Genetic variation of and environmental effects on inducibility of resistance in tomatoes *(Solanum lycopersicum* L.) to *Phytophthora infestans* (Mont) de Bary

Dissertation

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List of Abbreviation

AABA	DL-α-aminobutyric acid/ DL-2-aminobutyric acid
AUDPC	Area under the disease progress curve
BABA	DL-β-aminobutyric acid/ DL-3-aminobutyric acid
BTH	Benzothiadiazole-S-methyl ester (Bion) (ASM)
DAI	Days after inoculation
DF	Degree of freedom
DLA	Diseased leaf area
ET	Ethylene
GLM	General linear model
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
IE	Infection efficiency
INA	2,6-dichloro-isonicotinic acid
IR	Induced resistance
ISR	Induced systemic resistance
JA	Jasmonic acid
PEN	Penicillium extract
PGPR	Plant growth promoting rhizobacteria
PR	Pathogenesis related
PS	Plant strengtheners
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SC	Sporulation capacity

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Zusammenfassung

Wenn Pflanzen durch ein Pathogen attackiert werden, werden eine Reihe von Resistenzmechanismen mehr oder weniger schnell ausgelöst, die entweder das Eindringen oder die weitere Ausbreitung des Pathogens einschränken. Diese Reaktionen werden insgesamt als induzierte oder erworbene Resistenz (IR) zusammengefasst. IR bei Pflanzen kann allerdings nicht nur durch Infektionen sondern auch abiotische Faktoren ausgelöst (induziert) werden. Als Resistenzinduktoren können diverse chemische oder auch natürliche Substanzen, wie z.B. Pflanzenextrakte fungieren. Es gibt auch einige Arbeiten, die belegen, dass die Resistenz von Pflanzen durch das Anbausystem, vor allem den Einsatz bestimmter organischer Dünger gefördert werden kann. Dies ermöglicht es grundsätzlich, Pflanzen durch die Anregung eigener Abwehrmechanismen vor Pathogenen und anderen Schaderregern zu schützen und bietet damit eine Alternative zu den traditionellen Ansätzen im Pflanzenschutz mithilfe von traditionellen Pflanzenschutzmitteln.

Grundsätzlich wird angenommen, dass IR, wenn sie einmal ausgelöst wurde, generell gegen alle Rassen eines Erregers und häufig auch gegenüber einer breiten Palette von Erregern und mitunter auch Insekten wirksam ist. Während sehr viel über die Mechanismen der IR geforscht wurde und wird, weiß man aber nur wenig über die genetische Variation der IR. Es ist grundsätzlich davon auszugehen, dass die Induzierbarkeit von Resistenz wie alle anderen Resistenzmechanismen genetisch verankert ist und damit auch der genetischen Variabilität unterliegt. Damit sollte es möglich sein, für diese Eigenschaft zu züchten. Da es aber nicht klar ist, inwieweit die IR durch das Anbausystem, d.h. den Anbau mit chemisch synthetischen bzw. organischen Düngemitteln beeinflusst werden kann, muss, bevor dieses Zuchtziel angestrebt wird, erforscht werden, inwieweit die Resistenzinduktion vom Anbausystem abhängt.

Tomaten sind ein wichtiges Modellsystem zur Erforschung der IR gegenüber vielen Pathogenen, darunter auch dem Erreger der Braunfäule, *Phytophthora infestans*. Eine Vielzahl von Veröffentlichungen berichtet über den mehr oder weniger erfolgreichen Einsatz der unterschiedlichsten Substanzen, um Resistenz gegenüber *P. infestans* zu induzieren. Zum Einsatz kamen unter anderem die Chemikalien BABA (DL-3-amino butyric acid), Jasmonsäure und BTH (Benzothiadiazole-S-methylester, auch unter dem

Namen Bion bekannt), ein Extrakt von *Penicillium chrysogenum* (PEN), Chitosan, und eine Reihe von Erregern, bzw. Nutzorganismen, wie z.B. *P. infestans, Pseudomonas fluorescens, Bacillus pumilis,* Mycorrhizapilze und Tabac Necrose Virus (TNV). Je nach Substanz und Tomatensorte wurden von 20-95% Befallsreduktion durch IR gemessen. Allerdings wurden nur in wenigen Studien mehr als eine Tomatensorte zusammen getestet. Somit ist es nicht möglich, Unterschiede in der Befallsreduktion durch IR der Sorte oder dem eingesetzten Resistenzinduktor zuzuordnen.

In ökologischen Anbausystemen wird eine Vielzahl von Produkten beworben, die die Pflanzen durch verbesserte Nährstoffaufnahme und/oder durch IR stärken sollen. Ebenfalls werden viele organische Zusätze als gesundheitsfördernd („Plant Health Promotion“) beworben und einige Studien haben gezeigt, dass Tomaten in ökologisch gemanagten Böden insgesamt resistenter gegenüber *P. infestans* waren als in konventionellen Vergleichssystemen.

Chemische Resistenzinduktoren wie Bion, Jasmonsäure und BABA sind nicht für den Ökologischen Anbau geeignet. Eine Optimierung des ökologischen Tomatenanbaus in Hinblick auf die Reduktion der Braunfäule könnte aber durch die Kombination guter Induzierbarkeit von Resistenz mit den besten Bodensubstraten und Induktoren, die im Ökologischen Anbau zulässig sind, erreicht werden.

Die vorliegende Arbeit soll einerseits einen Beitrag zur ökologischen Pflanzenzüchtung leisten, indem der Frage nachgegangen wird, ob Induzierbarkeit der Resistenz ein Zuchtziel sein könnte. Andererseits soll ein Beitrag zur Entwicklung von erschwinglichen und umweltfreundlichen Strategien für die ökologische Tomatenproduktion geleistet werden.

Die **folgenden Fragen** wurden im Rahmen der vorliegenden Dissertation bearbeitet:

Gibt es bei Tomaten genetische Variation für die Induzierbarkeit von Resistenz?

Wie verhalten sich bestimmte Pflanzenstärkungsmittel (PS) im Vergleich zu BABA im Hinblick auf die IR? Hier wurden für die Hauptversuche PS gewählt, die leicht über den Boden applizierbar sind.

Interagiert die IR der Pflanzengenotypen mit den PS und verschiedenen ökologischen Düngemitteln?

In methodischen Vorarbeiten **(Kapitel 3)** wurden Fragen zur Inokulationsmethode geklärt. Die Inokulation von ganzen unbeschädigten Pflanzen ist zwar das Ideal, allerdings erfordert dies einen sehr hohen Platzaufwand und schwierige technische Hürden, da große Räume für längere Zeit bei nahe 100% relativer Luftfeuchtigkeit bei Tageslicht gehalten werden müssen. Aus diesem Grund wurden in einem ersten Schritt die Inokulation ganzer Pflanzen mit der Inokulation abgetrennter Fiederblättchen verglichen.

Die Versuche wurden mit zwei Tomatensorten und zwei Pathogeninsolaten and Blättern unterschiedlichen Alters durchgeführt. Alle Pflanzen wurden in Einheitserde angezogen und wöchentlich mineralisch (50ml pro Topf 8:8:6 NPK, 3 ml l^{-1}) gedüngt. Es wurden die PS *Fungend* (bestehend aus ätherischen Ölen, v.a. Thymian), AUSMA (ein wässriger Fichtennadelextrakt, Biolat, Salaspils, Latvia) und *BF enzyme* (ein Multikomponenten Extrakt aus unterschiedlichen Algen und anderen Pflanzen der Firma Agro bio products B.V. in den Niederlanden) getestet. Diese wurden ein bis zwei Tage vor der Inokulation tropfnass auf die Pflanzen gesprüht. Für *Fungend* wurde ein Emulgator zugesetzt, um den öligen Extrakt verteilen zu können. Kontrollpflanzen wurden unter denselben Bedingungen angezogen aber mit Wasser behandelt. Die Versuche wurden jeweils mit vier Wiederholungen vollständig randomisiert durchgeführt und jeder Versuch wurde insgesamt mindestens zwei Mal wiederholt.

Für die Inokulationen wurden *P. infestans* Isolate, die in den Jahren 2003-2004 am Standort Witzenhausen von Kartoffeln und Tomaten im Freiland isoliert wurden, genutzt. Alle Isolate wurden auf Erbsenextraktagar (125 g gefrorene Erbsen l^{-1} H_2O, 1,5% Agar) ca 3 Wochen lang angezogen. Sporulierende Kolonien wurden mit 3ml sterilem Wasser geflutet und die Sporangien vorsichtig abgeschabt. Die Sporangienlösungen wurden mithilfe eines Hämozytometers auf $5*10^4$ Sporangien ml^{-1} eingestellt und anschließend ca. zwei Stunden im Kühlschrank aufbewahrt, um ein Schlüpfen der Zoosporen zu fördern. Zum Vergleich ganzer Pflanzen und abgetrennter Blättchen wurde sprühnass inokuliert.

In einem weiteren Versuch wurden abgetrennte Blättchen und ausgestanzte Blattscheiben verglichen. Hier wurden mit BABA und Wasser behandelte Pflanzen verglichen und mit 20□l der Sporangienlösung pro Blättchen oder Blattscheibe mittig

beimpft. Ausgestanzte Blattscheiben haben den Vorteil, dass die Größe standardisiert ist und der Vergleich zwischen Sorten mit oft stark unterschiedlichen Blattformen und Größen deutlich einfacher ist.

Blattscheiben und abgetrennte Blättchen wurden in durchsichtigen Plastikschalen auf feuchtem sterilem Filterpapier Blattunterseite nach oben ausgelegt. Der Deckel der Schale wurde alle zwei Tage nass gesprüht, um die für die Sporulation notwendige Luftfeuchte zu erhalten. Beim Vergleich mit ganzen Pflanzen fand die Inkubation in der Gewächshauskabine statt. Ansonsten wurden abgetrennte Blättchen und Blattscheiben in einem klimatisierten Raum bei anfänglich 16h Dunkelheit und dann 17 ^{o}C und 16 h Licht pro Tag inkubiert.

Der Krankheitsbefall wurde grundsätzlich als % befallene Blattfläche von Tag 4 an bonitiert. Die Blattlänge und Breite der abgetrennten Blättchen wurden ebenfalls gemessen und die Fläche als Ellipse angenommen. Der Befall wurde als Fläche unter der Befallskurve (FUK) berechnet. Ebenfalls wurde für Blättchen und Blattscheiben der Befall am Tag 5 in cm^2 befallene Fläche berechnet.

Alle Datenanalysen wurden mit dem Statistikprogramm SAS durchgeführt. Ein- oder mehr-faktorielle Varianzanalysen wurden entweder mit GLM oder mit mixed Models gerechnet. Wo notwendig wurde eine Normalverteilung durch Log-Transformationen erreicht.

Der Befall wurde auf abgetrennten Blättchen und Blattscheiben bereits 4 Tage nach Inokulation (TNI) sichtbar, während es auf ganzen Pflanzen erst 5 TNI zur Sporulation kam. Ansonsten verhielten sich die Ganzpflanzen und abgetrennten Blättchen oder Blattscheiben in Bezug auf relative Anfälligkeit und ihre Reaktion gegenüber BABA und den PS Mitteln gleich. Damit konnte ein vereinfachtes System mit Blattscheiben für die detaillierten Versuche implementiert werden.

Die verwendeten PS Mittel induzierten Resistenz bei den Tomaten, allerdings variierte die Induktion zwischen PS-Mitteln, Isolaten und Sorten in diesen Vorversuchen. Aus diesem Grund wurden alle folgenden Versuche mit mindestens zwei Pathogenisolaten durchgeführt.

Ebenfalls war häufig eine stärkere Resistenzinduktion durch BABA auf jungen Blättern zu beobachten, die erst nach der Behandlung mit BABA, die 7 Tage vor der Inokulation

stattgefunden hatte, gewachsen waren als auf älteren Blättern, die direkt mit BABA behandelt worden waren. Aus diesem Grund wurden detaillierte Untersuchungen in Hinblick auf das Blattalter mit einbezogen.

Ein Screening von 32 Tomatensorten und Genbankakzessionen (**Kapitel 4**) auf die Variation der Induzierbarkeit von Resistenz gegenüber zwei Pathogenisolaten durch BABA wurde mit abgetrennten Blättchen in zwei Altersklassen durchgeführt. Der Platzverbrauch der abgetrennten Blättchen, die in großen Plastikschalen ausgelegt wurden war allerdings so hoch, dass die Sorten nicht parallel getestet werden konnten sondern zu insgesamt sieben Terminen. Zwei Kontrollsorten sorgten für eine interne Vergleichbarkeit. Die Ergebnisse zeigten erstens eine deutliche Variation in der Induzierbarkeit der Resistenz zwischen den Sorten, zweitens, dass Induzierbarkeit isolatspezifisch ist und drittens, dass jüngere Blätter insgesamt besser induziert wurden als Blätter, die bereits direkt mit BABA in Kontakt gekommen waren.

Durch die absätzigen Inokluationen waren aber statistische Vergleiche zwischen den Sorten nur teilweise möglich. Ebenfalls stellte die Variation in Blattformen und Blattgröße eine Schwierigkeit dar, da bei großen Blättern der Rand deutlich später erreicht wurde und die Berechnung der Blattgrößen zu ungenau war. Aus diesem Grund wurden die folgenden Versuche mit weniger Sorten und mit Blattscheiben durchgeführt, um sicherzustellen, dass die experimentellen Bedingungen immer gleich waren für alle Behandlungen.

Untern standardisierten Bedingungen mit Blattscheiben (**Kapitel 5)** wurden nun zunächst 13 Genotypen auf ihre Induzierbarkeit von Resistenz durch BABA gegenüber 2 *P. infestans* Isolaten getestet. Für eine Auswahl von sechs dieser Sorten wurden dann Blätter dreier Altersstufen (nach der Behandlung mit BABA gewachsene junge Blätter (=jung), die zum Zeitpunkt der BABA Behandlung gerade voll entwickelten Blätter (=mittel) und eine Etage tiefer (=alt) auf ihre Induzierbarkeit der Resistenz gegenüber 6 Pathogenisolaten getestet. Die Experimente wurden jeweils mit sechs Wiederholungen durchgeführt und jedes Experiment drei Mal. FUK, Sporulationskapazität (SK) und Infektionseffizienz (IE) wurden gemessen.

IR durch BABA hatte über alle Altersstufen den größten Einfluss auf FUK mit Reduktionen zwischen 43 und 100% auf den jüngsten Blättchen. SK wurde um 14-100% reduziert und IE um 0-100%. Die Tomatengenotypen unterschieden sich signifikant in ihrer Induzierbarkeit von Resistenz gegenüber *P. infestans* und die Stärke der Induktion nahm mit zunehmenden Blattalter ab, obwohl FUK und SK der mit Wasser behandelten Kontrollen sich zwischen Blättern unterschiedlichen Alters nur wenig unterschieden. Überraschend war, dass die Induzierbarkeit der Resistenz abhängig vom benutzten Pathogenisolat war. So gab es Sorten, die durch BABA vollständig resistent gegenüber einem Isolat wurden, während ein anderes Isolat immer noch infizieren und sporulieren konnte. Diese Ergebnisse zeigen, dass Induzierbarkeit von Resistenz gegenüber *P. infestans* eine selektierbare Eigenschaft darstellt, die allerdings isolatspezifisch ist.

Um die zweite und dritte Frage zu beantworten, wurden dieselben sechs Sorten, die vorher nur mit BABA behandelt worden waren und drei der sechs Isolate genutzt. Es wurden insgesamt vier Experimente durchgeführt (**Kapitel 6**). In Experiment I wurde der Einfluss der Düngung auf die Anfälligkeit der Tomatensorten getestet. Es wurden zwei komplexe organische Dünger: BioFeed Basis (7.5:2:4 NPK) (AgroBio Products, Wageningen, NL), and Bio-ILSA (12:0:2 NPK) (ILSA Group Arzignano, Vicenza, Italy), mit Hornmehl (13.7:0:2 NPK) und chemischem Dünger (27:46:40 NPK) verglichen. Alle Behandlungen wurden mit Superphosphat und K_2O ausgleichsgedüngt. In Experiment II wurden mit denselben sechs Sorten drei im Ökologischen Anbau zugelassene PS auf ihre Wirkung im Vergleich zur Behandlung mit BABA bzw. Wasser getestet. Zum Einsatz kamen das PS Mittel BioFeed Quality (Reg. Nummer 6536-00 (23.09.08), gem. Pflanzenschutzgesetz § 2 Nr. 10, http://pflanzenstaerkungsmittel.jki.bund.de/array1.php), PEN, ein wässriger Extrakt des kommerziellen Biodüngers Agrobiosol auf Basis von antibiotikafreien Penicilliumrückständen und Alfalfa Extrakt, das unter dem Namen ISLAC-ON angemeldet ist (Reg. Nummer 6804-00 (20.10.09) gemäß Pflanzenschutzgesetz, s. o. für Internet Link). Die Interaktionen der Düngemittel und PS wurden in Experiment III an zwei Sorten mit den drei Isolaten getestet. Um natürlichen Bedingungen näher zu kommen, unter denen so gut wie nie einzelne Pathogenisolate vorkommen, wurden in Experiment IV unter ansonsten denselben Bedingungen wie in

Experiment II die sechs Sorten nach Behandlung mit den verschiedenen PS mit den drei einzelnen Isolaten sowie deren drei zweier- und der dreier- Mischung inokuliert.

