supports the findings of Bailey and Lumsden (1998) that T-22 is able to induce systemic resistance when applied to the roots. The presented results also indicate that the observed failed control of Botrytis stem canker might be related to the inability to induce SAR by foliar applications of T-22. The observed control of *B. cinerea* on the leaf surface was probably related to T-22 ability to mycoparasitise or outcompete fungal pathogens (Paulitz and Bélanger, 2001).

Actigard (ASM) was included for the systemic acquired resistance experiments since it is a known inducer for tomatoes. Achuo et al. (2004) observed an increase in control of *B. cinerea* on tomatoes after foliar applications of ASM. The control effect is linked to the induction of systemic resistance which is activated via the salicylic acid defense pathway and should induce multiple PR-proteins, like chitinase, β-1,3-glucanase, or peroxidase. Nevertheless, other researchers detected only a weak induction of reactive oxygen species, including superoxide anions and hydrogen peroxide (Małolepsza, 2006), or no increase of peroxidase activity at all (Boughton et al., 2006). These observations are comparable with the results gained during the SAR experiments, where ASM expressed the lowest chitinase, β-1,3-glucanase, and SOD activity of all treatments, including the distilled water control. These results could be explained by the absence of *B. cinerea*, which seems to be necessary to stimulate the peroxidase activity in ASM treated plants (Małolepsza, 2006).

None of the biological control organisms had a negative effect on yield and since the disease pressure was not high enough to cause significant losses, an increase in yield could not be observed for plants treated with the biological control organisms either.

In conclusion: It appears that *B. mojavensis* isolate 203-7 and *B. mycoides* isolate BmJ are the best choices when it comes to control *B. cinerea* on tomatoes under greenhouse conditions. Their ability to induce pathogenesis-related defense compounds provides multiple advantages which make them superior to Serenade, T-22, and other systems. Also BmJ did not increase the activity of the tested PR-proteins, the results observed in previous experiments (Chapters 3 and 4; Bargabus et al., 2002, 2004; Neher et al., 2009) and the significant reduction of leaf lesions and stem cankers caused by *B. cinerea* support a control by means of SAR. In comparison to the *Pythium oligandrum* – tomato system, where PR-proteins only increase after *B. cinerea* attacked the leaf surface (Le Floch et al., 2003), an induction by 203-7 will elevate chitinase and β-1,3-glucanase levels in advance, enable the plant to react faster to an attack by *B. cinerea* and potentially to other bacterial, fungal, viral pathogens, and probably to insects as well. Other systems as described by Ramamoorthy et al. (2002) and Anfoka and Buchenauer (1997) were able to locally induce the above mentioned PR-proteins (*Pseudomonas fluorescens* isolate Pf1 mediated induction of the roots, localized induction of tomato leaf tissue by tobacco necrosis virus), but none were capable to provide a systemic induction throughout the plant.

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CHAPTER 6

EVALUATION OF *BACILLUS MYCOIDES* ISOLATE BMJ AND *BACILLUS MOJAVENSIS* ISOLATE 203-7 FOR THE CONTROL OF ANTHRACNOSE OF CUCURBITS CAUSED BY *GLOMERELLA CINGULATA* VAR. *ORBICULARE*

Abstract

Bacillus mycoides isolate BmJ (BmJ) and *Bacillus mojavensis* isolate 203-7 (203-7) were tested in the greenhouse for their ability to control *Glomerella cingulata* var. *orbiculare* the causal agent of anthracnose of cucumber by induced systemic acquired resistance (SAR). BmJ and 203-7 delayed disease onset and reduced total (43 % and 56 %) and live spore production (38 % and 49 %) per mm ^{2} of lesion area when used to induce SAR in cucumber. 203-7 also reduced lesion diameter. Induction by *G. cingulata* conidia resulted in delayed disease onset, reduction of number of lesions per leaf and lesion diameter. Assays of cucumber apoplastic proteins extracted 6 days after induction showed that BmJ increased β-1,3-glucanase activity by 135 %, and 203-7 increased β-1,3-glucanase activity by 72 % and peroxidase activity by 79 % when compared to the water control. Acibenzolar-S-methyl induced the highest (P=0.05) levels of chitinase (950 %) and peroxidase (420 %) activity. 2004 and 2005 field experiments evaluated applications of BmJ and fungicides for the control of anthracnose in cucumber (var. 'General Lee') and cantaloupe (var. 'Athena'). BmJ was compared to full and half labeled rate alternate applications of azoxystrobin and chlorothalonil, and BmJ with half

rate of azoxystrobin and chlorothalonil. BmJ applied seven days before inoculation provided disease reduction of 41 % in cucumber in 2004 and 24 to 21 % reduction in cantaloupe for both years which was statistically equal to the fungicide treatments. The full and half rate fungicide program provided the best disease control (P=0.05) in both years (97 to 37 %). BmJ applied one week before inoculation reduced AUDPC in cucumber compared to the water control in 2004 in cantaloupe for both years while the full and half rate fungicide program were equivalent and provided the lowest AUDPC. No yield reduction was noted as a result of the disease or treatment for either cantaloupe or cucumber.