Die Befallsschwere wurde durch den Einsatz von Bio-ILSA und BioFeed Basis im Vergleich zu Hornmehl und chemischer Düngung auf allen Sorten und mit allen Isolaten signifikant reduziert ohne Interaktion zwischen Sorten oder Isolaten mit Düngern. Alle PS reduzierten die Anfälligkeit der Tomaten signifikant. Allerdings interagierten die PS sowohl mit den Sorten als auch mit den Isolaten. Die Reduktionen der FUK betrugen für Alfalfa Extrakt 23-78 %, für PEN 21-77%, für BioFeed Quality 17-66 % und für BABA 37-100 % im Vergleich zur Wasserkontrolle. Ähnliche, aber etwas geringere Reduktionen wurden bei der Sporulationskapazität gemessen. Der Einfluss der Düngemittel auf die Anfälligkeit konnte nur bei Behandlung mit Wasser aber nicht, wenn PS Mittel oder BABA eingesetzt wurden festgestellt werden. Dies deutet darauf hin, dass die Wirkung der PS Mittel unabhängig vom Bodensubstrat zu erwarten ist. Im Gegensatz zu den fehlenden Interaktionen oder additiver Effekte zwischen Düngemitteln und PS, veränderten sich die Ergebnisse deutlich, wenn Isolatemischungen eingesetzt wurden. Insgesamt waren alle Pflanzen weniger anfällig gegenüber Isolatemischungen im Vergleich zu Einzelisolaten und die PS waren deutlich wirksamer in Kombination mit den Isolatemischungen als mit Einzelisolaten. So war die Befallsreduktion durch BABA bei Einsatz von Einzelisolaten in 34 von 54 Vergleichen (65 %) signifikant größer als die durch die PS erreichte Reduktion. Im Gegensatz dazu war BABA beim Einsatz von Mischungen zweier Isolate nur noch in 25 von 54 Fällen (45 %) besser, während bei den Drei-Isolate-Mischungen BABA nur noch in 6 von 18 Fällen (33 %) besser abschnitt als die PS.

Insgesamt haben die im Rahmen der Dissertation durchgeführten Arbeiten mit einer großen Anzahl von Kombinationen von Wirts- und Pathogengenotypen mit unterschiedlichen Behandlungsmitteln und in unterschiedlichen Düngesystemen eine Reihe neuer Ergebnisse ergeben, die sowohl für Züchter als auch für die landwirtschaftliche Praxis von Interesse sind.

Wenn die vielen unterschiedlichen Mechanismen der IR auf Pflanzenseite, die bekannt sind und die unterschiedlichen Pathogenitätsfaktoren in Betracht gezogen werden, ist es nicht weiter überraschend, dass es sowohl sorten- als auch isolatspezifische Interaktionen

bei der Resistenzinduktion gibt. Die Tatsache, dass die unterschiedlichen eingesetzten Mittel unterschiedlich auf die verschiedenen Wirtsgenotyp-Isolate Kombinationen reagierten macht es fragwürdig ob es sinnvoll ist, Resistenzinduktion als Züchtungsziel zu definieren, da Sorten dann abhängig von spezifischen Induktoren würden. Die Isolatspezifität der IR legt nahe, dass zumindest *P. infestans* grundsätzlich in der Lage sein sollte, sich an IR anzupassen. Dies steht im Gegensatz zur Lehrbuchmeinung, dass IR nicht isolatspezifisch ist und eine Anpassung der Pathogen nicht zu erwarten ist. Hier sind die Ergebnisse mit den Isolatemischungen von hoher Relevanz. Selbst die sehr einfache Mischung nur zweier virulenter Isolate erhöhte die Wirksamkeit aller Induktoren signifikant und die sorten-, isolat- und induktorspezifischen Effekte wurden deutlich verringert. Damit sollte die Gefahr der Anpassung im Feld auch verringert werden. Geht man davon aus, dass in natürlichen Populationen von *P. infestans* auch avirulente Isolate vorkommen, dann ist zu erwarten, dass insgesamt die Feldanfälligkeit noch weiter reduziert werden sollte und möglicherweise die Wirksamkeit der Induktoren weiter erhöht wird. Diese Ergebnisse müssen aber mit weiterführenden Experimenten verifiziert werden. Werden sie so bestätigt, wäre eine Konsequenz, dass Maßnahmen, die die Pathogenvielfalt fördern grundsätzlich auch die Resistenz der Wirtspopulation fördern sollten.

Die Interaktion zwischen den PS, Sorten, und Isolaten legt nahe, auch Kombinationen von PS auszuprobieren. Hier könnten einerseits unterschiedliche und komplementäre Resistenzmechanismen ausgelöst werden, die die IR verbessern. Andererseits müsste aber auch getestet werden, inwieweit Pflanzen mit Stressreaktionen auf multiple Induktion reagieren und es möglicherweise zu negativen Interaktionen kommen kann.

Für den ökologischen Tomatenanbau sind die erzielten Ergebnisse insofern relevant als in dieser Arbeit klar gezeigt wurde, dass es Unterschiede in den Wirkungen sowohl verschiedener PS als auch von Düngemitteln gibt und es lohnend sein kann, das System im Hinblick auf die verwendeten Hilfsmittel zu optimieren. Vor allem auch, weil einige der genutzten PS in anderen Versuchen positive Ertrags- und Qualitätswirkungen gezeigt haben. Der Anbau von moderat resistenten Tomaten unter Einsatz positiv wirkender Düngemittel und PS könnte insgesamt den Befallsdruck reduzieren und damit zur Ertragssicherung beitragen.

Summary

Induced resistance (IR) offers the prospect of broad spectrum disease control using plant's own defences. Much research has been conducted to develop and identify different synthetic and biological resistance inducers such as Plant Growth Promoting Rhizobacteria (PGPR) and on the mechanisms of resistance induction. However, IR is not yet made use of widely in practical agriculture. One reason for this is that there is little knowledge about the effects of host genetic background on the expression of IR. As there are many different resistance mechanisms involved in resistance induction it is to be expected that the inducibility of resistance and thus its usefulness to practical agriculture could be improved by breeding for this trait. IR is an especially interesting approach to disease management in organic agriculture provided the compounds used for resistance induction are compatible with organic regulations. Many so-called plant strengtheners (PS) which are supposed to induce resistance are available, however, often systematic knowledge about their effectiveness is missing nor is it known if and how growing conditions, plant strengtheners and host variety interact.

Using the model system of tomatoes (*Solanum lycopersicum* L.) and late blight (*Phytotphthora infestans* Mont. De Bary) the presented thesis was aimed at determining if there exists variation for inducibility of resistance in tomatoes. The second aim was to compare compounds that can be used in organic farming for their ability to induce resistance in tomatoes with an emphasis on products that are easy to be applied, preferably via the soil. The third aim was to determine, how inducibility is affected by the use of different organic fertilisers.

In a first methodological study whole plant and detached leaf inoculations were compared and it was shown that IR can be identified using detached leaves instead of whole plants. In a first series of trials a total of 32 tomato accessions were screened for variation in inducibility of resistance by the chemical inducer BABA (DL-3-amino butyric acid) a potent inducer of broad-spectrum disease resistance in different plant species using a detached leaf test. One-month-old plants were sprayed to run-off with 1g l^{-1} demin. water BABA or water and inoculated seven days later. Leaves directly treated with BABA (2^{nd} leaf) and newly grown leaves (1^{st} leaf) were included in the test. Leaves were drop inoculated on the lower side with 20 µl ($5*10^4$ sporangia ml^{-1}) of two *P. infestans* isolates. Percent diseased leaf area (DLA) was assessed from day 5 to 7. As multiple

inoculations had to be carried out the varieties Supermarmande and Matina were used as standard for all inoculations.

Disease severities on the standards varied among inoculation dates but Supermarmande was consistently more susceptible than Matina. Disease reductions through BABA varied significantly among accessions and depended on the isolate the plants were challenged with. Also, resistance induction on young leaves was generally greater than on old leaves. Due to the great variation among inoculations and because different accessions were tested on different dates only the very general conclusion that inducibility is subject to genetic variation and that it may not be the same against all isolates of *P. infestans* could be drawn from these results.

A further standardisation of experimental conditions was reached by using excised leaf discs of 18mm diameter in the subsequent experiments. This method allowed to directly compare the reaction of many different plant genotypes to resistance inducers and different pathogen isolates without confounding effects of leaf size and 13 of the 32 tomato accessions were included in a test with excised leaf discs again using BABA as inducing agent and two pathogen isolates.

The results confirmed that inducibility of resistance depends on host and pathogen genotype. In a more detailed trial, six of the accessions were assessed for their inducibility of resistance to six *P. infestans* isolates on three leaves of different age per plant. Area under the disease progress curve (AUDPC), sporulation capacity (SC), and infection efficiency (IE) were all affected by treatment with BABA. On leaves of all ages AUDPC was most affected by induction (43-100% reduction on the youngest leaves) followed by SC (14-100%) and IE (0-100% reduction). Tomato accessions varied significantly in inducibility of resistance against *P. infestans* and the degree of induction generally decreased with increasing leaf age while the absolute susceptibility with respect to AUDPC and SC rarely changed.

The level of induction was not always related to the resistance level of the tomato accessions and it was significantly influenced by the pathogen isolate used for challenge inoculation.

The same six tomato cultivars were used in further experiments to determine their inducibility by three different organic plant strengtheners (PS) and if and how IR is affected by different growth substrates. Three organic fertilizers, Horn meal, BioFeed Basis, and Bio-ILSA were used in comparison to chemical fertilizer application and three

PS Alfalfa extract, PEN, and QUALITY, applied to the soil weekly for four weeks were tested in comparison to BABA and water using three isolates of *P. infestans*.

Late blight severity was significantly reduced on plants fertilized with Bio-ILSA and BioFeed Basis as compared to plants fertilized with horn meal and chemical fertilizer. There were no interactions between fertilizers and isolates or fertilizers and varieties. All PS significantly reduced the susceptibility of all tomato cultivars, however, PS interacted as well with isolates as with cultivars. The reductions in area under the disease progress curve relative to the water control for the different tomato cultivars and isolates ranged between 23-78%, 21-77%, 17-66%, and 37-100% for Alfalfa extract, PEN, QUALITY, and BABA, respectively. Similar but somewhat smaller reductions were observed for sporulation capacity.

Pathogens usually occur in mixed populations in nature. Therefore, plants treated with PS were also challenged with two-way and three-way mixtures of the pathogen isolates. The PS were more effective in inducing resistance on plants challenged with isolate mixtures than with single isolates. Thus, BABA performed significantly better than the PS in 34 out of 54 (65 %) cases tested, when single isolates were used. When two-way isolate mixtures were used, the percentage was reduced to 45 % (25 out of 54 cases and with the three-way mixtures to 33%, (6 out of 18 cases).

In conclusion, in this thesis it was shown that induced resistance of tomatoes against *P. infestans* is host and pathogen dependent and different compounds used in this study not only vary in the degree of resistance induced but are also host-genotype and isolate specific. These results put into question if breeding for inducibility will be useful in practice as it could make varieties dependent on specific inducers or growing conditions. In this context, it might be interesting to further test the combination of different inducers for their usefulness in practice to enhance plant performance. However, care has to be taken to avoid negative effects on plants. It is also unclear, how long induction will remain effective. Mixed inoculation experiments suggest that the isolate specificity may not be important in the field but rather that overall performance of inducers might be enhanced. However, these results will need to be confirmed in repeated experiments with different types of isolate mixtures also including avirulent isolates. Thus, before recommending the PS used in this study alone or in combination they have to be evaluated in a commercial type of setup of greenhouse and/or field grown tomatoes challenged with *P. infestans* and other relevant pathogens.

1. General introduction

1. 1. Introduction and aims

Exploitation of induced resistance (IR) is a desirable strategy in plant protection since it involves enhancing natural defense mechanisms in plants. Therefore, IR is especially interesting for organic farming provided it can be induced with substances compatible with organic principles. However, despite the numerous instances in which induced plant responses have been achieved by the use of a large number of different substances only little use is made of these in crop protection so far.

Much research has been conducted on the mechanisms of induction while little systematic information exists on the genetic variation of inducibility. Also, while there is evidence that depending on the growth substrate resistance may be more or less pronounced, it is not known how different organic amendments interact with IR.

Tomatoes have served as a successful model system for induction of resistance to many pathogens including *Phytophthora infestans*, causal agent of late blight. Different researchers have used different cultivars of tomato plants and different substances to test for induction of resistance against late blight with protection levels ranging from 20% to 95% but only in a few studies more than one variety has been used (Table 1.1). It is thus unclear if different protection levels reported were only due to differences in the inducers and experimental conditions or due to the genetic background of the tomato cultivars and/or pathogen isolates used.

In organic farming systems, many products are being promoted for their supposed plant strengthening effects reaching from enhanced growth through improved nutrient uptake to improved plant health through induced resistance. Also, many organic amendments are promoted as being plant health promoting and there are reports of tomatoes being more resistant to late blight when grown in organic rather than in conventionally managed soils (Berner et al. 2002; Wang et al. 2000).

Bringing together good inducibility of resistance with the best growing substrates and inducing agents acceptable to organic farming could contribute to plant health management in a system where most chemical inducers or pesticides are not an option.

1.1.1. Objectives and aims

The overall long-term goal of the research of this thesis is to provide new tools to breeders for breeding for inducibility of resistance on the one hand and, on the other

hand, to develop environmentally friendly and affordable management strategies for the production of tomatoes, especially in low-input and organic farming.

The first aim of the PhD project is to determine, if there exists variation for inducibility of resistance in tomatoes and if inducibility is affected by pathogen genotype and leaf age. The second aim is to determine how some of the available organic inducers perform compared to the chemical inducer BABA (DL-3-amino butyric acid) with an emphasis on products that are easy to be applied, preferably via the soil. The third aim is to determine if and how organic fertilizers and plant strengtheners interact with host and pathogen genotype.

1.1.2. Structure of this thesis

The thesis is divided into seven chapters. The scientific background is summarized in chapter 2. Chapter 3 describes the development of the methodology that was used in the research.

In chapter 4, the results of a preliminary trial are presented in which leaves of different ages of 32 tomato (*Solanum lycopersicum* L.) varieties and accessions were screened for variation in their inducibility of resistance to two isolates of *P. infestans* by the well studied chemical inducer BABA (DL-3-amino butyric acid).

In more standardized and repeated trials using detached leaf discs the inducibility of thirteen selected tomato accessions towards up to six pathogen isolates were then studied in detail (Chapter 5) using three different leaf ages. This work is accepted in Plant Pathology journal.

Six selected varieties and three pathogen isolates were then used to test if and how some organic fertilizers and plant strengtheners affect the susceptibility of tomatoes to *P. infestans* and how they interact with varieties and isolates (Chapter 6). The aim was to determine if effects of growth substrate and inducers depend on variety and/ or pathogen isolate and if they are additive. In order to determine if such a complex system of IR can be useful for breeders or in practice, plants were challenged with isolate mixtures to simulate a situation more close to real life. This work is submitted to European Journal of Plant Pathology and is under reviewing process.

Some concluding remarks finalize the thesis in chapter 7.

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Table 1. 1. Inducing agents and tomato varieties used in various studies on resistance induction against *P. infestans*

Tomato varieties	**Inducing agent**	**%Disease reduction**	**Reference**
Baby	Jasmonic acid	29-54	Cohen et al.1993
	Jasmonic methyl ester	34	
Bonny Best	*P. infestans*	85 [1]	Heller & Gessler 1986
Florida Basket	BABA	75	Cohen 1994
Baby		90	
Harzfeuer	Penicillium extract (PEN)	90	Unger et al. 2006
Baby	*P. infestans*	55 [2]	Enkerli et al. 1993
Pieralbo		45 [2]	
Pieraline		20 [2]	
Supermarmande		40 [2]	
Solar Set	*Pseudomonas fluorescens*	34	Yan et al. 2002
	BABA	65	
	P. infestans	47	
	Bacillus pumilus	42	
Supermarmande	PEN	71	Thuerig et al. 2006
	Benzothiadiazole-S-methyl ester (Bion/BTH)	41	
Tip-top	Chitosan	95 [1]	Atia et al. 2005
Vollendung	Tobacco necrosis virus	67	Anfoka & Buchenauer 1997
Vollendung	Tobacco necrosis virus	na [3]	Jeun & Buchenauer 2001
	BABA	na	
Vollendung	BABA	na	Jeun 2000
Early mech	Mycorrhiza	na	Pozo et al. 1998

[1]Protection on non induced leaves

[2]Approximate values were calculated from graph, Pieraline was field resistant, others susceptible

[3]Not available

2. Scientific background

2. 1. The Pathogen *Phytophthora infestans*

2. 1. 1. Nomenclature, taxonomy and biology

Dr. Jean Francis Camille Montagne was the first person to describe the late blight fungus as *Botrytis infestans*, but the name was changed to *Phytophthora infestans* by the German scientist Anton de Bary 1876 (Turner 2005). He observed the motile zoospores and described the life cycle of the late blight fungus. *Phytophthora* is derived from the Greek, *phyto* means plant and *phthora* means destroyer, and the species name, *infestans*, suggests the devastating infestation (Turner 2005).