Introduction

Bacillus-based biological controls have become important tools for control of pests and diseases affecting agricultural and horticultural production (Jacobsen et al., 2004, Jacobsen, 2006). Some researchers (Jetiyanon and Kloepper, 2002; Jetiyanon et al., 2003; Kloepper et al., 2004; Mahaffee and Backman, 1993; McSpadden-Gardner and Driks, 2004; Raupach and Kloepper, 1998; Raupach and Kloepper, 2000; Turner and Backman, 1991; Wei et al., 1991; Wei et al., 1996) have focused on *Bacillus* - based plant growth-promoting rhizobacteria (PGPR) seed or root treatments while others have focused on foliar or post harvest disease control (Jacobsen, 2006). Foliar applications of *Bacillus mycoides* isolate BmJ (Bargabus et al., 2002) provided 38-91% control of Cercospora leaf spot (*Cercospora beticola* Sacc.) of sugar beet in a six year study using susceptible sugar beet varieties. Jacobsen et al. (2004) showed that BmJ provides control

of Cercospora leaf spot equivalent to the standard fungicides on sugar beet varieties with moderate levels of resistance. Bargabus et al. (2002, 2003, 2004) and Bargabus-Larson and Jacobsen (2007) showed that *B. mycoides* isolate BmJ and *B.* (*pumilus*) *mojavensis* isolate 203-7 reduced Cercospora leaf spot and bacterial vascular necrosis (*Erwinia carotovora* pv. *betavasculorum*) of sugar beets by inducing systemic acquired resistance (SAR). Systemic acquired resistance has been demonstrated to control *Colletotrichum orbiculare* (Berk. & Mont.) Arx (*Colletotrichum lagenarium* (Pass.) Ellis & Halst) on cucumber (*Cucumis sativus* L.) by induction of lower leaves by foliar applications of conidial suspensions of *C. orbiculare* (Dean and Kuc, 1986; Kubota and Abiko, 2000). Applications of low concentrations of chemical inducers such as Actigard (acibenzolar-S-methyl), DCINA (dichloroisonicotinic acid), salicylic acid, and dibasic potassium phosphate Lopez and Lucas (2002) provided good control of *Colletotrichum gloeosporioides*, the causal agent of anthracnose of cashew (*Anacardium occidentale*) without phytotoxic effects. The ability of dibasic potassium phosphate to control *C. orbiculare* in cucumber was further investigated by Irving and Kuć (1990), and by Orober et al. (1999, 2002). Only Orober et al. (2002) reported hypersensitive-reaction like lesions on leaves previously treated with dibasic potassium phosphate. In comparison to phosphate and acibenzolar-S-methyl (Baysal et al., 2003; Brisset et al., 2000; Lemay et al., 2002; Maxson-Stein et al., 2002), *B. mycoides* isolate BmJ induces SAR without causing hypersensitive cell death in sugar beet (Bargabus et al., 2003) and other plants such as pepper, tomato, potato, geranium, and cucumber (Jacobsen, unpublished).

The objective of this research was to test *B. mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 for their ability to control anthracnose of cucumber and cantaloupe (*Cucumis melo* L.) caused by *Glomerella cingulata* var. *orbiculare*, the teleomorph of *Colletotrichum orbiculare* (Berk. & Mont.) Arx, and to further investigate the potential to integrate BmJ with low rates of fungicides in field studies.

Materials & Methods

Bacterial Cultures

B. mycoides isolate BmJ (BmJ) was originally isolated from sugar beet leaves. *B. mojavensis* isolate 203-7 (203-7) originally isolated from sugar beet seed embryos was included in the greenhouse experiments since it showed good induction of SAR in previous experiments (Bargabus et al., 2004). Both isolates were stored at -80 °C in 10 % glycerol and 1 % tryptic soy broth (TSB, EMD Chemicals Inc., Darmstadt, Germany). Bacteria were cultured in 3 % TSB for 24 h at room temperature (22 °C) on an orbital shaker (Model OS-500, VWR International, West Chester, PA) at 250 rpm. Fresh cells were harvested by centrifugation for 20 min at 5,000 rpm at 4 °C. The pellet was re-suspended in sterile-distilled water and pelleted twice by centrifugation for 20 min at 5,000 rpm at 4 °C to assure that all fermentation beer was separated from the cells. The inoculum density was adjusted to 10^8 colony forming units (CFU)/ml with distilled water for greenhouse and field experiments.

Fungal Culture

A Montana isolate of *Glomerella cingulata* var. *orbiculare* (isolate MT-1) was maintained for long-term storage at - 80 °C as described above. For the experiments the cultures were transferred to potato dextrose agar (PDA, EMD Chemicals Inc., Darmstadt, Germany) and incubated for 1 week at 28 °C in the dark. Cultures were scraped with a sterile glass rod to loosen conidia. The plates were subsequently flooded with sterile-distilled water and the conidia suspension was decanted and filtered through two layers of cheesecloth to remove mycelium and agar pieces. Inoculum density was adjusted to 10^5 conidia/ml for the greenhouse and field experiments.

Chemical Inducer and Fungicides

Acibenzolar-s-methyl (ASM) (Actigard 50WG Fungicide, Syngenta, Greensboro, NC) was used as the chemical inducer in the greenhouse experiments at a rate of 50 µg/ml and was applied using a Crown aerosol sprayer (Aervoe Industries Inc., Gardnerville, NV) with applications made to run-off.

For the field experiments, the fungicide treatments consisted of alternate applications of azoxystrobin (Quadris, Syngenta, Greensboro, NC) at a rate of 1.08 or at 0.54 kg of product/ha and chlorothalonil (Bravo WeatherStik, Syngenta, Greensboro, NC) at 2.20 or 1.10 kg of product/ha. Fungicides and BmJ were applied with a CO_2 pressurized (207 kPa) sprayer outfitted with Teejet 8002VS nozzles (Spraying Systems Co., Wheaton, IL) with a total spray volume of 234 L/ha.