P. infestans belongs to the oomycetes of the kingdom Chromista that includes various plant and animal pathogens as well as saprophytic species (Agrios 2005). The oomycetes are referred to as fungi because they have a fungal like morphology and physiology, but they are more related to heterokont algae and diatoms (Dick 2001). The presence of a non-septated mycelium and motile zoospores with two flagella separate them from the true fungi. The cell wall of oomycetes mainly consists of cellulose and glucans (Agrios 2005), while chitin is the major cell wall components of true fungi. Within the oomycetes, *Phytophthora* lacks the ability to synthesize sterol and thiamine. Therefore it depends on the host to acquire these essential compounds (Erwin & Ribeiro 1996).

2. 1. 2. Infection cycle

The infection cycle of *P. infestans* is well described (Erwin & Ribeiro 1996; Agrios 2005). Infection is initiated when sporangia come into contact with a moist leaf surface. The sporangia will either germinate directly at temperatures above 15 ºC or release 5-10 biflagellate zoospores per sporangium at temperatures below 15 ºC (Harrison 1992). The infection can take place either directly by the sporangium itself (above 15 ^{o}C) or indirectly (below 15 ^{o}C) by the zoospores which each can infect the host plant. The zoospores encyst and form germ tubes which swell to form appressoria. Following appressorium formation, infection tubes emerge and penetrate epidermal cells. After penetration, an infection vesicle is formed and mycelium grows both inter and intracellularly. In susceptible plants (compatible interactions), hyphae spread into the

mesophyll layer, occasionally forming haustorium like feeding structures. After a latent period of 3 to 4 days new sporangia are formed and emerge through the stomata on the lower leaf surface and spread to infect new plants via wind and splash dispersal (Agrios 2005). Infected foliage becomes yellowish, water soaked and ultimately turns black. Unprotected crops in favourable weather conditions and in the presence of an inoculum source can be destroyed within 10 to 14 days resulting in tremendous yield loss (Agrios 2005; Lebecka 2008).

2. 1. 3. The sexual cycle and origin of P. infestans

P. infestans is heterothallic with two distinct mating types called A1 and A2 and can produce sexual resting spores (oospores) when the two mating types meet (Knapova & Gisi 2002). Sexual reproduction changes the epidemiology of the fungus by increasing genetic variation by recombination (Dahlberg et al. 2002) and giving the pathogen the possibility of surviving between seasons in the soil in the form of oospores that can survive under adverse climatic conditions. Oospores are formed more abundantly in the stem than in foliage of potatoes as stems survive blight attack longer than the leaves allowing for more contact between isolates (Mosa et al. 1991). For the same reason, more oospores are produced on the leaves of moderately resistant cultivars than on the leaves of highly susceptible cultivar (Hanson & Shattock 1998). In tomatoes, oospores form in the fruit and may be seed transmitted (Rubin & Cohen 2004).

It is believed by most researchers that *P. infestans* originates from the Mexican highlands. Originally, it was only here that both mating types could be found at a 1:1 ratio. The population of *P. infestans* in this area has been found to be very diverse, both phenotypically and genetically (Grünwald et al. 2001). Also, the numerous native *Solanaceae* species possessing resistances to *P. infestans* found in Central Mexico are suggestive that this area might be the region of origin of the pathogen. However, based on studies of mitochondrial and nuclear loci it has been suggested that *P. infestans* has its origin in the Andean parts of South America (Gómez-Alpizar et al. 2006).

2. 2. Late blight of tomatoes

The pathogen has a wide range of solanaceous hosts: *Lycopersicon esculentum, Solanum sarrachoides, S. triflorum, S. dulcamara, S. sisymbriifolium, Nicotiana benthamiana*, and plants of genus *Calibrachoa* and *Petunia* (Dandurand et al. 2006) and is economically significant on potato and tomato. Flier et al. (2003) described non-cultivated species that can be an important source of inoculum reservoir for A1 and A2 mating types ultimately leading to sexual reproduction. There is always the likelihood that inoculum produced in non-cultivated plants can move to potato and tomato plants.

Cross-infection of potato and tomato by *P. infestans* is of practical significance in areas where both hosts are cultivated in close vicinity. The dynamics of primary and secondary inocula of *P. infestans* on tomato and potato are greatly influenced by climate and differ between temperate, sub-tropical and tropical regions. In temperate regions with severe winters asexual structures of *P. infestans* survive poorly in the field. Soilborne or possible seedborne (in tomatoes) oospores or tuber borne mycelium in seed potatoes act as primary inoculum for late blight epidemics (Andersson et al. 1998). However, in tropical and subtropical regions, sporangia and mycelia act as the primary inoculum, and the availability of inoculum to start late blight epidemics is high because of the abundant airborne inoculum in addition to oospores (Lima et al. 2009). In those regions, airborne inoculum is more important for late blight epidemics than inoculum from crop debris or alternate hosts (Lima et al. 2009). Late blight epidemics are favoured by monoculture, planting of year round successive crops, conducive weather conditions (moderately cool ~18°C and high air humidity >90% RH) and the lack of harsh winters. Because of the high epidemic potential of *P. infestans* and their sensitivity to temperatures below 10^{o}C tomato production in the temperate climatic regions is almost always done in glass houses or plastic tunnels.

Late blight of tomatoes has dramatically increased in importance during the past three decades due to an intercontinental migration of severe strains of the pathogen (Fry & Goodwin 1997). While the pre-1980s *P. infestans* populations outside Mexico were little to non-aggressive on tomatoes the new immigrant genotypes were composed of A1 and A2 mating types and were able to infect potato and/ or tomato (Legard et al. 1995). Isolates originating from potato are often less aggressive to tomato while those taken

from tomato often are equally aggressive to tomato and potato (Legard et al. 1995). Also, the observation of a high diversity among tomato isolates, together with the simultaneous presence of A1 and A2 isolates on the same crop, suggests that sexual reproduction may be more frequent on tomato plants than in potato (Lebreton & Andrivon 1998). In addition, tomato fruits also play a major role in late blight epidemics and in the evolution of recombinant genotypes of *P. infestans* as they produce abundant infectious oospores (Turkensteen et al. 2000) in contrast to potato tubers (Medina et al. 2000).

2. 3. Plant resistance to pathogen infection

Plants have developed mechanisms to successfully co-exist with their pathogens. Resistance may be due to morphological features of the host which act as preventive mechanism to avoid infection or to physiological defense mechanisms which make the infection unsuccessful.

Plant resistance can be broadly defined as the plant's ability to suppress or slow down the damaging activity of the pathogen (Agrios 2005). Resistance operates at different levels and can accordingly be subdivided into different classes (Mauch-Mani 2002). A plant species not affected by certain pathogens is considered to be a non-host for those pathogens and its resistance as non-host resistance. Non-host resistance protects the plant completely against pathogen infection and is expressed when a plant comes into contact with different micro-organisms.

If a species can be infected by a pathogen it may possess a general resistance conferring partial and quantitative protection also known as field or horizontal resistance which is usually not race-specific (Mauch-Mani 2002). Upon infection, the rate of disease progress in plants showing quantitative resistance is reduced compared to susceptible plants. In contrast, gene-for-gene resistance (vertical resistance) is based on the specific interaction between the products of avirulence genes in the pathogen and resistance genes in the host and is race specific (Agrios 2005). In many of the race-specific incompatible reactions a hypersensitive response (HR) is triggered, i.e. initially infected and surrounding cells die and disease is completely inhibited. Cytological studies have demonstrated that the HR is associated with all forms of resistance to *P. infestans* at

different rates of induction (Vleeshouwers et al. 2002). In race specifically resistant hosts and in non-host plants, induction of the HR is limited to one or a few cells and results in the arrest of pathogen growth in the early stages of infection (Vleeshouwers et al. 2002). In contrast, in partially resistant plants HR occurs as a trailing type of necrosis (Vleeshouwers et al. 2002).

Like with potatoes, the late blight pathogen is able to adapt to race specific tomato resistances very quickly and there is a lack of commercially acceptable resistant tomato cultivars. In a recent screening study of more than 100 tomato varieties and gene bank accessions it was shown that variation for quantitative resistance to late blight is high and not totally race-non specific. Thus, varieties can be separated into different groups based on specific interactions with pathogen strains (Butz 2010). Recombining the genetic background of the different groups, especially if different resistance mechanisms are involved might result in broader resistance than has been achieved until now.

2. 3. 1. Constitutive defence mechanisms

Constitutive defenses are always present in plants. There is a wide variation in the composition and concentration of constitutive defenses ranging from mechanical defenses to digestibility reducers and toxins. Most external mechanical defenses and large quantitative defenses are constitutive, as they require large amounts of resources to produce and are difficult to mobilize (Mauch-Mani 2002). This type of defense response is due to the presence of some structural components or some type of metabolites present in the body of the plant. The outer covering of the plant surface may be a special type such as cuticle or wax, which cannot be attacked or digested by the infecting fungus or bacteria. The presence of strong material such as lignin, tough bark, cuticle, etc. can effectively prevent the organisms from penetrating the plant surface. Also, crop architecture such as leaf angle or hairiness, e.g., might have an effect on spore deposition and microclimate and thus affect the plant's susceptibility to pathogens (Agrios 2005). There are a large number of secondary metabolites such as alkaloids, tannins, phenols, resins, etc., which are toxic to pathogens (Agrios 2005). Some of these compounds may have antimicrobial, antibacterial, or insecticidal properties.

2. 3. 2. Inducible defence mechanisms

Plants activate various defense mechanisms upon recognition of a pathogen. For example, plants protect themselves with additional structural barriers such as formation of papillae, tyloses, abscission zones or lignifications can improve plant resistance to fungal penetration (Agrios 2005; Mauch-Mani 2002). Antifungal phytoalexins are not present in healthy plants but are synthesized after pathogen attack or stress as part of the plant defense response and are restricted to the cells surrounding the infection site. Similarly, pathogenesis related (PR) proteins, such as antimicrobial proteins and hydrolytic enzymes are synthesized in the early events of the plant defense response (Agrios 2005; Mauch-Mani 2002).

Plant pathogen derived molecules (commonly called elicitors) are secreted on the surface of the pathogen. They may help in recognizing the plant as a host and they may also help in avoiding recognition by the host plant (Agrios 2005). The common elicitors from *P. infestans* are cell wall glucans (Andreu et al. 1998), arachidonic acid (Bostoc et al. 1983) and elicitins (Kamoun et al. 1997). However, recognition of the elicitors of the pathogen on the plant cell surface by the plant may also induce defence responses (Agrios 2005). These include additional intracellular signals and synthesis of phenolics and proteins in the cell wall, rapid cell collapse and death, accumulation of antimicrobial compounds and the synthesis of hydrolytic enzymes e.g. chitinases and glucanases (Agrios 2005). Thus, to be able to colonize or invade the plant host tissue, a pathogen has to overcome the plant defences by evasion of recognition, suppression of the plant defence response and/or detoxification of antimicrobial compounds (Agrios 2005). Glucans (Andreu et al. 1998) and extracellular protease inhibitors (Tian et al. 2004) are suppressors so far identified from *P. infestans*.

2. 4. Induced resistance

The inducible defence mechanisms described above can be triggered before infection by pre-treatment of plants with a variety of organisms or compounds, a phenomenon known as induced resistance (IR).

Chester (1933) first observed and described IR but only in 1959 Kuć et al. (1959) verified it. At that time the phenomenon was largely ignored and often thought to be "somehow mistaken". By now the concept is considered to be "self-evident and obvious" (Kuć 2000). IR exists in two different forms: localized and systemic. Localized IR can be detected only in the area immediately adjacent to the site of attempted penetration by the pathogen (Kessmann 1994). This type of resistance is often accompanied by rapid collapse and desiccation of the host tissue and a reaction called hypersensitive response (Agrios 2005). Systemic IR refers to resistance that occurs at sites in the host distant from the point of initial interaction with a potential pathogen (Kessmann 1994).

IR can be split broadly into systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR develops locally or systemically in response to, for example, pathogen infection (virulent, avirulent and non-pathogenic micro-organisms) or treatment with certain chemicals and is mediated by salicylic acid (SA) dependent processes (Zimmerli et al. 2001; Jakab et al. 2001, Cohen 2002). In contrast, ISR develops, for example, as a result of colonization of plant roots by plant growth promoting rhizobacteria (PGPR) such as several species of *Pseudomonas* and *Bacillus* and is mediated by the jasmonic acid (JA) or ethylene (ET) pathway (Van Wees et al. 1997; Yan et al. 2002). SAR is effective against a wide range of pathogens, whereas certain PGPR have demonstrated specificity in their ability to elicit ISR on certain plants species and genotypes (Van Wees et al. 1997; Yan et al. 2002).

The use of IR to protect crops in the field is highly attractive because of its systemic effect and broad range of effectiveness against many pathogens and herbivores (Agrios 2005). Also, it is thought that pathogens do not develop resistance to IR as easily as they do to traditional fungicides because it involves enhancing natural defense mechanisms in plants (Walters et al. 2005). As IR relies on the triggering of the plants' responses rather than the coverage of plants with chemicals or the systemic introduction of foreign chemicals into plants, disease control based on IR could potentially lead to a massive reduction of pesticide inputs in agriculture.

Various natural or synthetic compounds such as 2,6-dichloro-isonicotinic acid (INA) (Dan et al. 1998), Benzothiadiazole-S-methyl ester (BTH/Bion) (Sticher et al. 1997), oligosaccharides (Walters et al. 2005), proteins (Chen et al. 2008), probenazole (Sticher

et al. 1997), phosphate salt (Orober et al. 2002), BABA (Cohen 2002), fungal cell walls (Thuerig et al. 2006) or yeast extracts (Reglinski et al. 1994), various crude extracts from microorganisms or plants (Stephan et al. 2005) as well as certain non pathogenic root colonizing PGPR (Van Wees et al. 1997; Yan et al. 2002) have been used as inducers in glass house and field conditions. The use of pathogenic microorganisms is hardly feasible for agricultural practice because large-scale, outdoor application will require additional scientific and technical progress in the areas of production, storage, formulation and application. In contrast, application of SA is not feasible because SA is not stable and can be toxic to the plants in the doses required to induce resistance (Kessmann et al. 1994). Some of the synthetic products e.g. ASM or BTH registered as Bion or Actigard, Probenazole registered as Oryzemate, (Sticher et al. 1997) and natural products from giant knotweed *Reynoutria sacchaliensis* as Milsana (Daayf et al. 1997), bacterial protein Harpin as Messenger (Chen et al. 2008) are effective against various pathogens under field conditions and some of these are sold commercially.

2. 4. 1. Mechanisms of induced resistance

Induced resistance follows different biochemical pathways, which include 'cascades' of induced responses. These cascades of resistance are induced when a plant recognizes that a potential pathogen is present. The compounds, which are capable of triggering such responses, are termed elicitors (Agrios 2005). When receptors in the host plant recognise pathogen elicitors, a series of alarm signals are sent out to the host cell proteins and many biochemical reactions, altered cell functions, structural changes and the formation of new or greatly activated defence-related compounds take place with an effort to fend off the pathogens, its enzymes, toxins etc. (Agrios 2005).

The signal for IR may be generated within 4-6 hours and the expression of IR occurs within 24 hours after treatment. Some plant responses are apparent very quickly within an hour after induction, while some others are seen in the season following induction (Agrios 2005; Kessmann 1994). These factors suggest that induced resistance against pathogens and herbivores involves multiple mechanisms. Systemic transportation of signals is carried out via the phloem.

The effects of IR include a rapid oxidative burst, which involves the production of reactive oxygen species (ROS), super oxide, and hydrogen peroxide (H_2O_2) (Agrios 2005). As a result, changes in the membrane permeability take place with increased ion movement, such as potassium (K^+), hydrogen (H^+), calcium (Ca^{2+}) ions through the cell membrane, disruption of membranes and loss of cellular compartmentalization, activation of the antimicrobial substances like pathogenesis related (PR) proteins, phenolics, and phytoalexins (Agrios 2005). Rapid generation of superoxide and accumulation of H_2O_2 causes cell collapse, death, and HR.

The success of hypersensitive death as a resistance mechanism depends on the nutritional requirements of the specific pathogen and the timing, magnitude and location of the host (Mauch-Mani 2002). HR occurs only in specific host pathogen combinations when the host and pathogen are incompatible. It happens because of the presence of the resistance gene (R) in the plant, which recognises the elicitor of a pathogen (Agrios 2005, Mauch-Mani 2002). The pathogen-produced elicitor is the product of pathogen gene, which triggers the development of resistance in the host and makes that pathogen avirulent; therefore, this is called an avirulence gene.

Later events (but prior to the synthesis of defensive compounds) include signalling pathways in which the hormonal signals SA, JA, and ET play a major role (Agrios 2005). Induced resistance against many pathogens is initiated via SA and leads to HR and oxidative bursts in which plant cells around the site of infection die and might effectively trap and kill the pathogen (Agrios 2005; Kessmann 1994). In other cases SAR against pathogens occurs without HR. In most cases, the induced resistance against herbivores lacks HR and oxidative bursts (Agrios 2005; Kessmann 1994) but it leads to the accumulation of defensins via JA. IR against pathogens is mostly localised (Mauch-Mani 2002).

2. 4. 2. BABA (DL-3-amino butyric acid) as chemical inducer of resistance

A product is considered to be an inducer of resistance when neither the substance nor its metabolites demonstrate direct antibiotic activity in vitro or in vivo in plants. Additionally, the compound has to be efficient against a broad spectrum of pathogens, with similar protection at phenotypic and genetic levels (Kessmann et al. 1994).

BABA (DL-3-amino butyric acid) is a simple non-protein amino acid which, when sprayed onto the leaf surface or drenched into the soil, induces resistance against various foliar and root pathogens in many hosts. Although BABA is rarely found as a naturally occurring compound in plants, it is a potent inducer of broad-spectrum disease resistance in different plant species (Table 2.1). There are different forms and isomers of aminobutyric acid such as DL-2-amino-butanoic acid (AABA), 2-amino-isobutanoic acid (iso-AABA), DL-3-amino butyric acid (BABA), DL-3-amino-isobutanoic acid (iso-BABA), 4-aminobutanoic acid (GABA), (R)-3-amino-butanoic acid (R-BABA) and (S)-3-amino-butanoic acid (S-BABA) (Cohen et al. 2010). Nevertheless, the resistance-inducing activity of aminobutyric acid depends not only on the specific structure of the molecule but also on the host parasite system used (Cohen et al. 1999; Cohen 2002; Hwang et al. 1997; Pajot et al. 2001; Silue et al. 2002).

In 1958, Van Andel described the ability of different amino acids including BABA (DL-3-amino butyric acid) to induce resistance against *Cladosporium cucumerinum* in cucumber (reviewed in Cohen 2002). Two years later, Oort and Van Andel (1960) first noted induced resistance to tomato late blight following BABA treatment (reviewed in Cohen 2002). Similar observations were made around the same time on peas which were protected against the oomycetes *Aphanomyces euteiches* by BABA (Papavizas & Davey 1963). Soil drench application of BABA at a concentration of 100 ppm three days before inoculation was sufficient to reduce the root rot severity.

Since then, systemic protection against many pathogens on different crops has been reported. The possible direct toxicity of BABA on many plant pathogens has been repeatedly tested in vitro and in vivo by different research groups who could not find a direct antimicrobial activity of this chemical (Cohen 2002, Hong et al. 1999, Tosi et al. 1999). However, Fisher et al. (2009) who found direct effects on fungi, since BABA inhibited the mycelial growth of *Botrytis cinerea* and affected *Saccharomyces cerevisiae* growth in a concentration dependent manner. Similarly, Porat et al. (2003) suggested a direct antifungal effect against *Penicillium digitatum*. Thus, BABA mediated resistance is probably mostly based on the activation of host resistance mechanisms.

BABA (DL-3-amino butyric acid) has been applied successfully as foliar sprays, soil (root) drenches, and seed soakage. It is also effective when incorporated (as a powder)

into the soil, injected into the stem, or applied as a solution to bare roots, cut stems, or cut leaves. Higher concentrations of BABA are required on leaves in contrast to small doses that are required when applied to the root system (Cohen & Gisi 1994). Local treatments with BABA systemically protect tomato and potato against *P. infestans* and tobacco against *P. tabacina* (Cohen 2002). In tobacco, spraying of BABA at higher concentrations induced necrosis on leaves (Cohen & Gisi 1994; Siegrist et al. 2000). A unique feature of BABA is that it can translocate in the plant in both basipetal and acropetal directions, therefore foliar spray is effective against root diseases (Cohen & Gisi 1994). For example, Oka & Cohen (2001) observed protection of cereals against nematodes not only by a soil drench but also by foliar spray.

Persistence of resistance induced by BABA (DL-3-amino butyric acid) depends on the pathosystem and the mode of application. Cohen (2002) reported that a single foliar spray on tomato was effective for twelve days against *P. infestans*, whereas Shailasree et al. (2001) reported a seed treatment of pearl millet was effective against *Sclerospora graminicola* for 30 days. Cohen (2002) also observed that BABA protects grape leaves from mildew (*Plasmopara viticola*) when applied after infection. Even when applied 48h after inoculation, protection was achieved compared to the control.

2. 4. 3. Mechanisms of resistance induction by BABA (DL-3-amino butyric acid)

The diversity of resistance mechanisms induced by BABA (DL-3-amino butyric acid) is huge and has been previously reviewed by several authors (Jakab et al. 2001; Cohen 2002).

BABA (DL-3-amino butyric acid) operates via a variety of defense mechanisms, including physical barriers and biochemical changes leading to resistance. It has been speculated that BABA deteriorates the fungus-penetrated host cells so that translocation of nutrients into the haustoria is blocked, thus preventing further mycelial growth and sporangia production (Steiner et al. 1988). Zimmerli et al. (2001) observed that the protective effect of BABA is due to a potentiation of natural defense mechanisms against biotic and abiotic stresses. However, protection of *Arabidopsis thaliana* against the necrotrophic fungal pathogen *Botrytis cinerea* by BABA was effective in mutants impaired in jasmonic acid (JA) and ethylene (ET) pathways but not in mutants impaired

in the salicylic acid (SA) pathways indicating that the effects are very specific (Zimmerli et al. 2001).

Overall, BABA (DL-3-amino butyric acid) induced defence mechanisms may depend not only on plant species but also on the elicitors released by specific pathogens. Even within pathogen groups a diversity of reactions may be observed and the effect of BABA also depends on the developmental stage of the plant as well as the cultivar used (Altamiranda et al. 2008; Andreu et al. 2006). Both research groups found the highest level of BABA protection against late blight at the early stages of crop development (30 days after crop emergence), and the best effect was found on a moderately resistant cultivar in comparison to a highly susceptible cultivar of potato.

BABA (DL-3-amino butyric acid) does not inhibit penetration of *Phytophthora capsici* into pepper stem tissue but severely suppresses hyphal growth and sporulation by inducing the formation of electron dense cell wall appositions. These encase the haustoria and inhibit the further growth of the pathogen in a similar manner to the incompatible interaction of a resistant host (Lee et al. 2000). Cauliflower leaves treated with BABA and then inoculated with *Peronospora parasitica* develop callose that encases haustoria (Silue et al. 2002). Jeun (2000) showed similar defence mechanisms expressed in leaf tissue but not on leaf surfaces of tomato plants challenged with *P. infestans*, while Cohen (2002) observed both callose and lignin. Development of *P. infestans* in tomato leaves expressing IR was delayed by the accumulation of PR-proteins (Enkerli et al. 1993). In grapes, lignin was accumulated following inoculation with *Plasmopara viticola* (Cohen 2002), whereas in tobacco inoculated with *Peronospora tabacina*, neither callose nor lignin was formed (Cohen 2002). Thus, it appears that the physical barriers induced by BABA are pathosystem-specific.

2. 4. 4. Mechanisms of resistance induction in tomatoes

Tomatoes have served as a successful model system for induction of resistance to many pathogens including *P. infestans* (Cohen 2002; Malolepsza & Rozalska 2005). The most commonly used substance used for resistance induction in tomatoes is BABA (DL-3-amino butyric acid) (Chapter 1: Table 1.1). Other substances that have been used for

induction include JA, SA and its derivates, Penicillium extract (PEN), Benzothiadiazole-S-methyl ester (Bion), and various avirulent microorganisms (Chapter 1: Table 1.1).

Various inducible defense responses such as accumulation of reactive oxygen species (ROS), pathogenesis related proteins, phytoalexins, and physiological changes in the cell walls have been documented in tomato plants during the interaction with pathogenic fungi and resistance induction. Malolepsza and Rozalska (2005) demonstrated that the generation of higher amounts of ROS, mostly hydrogen peroxide (H_2O_2), in tomato plants pretreated with an inducer and inoculated with the pathogen caused higher resistance of these plants in comparison to non-induced plants. The ROS, especially H_2O_2, are involved both directly and indirectly in the restriction of fungal growth, and are known to be an important disease resistance mechanism (Malolepsza & Rozalska 2005).

PR proteins have been reported as an important factor in tomato plants exhibiting IR. Christ and Mösinger (1989) detected 11 PR proteins in tomato leaves infected by *P. infestans*. Activity of β-1, 3-glucanase (PR2) and chitinase (PR4) was enhanced in tomato plants expressing IR against fungal infection (Christ & Mösinger 1989; Cohen et al. 1994; Jeun & Buchenauer 2001). Cohen et al. (1994) demonstrated rapid and strong enhancement of PR protein accumulation in tomato plants after applying BABA as a foliar application. Similarly, Anfoka and Buchenauer (1997) demonstrated an inhibitory effect of PR proteins on the release of zoospores and germination of sporangia of *P. infestans*. Jeun (2000) observed both local and systemic accumulation of PR proteins in non-inoculated BABA treated tomato plants.

Besides stimulating production of PR proteins BABA also stimulates the production of phytoalexins. For example, Raviv (1994, cited in Cohen 2002) observed enhanced autofluorescence of phytoalexins of tomato leaf discs 20 h after inoculation with *P. infestans* sporangia mixed with BABA in comparison to water.

Alterations in cell wall structures such as cell wall appositions (papillae) and callose depositions are also important resistance mechanisms. These are triggered in tomatoes by BABA treatment (Cohen & Gisi 1994). Changes in cell wall structures interact with PR proteins which accumulate in papillae formed against pathogen ingress and in fungal structures penetrating plant tissues (Jeun 2000; Jeun & Buchenauer 2001).

2. 4. 5. Environmental and genetic effects on induced resistance

Many attempts at using IR in practice have resulted in inconsistent or unsatisfactory disease control under varying environmental conditions and locations (see Vallad & Goodman 2004; Walters et al. 2005; Walters 2009; Walters & Fountaine 2009). IR is a plant response to attempted infection and thus the expression of this response can be affected by a range of factors such as host and pathogen genetics and environmental conditions (see Vallad & Goodman 2004; Walters et al. 2005; Walters 2009; Walters & Fountaine 2009). This inconsistent performance may be related to a general lack of understanding of how IR works and under what conditions IR may or may not be expected to function. E.g. little is known if and how IR is affected by pathogen isolates, plant genotype and changing environmental conditions.

Genotypic effects on the expression of induced resistance have been investigated only in few studies. Steiner et al. (1988) reported that the reduction in powdery mildew of wheat (caused by *Blumeria graminis* f.sp. *tritici*), following treatment with *Bacillus subtilis* culture filtrate, was cultivar specific and strongest in partially resistant cultivars. Similarly, Hijwegen & Verhaar (1994) reported differences in inducibility of resistance to powdery mildew, caused by *Sphaerotheca fuliginea* between susceptible and partially resistant cucumber genotypes when induced with 2, 6-dichloroisonicotinic acid (INA). In the partially resistant genotype, powdery mildew could be effectively controlled with INA (at a low dose), but this was not the case in the susceptible genotype, even at a high dose of INA. More recently, Olivieri et al. (2009) found that resistance induction by BABA (DL-3-amino butyric acid) against *Phytophthora infestans* was greater in the moderately resistant potato cultivar Pampeana than in the susceptible cultivar Bintje. In contrast, induction of resistance in soybean to *Sclerotinia sclerotiorum* with INA or benzothiadiazole-S-methyl ester (Bion) was greatest in susceptible accessions (Dann et al. 1998). In this case, the authors suggested that the defence mechanisms of the more resistant accessions already resisted infection and colonization to a high degree masking any further enhancement of physiological resistance by chemicals.

When working with avirulent isolates as inducers, Martinelli et al. (1993) found that the reduction in the number of powdery mildew (*Blumeria graminis* f.sp. *hordei*) colonies on

barley differed among three sets of near-isogenic lines, which differed in their quantitative resistance level, but all possessed the same four different race specific resistance genes. Resistance induction was strongest in the most resistant and weakest in the most susceptible barley genotype. In addition, however, the expression of IR was most pronounced in lines with the *Mla7* gene and least in lines with *Mla13,* suggesting specific effects of these resistance genes on the inducibility of resistance. Thus, race specific and quantitative resistances affected the expression of induced resistance differently. Specific effects of specific genes on inducibility of resistance have also been found in *Arabidopsis thaliana.* There, the PGPR strain *Pseudomonas fluorescens* WCS417r induced systemic resistance to *Pseudomonas syringae* pv. *tomato* and *Fusarium oxysporum* f. sp. *raphani* in two out of three ecotypes (Van Wees et al. 1997). Subsequent studies showed that a recessive trait in the non-inducible ecotype affected IR by disrupting ethylene signaling (Ton et al. 2001). Only in one recent study Cohen et al. (2010) studied the inducibility of resistance against *Bremia lactucae* of lettuce cultivar Noga and Cobham Green (Dm 0) by BABA using six different pathogen isolates on detached cotyledon leaves. The authors concluded that protection induced by BABA was independent of the isolates or the cultivars used for inoculation. For all isolates spore yield per cotyledon at seven days after inoculation was significantly suppressed in BABA-treated leaves relative to the controls in both cultivars. While it seems that there is a small interaction between cultivar*isolate*resistance induction by BABA (interpretation drawn from graphs), information on these aspects is not given.

Environmental conditions such as temperature, light, water availability, and nutritional status all may affect the inducibility of resistance. For example, resistance induced by microbial metabolites against powdery mildew on barley was more effective under field conditions than when plants were grown with constant temperature, light and humidity (Falkhof et al. 1988). In the presence of light, resistance induction in *Arabidopsis thaliana* against bacterial leaf spot caused by *Pseudomonas syringae* pv. *maculicola* through an avirulent strain was successful, whereas in the dark susceptibility was increased (Zeier et al. 2004). Water stress also has been reported to increase susceptibility to several foliar pathogens (Oerke et al. 1992), while it may enhance resistance to powdery mildew in older leaves of barley (Ayres & Woolacott 1980). The expression of

constitutive and induced resistance in *Arabidopsis thaliana* was significantly lower under limiting nitrogen supply (Heil et al. 2000).

In addition to direct effects of the environment on inducibility of resistance, soil management and some organic amendments may affect plant resistance to root as well as foliar plant pathogens (see Vallad & Goodman 2004). For example, cucumbers and *Arabidopsis* grown in composted pine bark potting mixture had reduced *Colletotrichum lindemuthianum* and *Pseudomonas syringae* pv. *maculicola* severity compared to plants grown in non-amended soils. Further, composted paper mill residue suppressed the severity of *Pseudomonas syringae* pv. *syringae* on cucumber as compared to the not composted paper mill residues (Stone et al. 2003). Wang et al. (2000) also found that severity of late blight on tomatoes in organically managed soil was significantly reduced in comparison to plants given chemical fertilization. In a field trial with organic fertilisers and plant strengtheners at the University Kassel in 2005 and 2006, the commercially available organic fertiliser Bio-feed Basis and the plant strengthener Bio-Feed QUALITY improved quality and yield, and reduced severity of *P. infestans* in tomatoes when applied to the soil (Schulte Geldermann 2008).

2. 5. Concluding remarks

The review of the literature shows that there is a lack of understanding about the genetic and environmental effects on inducibility of resistance. Especially, there are no studies which systematically assessed the effects of host genotype or pathogen isolates on the inducibility of resistance. The few studies including environmental effects and organic amendments clearly show that these do affects the inducibility of resistance. This warrants further studies of these topics in detail.

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Table 2. 1. Pathosystem in which resistance induced by BABA (DL-3-amino butyric acid) was studied

Plant	Pathogen protected against	Reference
Apple fruit	*Alternaria alternata*	Reuveni et al. 2003
Arabidopsis	*Botrytis cinerea*	Zimmerli et al. 2001
Cauliflower	*Peronospora parasitica*	Silue et al. 2002
Broccoli	*Pseudomonas marginalis*	Pajot & Silue 2005
	Pseudomonas fluorescens	Pajot & Silue 2005
Cereals	*Heterodera lalipons*	Oka & Cohen 2001
	Heterodera avenae	Oka & Cohen 2001
Cotton	*Verticillium dahliae*	Li et al.1996
Cucumber	*Sphaerotheca fuliginea*	Vogt & Buchenauer 1997
	Meloidogyne javanicum	Oka & Cohen 2001
	Sphaerotheca fuliginea	Vogt & Buchenauer 1997
	Cladosporium cucumerinum	Van Andel 1958 (reviewed in Cohen 2002)
Grapes	*Plasmopara viticola*	Cohen et al. 1999; Reuveni et al. 2001
Lettuce	*Bremia lactucae*	Pajot et al. 2001; Cohen et al. 2010
Pea	*Aphanomyces euteiches*	Papavizas & Davey 1963
Peanut	*Cercosporidium personatum*	Zhang et al. 1998; 2001
Pearl millet	*Sclerospora graminicola*	Shailasree et al. 2001
Pepper	*Colletotrichum coccodes*	Hong et al.1999
	Phytophthora capsici	Hwang et al.1997
Potato	*Phytophthora infestans*	Andreu et al. 2006; Olivieri et al. 2009; Liljeroth et al. 2010
	Fusarium sambucinum	Greyerbiehl & Hammerschmidt 1998
	Fusarium solani	Olivieri et al. 2009
Squash	*Phytophthora capsici*	Kone et al. 2009
Sunflower	*Plasmopara halstedii*	Tosi et al. 1998
	Puccinia helianthi	Amzalek & Cohen 2007
Tobacco	*Peronospora tabacina*	Cohen 2002
	Tobacco mosaic virus	Siegrist et al. 2000
Tomato	*Phytophthora infestans*	Cohen 2002, Cohen & Gisi 1994; Oort & Van Andel, 1960 (reviewed in Cohen 2002)
	Fusarium oxysporum f. sp. *lycopersici*	Li et al. 1996
	Meloidogyne javanicum	Oka & Cohen 2001

3. Effects of inoculation methodology on the expression of resistance to *Phytophthora infestans* in tomatoes treated with various plant strengtheners

Abstract

Whole plant, detached leaf, and leaf disc inoculations were evaluated to determine if these methods affect the expression of resistance in tomatoes against late blight, caused by *Phytophthora infestans* (Mont) de Bary. Depending upon the test one or two tomato varieties and two or three isolates of *P. infestans* were used. In the trials comparing whole plant with detached leaf inoculations plants were treated one or two days before inoculation with water and three commercially available plant strengtheners Fungend, BF enzyme and Ausma. For the comparison of detached leaf and leaf disc inoculations plants of one variety were treated seven days before inoculation either with water or with the chemical inducer BABA (DL-3-amino-n-butyric acid) and challenged with two *P. infestans* isolates. Plants or leaflets were inoculated either by spraying to run-off or by point inoculation with 20 µl of a sporangial solution of 10^5 spores ml^{-1}. Assessments were carried out during eight and five days on detached leaves and leaf discs, respectively, and during ten days on whole plants. Disease started slightly earlier on detached leaves than on the whole plants. The clearest and most consistent differences in disease development were due to isolate followed by plant strengtheners in both systems. The isolate effects remained significant throughout the complete assessment periods in almost all experiments. In contrast, the effects of plant strengtheners on whole plants wore off at the latest after eight days. This could not be compared with detached leaf tests as these did not remain intact long enough. As well in detached leaflet as in leaf disc inoculation assays infection and sporulation in the control plants started 4 days after inoculation (DAI) while in induced plants it started 5 DAI. Infection efficiency was same in both assays irrespective of isolate and treatment used. Based on these tests it appears that leaf disc inoculations are an adequate method to assess tomato plants for their ability to be induced for resistance against *P. infestans*.