Greenhouse Experiments: Plant
Culture, Treatments and Inoculation

Cucumber plants (*C. sativus* L. variety 'General Lee') were grown in 10 x 10 x 10cm plastic pots filled with equal parts (by volume) of PGC Soil Mix (1/3 loam soil, 1/3 washed concrete sand, 1/3 Canadian Sphagnum peat moss plus AquaGro 2000 G [Aquatrols, Paulsboro, NJ] wetting agent, aerated steam pasteurized at 80 °C for 45 min) and Sunshine Mix #1 (Sun Gro Horticulture Inc., Bellevue, WA). Plants were grown for 3 weeks or until the first true leaf was fully expanded under greenhouse conditions at 24 ± 2 °C day and 18 ± 2 °C night temperature with a 16 h photoperiod. Supplemental lighting was provided by SON AGRO 430 WATT high pressure sodium lights (Philips Lighting Company, Somerset, NJ). To maintain vigorous growth, plants were watered daily and fertilized twice a week with Peters Professional 20-20-20 General Purpose (The Scotts Company, Marysville, OH) at a rate of 200 µg/ml nitrogen.

The first true leaves of 10 cucumber plants per replicate were induced with either distilled water, BmJ, 203-7, or MT-1.

The bacterial suspensions at 1 x 10^8 cfu/ml as well as the sterile-distilled water were sprayed using a Crown aerosol sprayer (Aervoe Industries Inc., Gardnerville, NV) onto the first true leaves until run-off while covering the rest of the plant with a plastic bag. For the pathogen induced resistance treatment, the first true leaf was inoculated with 10 evenly spaced 10 µl drops of MT-1 conidia suspension adjusted to 10^5 conidia/ml. Plants were placed in a dew chamber at 24 ± 2 °C and 100 % relative humidity (RH) for 48 h, and then transferred to the greenhouse.

Five days after the induction, the second true leaves of all plants were challenge inoculated with the anthracnose pathogen as described above and placed in a dew chamber for 48 h at 24 ± 2 °C under 100 % RH. The experimental design was a randomized complete block with 10 replications per treatment. The experiment was repeated three times.

Plants were examined daily for disease development. The number of lesions was counted 10 days post inoculation and the lesion diameter was recorded in millimeters by making two perpendicular measurements and taking their average.

Two weeks post inoculation plants were placed in a dew chamber for 48 h at 24 ± 2 °C under 100 % rh to induce sporulation. After removal from the dew-chamber, two lesions were removed from each cucumber leaf and placed into a test tube filled with 10 ml of sterile-distilled water. The samples were vortexed (Fisher Vortex Genie 2, Fisher Scientific, Pittsburgh, PA) for 10 s at highest speed and the lesion bearing tissue was removed from the water. Number of conidia per ml was estimated using a hemacytometer (Hausser Scientific, Horsham, PA). The number of conidia per ml and the lesion area were used to calculate the conidia per square millimeter of lesion area. Samples of this conidial solution were also plated onto PDA supplemented with 50 µg/ml tetracycline hydrochloride (OmniPur*, EMD Chemicals Inc., Darmstadt, Germany) with a spiral plater (model D, Spiral Biotech Inc., Norwood, MA.). Developing colonies were counted after 4 days to estimate the number of viable conidia per lesion area.

For all described experiments, collected data were analyzed statistically by conducting an analysis of variance (Madden et al., 1982) using the general linear model

procedure of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at P=0.05.

Apoplastic Fluid Extraction
and Protein Quantification

The first true leaf of three- to four-weeks-old cucumber plants were induced, in replicates of five, with distilled water, BmJ, 203-7, ASM and *G. orbiculare* as described above. Plants were placed for 48 h in a dew chamber at 24 ± 2 °C and 100 % RH, and then transferred to the greenhouse. Plants were kept under greenhouse conditions as described above. Six days post induction the second true leaf and the consecutive leaves of each replication were collected and submerged in chilled distilled water on ice. Leaves were then transferred to a filtering flask containing 150 mM NaCl and 25 mM MES in double-distilled water at pH 6.2. A vacuum was applied for 15 min and the buffer was forced into the leaf tissue by releasing the vacuum and swirling the flask content every 3 min. Leaves were removed from the flask, blotted dry between paper towels and rolled into 20 ml syringe barrels with petioles facing the syringe tip. Samples were then centrifuged at 2000 rpm for 10 min at 4 °C. Apoplastic fluid was collected in a 2 ml microcentrifuge tube and frozen at -80 °C until analyzed. Protein amount was quantified using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) per manufacturer's instructions using bovine serum albumin (EMD Chemicals Inc., Darmstadt, Germany) as standards.

Chitinase and β-1,3-glucanase
Activity Plate Assay

Chitinase activity was measured using the method described by Bargabus et al. (2002). Three 3-mm wells per sample and standard were made in a 14-cm glass Petri dish containing 30 ml of sodium phosphate buffer (100 mM at pH 5.0) with 0.3 ml of 0.1 % glycol chitin and 1 % agarose (OmniPur*, EMD Chemicals Inc., Darmstadt, Germany). Wells were filled with 4 µl of apoplastic fluid or chitinase standards (0.00484 units/µl, 0.0484 units/µl, and 0.484 units/µl, *Streptomyces griseus*, Sigma). Plates were incubated overnight at 37 °C and then flooded with 50 ml of 0.01 % calcofluor white (Fluorescent Brightener 28, Sigma) in 500 mM Tris-HCl (pH 8.9). Following incubation on an orbital shaker at 100 rpm for 10 min, plates were rinsed multiple times with distilled water, flooded and destained overnight. Under UV light (365 nm) the non-fluorescence area of enzyme activity around each well was recorded in millimeters by averaging two perpendicular measurements of the diameter. Specific activity of protein samples in apoplastic fluid (mg of *N*-acetyl-D-glucosamine released /24h/ug of apoplastic protein) was estimated by comparison to the non-fluorescence zones produced by the chitinase standards. The R^2 for the regression of the chitinase standards versus the non-fluorescence zones was 0.9856.