Keywords

BABA, *P. infestans*, induced resistance, leaf disc, detached leaf

3. 1. Introduction

Several methods such as field tests (Fry 1978; Colon & Budding 1988), whole plant greenhouse test (Stewart et al. 1983), and laboratory tests on detached leaves (Lapwood 1961; Goth & Keane 1997; Huang et al. 2004), leaflets (Malcolmson 1969) and leaf discs (Hodgson 1961; Sedegui et al. 1999; Daayf & Platt 2003) have been used to assess foliar resistance to *P. infestans*. While field tests as described by Fry (1978) and Colon and Budding (1988) correspond to the natural conditions under which late blight resistance is important they can be performed only once a year during the growing season and they are strongly weather dependent. Little is known about the differences between detached leaf and whole plant inoculations, however. Dorrance & Inglis (1997) reported that in greenhouse tests intact plants reacted more susceptible than leaflets and leaf discs in the laboratory. Overall, however, it has been reported that greenhouse and laboratory tests of several cultivars are comparable with respect to relative resistance levels in the field (Singh & Birhman 1994; Vleeshouwers et al. 1999).

From the practical point of view, tests with detached leaves, leaflets or leaf discs are very attractive. Especially, when comparing varieties differing in leaf size, leaf disc assays would be attractive as no leaf area measurements are necessary. In addition, the duration of the test is shorter and space requirements are greatly reduced as compared to tests with detached leaves or leaflets. However, it is unclear whether these methods can be used to test for resistance induction as the plants might be induced by the wounding process itself.

In a series of experiments the effects of inoculation methodology on the expression of resistance in tomatoes treated with various plant strengtheners or with the chemical inducer BABA were evaluated. The questions addressed were: (i) are results obtained through spray inoculation on whole plants and detached leaves comparable?, (ii) are

effects of plant strengtheners the same in detached leaves as on whole plants?, and (iii) do excised leaf discs show the same qualitative responses as detached leaflets?

3. 2. Materials and Methods

3. 2. 1. Comparison of whole plants and detached leaves and effects of plant strengtheners

The experiment was repeated four times from January to April 2005 in a greenhouse with temperature and humidity control.

Growing of plants

Two tomato varieties Cerise Rot (CR) and Celsior (C) obtained from Dreschflegel e. V. Seed Company in Witzenhausen, Germany were used for the experiments.

Plants were grown in a peat substrate containing no added nutrients in the greenhouse under controlled climatic conditions (day/night temperature 23/18 °C, respectively). For experiment 1, four plants per treatment, variety and isolate were used while in experiment 2-4, six plants per treatment, variety and isolate were used. Additional plants were grown for the detached leaf tests. Ten day old seedlings were transplanted (one plant per pot) into square containers 9*9*9.5 cm^3 and fertilized weekly with 50 ml mineral fertilizer (8:8:6 NPK, 3 ml l^{-1}) each. The plants were randomly distributed on the tables. Day temperature of 23°C and night temperature of 18°C was maintained until inoculation.

Preparation of pathogen inoculum

Isolate 48/58 and 72/69, collected from tomatoes in 2003 and isolate 108, collected in 2004 were used in the first experimental run. Only isolates 108 and 48/58 were used for the three subsequent experimental repeats.

P. infestans was grown and maintained at 17 °C in Petri dishes on pea agar (125g frozen peas l^{-1} H_2O, 1.5% agar) in the dark. Sporangial suspensions were prepared from about three week old cultures. Three 3 ml sterile water added to the Petri plate and with the thin end of a Pasteur pipette that had been bent at a right angle over a flame and the sporangia were dislodged from the mycelium. The sporangial concentration was determined with a haemocytometer and adjusted to $5*10^4$ sporangia ml^{-1}. The suspensions were incubated

for 2 h at 5 °C to induce the release of zoospores. This was followed by passing the spore solution through a plastic strainer to avoid clogging of the spray nozzle during the inoculation.

Plant strengtheners and their application

The plant strengtheners used were 1) *Fungend* provided by a person named Blumenstein who operated from Turkey. 2) *Bio Feed enzyme (BF enzyme)* provided by Agro bio products B.V., Netherlands, and 3) *Ausma* provided by BIOLAT, Latvia. All were formulated as liquids and stored in the refrigerator until use.

Fungend is produced from essential oils derived from herbs (mostly thyme). Fields of application include fruits such as stone fruit, soft fruit, strawberry, grapes, vegetables and ornamental plants. It is supposed to stimulate the metabolic activities of the plants, to increase the robustness of the plants against non-parasitic and parasitic stress, and to activate micro-organisms. *BF enzyme* is a multi compound extract derived from several seaweed species and other plant materials (Agro bio products B. V. 2007). It can be used as spray on plants, soil, and compost or it can be used with irrigation water. Its regular use is supposed to increase the strength and resistance of the crops, enhance the turnover of residual soil elements and is registered for use in organic agriculture according to EU regulation 2092/91 annex II (Agro-bio products B. V. 2007). *Ausma* is obtained by water extraction from pine and spruce needles and is a stimulator of plant rooting, germination, growing, flowering and productivity (Biolat, Salaspils, Latvia). Production technology and properties of "*Ausma*" are up to the mark of organic agriculture (BIOLAT 2003). It contains resin acids, terpenes and some essential oils that act against fungi and possesses insecticidal and repellent properties. The manufacturer claims that *Ausma* has inhibited the development of diseases like mildew (retrieved at http://www.biolat.lv/87/section.aspx/61 on 06.22.2010).

As the plant strengtheners were thought to activate the plant's own defence mechanisms, they were applied prior to inoculation. *Fungend* (0.05 %), *BF enzyme* (1%), *Ausma* (0.1%) and demineralised water were applied one day before inoculation (DBI) in experiment 1. For experiment 2, 3 and 4, *Fungend* was applied two DBI following the recommendation of the producer.

In experiment 1, *Fungend* was somehow greasy and oily in nature and was not adhering to the plant surface when mixed with water and sprayed. Therefore, for experiments 2-4, an emulsifier was added to *Fungend* at three parts of *Fungend* to one part of emulsifier according to the producer's recommendation. The required volume to achieve run-off for each treatment was altogether 200ml. A separate sprayer for flowers was used for each treatment to avoid mixing of different strengtheners.

Inoculation

For detached leaf inoculations, square shaped plastic dishes were lined with sterilised filter paper and 4 ml of demineralised water added. Non-terminal lateral leaflets from fully expanded leaves from the middle canopy of the tomato plants were used. In each plate, four leaflets were placed with the lower side of the leaf facing upwards (Fig. 3.1.). The leaflets originated from plants of the same variety that had been treated with the different plant strengtheners or water.

The youngest leaves of the whole plants at the time of treatment were marked by hanging paper clips, so that the leaves grown after treatment and inoculation could be identified. Whole plants and detached leaves were inoculated together. The plants as well as the Petri plates were placed randomly to minimise error. Inoculations were carried out by spraying the sporangial solution to run-off in such a way that each and every plant and the detached leaves in the Petri plates were exposed as evenly as possible. After inoculation the plates were covered with a lid to avoid drying and to create a humid environment. The plants and Petri plates were kept in a greenhouse cabin where humidity was continuously assured through an automatic spraying system. On the second day, all plants were rearranged randomly.

Disease assessments

Disease severity was assessed visually and the percentage diseased leaf area (% DLA) was estimated on the detached leaves while tip infection, stem infection and %DLA was assessed on the whole plants. For the detached leaves, assessments were done daily from day four to eight after inoculation (DAI). The length and the width of all detached leaves were measured with a ruler three DAI.

Assessments of the whole plants were conducted from five to ten DAI. Total number of inoculated leaflets, number of infected leaflets and % diseased leaf area (DLA) were recorded.

Data analysis and statistics

Leaf area (LA) of the detached leaves was approximated by using the formula of an ellipse. DLA was calculated by multiplying percentage of diseased leaf area by leaf area (DLA= %DLA/100*LA). Area under the disease progress curve (AUDPC) was calculated according to Campbell & Madden (1990).

Percent data were square root transformed to improve the normality and homogeneity of variance. Statistical analysis was carried out with the GLM procedure (SAS institute, Inc., Cary, NC) as factorial design with the factors variety, isolate, and plant strengthener using AUDPC for leaf infection. Mean separations were generally based on the t-test (LSDs). A repeated measures analysis was performed to determine if treatment differences changed over time.

3. 2. 2. Comparison of detached leaves and excised leaf disc inoculations

The tomato variety Supermarmande and *P. infestans* isolates 108 and 101, locally collected from tomato in 2004 were used. Growing of plants and preparation of pathogen inoculum were as described above. The experiment was conducted once (August 2007) with twelve replications. Two leaf discs per plant and treatment were used. Thus, there were 24 leaf discs from twelve plants per treatment.

BABA treatment

BABA (DL-3-amino-butyric acid) which is known to readily induce tomatoes for resistance to late blight (Cohen 1994) was used as a reference inducer. Twenty days after transplanting, when plants had five to six fully developed compound leaves and seven days before inoculation, plants were sprayed near run off with a solution of 1 g l^{-1} BABA in demineralised water while control plants were sprayed with demineralised water only. The youngest fully expanded leaf at that time was marked by hanging a plastic clip on the leaf stem. Lateral leaflets from leaves grown in the week following BABA treatment (termed 1st leaves) were included in the test.

Inoculation

Detached leaves were prepared as described above. Leaf discs (18 mm diameter) were excised with a sterile cork borer. In each plate, detached leaflets and leaf discs originating from the same plants were placed lower side up (Fig. 3.1.D). For each replicate control and induced leaflets and leaf discs were placed together into one Petri plate. Separate Petri plates were used for different isolates.

Inoculation was done with a 20 µl drop of $5*10^4$ sporangia ml^{-1} of the sporangial solution. After inoculation, Petri plates were kept in the dark for 24 h at 17 °C and afterwards a 16 h light/ 8 h dark cycle was maintained and leaf discs were sprayed with sterile demineralised water every two days.

Disease assessments, data analysis and statistics

Percent diseased leaf area was assessed daily from four to six DAI. Length and width of each leaflet were measured and leaf area was calculated as an ellipse. The lesion area in cm^2 was calculated from the percentage diseased leaflet or disc.

AUDPC data were log-transformed and statistical analysis was carried out with the GLM procedure (SAS institute, Inc., Cary, NC) as factorial design with the factors isolate and treatment. Mean separations were generally based on the Tukey-test.

3. 3. Results and discussion

3. 3. 1. Comparison of whole plants and detached leaves

Whole plants continued to grow during incubation. This reduced the overall relative but not the absolute disease increase. Disease appeared in detached leaves four DAI, followed by sporulation while in whole plants disease symptoms were apparent only five DAI.

The shorter incubation time in detached leaves might be caused by environmental conditions such as optimised humidity in the Petri plates rather than by leaf detachment (Vleeshouwers et al. 1999). The relative amount of inoculum per leaf area could be another reason for the shorter incubation period on detached leaflets. Even though the whole plants were sprayed to run-off with the same concentration of inoculum as the detached leaflets and at the same time, it is possible that less inoculum adhered to the

surface of the whole plants because the cut leaflets were laid out horizontally (See Fig. 3.1.A and B). Furthermore, on whole plants the upper side of the leaves was inoculated while the lower side of the detached leaflets was inoculated. Stomata are more numerous on the lower side of leaves providing easier access for *P. infestans* (Agrios 2005).

Overall, reactions of detached leaves and whole plants were comparable (Fig. 3.2). Isolate 108 was generally more aggressive than isolate 48/58. One exception was the reaction of the whole plants in expt. 4 in April where isolate effects were not significant (Table A-3.1). This could have been due to the very high temperatures in the greenhouse where the tomatoes were grown before inoculation. Temperatures rose repeatedly above 27 ^{o} C during the ten days before inoculation. It could be that at such temperatures resistance reactions could be induced and that these are isolate specific (personal communication L. Tamm, Research Institute of Organic Farming, Switzerland).

In the whole plant tests the two varieties did not differ in susceptibility and except for experiment 4 effects of isolates and plant strengtheners were highly significant (Table A-3.1 and A-3.3). In contrast, on detached leaves varieties and isolates interacted in experiments 1, 2, and 4 (Table A-3.2 and A-3.3). In all four experiments the varieties were significantly more susceptible to isolate 108.04 than to 48/58, however, in experiments 1 and 4, isolate 48/58 was significantly less aggressive on Cerise Rot than on Celsior while in the other two experiments there were no differences (Table A-3.3).

The plant strengtheners consistently reduced disease on whole plants and detached leaves but the effects were not always significant (Fig. 3.2). *Fungend* significantly reduced disease in experiments 2, 3 and 4, when applied 2 days before inoculation in combination with the emulsifier as recommended by the manufacturer. The fact that application time was only one day before inoculation might be the reason that the effects of the other plant strengtheners were not always significant in the experiments especially if their mode of action was resistance induction as supposed by the manufacturers. It is well-known that usually there must be a time interval between application of resistance inducers and the onset of protection in the plant through induction of systemic resistance (e.g. Kilic-Ekici & Yuen 2003).

The changes in detectable reactions in whole plants and in detached leaves followed a similar time course (Tables 3.1, 3.2, A-3.4 and A-3.5). On whole plants plant

strengthener effects were clearest 6 DAI and wore off after this time. Isolate effects were clearest 6-7 DAI and started to wear off by 10 DAI. On detached leaves, plant strengthener effects were detectable from 5 DAI on. Isolate effects remained constant from 4-8DAI (after that date assessments were no longer possible).

Overall, the detached leaflet assay was found to be a reasonably accurate predictor of the plants' reactions to isolates and plant strengtheners under greenhouse conditions up to 6 DAI compared to whole plant results. Therefore, detached leaf experiments can be accepted as a method of inoculation to test for inducibility of resistance during early stages.

3. 3. 2. Comparison of detached leaflet and excised leaf disc inoculations

There were no qualitative differences between the detached leaflet and the leaf disc assay method (Figure 3.3). However, the leaf discs were 100% diseased after day six while on the detached leaflets there were still differences. In the controls, isolate 108 was significantly more aggressive than isolate 101 while the difference between the two isolates was no more significant when treated with BABA (Fig. 3.3). In both assays infection and sporulation in the control plants started 4 DAI while in induced plants it started 5 DAI. Infection efficiency (IE) was the same irrespective of isolate and treatment used (Detached leaflet and leaf disc: 100 and 83.6 % IE in controls and induced treatments, respectively for isolate 108 and 101). It appears, based on this study, that leaf discs are a reliable method to detect differences in resistance of tomato plants to different isolates and also induced resistance against *P. infestans*.

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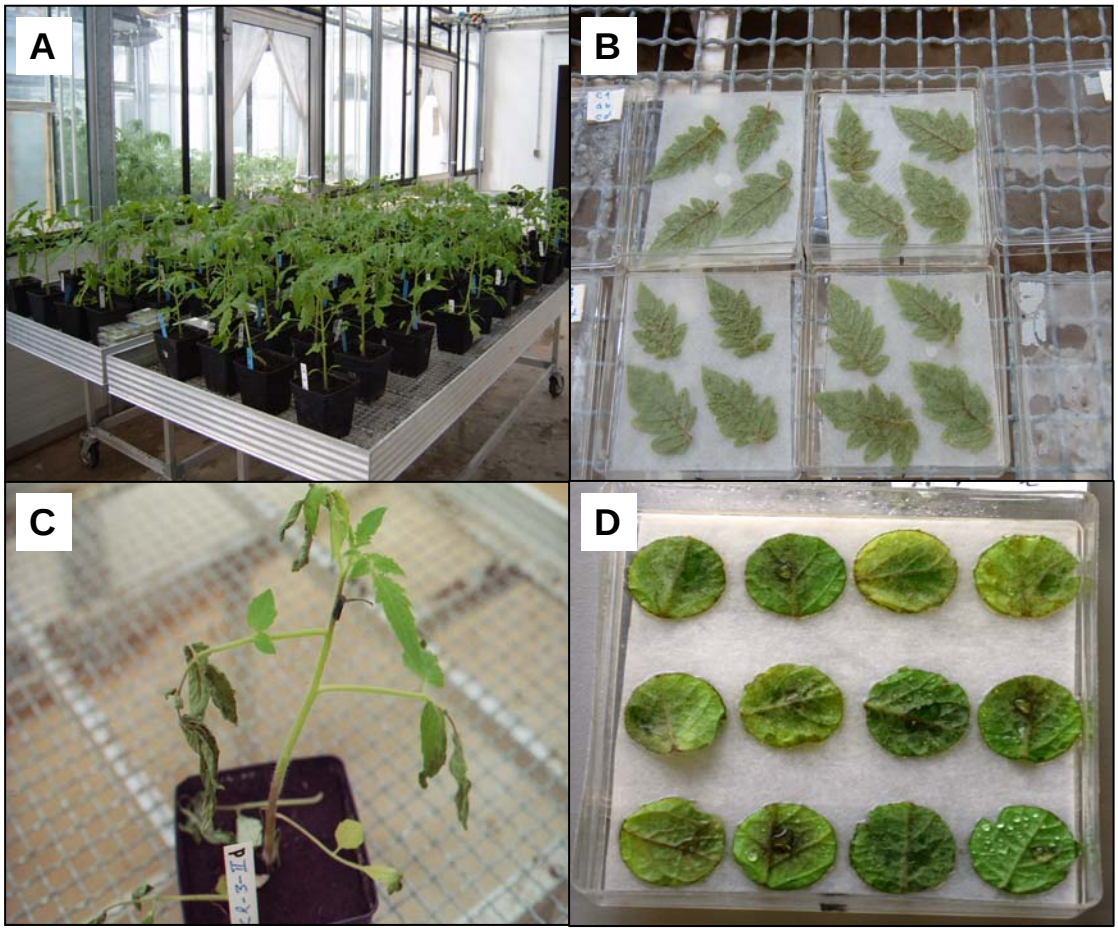

Figure 3. 1. (A) Whole plants and detached leaflets after inoculation, (B) Detached leaflets on Petri plates eight days after inoculation in green house, (C) A whole plant infected by *P. infestans* (late blight on stem and leaves), (D) Leaf discs arranged in Petri plates

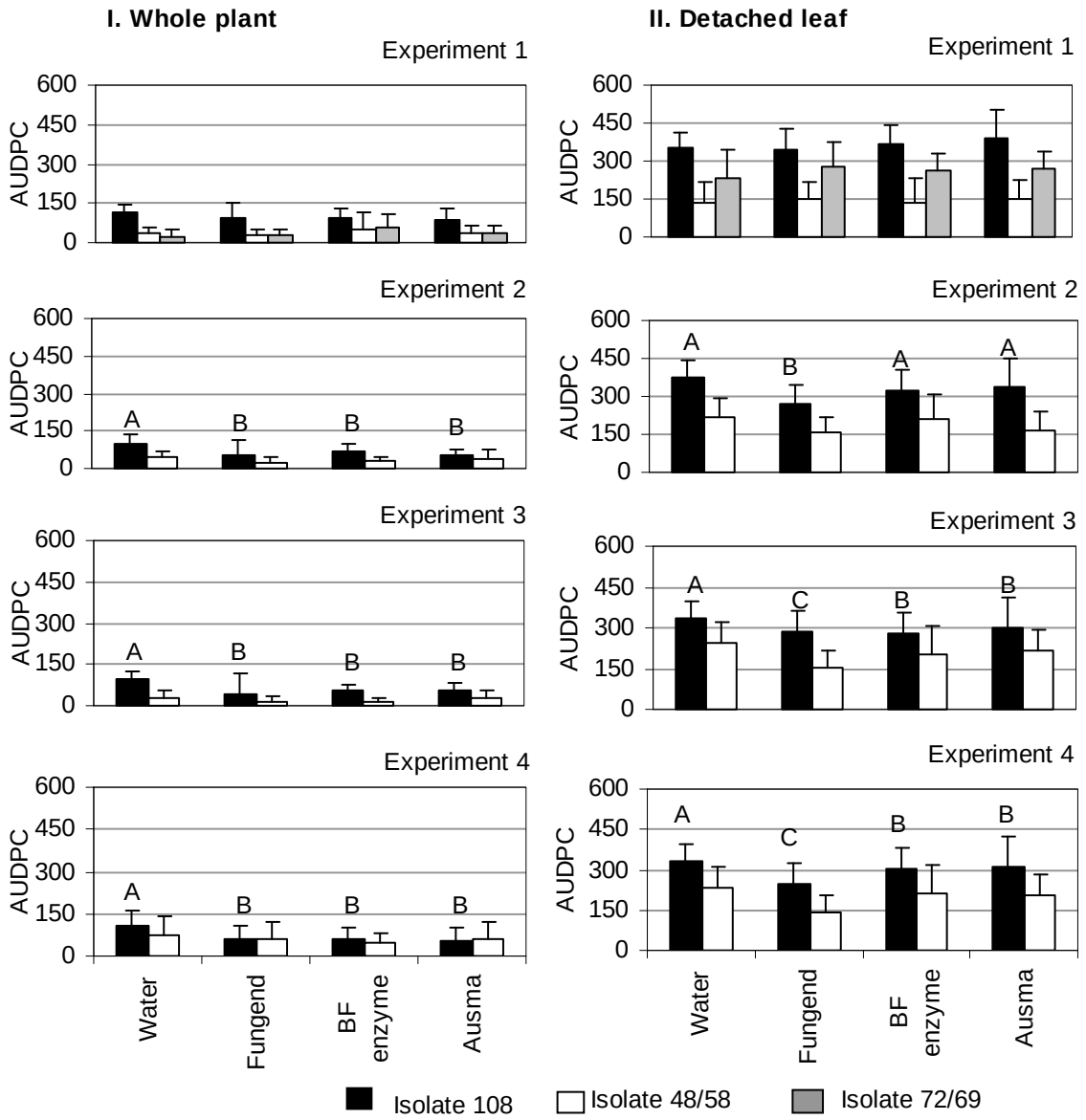

Figure 3. 2. Comparison of whole plant (left) and detached leaf (right) reactions of tomatoes to three isolates of *P. infestans* treated with water or the plant strengtheners *Fungend, BF enzyme,* or *Ausma*. Area under the disease progress curve (AUDPC) over 10 days (whole plants) and six days (detached leaves). Means of the two tomato varieties Cerise Rot and Celsior are shown. Significant differences between plant strengtheners across isolates are marked with different letters ($P \leq 0.05$, t-test, LSD for whole plant inoculation and $P \leq 0.05$, LS means for detached leaf inoculation).

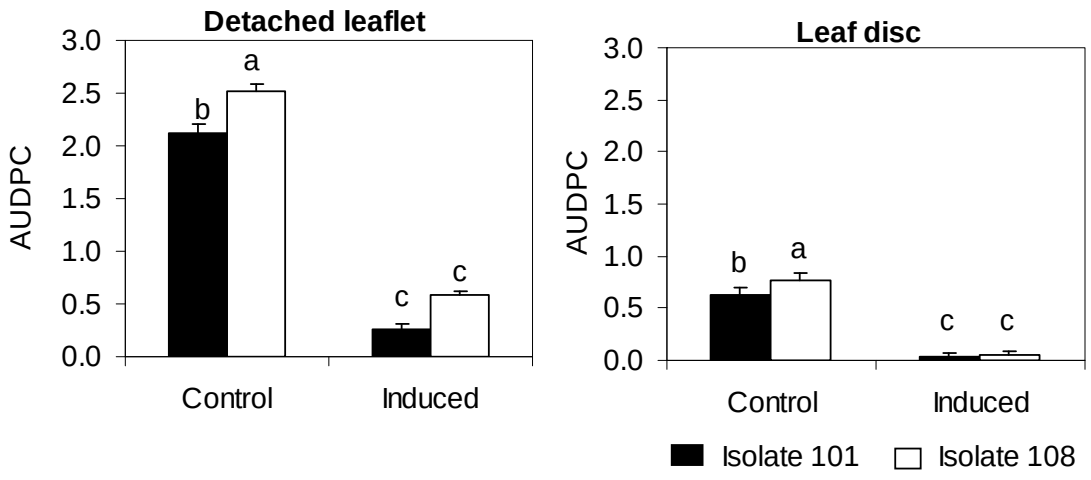

Figure 3. 3. Area under the disease progress curve (AUDPC, back-transformed data) of *P. infestans* isolate 101 (black bars) and 108 (open bars) on tomato variety Supermarmande when induced with BABA or not either on detached leaflets or on leaf discs. Different letters above bars indicate significant differences (Tukey-test, $P<0.05$).

Table 3. 1. Repeated measures analysis for % DLA over time on whole plants. Significant effects on the different dates are shown for main effects and interaction terms (see Table A-3.5 for complete ANOVA Table)

Source of Variance	5 DAI[1]				6 DAI				7 DAI				8 DAI				9 DAI				10 DAI				Number of times an effect was significant
	Experiment				Experiment				Experiment				Experiment				Experiment				Experiment				
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
Tomato			**				**				(+)														2
Isolate		*	**		**	*	**		**	**	**	*	**	**	**		**	**	**		**	*	*		18
PS[2]		(+)	**	*		**	**	*		**	**			**	(+)				*						9
Tomato*Isolate			**				**																		2
Tomato*PS			**				(+)				(+)														1
Isolate*PS			**				**			*	*			*											5
Tomato*Isolate*PS			*					(+)																	1

[1] Days after inoculation

[2] PS-Plant strengtheners

**, * and (+) indicate that effects were significant at P<0.01, P<0.05 and P<0.1 respectively.

Table 3. 2. Repeated measures analysis for % DLA over time on detached leaves. Significant effects on the different dates are shown for main effects and interaction terms (see Appendix II, Table A-3.6 for complete ANOVA Table)

Source of Variance	4 DAI[1]				5 DAI				6 DAI				7 DAI				8 DAI				Number of times an effect was significant
	Experiment				Experiment				Experiment				Experiment				Experiment				
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
Tomato		**			*	(+)	**	**	**		(+)	*	**			(+)	**				8
Isolate	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	*	20
PS[2]			**	**		**	**	**		**	**	**		(+)	**	**		(+)	**	**	12
Tomato*Isolate		**			**				**			**	(+)			*	**				6
Tomato*PS						(+)				(+)								*			1
Isolate*PS			**	**		*		(+)			(+)	**		(+)	*			(+)	(+)		5
Tomato*Isolate*PS																		*			1

[1] Days after inoculation

[2] PS-Plant strengtheners

**, * and (+) indicate that effects were significant at P<0.01, P<0.05 and P<0.1 respectively

4. Variation in inducibility of resistance to *Phytophthora infestans* among 32 tomato accessions

Abstract

The potential for use of resistance induction in plant protection can be greatly increased by breeding for inducibility. However, to make use of this trait there is a need to determine if there is genetic variation within species for inducibility and if inducibility is affected by isolate-host genotype interactions. A total of 32 tomato accessions were screened for their inducibility of resistance to two *Phytophthora infestans* isolates by BABA (DL-3-amino butyric acid). Plants were fertilised once a week with mineral fertilizer. One-month-old plants were sprayed to run-off with 1g l^{-1} demin water BABA or water and inoculated seven days later. Leaves directly treated with BABA (2^{nd} leaf) and newly grown leaves (1^{st} leaf) were included in the test. Leaves were drop inoculated on the lower side with 20 µl ($5*10^4$ sporangia ml^{-1}) of two *P. infestans* isolates. Percent diseased leaf area (DLA) was assessed from day 5 to 7. As multiple inoculations had to be carried out the varieties Supermarmande and Matina were used as standard for all inoculations. Disease severities on the standards varied among inoculation dates but Supermarmande was consistently more susceptible than Matina. Disease reductions through BABA varied significantly among accessions and depended on the isolate the plants were challenged with. Also, resistance induction on young leaves was generally greater than on old leaves. Due to the great variation among inoculations and because different accessions were tested on different dates only the very general conclusion that inducibility is subject to genetic variation and that it may not be the same against all isolates of *P. infestans* can be drawn from these results.

Keywords

AUDPC, tomato, induced resistance, isolate effect

4. 1. Introduction

Considerable knowledge has accumulated in recent years on the potential use of resistance induction in plant protection. Especially the mechanisms of induction and potential inducers are being focused on in research (Jeun & Buchenauer 2001; Zimmerli et al. 2001; Cohen 2002; Unger et al. 2006). Despite the numerous instances in which induced responses have been demonstrated, they have not found their way into practical plant production. One reason might be that inducibility for resistance is not a trait that breeders currently select for. This is also due to the fact that there is almost no knowledge about genetic variation within species for inducibility of resistance, a prerequisite for breeding for this trait.

Tomatoes have served as a successful model system for induction of resistance to many pathogens including *Phytophthora infestans*, the causal agent of late blight (e.g. Oka et al. 1999; Cohen 2002; Silva et al. 2004; Atia et al. 2005; Romeiro et al. 2005; Thuerig et al. 2006). Different researchers have used different cultivars of tomatoes to test inducibility of resistance against late blight. They achieved different protection levels but only in a few studies more than one variety was used (see Table 1.1., chapter 1). It is unclear if different protection levels reported were only due to differences in the inducers and experimental conditions or due to the genetic background of the tomato cultivars themselves.

The main purpose of this study was to determine if tomato accessions differ in their ability to be induced for resistance against *P. infestans*.

4. 2. Materials and Methods

4. 2. 1. Plant material, BABA treatment and inoculation

A total of 32 tomato accessions (*Solanum lycopersicum* L.) obtained from breeders, gene banks and commercial shops (Table 4.1) were selected to represent a broad spectrum of variation in their response to twelve *P. infestans* isolates tested in our laboratory (Butz 2010).

Plants were transplanted 10 days after sowing into square containers 9*9*9.5 cm^3 (one plant per pot), watered daily and fertilized once a week with 50 ml mineral fertilizer (8:8:6 NPK, 3 ml l^{-1}) (8:8:6 NPK) each. Four weeks after transplanting, plants were

sprayed near run off with 1g l^{-1} BABA (DL-3-amino butyric acid) in demineralized water while control plants were sprayed with demineralized water only (four replicate plants per treatment). Leaflets from leaves directly treated with BABA (termed 2nd leaves) and from leaves grown in the week following BABA treatment (termed 1st leaves) were included in the test.

Seven days after treatment with BABA, the two first lateral leaflets of the compound leaves were detached and placed lower side up in 38*28 cm² plastic trays lined with wet fleece and filter paper and covered with plexi glass (Fig. 4.1). Per tray leaflets of eight accessions were arranged with the control and induced treatments paired within the same tray, while old and young leaflets were placed into separate trays. *P. infestans* isolates 101 and 108, collected from tomatoes in 2004, were used. Each leaflet was inoculated with a 20 µl drop of a solution of 5*10^{4}sporangia ml^{-1}. Trays were kept in the dark for 24 h at 17 °C. After 24 h a 16-h light/ 8-h dark cycle was maintained and leaves were sprayed with sterile demineralized water every 2 days. Percent diseased leaf area was assessed daily from day 5 to 7. Length and width of each leaflet were measured and approximate leaf area was calculated as an ellipse.

Due to uneven germination and space limitations only six to eight accessions could normally be handled at a time. Therefore, Supermarmande and Matina were used as an internal control and included in each set of inoculations. Six separate inoculations with four replications were carried out between November 2006 and January 2007. Isolate 101 was not successful in set 5 on the two control accessions. In addition, while the controls were diseased as expected in set 3, almost all accessions tested in this set against isolate 101 were almost completely resistant. Therefore, the accessions from set 3 and 5 were included in the additional set 7 in February 2007 (Figure A-4.1). The results of set 7 showed that the data of set 3 were alike while in set 5 the inoculum of both isolates was considerably less aggressive than normal for unknown reasons. Therefore, the data of set 5 were considered invalid. For consistency, the data of set 3 and 5 were excluded for both isolates and replaced with the data of set 7 for the overall analysis.

4. 2. 2. Data analysis

The diseased leaf area in cm^2 was calculated by multiplying percentage of diseased leaf area by the calculated leaf area. This was then used to calculate the area under the disease progress curve (AUDPC) (Campbell & Madden, 1990). Data were log-transformed and then analyzed with SAS PROC mixed.