β-1,3-glucanase activity was determined using the method described by Bargabus et al. (2004) with following modifications: Three 3-mm wells per sample and standard were made in a 14-cm glass Petri dish containing 40 ml of 0.1 M citrate buffer (pH 4.8) with 1 % agarose, 0.05 % laminaran (from *Eisenia bicyclis*, TCI , Portland, OR), and 0.05% CM-Pachyman (Megazyme International Ireland Ltd, Ireland). Wells were filled

with 4 µl of apoplastic fluid or the laminarinase standards (0.00025 units/µl, 0.00125 units/µl, and 0.0025 units/µl, from *Trichoderma* sp., Sigma). Plates were incubated for 24 h at 37 °C, then flooded with 0.1 % Congo red (Sigma) solution and incubated overnight on an orbital shaker at 100 rpm. Gels were destained by rinsing with 1 M sodium chloride solution and the area of enzyme activity indicated by a cleared zone around the wells was determined as described above. The specific activity of apoplastic fluid (mg of glucose released/24h/ug of apoplastic protein) was estimated by comparing the cleared zone diameter to that produced by the laminarinase standards. The R^2 for the regression of the laminarinase standards versus the cleared zones was 0.9987.

Peroxidase Activity Assay

Peroxidase activity was determined by measuring the oxidation of guaiacol to tetra-guaiacol based on the protocols of Chance and Maehly (1955) and Geng et al. (2001). The reaction mixture contained 166.7 µl diluted apoplastic fluid sample (1:1000 with 0.1 M sodium phosphate buffer [NaH_2PO_4/NaOH, pH 6.7]) and 62.5 µl guaiacol solution (Sigma, 1% v/v in 0.1 M sodium phosphate buffer). The reaction was started by adding 20.8 µl hydrogen peroxide solution (Sigma, 0.3 % v/v in distilled water) to the mixture and the change in absorbance was measured at 470 nm over a 5 min period with a SpectraMax plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA) running the kinetics program. The specific activity (enzyme activity in units/mg of apoplastic protein) of the apoplastic fluid samples was determined by comparison to horseradish peroxidase standards (3×10^{-3}, 1.8×10^{-3}, and 6×10^{-4} units/well, Calbiochem, Darmstadt,

Germany). The R^2 for the regression of the horseradish peroxidase standards versus the absorbance at 470 nm over a 5 min period was 0.9984.

Field Experiments: Plant
Culture, Treatments and Inoculation

Field experiments were conducted at the Montana Agricultural Experiment Station Southern Agricultural Research Center at Huntley, MT in 2004 and 2005. Cucumber plants (variety 'General Lee') and cantaloupe plants (*C. melo* L. variety 'Athena') were started in 10 x 10 x 10 cm peat pots filled with Sunshine Mix #1 and grown for 4 weeks under conditions described above. Plants were transplanted (15 June 2004, 02 June 2005) into beds covered with infrared transmissible (IRT 76) mulch film and irrigated with drip tape (model T-Tape®, Dripworks, Willits, CA). Plants were spaced 0.45 m apart and plots were 6 m long and separated by a 0.9 m buffer zone between plots within the row. The experimental design was a randomized complete block design with six replications.

Treatments included a water control applied one week prior to pathogen inoculation and at one week intervals, BmJ applied at time of pathogen inoculation and at one week intervals, BmJ applied one week prior to inoculation and at one week intervals, alternating fungicide treatments of azoxystrobin (1.079 kg of product/ha) and chlorothalonil (2.242 kg of product/ha) on a weekly schedule, BmJ one week prior to inoculation followed by half the application rate of the weekly alternating fungicides, and half the rate of the fungicides alone at one week intervals. Due to spatial restrictions only BmJ could be included into the field experiments. All treatments were applied using a

CO_2 pressurized (207 kPa) sprayer with a total spray volume of 234 L/ha. After cucumber and cantaloupe plants were well established, they were challenged (27 July 2004, 13 July 2005) with MT-1 by spraying the plants to run-off with a suspension containing 2.5 x 10^5 conidia/ml and 0.05 % Silwet L-77 surfactant (Helena Chemical Company, Collierville Tennessee). The challenge inoculation was followed by an overhead irrigation to wet the plants and to increase the humidity. Plots were overhead irrigated at least twice a week to facilitate infection and to promote spread via conidia splash.

The first BmJ treatments were applied one week prior (20 July 2004, 6 July 2005) to the pathogen inoculation. The first fungicide sprays and the second BmJ sprays were applied one day after inoculation with MT-1. All successive spray treatments were applied weekly for four weeks (until 24 August 2004 or 10 August 2005).

Starting three weeks after inoculation with MT-1, weekly yield data were collected by harvesting and weighing cucumbers ranging in size from 5 to 20 cm. Disease data were collected weekly over a three week period beginning five weeks post inoculation by counting lesions on 25 randomly chosen individual leaves per plot. By combining three weeks of disease ratings in the cucumber plots, the area under the disease progress curve (AUDPC, Bjarko and Line, 1988) was calculated. Data was analyzed for homogeneity over years using Levene's test and treatment effects were analyzed statistically by conducting an analysis of variance (Madden et al., 1982) using the general linear model procedure of the SAS program (SAS system, Version 9.00, SAS

Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at P=0.05.

Results

Greenhouse Experiments

The disease onset on cucumbers was observed as faint necrotic lesions 2-3 days after inoculation. Anthracnose lesions on plants treated with BmJ and 203-7, or on MT-1 self-induced plants appeared on average 12 to 18 h later than on plants treated with sterile-distilled water (Table 6.1).