There were significant effects of the date of inoculation on the AUDPC of the control accessions Matina and Supermarmande. In the five sets that were used,, AUDPC on the first leaf of Supermarmande was overall most consistent among inoculations varying from 8.0 to 24.3 cm² for isolate 108and 5.9 to 12.8 cm² for isolate 101 (Table 4.2, Figure A-4.1). Therefore, within each set the AUDPC of each accession was divided by the AUDPC of Supermarmande. This resulted in values relative to Supermarmande (RAUDPC). The combined RAUDPC data across sets 1,2,4,6, and 7 were analysed together to compare all tested accessions.

4. 3. Results and Discussion

In the water controls, isolate 108 was able to infect at least some of the 1st leaves of all accessions except T79. In contrast, isolate 101 caused no infections on the 1st leaves of six accessions (T88, T131, T133, T134, T22 and T11). Isolate 108 was able to cause infection on at least some of the 2nd leaves of all accessions while in addition to the six accessions above, isolate 101 also failed to infect the 2nd leaves of T127 (Fig. 4.2).

Resistance induction on the 1st leaves that had emerged after BABA was applied was generally stronger than on the older 2nd leaves that had been directly treated with BABA (Fig, 4.2, Table A-4.1). Thus, while for isolate 108 induction for the 1st leaves was in almost all cases near 100% this was not uniform for the 2nd leaves. For isolate 101, the reactions were much more variable. Depending on isolate and leaf age used disease reductions through BABA treatment were significant or not (Fig.4.2).

The generally higher degree of induction on young leaves might be because of a combined effect of local and systemic induction of resistance since the young leaves were not fully developed while spraying BABA. However, in some cultivars BABA apparently gave better induction on old leaves. While local induction levels may be more important

in these cultivars on the six cultivars that were used for comparison of leaf age effects using leaf disc inoculations in chapter 5 induction was consistently greater on the youngest leaves and decreased with increasing leaf age.

Disease reductions through BABA were not always the same on accessions of the same relative level of susceptibility especially on the older leaves and when considering isolate 101 (Fig.4.2, Table A-4.1, and Table A-4.2). For example, the first leaves of T68, T125, and T10 reacted equally susceptible to isolate 101 but reduction through BABA was considerably less on T125 in comparison to the two other accessions. However, no final conclusions can be drawn from these results. Several of the accessions used in the experiments (T10, T54, T61, T72, T74 and T121) were also used in later repeated experiments with leaf discs. These experiments confirmed the qualitative differences in the degree of inducibility among accessions and also the isolate specificity of resistance induction (chapter 5). However, quantitatively, the differences were somewhat different.

The inconsistencies in results from the detached leaflet tests reported here and the later experiments (chapters 5 and 6) are likely due to the inoculation conditions and the use of only roughly approximated leaf areas. The results of the different inoculations were highly variable suggesting that the inoculation conditions were not uniform over time. Thus, temperatures may not have been uniform in different parts of the trays as some parts of the trays were near the wall while others farther away. Also, the plexi glass cover did not close the trays perfectly well and this might have led to uneven humidity inside the trays. Measurement of the leaf area could be another reason for the variable results over time. Leaf area of all the accession was calculated using the formula of an ellipse, but the leaf shapes of the different accessions were rather different.

Overall, the results suggest that differences among tomato accessions in inducibility of resistance to late blight exist and that inducibility is isolate specific. However, more standardised conditions are needed to confirm these results (see chapter 5).

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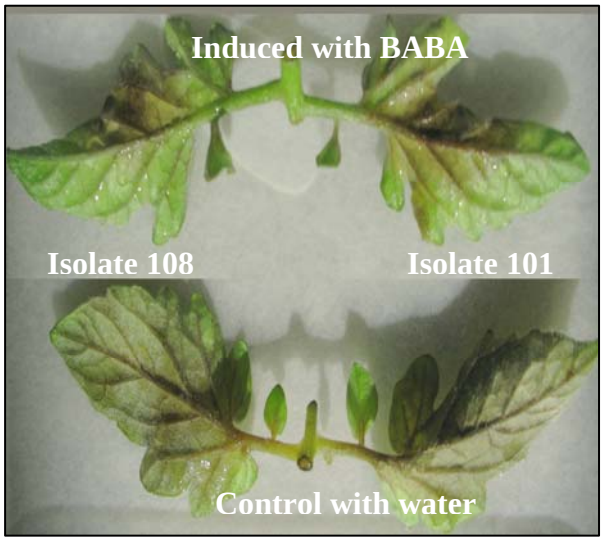

Figure 4. 1. Detached leaf experiment in tray 7 days after inoculation with isolate 108 (left leaflet) and 101 (right leaflet) and treated with BABA (upper leaflets) seven days before inoculation and control treatment water (lower leaflets)

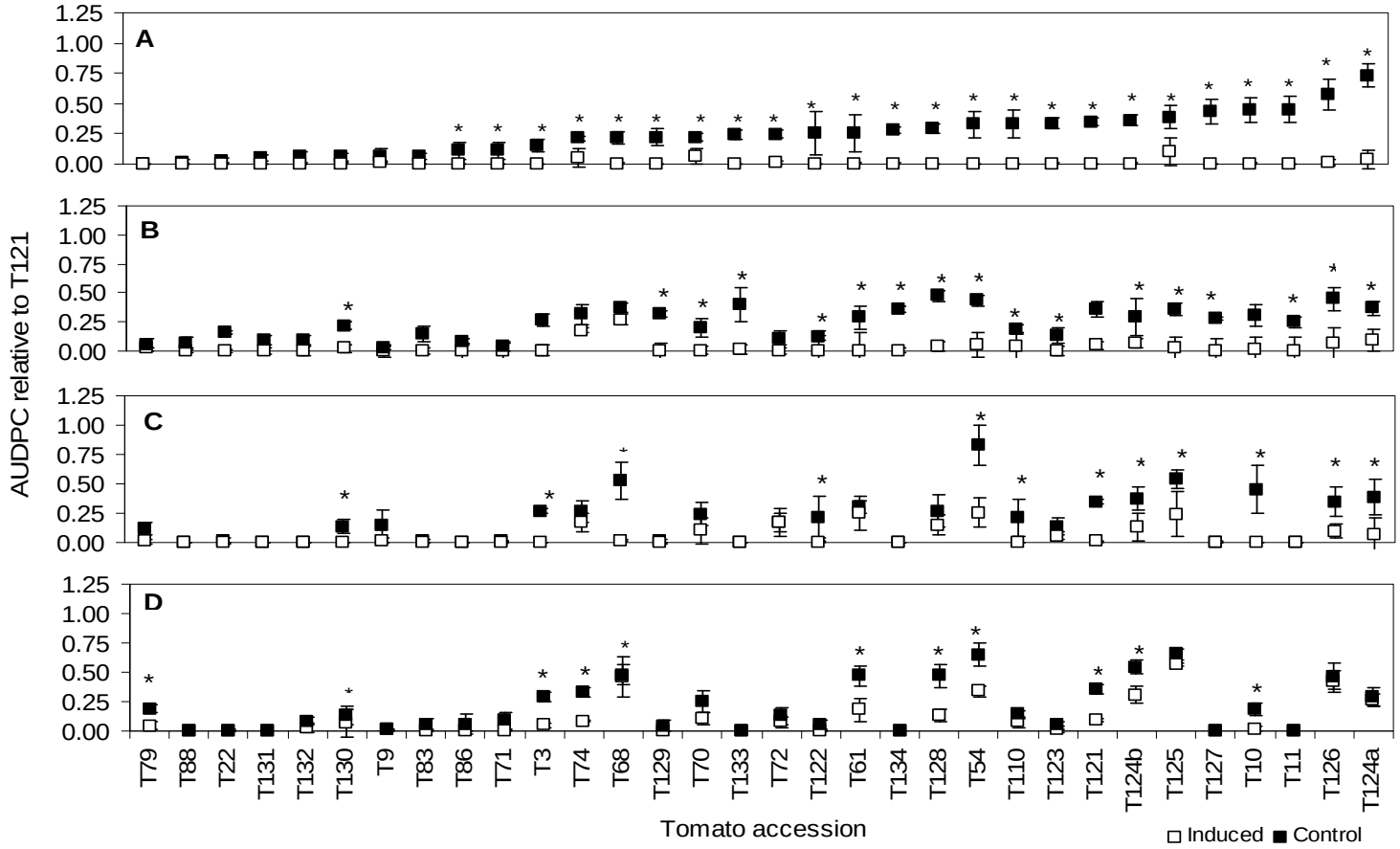

Figure 4. 2. Area under the disease progress curve (AUDPC) relative to Supermarmande (accession T121) on 32 tomato accessions when induced with BABA (white) or not induced (black) (A) for isolate 108 on 1st leaf (B) for isolate 108 on 2nd leaf (C) for isolate 101 on 1st leaf and (D) for isolate 101 on 2nd leaf (see Table A-4.2 for detailed ANOVA) (log-transformed data). * indicates that differences between induced and non-induced were significant (Linear contrast, $P<0.05$); bars represent ±SD. Data on the figures are the means of four replication of the preliminary screening trial. For names of accessions see Table 4.1.

Table 4. 1. Origin of tomato accessions used and their codes

Common name	Code	Source
Matina	T3	Bingenheim, Germany
Harzfeuer	T9	Bingenheim, Germany
Balkonzauber GS	T10	Erfurter Samen, Germany
Quadro	T11	Bingenheim, Germany
LYC2466/03	T22	IPK, Gatersleben, Germany
Berner Rose	T54	Bingenheim, Germany
Marmande	T61	Bingenheim, Germany
Delicado F1 Hybride	T68	Sperli & Co., Germany
Sweet Million F1	T70	Chrestensen, Germany
Tomaten Hellfrucht	T71	Erfurter Samen, Germany
Zuckertraube	T72	Bingenheim, Germany
C1131	T74	Kathmandu, Nepal
T329/79	T79	IPK, Gatersleben, Germany
LYC2468/03	T83	IPK, Gatersleben, Germany
LYC2458/88	T86	IPK, Gatersleben, Germany
Philovita	T88	Bruno Nebelung, Germany
LYC2524	T110	IPK, Gatersleben, Germany
Supermarmande	T121	Thompson & Morgan Ltd, UK
Solar set	T122	IPK, Gatersleben, Germany
Florida Basket	T123	Seeds by size company, UK
Vollendung	T125	IPK, Gatersleben, Germany
Tip-top	T126	IPK, Gatersleben, Germany
Heline	T127	INRA, France
Pieralbo	T128	INRA, France
Hecline	T129	INRA, France
Flora Dade	T130	INRA, France
Early mech	T131	INRA, France
Heinz 1706	T132	INRA, France
Fline	T133	INRA, France
Pieraline	T134	INRA, France
Bonny Best	T124a	Seeds by size company, UK
Bonny Best	T124b	IPK, Gatersleben, Germany

Table 4. 2. AUDPC of two isolates of *P. infestans* on detached leaflets of Supermarmande (T121) and Matina (T3) in seven sets of inoculations. One week before inoculation plants were either treated with BABA (induced) or with water.

Set	Tomato variety	Isolate 108				Isolate 101			
		1st leaf[1]		2nd leaf[1]		1st leaf[1]		2nd leaf[1]	
		Control	Induced	Control	Induced	Control	Induced	Control	Induced
1	T3	2.13	0.00	11.91	0.00	2.56	0.00	4.87	1.43
	T121	9.00	0.08	26.48	10.53	6.20	0.38	17.09	9.09
2	T3	7.85	0.00	10.86	2.32	6.99	0.01	11.62	1.43
	T121	24.30	0.19	23.04	9.33	6.36	0.32	13.84	3.99
3[2]	T3	3.60	0.00	4.74	0.08	0.05	0.16	2.69	0.28
	T121	5.94	0.01	7.55	0.56	2.13	0.40	1.41	0.54
4	T3	1.45	0.00	5.67	0.00	1.65	0.16	4.04	0.51
	T121	14.53	0.09	11.81	1.83	8.38	1.14	13.72	4.76
5[2]	T3	0.40	0.00	2.73	0.04	0.05	0.16	2.69	0.28
	T121	7.43	0.06	13.23	0.17	2.13	0.40	1.41	0.54
6	T3	2.08	0.00	11.15	0.02	2.13	0.01	4.83	1.07
	T121	7.99	0.05	26.27	4.86	5.89	0.14	15.89	3.24
7	T3	8.37	0.00	9.71	0.01	9.50	0.01	10.77	1.45
	T121	14.74	0.00	10.44	0.14	12.83	0.00	11.43	1.79

[1] 1st leaves: leaves grown after treatment with BABA; 2nd leaves: leaves directly treated with BABA

[2] Data from the grey shaded sets 3 and 5 were excluded.

5. Effects of host and pathogen genotypes on inducibility of resistance in tomato (*Solanum lycopersicum* L.) to *Phytophthora infestans*

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Abstract

The potential for use of resistance induction in plant protection could be greatly increased by breeding for inducibility. However, to make use of this trait, there is a need to determine if there is genetic variation within species for inducibility and if inducibility is isolate specific. Thirteen tomato (*Solanum lycopersicum* L.) accessions were tested for inducibility of resistance against two isolates of *Phytophthora infestans* (Mont.) de Bary using BABA (DL-3-amino butyric acid) as inducing agent. In a more detailed trial, six of the accessions were assessed for inducibility of resistance to six *P. infestans* isolates on three leaves of different age per plant. Plants were inoculated one week after treatment with BABA. Area under the disease progress curve (AUDPC), sporulation capacity (SC), and infection efficiency (IE) were all affected by treatment with BABA. On leaves of all ages AUDPC was most affected by induction (43-100% reduction on the youngest leaves) followed by SC (14-100%) and IE (0-100% reduction). Tomato genotypes varied significantly in inducibility of resistance against *P. infestans* and the degree of induction generally decreased with increasing leaf age while the absolute susceptibility with respect to AUDPC and SC rarely changed. The level of induction was not always related to the resistance level of the tomato accession and it was significantly influenced by the pathogen isolate used for challenge inoculation. The results show that inducibility of resistance is a selectable trait that is, however, isolate specific.

Keywords

BABA, genetic variation in inducibility, induced resistance, isolate effects

5. 1. Introduction

Exploitation of induced resistance (IR) is a potentially desirable strategy in plant protection since it involves enhancing natural defence mechanisms in plants (Walters *et al*., 2005). Different biological (Pozo *et al*., 1998; Zehnder *et al*., 2001; Yan *et al*., 2002), physical (i.e. abiotic e.g. wounding, water drops or various types of stress) (Walters *et al*., 2005) as well as chemical inducers (Dann *et al*., 1998; Cohen 2002; Faoro *et al*., 2008) can be used to activate or boost natural disease resistance in non-infected plant tissue.

Despite the numerous times in which induced plant responses to pathogens have been demonstrated (see Vallad & Goodman, 2004 for review), only few studies have investigated differences in inducibility of resistance among genotypes (e.g. Steiner *et al*., 1988; Martinelli *et al*., 1993; Hijwegen & Verhaar, 1994; Dann *et al*., 1998; Olivieri *et al*., 2009).

Tomatoes have served as a successful model system for induction of resistance to many pathogens including *Phytophthora infestans* (e.g. Oka *et al*., 1999; Cohen, 2002; Silva *et al*., 2004; Atia *et al*., 2005; Romerio *et al*., 2005; Thuerig *et al*., 2006). With respect to *P. infestans*, between 36 and 95% reductions in disease severity have been reported depending on tomato genotype, inducing agent used and study (Heller & Gessler, 1986; Cohen *et al*., 1993; Enkerli *et al*., 1993; Cohen, 1994; Anfoka & Buchenauer, 1997; Pozo *et al*., 1998; Jeun *et al*., 2000; Jeun & Buchenauer, 2001; Yan *et al*., 2002; Atia *et al*., 2005; Thuerig *et al*., 2006; Unger *et al*., 2006). It is unclear, however, if the different protection levels reported were only due to differences in the inducers and experimental conditions or to the genetic background of the tomato accessions and/or pathogen isolates used. In two studies where more than one tomato genotype was used, different levels of disease reduction through IR were found (Enkerli *et al*., 1993; Cohen, 1994). Overall, no systematic information is available on the genetic variation in inducibility of resistance in tomato.

In a preliminary trial, inducibility of resistance against two isolates of *P. infestans* was tested on detached leaves of 32 tomato accessions using BABA (DL-3-amino butyric acid) as inducer. The data indicated considerable accession by treatment interactions. Disease reductions through BABA were not the same on accessions of the same level of

susceptibility and both, leaf position and isolate interacted with inducibility (Sharma *et al*., 2009). In that trial, only eight accessions could be handled at a time and inoculations were carried out over several months. This, and variations in leaf size made direct comparisons across all accessions difficult, however.

The objective of this research was to quantify the extent of variation for inducibility of resistance in tomatoes in detail. The following questions were addressed in this study: (i) To what extent does inducibility of resistance vary? (ii) Is inducibility affected by different isolates of *P. infestans?* And, (iii) how is inducibility affected by leaf age? Results of two fully repeated trials using a standardised set up with leaf discs are presented. The previously observed host and isolate effects were confirmed using 13 accessions and two isolates while effects of leaf position and isolates were determined for six of the accessions challenged with six different pathogen isolates.