Plants self-induced with MT-1 developed significantly (P=0.05) fewer lesions per leaf when compared to the sterile-distilled water treatment, but were not significantly different from the bacilli treatments. There were no significant differences among the bacilli treatments and plants treated with sterile-distilled water in regard to the number of lesions developed per leaf (Table 6.1).

Significant differences between the treatments were observed for conidia production per mm^2 of lesion area (Table 6.1). Lesions from plants induced with MT-1, BmJ and 203-7 yielded significantly (P=0.05) fewer conidia per mm^2 of lesion area when compared with the sterile-distilled water treated controls. The total number of conidia counted using the hemacytometer was considerably higher than the colony forming units counted after spiral plating. 203-7-treated plants had significantly (P=0.05) lower counts of colony forming units when compared to plants treated with sterile-distilled water alone, but were not significantly different from BmJ- or MT-1-treated plants. A reduction of colony

forming units by 49 % was observed for 203-7-treated plants, as was a 38 % and 35 % reduction for BmJ and MT-1.

Plants treated with sterile-distilled water alone or BmJ showed no significant differences in lesion diameter, whereas lesions on plants treated with 203-7 or self-induced with MT-1 exhibited significantly smaller lesion diameter compared to BmJ and the sterile-distilled water treated controls (12 % or 17 %).

Plants treated with BmJ and 203-7 had significantly fewer conidia (43 % and 56 %) and lower colony forming units (38 % and 49 %) per mm^2 of lesion area in comparison to plants treated with sterile-distilled water alone. Plants self-induced with MT-1 were not significantly different from the bacilli treatments for total and viable conidia production or from the sterile-distilled water treatment in regard to reduction of viable conidia (Table 6.1).

Table 6.1: Effect of *B. mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 induced resistance on latent period, lesion number, and size, and on spore production of *G. cingulata* var. *orbiculare* isolate MT-1 on cucumbers variety 'General Lee'.

Treatment	Disease onset (days)	Number of lesions per leaf	Lesion diameter in mm	Conidia per mm^2 of lesion area (hemacytometer)	(cfu) [z]
Sterile-distilled water	4.00 b [y]	9.83 a	7.15 a	3.56×10^5 a	2.11×10^4 a
B. mycoides isolate BmJ	4.50 a	9.71 ab	6.86 a	2.04×10^5 b	1.30×10^4 b
B. mojavensis isolate 203-7	4.67 a	9.75 ab	6.29 b	1.54×10^5 b	1.08×10^4 b
G. cingulata isolate MT-1	4.54 a	9.25 b	5.91 b	1.84×10^5 b	1.37×10^4 ab

[z] Colony forming units (cfu) determined by plating conidia solution onto PDA supplemented with 50 µg/ml tetracycline with a spiral plater, counting developing colonies after 4 days, and calculating the number of colony forming units per mm^2 of lesion area.

[y] Means followed by the same letter are not significantly different according to Fisher's protected LSD (P=0.05).

Chitinase, β-1,3-glucanase
and Peroxidase Activity Assays

Plants treated with the chemical inducer ASM had significantly higher (P=0.05) chitinase activity than other treatments and showed an increase of enzyme activity by 950 % (Table 6.2). Chitinase activity in MT-1-induced plants (170 %) was significantly lower than ASM-treated plants but higher than sterile-distilled water (P=0.05). The chitinase activity of plants sprayed with BmJ and 203-7 showed an increase of activity by 105 % for BmJ and 62 % for 203-7, but was similar to plants induced with MT-1 or sterile-distilled water (P=0.05).

The highest (P=0.05) β-1,3-glucanase activity was found in plants induced with BmJ (135 %). Plants induced with 203-7 and ASM were significantly lower than the BmJ-treated plants, but showed elevated levels of 72 % and 49 %. All plants induced with BmJ, 203-7, or ASM had significantly higher β-1,3-glucanase activity when compared to plants treated with sterile-distilled water (Table 6.2).

Acibenzolar-S-methyl induced the highest (P=0.05) peroxidase activity (420 %) in comparison to the other treatments. Plants treated with 203-7 or MT-1 showed a significant increase of 79 % or 54 % in peroxidase activity when compared to plants treated with sterile-distilled water alone. The BmJ treatment did not result in an increase in peroxidase activity when compared to the sterile-distilled water control (P=0.05).

Table 6.2: Chitinase, β-1,3-glucanase and peroxidase activity assays for apoplastic fluids of cucumbers, extracted after foliar treatments with of *B. mycoides* isolate BmJ and *B. mojavensis* isolate 203-7.

Treatment	Chitinase assay [z]	β-1,3-glucanase assay [y]	Peroxidase assay [x]
Distilled water	0.943 c[w]	0.423 d	17.760 c
B. mycoides isolate BmJ	1.936 bc	0.995 a	17.839 c
B. mojavensis isolate 203-7	1.528 bc	0.727 b	31.835 b
Acibenzolar-S-methyl	9.902 a	0.632 bc	92.379 a
G. cingulata isolate MT-1	2.546 b	0.481 cd	27.426 b

[z] Specific enzyme activity in mg of N-acetyl-D-glucosamine released/24h/µg of apoplastic protein [v].
[y] Specific enzyme activity in mg of glucose released/24h/µg of apoplastic protein [v].
[x] Specific enzyme activity in units/mg of apoplastic protein [v]compared to the horseradish peroxidase standard.
[w]Means followed by the same letter are not significantly different according to Fisher's protected LSD (P=0.05).
[v] Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

Field Experiments

Anthracnose disease was observed in the field two weeks after the challenge inoculation in both years. In the first year (2004) of this study, the plots showed low levels of disease at which the distilled water control exhibited on average 0.2 lesions per leaf with a range of 0 to 2 lesions. In 2005, the plots had a moderate to high level of disease with the distilled water control showing on average 1.5 lesions per leaf with a range of 0 to 24 lesions at the first rating to an average of 7 lesions with a range of 2 to 26 lesions per leaf at the last rating. The area under the disease progress curve (AUDPC) of

all fungicide treatments and the integrated treatment were significantly different (P=0.05) from the distilled water control in both years for cucumber (Table 6.3). At P=0.05, the AUDPC for BmJ applied one week prior to inoculation was significantly different and showed a 41 % reduction from plants sprayed with distilled water for cucumber in 2004. In 2005, BmJ applied at inoculation had a 35 % lower AUDPC than distilled water at P=0.1. There were no significant AUDPC differences in timing of BmJ treatments over the two years on cucumber (P=0.05). Data were homogeneous over the two years of experiments.

For the cantaloupe experiments, the AUDPC for all treatments were significantly (P=0.05) different from the distilled water control. Both BmJ treatments were significantly different from the distilled water control but not from each other while BmJ applied one week before inoculation had the lowest AUDPC in 2004 but not 2005. BmJ applied one week before inoculation was not significantly different to both full and half rate fungicide treatments at P=0.05 in 2004 (Table 6.3). Data were not homogenous over the two years of experiments.

In both cucumber and cantaloupe plots, the full and half rate fungicide treatments provided the best control as measured by the AUDPC and showed a reduction of 97 to 36 %, but were not statistically different from the AUDPC calculated for plants treated with BmJ in combination with half rate fungicides or plants treated with BmJ one week before inoculation in 2004 but not 2005. There was no difference in either cucumber or cantaloupe yield between any of the treatments and the untreated control at any time (Table 6.3).

Table 6.3: Effect of weekly applications of *Bacillus mycoides* isolate BmJ (BmJ) , azoxystrobin [z] alternated with chlorothalonil [y] and an integrated BmJ/half rate fungicide treatment on anthracnose disease severity of cucumber variety 'General Lee' and cantaloupe variety 'Athena' in 2004 and 2005.

Treatment	AUDPC [x] for cucumber		AUDPC [x] for cantaloupe	
	2004	2005	2004	2005
Distilled water…….	92.60	103.79	24.27	63.75
BmJ applied at inoculation……..	75.63	67.97	19.37	42.51
BmJ applied 1 wk before inoculation……..	54.83	85.70	18.53	49.70
Azoxystrobin (1.08kg/A) alternated with chlorothalonil (2.20 kg/A)……	21.27	3.27	15.26	8.31
BmJ 1 wk before inoculation followed by Azoxystrobin (0.54 kg/A) alternated with chlorothalonil (1.10 kg/A)……	45.77	5.18	17.27	7.19
Azoxystrobin (0.54 kg/A) alternated with chlorothalonil (1.10 kg/A)…….....	37.03	3.52	15.45	9.80
LSD (0.05) [w]	34.82	40.13	3.27	10.76
LSD (0.1) [v]	34.58	33.28	2.74	9.03

[z] Azoxystrobin at 1.08kg or 0.54 kg of product/A (Quadris, Syngenta, Greensboro, NC).
[y] Chlorothalonil at 2.20 kg or 1.10 kg of product/A (Bravo WeatherStik, Syngenta, Greensboro, NC).
[x] Area under disease-progress curve (AUDPC) calculated for three leaf lesion ratings.
[w] LSD = least significant difference (probability = 0.05).
[v] LSD = least significant difference (probability = 0.1).

Discussion

The observed reduction in anthracnose development on cucumber plants treated with *B. mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 is likely due to the induction of SAR based on the separation of bacillus induced leaves from pathogen challenged leaves in greenhouse experiments. Estimates of protein activity for apoplastic fluid indicates that PR-proteins (chitinase, β-glucanase, and peroxidase) commonly associated with SAR were present in elevated levels in leaves distal to those induced by the bacilli. This study also demonstrates that foliar applications of BmJ and 203-7 are able to reduce not only Cercospora leaf spot and bacterial vascular necrosis of sugar beets (Bargabus et al. 2002, 2003), but also anthracnose in cucumber by inducing SAR.

Both bacilli treatments showed similar increases in chitinase activity (105 % for BmJ and 62 % for 203-7) which were not significantly different, but still twice the activity of the sterile-distilled water control. BmJ-treated plants showed higher β-1,3-glucanase activity (135 %), whereas plants sprayed with 203-7 had a79 % increase in peroxidase activity when compared to the sterile-distilled water control. These differences in PR-protein activity might result from the activation of separate pathways in the plant defense system. Bargabus-Larson and Jacobsen (2007) showed that SAR induced by BmJ in sugar beets is independent of the salicylic acid pathway, but depends on the activation of the non-expresser of pathogenesis-related genes (NPR1).

Other evidence for SAR induction by BmJ and 203-7 are the delays in disease onset, and the reductions in the number of total conidia and colony forming units per mm^2 of lesion area. Induction by 203-7 and MT-1 significantly reduced the size of

lesions (12 % and 17 %) when compared to the sterile-distilled water control. The data collected for disease onset and lesion diameter were similar to earlier findings of Dean and Kuć (1986) showing that plants protected by systemic resistance expressed a delayed disease onset and had smaller lesions than unprotected plants. A reduction of viable spores was also observed by Zhang et al. (2002) who reported a significant decrease of *Peronospora tabacina* sporangia on tobacco cv. Xanthi-nc after treatments with five different PGPR strains.

Two field experiments were performed to test the ability of BmJ to control anthracnose of cucumber under different environmental conditions. While environmental conditions during the course of the experiments in 2004 and 2005 were similar, disease severity was higher on both cucumber and cantaloupe in 2005. It was expected based on applications of BmJ for control of Cercospora leaf spot of sugar beet (Bargabus et al. 2002), that BmJ applications four to seven days prior to inoculation would provide the best disease control. BmJ treatments applied one week prior to inoculation significantly reduced the area under disease-progress curve (AUDPC) by 41 % in 2004 for cucumber and by 24 to 22 % in both years for cantaloupe when compared to the distilled water control while the AUDPC for BmJ applied at inoculation was statistically similar to BmJ applied seven days before inoculation over both years on both crops. Bargabus et al. (2002) showed that there is a five to seven day period between application of BmJ and maximum level of PR-protein expression in sugar beet. Parallel studies for PR-protein expression have not been done on cucumber or cantaloupe. The minimal differences between 'BmJ one week prior to inoculation' and 'BmJ at inoculation' could be

explained by delayed disease development related to unfavorable environmental conditions following inoculation since symptoms appeared two weeks after challenge inoculation in the field compared to the typical four to five days seen in greenhouse experiments. This delay would allow the plant to express elevated levels of PR-proteins in both of these BmJ treatments. The percentage anthracnose disease reduction by BmJ treatments on cucumber ranged from 41-18 % on cucumber and 24-22 % on cantaloupe. This is compared to 91-38 % reductions of Cercospora leaf spot of sugar beet observed over six years (Bargabus et al., 2002). On pecan scab in a single year of observation BmJ reduced disease severity by 62 % and in two location years BmJ reduced the severity of early blight of potato by 63-50 % (Jacobsen et al., 2007). These reductions are lower and more variable than those of fungicide treatments in these experiments where fungicides reduced disease by 97-77 % on cucumber and 87-38 % on cantaloupe. On sugar beet fungicides resulted in 97-27 % control (Bargabus et al., 2002) while fungicides reduced the severity of pecan scab by 100-68 % and potato early blight by 95-70 % .

While antibiosis cannot be excluded as a mode of action for BmJ or 203-7 in controlling anthracnose in the field, the greenhouse SAR experiments discussed previously and the use of washed bacillus cells free of fermentation beer suggest that SAR is likely involved as the mode of action in the field experiments. Antibiosis depends strongly on the nutrients existing in/on the substrate (Jacobsen, 2006). *In vitro*, BmJ produces antimicrobial compounds only in small amounts (Jacobsen unpublished), depending on the available nutrients. *In planta*, as described earlier, BmJ was applied as washed cells in distilled water only so that no additional nutrients or metabolites found in

fermentation beer were applied to the plants. Also antibiotic production in the phyllosphere is not likely since colony forming units of both 203-7 and BmJ decline linearly from 10^8 to 10^1 cfu over 14 days (Neher, unpublished). It might be possible that other environmental factors (irrigation water, heat stress, pathogen infection) also induced systemic resistance in plants grown under field conditions, but they would have affected all plant treatments equally.

The combination of a synthetic fungicide with a biological control agent can reduce the variability in disease control associated with the use of biological control agents used alone (Jacobsen 2006). It also offers the ability to reduce fungicide rates without compromising control, as shown by Buck (2004) and Buck and Jeffers (2004). The combination of BmJ and half rate fungicides was not as effective as expected from earlier studies, where Jacobsen et al. (2004) demonstrated that a combination of BmJ and tetraconazole provided significantly increased control of Cercospora leaf spot of sugar beet. It would be important to repeat the integrated treatment with even lower concentrations of fungicides, since the half rate fungicide treatment provides the same level of control as the full rate treatment and therefore no conclusion can be drawn regarding the effectiveness of the integrated treatment. Nevertheless, this is important information from the perspective that it offers the opportunity to reduce fungicide use for control of anthracnose of cucurbits.

The findings of the greenhouse and field experiments support the conclusion that *B. mycoides* isolate BmJ may represent an important tool for integrated management due to its ability to control cucurbit anthracnose by delaying disease onset and reducing the

production of colony forming units by induction of SAR. The reduction in number of colony forming units per mm^2 lesion area and the resulting reduction of secondary inoculum on induced plants is potentially of great importance, since this could reduce the rate of an epidemic development for a polycyclic disease such as anthracnose (Madden et al., 2007). This is demonstrated by the reduced AUDPC for biological control treatments.

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7. SUMMARY

This dissertation showed that systemic resistance induced by *Bacillus mojavensis* isolate 203-7 and measured by disease control of *Botrytis cinerea* and induction of PR-proteins is controlled by salicylic acid (SA) and non-expresser of pathogenesis-related proteins gene1 (NPR1) independent pathways, but jasmonic acid (JA) dependent pathways without the involvement of ethylene (ET).

This conclusion is supported by the significant disease reduction and the increase in enzyme activity observed in *Arabidopsis thaliana npr1-1*, *ndr1-1/npr1-2*, *NahG*, and *ein2-1* mutants. The defense pathway stimulated by 203-7 appeared to be JA dependent as evidenced by reduced disease control and decreased enzyme activity in *jar1-1* mutants when compared to the chemical inducer methyl jasmonate. A similar induction in Arabidopsis via the JA dependent, SA- / NPR1-independent pathway was observed for the gram-negative plant growth-promoting rhizobacteria *Serratia marcescens* strain 90-166 which required, in contrast to 203-7, responsiveness to JA and ET (Pieterse et al., 1998).

The findings reported by Bargabus-Larson and Jacobsen (2007) that the induction of systemic resistance in sugar beets via foliar applications of *Bacillus mycoides* isolate BmJ is SA independent, but is regulated by NPR1 genes, was confirmed and even extended for the Arabidopsis system where BmJ involvement in the ethylene pathway was shown. The induction by BmJ resulted in significantly increased disease control and chitinase, β-1,3-glucanase, and SOD enzyme levels in *jar1-1* and *NahG* mutants. Arabidopsis *npr1-1* and *ndr1-1/npr1-2* mutants induced with BmJ showed an increase in

disease caused by *B. cinerea* and also expressed significantly lower levels of enzyme activity, indicating a regulation of the tested PR-proteins by the NPR1 genes. Surprisingly, the Arabidopsis *ein2-1* mutant also showed a decrease in disease control and enzyme activity, supporting the conclusion that the pathway induced by BmJ is ET dependent. It is proposed that defense responses activated by foliar applications of BmJ are SA independent, but dependent on ET and NPR1 regulated pathways.

The induction of pathogenesis-related (PR) proteins such as chitinase and β-1,3-glucanase and the resulting disease control of *B. cinerea* appears to depend strongly on the applied dose of the biological or chemical inducers. 203-7 and BmJ applied at lower concentrations (similar to a 3 leaves application) had the highest disease reduction of *B. cinerea* leaf spot of all treatments when compared to the water control. However, the chemical inducer Acibenzolar-s-methyl (ASM) when applied at lower concentrations similar to 3 leaves only showed less disease reduction when compared to the higher concentration or whole plant application. The non-significant differences between the induced and tested PR-proteins of plants treated with the BCAs, suggest however, that other than the tested PR-proteins are expressed and might be involved in the disease reduction.

Similar observations could be made for the control of Botrytis grey mold (*B. cinerea*) in greenhouse tomatoes and for the control of anthracnose of cucurbits (*Glomerella cingulata* var. *orbiculare*). In the case of BmJ used to control Botrytis grey mold, the tested PR-proteins showed only an insignificant increase of chitinase and SOD activity when compared to the water control. Nevertheless, the BmJ treatment resulted in

significant reduction of leaf lesions and stem cankers caused by Botrytis without producing abundant antimicrobial compounds. Since only chitinase, β-1,3-glucanase, and SOD activity and no other PR-proteins such as defensins were tested, an induction of systemic resistance cannot be ruled out. As previously described in chapters 3 and 4, BmJ is able to control *B. cinerea* by inducing the ET dependent pathway resulting in SAR in *A. thaliana* plants. Díaz et al. (2002) showed that the ethylene dependent pathway is important for the resistance of tomato plants to *B. cinerea*, since this pathway may induce PR-proteins and phytoalexins (Fan et al., 2000; Rodrigo et al., 1993).

203-7 treatment resulted in a significant increase in disease reduction as measured by the reduction of leaf lesions caused by *B. cinerea*. However the increase in chitinase, β-1,3-glucanase, and SOD suggest a disease control by means of SAR.

The observed control of anthracnose measured as delay in disease onset, reduction of lesion size, reduction of total conidia count, and colony forming units per mm^2 of lesion area on cucumber plants treated with BmJ and 203-7 is likely due to the induction of SAR based on the separation of bacillus induced leaves from pathogen challenged leaves in greenhouse experiments. This conclusion is supported by the estimates of protein activity for apoplastic fluid indicating that PR-proteins (chitinase, β-glucanase, and peroxidase) commonly associated with SAR were present at elevated levels in leaves distal to those induced by the bacilli. The data collected for disease onset and lesion diameter were similar to earlier findings of Dean and Kuć (1986), showing that plants protected by systemic resistance expressed a delayed disease onset and had smaller lesions than unprotected plants. A reduction of viable spores was also observed by

Zhang et al. (2002) who reported a significant decrease of *Peronospora tabacina* sporangia on tobacco cv. Xanthi-nc after treatments with five different plant growth-promoting rhizobacteria (PGPR) strains.

The reduction in number of colony forming units per mm^2 lesion area and the resulting reduction of secondary inoculum on induced plants is potentially of great importance, since this could reduce the rate of an epidemic development for a polycyclic disease such as anthracnose (Madden et al., 2007).

None of the biological control organisms had a negative effect on yield, since the disease pressure was not high enough to cause significant losses, an increase in yield could not be observed for plants treated with the biological control organisms.

The findings presented in this dissertation demonstrate that the differentiation of induced resistance into ISR and SAR needs to be reconsidered. We suggest the term "induced resistance" should be used since the old definitions for SAR and ISR are no longer appropriate as the underlying mechanisms are being revealed by other researchers (Pieterse et al., 1998, Spoel et al., 2003) and this dissertation.

The limitations (especially sample size and number of tested PR-proteins) imposed by measuring the activity of PR-proteins with either gel diffusion assays or colorimetric methods also need to be overcome by using molecular based methods. As described by Desmond et al. (2006) the quantification of mRNA of selected PR-proteins via quantitative real-time PCR (QPCR) provides a powerful tool to assess the induction of multiple PR-proteins in a short period of time. A QPCR method would be especially helpful to determine, if other PR-proteins than the SAR marker genes (PR-1, PR-2, and

PR-5(Ryals et al., 1996; Sticher et al., 1997; Uknes et al., 1992) are being expressed. A disadvantage of this method is that only mRNA is measured and not the levels of specific PR-proteins or defense compounds. If this limitation is overcome, then more in depth studies of the interaction of the different defense pathways, activated by 203-7 or BmJ, could be conducted.

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