5. 2. Materials and methods

5. 2. 1. Growing of plants and BABA treatment

For simplicity, the term accession is used in this paper for varieties, gene bank, and breeding materials. The tomato accessions used were selected from a collection of more than 100 accessions that were obtained from breeders, gene banks and commercial shops. Generally, almost no information is available on the pedigrees or specific resistances of tomatoes as tomato breeding is an exclusively private business. The selection was therefore based on the reactions of the accessions to ten *P. infestans* isolates tested in our laboratory (Butz & Finckh, unpublished). Only accession compatible to the isolates used were selected. Thus, the isolates varied in their aggressiveness to the tomato accessions used (Butz & Finckh, unpublished), however, there were no qualitative avirulent reactions.

Plants were grown in a peat substrate containing no added nutrients in the glasshouse under controlled climatic conditions (day/night temperature 23/18 °C, respectively). For each experiment, six plants were used per treatment and accession. Ten day old seedlings were transplanted (one plant per pot) into square containers 9*9*9.5 cm^3 and fertilised

weekly with 50 ml mineral fertilizer (8:8:6 NPK, 3 ml l^{-1}) each. The plants were randomly distributed on the tables.

BABA (DL-3-amino butyric acid) was used for resistance induction in all experiments. Twenty days after transplanting, when plants had five to six fully developed compound leaves and seven days before inoculation, plants were sprayed near run off with a solution of 1 g l^{-1} BABA in demineralised water while control plants were sprayed with demineralised water only. The youngest fully expanded leaf at that time was marked by hanging a plastic clip on the leaf stem.

5. 2. 2. Preparation of pathogen inoculum and inoculations

P. infestans was grown and maintained at 17 °C in Petri dishes on pea agar (125g frozen peas l^{-1} H_2O, 1.5% agar) in the dark. All isolates used were re-isolated before use from lesions on tomato leaves to keep them vigorous and maintain pathogenicity. For this, a 1 cm^2 piece was excised from the edge of a sporulating lesion on a tomato leaf and sandwiched between two potato tuber slices of the cultivar Nicola which is susceptible to all isolates used. Seven days later, mycelia with sporangia which appeared on the upper and lower surfaces of the sandwiches were re-isolated onto pea agar containing per l 100 mg Ampicillin, 30 mg Rifamycin, 10 mg Benomyl and 0.4 ml Pimaricin. Once free of contaminants cultures of *P. infestans* were transferred onto pea agar without antibiotics.

Sporangial suspensions were prepared from 21 day old cultures by adding 3 ml of sterile water into the Petri plate. The thin end of a Pasteur pipette was bent at a right angle over a flame and was then used to dislodge the sporangia from the mycelium. The sporangial concentration was determined with a haemocytometer and adjusted to $5*10^4$ sporangia ml^{-1}. The suspensions were incubated for 2 h at 5 °C to induce the release of zoospores. While the number of zoospores per sporangium was not verified, typically between two and twenty five zoospores are released per sporangium (Ullrich & Schöber, 1972). Depending upon the test, isolates 66, 75, 85, 101 and 108 that were locally collected from tomatoes in 2004 and isolate 19, collected from potatoes, respectively, were used.

As resistance might be induced by wounding, before starting the trials reported here, detached leaves and whole plants of two tomato varieties were inoculated with two isolates in four independent trials with four to six replications each. Overall, infection success was higher on detached leaves and Sporulation was observed a day earlier,

however, the relative difference in diseased leaf area among the two isolates and the two varieties were very much alike. Additional experiments were conducted to confirm that standardised leaf discs also resulted in qualitatively identical results (Data not shown). Only the two lateral leaflets nearest the tip of the compound leaves were used for inoculations.

Leaf discs were prepared using a cork borer (Ø: 18 mm) to standardize the inoculated leaf area and placed in square Petri plates (10*10 cm^2) lower side up on moistened sterilised filter paper. Each leaf disc was inoculated with a 20 µl drop of the sporangial solution. Inoculated leaf discs were kept in the dark for 24 h at 17 °C and afterwards a 16 h light/ 8 h dark cycle was maintained and leaf discs were sprayed with sterile demineralised water every two days. From day three after inoculation (DAI), the leaf discs were checked every day microscopically for sporulating lesions. The earliest sporulating lesions were visible only four DAI in all cases. Percent diseased leaf area was assessed on day four and five (after day five, the controls were 100% sporulating).

5. 2. 3. Trial I: Screening of 13 accessions

Thirteen tomato accessions (Table 5. 1) were selected based on their variation in inducibility and susceptibility in the preliminary trial (Sharma et al., 2009). Lateral leaflets from leaves grown in the week following BABA treatment (termed 1st leaves) were included in the test.

P. infestans isolates 75 and 108 were used and the experiment was repeated three times on 12.11.2007, 14.11.2007 and 15.11.2007. Each experiment was replicated six times.

To determine Sporulation capacity (SC) immediately after the final disease assessments, the leaflets were frozen at –20°C in the Petri plates. For counting, the plates were thawed and kept at room temperature for about 20 min. Each leaf disc was placed in a test tube to which 1 ml of sterile distilled water was added, vortexed strongly for 30 seconds and sporangia were counted with a haemocytometer. Data for SC were obtained for four replications from the first experiment only.

5. 2. 4. Trial II: Effects of isolate, and leaf age on inducibility

Six tomato accessions (Table 5. 1) were used to determine isolate and leaf age effects. Isolates 19, 66, 75, 85, 101 and 108 were used and leaf age effects were tested on the 1st leaf as described in Trial I. In addition, leaflets from leaves directly treated with BABA (termed as 2nd leaves) and also from the leaf below the 2nd, i.e. the 3rd leaves were included in the tests. The entire experiment was done with leaf discs as described for Trial I. Six leaf discs per accession per treatment per leaf age were prepared. In total, there were 36 leaf discs from six plants per accession per treatment per leaf age. Again, the experiment was repeated three times on 21.11.2007, 22.11.2007 and 23.11.2007 with six replications per experiment except for isolate 101 which was included only in the first two experiments. SC was determined as described above for four replications of the first experiment.

5.2.5. Data analysis

The lesion area in cm^2 was calculated from the percentage diseased leaf disc. From this, area under the disease progress curve (AUDPC) (Campbell & Madden, 1990) and the sporulation capacity per cm^2 lesion (SC) were calculated. The infection efficiency (IE) on the non-induced controls was always 100%. For the induced treatments, IE was calculated as the proportion of inoculations that developed into sporulating lesions.

Data were either log (x+1) or square root transformed when necessary to improve the normality and homogeneity of variance. Combined data from the repeated trials were analysed with the experimental date as a factor to determine effects or interactions due to experiment. As there were neither effects of the experimental date nor significant interactions between date and the other factors, the analyses were performed across experiments resulting in 18 replications per treatment (Table A-5.1).

All experiments were analysed with the GLM procedure and PROC mixed of the statistical analysis system version 9.1 (SAS institute, Inc., Cary, NC) as factorial design with the factors treatment, accession, and isolate in trial I and, in addition, leaf age in trial II. Mean separations were generally based on the Tukey-Kramer test. Linear contrasts were used to determine significant differences between control and induced treatments.

5. 3. Results

5. 3. 1. Trial I: Screening of 13 accessions

While disease on induced plants was significantly reduced on all accessions tested, the level of induction achieved varied and was not related to the degree of susceptibility of an accession (Table 5. 2). Also, the degree of induction depended on the isolate used (significant accession x treatment interactions) ($F=16.86$, $P<0.0001$).

BABA significantly reduced SC on the infected leaves with the degree of reduction depending on accession and isolate. While the reduction patterns for AUDPC and SC were more or less similar for isolate 75 on most accessions tested, for isolate 108, there were some distinct deviations. For example, with isolate 108 accession T128 had the highest AUDPC (0.75) among the thirteen accessions and produced about $19 * 10^3$ sporangia cm^{-2}. In contrast, AUDPC on accession T124b was significantly lower (0.56) while the sporulation capacity was significantly higher ($30 * 10^3$ sporangia cm^{-2}). After induction, SC of the two accessions were no more significantly different (T128: $SC=9.5 * 10^3$, T124b: $SC = 11 * 10^3$) (Table 5. 2).

Reductions in infection efficiency (IE) through induction varied among accessions and isolates. While induction reduced IE of isolate 75 on six accessions, for isolate 108 it was only reduced on three accessions (Table 5. 2).

5.3.2. Trial II: Effects of isolate, and leaf age on inducibility

Both, pathogen isolates, and leaf age affected the inducibility of resistance through BABA depending on host genotype. The susceptibility, degree of induction and effects on IE and SC in trials I and II were very similar for isolates 75 and 108. Like in trial I, there was a strong interactive effect of isolate and accession on inducibility in trial II ($F=52.07$, $P<0.0001$).

Overall, isolate 85 and 66 were the most aggressive with mean AUDPC on the controls of 0.83 and 0.92 and isolate 19 the least aggressive (mean AUDPC=0.33) and isolates interacted significantly with resistance induction by BABA ($F=743.49$, $P<0.0001$). Disease reduction on the first leaves through BABA was significant in all combinations

tested. Reductions relative to the control treatment were highest for isolate 19 ranging between 80-100 % on the youngest leaves and lowest for isolate 85 ranging from 43-97 % (Table A-5. 2A).

Again, level of induction was not related to the degree of susceptibility of an accession to the isolate used (Fig. 5. 1). For example, tomato accession T72 was similarly susceptible to isolates 75, 85, 101, and 108, but the level of induction achieved by BABA was variable (Fig. 5. 1d). T74 appeared to be the most readily inducible of the six tomato accessions independent of isolate used (Fig. 5. 1e). There were also some cases where the level of induction decreased with increased susceptibility of the accession (e.g. T54, T61, and T121, Fig. 5. 1b, 5. 1c and 5. 1f). In contrast, on T10 greater disease reductions through resistance induction by BABA were achieved for the more aggressive isolates 85 and 66 than for the less aggressive isolate 75 (Fig. 5. 1a).

While AUDPC in the controls was similar across leaf ages (Fig. 5. 1) the protection achieved by induction with BABA with respect to AUDPC was significantly higher on the 1st leaf (mean reduction: 72%; range: 43-100%) than on the 2nd (mean: 48%; range: 22-100%) or 3rd leaf (mean: 32%; range: 15-100%) across all tomato accession * pathogen isolate combinations (Fig. 5. 1, Fig. A-5. 1, Table A-5. 2A). Some reductions were not significant for the 2nd and 3rd leaves while they had been significant on the 1st leaves (Fig. 5. 1, Fig. A-5. 1). Overall, the correlation between susceptibility and inducibility was visibly higher in the 2nd (Pearson correlation coefficient=0.77, $P<0.001$) and 3rd (Pearson correlation=0.83, $P<0.001$) leaves as compared to the 1st (Pearson correlation=0.60, $P<0.001$).

BABA significantly reduced SC of most of the infected leaves ($F=1914.72$). SC was also significantly affected by isolates ($F=280.94$) and tomato accessions ($F=143.03$). SC of the isolates decreased from 85>66>101>108>75>19 with the SC on T72>T10>T54>T121>T61>T74, respectively. The isolate*accession*treatment interaction was highly significant ($F=9.09$, $P<0.0001$), however, the F-value is an order of magnitude smaller than that of the main effects. Overall, SC was somewhat higher on the older leaves ($21.3*10^3$ sporangia cm^{-2} on the first leaves versus $25.5*10^3$ sporangia cm^{-2} on the third leaves) when not induced and the mean reduction decreased from more than two thirds on the first leaves ($8.7*10^3$ sporangia cm^{-2}) to roughly one third on the

third leaves ($15.6*10^3$ sporangia cm^{-2}) when induced (Fig. 5. 2, Table A-5. 2B). There were also some cases where effects of BABA on SC were insignificant, for example, isolate 101 on accession T54 on all three leaves (Fig. 5. 2b) or only significant on older leaves (T10, isolate 85, Fig. 5. 2a; T61, isolate 75, Fig. 5. 2c).

As for AUDPC and SC, effects of BABA on IE were affected by host and pathogen genotype, however only on the 1st leaves. Thus, IE of isolate 19 was reduced on T10, T74, T121; IE of isolates 75 and 101 on T74, and that of isolate 108 on T74 and T121 (Table A-5. 2C). IE of isolates 66 and 85 was unaffected and IE of isolate 19 was most affected by BABA treatment. There were no reductions of IE on the 2nd and 3rd leaves except for isolate 19 on T74 (Table A-5. 2C). For this host-isolate combination, BABA treatment completely suppressed infection (Fig. 5.1e).

5. 4. Discussion

The results presented in this paper confirm that tomato accessions vary considerably in inducibility of resistance against *P. infestans* and the degree of induction generally decreases with increasing leaf age. The level of induction is not always related to the resistance level of the tomato accessions and it is significantly affected by the pathogen isolate used for challenge inoculation. Similar interactions apply to leaf age effects. While AUDPC, SC, and IE were all affected by treatment with BABA, effects were greatest on AUDPC and least on IE, which was only affected on the youngest leaves except in one case.

The leaf disc assays used produced highly repeatable results. The advantage of the leaf discs is that conditions are more reproducible, the duration of the test is shorter and space requirements are greatly reduced. Thus, multiple isolate * host interactions can be assessed simultaneously and precise data can be obtained. Laboratory assays with leaf discs therefore appear to be a good method for studying particular aspects of resistance induction and for eliminating confounding influences of whole-leaf architecture.

Inoculations were performed with inoculum droplets containing 1000 sporangia which had been given the chance to hatch zoospores before inoculation by cooling down the inoculum for two hours only. In a later trial leaf discs of accessions T10 and T121 that

were BABA induced and controls were inoculated with isolates 75, 101, and 108 and destained with KOH before staining with Aniline blue. No germinated zoospores were found and all infections were established by directly germinated sporangia. It could be that the two hours cold storage were not enough to allow for zoospore hatching. Thus, potential differences among the isolates in the number of zoospores per sporangium are unlikely to play a role in this study. However, it is well possible, that with lower inoculum density, effects on infection efficiency might have been more visible.

IE was affected almost exclusively in the youngest leaves. While this could indicate that systemic induced resistance is mostly responsible for inhibition of infection this would have to be tested with much lower inoculum density or by spray inoculation counting successful infections under the microscope. The complete suppression of infections on all leaf ages on accession T74 through BABA when challenged by isolate 19 deserves therefore closer investigation. While isolate 19 was the least aggressive it had still 100 % IE in the controls and most other induced accessions even on the youngest leaves. While induction by BABA made no difference in the percentage of germinated sporangia on T10 and T121, depending on isolate and host the successful establishment of infections increased, decreased or remained unaffected by BABA treatment (unpublished data).

The effect of induced resistance in reducing diseased leaf area could be direct, in that the growth of young mycelia might be restricted, so that they are killed or otherwise fail to form large visible lesions. Induced resistance with BABA was shown to be effective for up to twelve days after infection by *P. infestans* under the conditions used by Cohen (1994). Thus, it is possible that the compounds which reduce the diseased leaf area persist in the leaf, thereby reducing the size or vigour of colonies and so causing decreased sporulation capacity.

Higher resistance induction by BABA against *P. infestans* on younger leaves as compared to older leaves has been reported before (Cohen, 1994; Cohen & Gisi, 1994) and can be explained by an acropetal systemic effect of BABA (Cohen & Gisi, 1994). The fact that induction by BABA was highest on young leaves might be due to a combination of local and systemic induction of resistance since the young leaves were not fully developed while spraying BABA. However, in some cases induction by BABA was almost the same on old leaves suggesting that local induction levels may be more

important in these accessions. It is therefore essential for reliable comparison of different tomato accessions and treatments that leaves of the same age are tested for resistance induction.

The variation in inducibility observed was within the ranges reported in earlier studies (e.g. Enkerli *et al*., 1993; Cohen, 1994). Little is known about the tomato genotypes and the type of resistance reactions induced on them, making speculations about the resistance mechanisms that may be activated and important in the containment of infections difficult. IR through BABA can be due to the triggering of a variety of mechanisms, including physical barriers and biochemical changes leading to resistance (Jakab *et al*., 2001; Cohen, 2002). As well callose as lignin formation have been found in BABA treated tomato leaves challenged with *P. infestans (*Jeun *et al*, 2000; Cohen, 2002). In potatoes differing in race-non specific resistances, Olivieri *et al*. (2009), found significantly higher levels of phenol, phytoalexin and aspartyl protease *StAp1* accumulation after treatment with BABA in a more resistant potato cultivar than in a less resistant one. They concluded that BABA treatment increases the resistance of potatoes but the degree of increase depends on the original level of resistance present in each cultivar.

The lack of a clear relation between the level of resistance and the level of resistance induction does not fit the results from other host-pathogen systems. For example, induction of resistance in soybean to *Sclerotinia sclerotiorum* with INA or acibenzolar-S-methyl (ASM) was greatest in susceptible accessions (Dann *et al*., 1998). In contrast, resistance induction to *B. graminis* was strongest in the most resistant and weakest in the most susceptible barley genotype (Martinelli *et al*., 1993). The differential resistance induction could be due to defence mechanisms and responses to BABA specific to the accessions used which work differentially against different isolates possessing different virulence mechanisms. Specific effects of specific genes on inducibility of resistance have also been found in *Arabidopsis thaliana.* There, the PGPR strain *Pseudomonas fluorescens* WCS417r induced systemic resistance to *Pseudomonas syringae* pv. *tomato* and *Fusarium oxysporum* f. sp. *raphani* in two out of three ecotypes (van Wees *et al*., 1997). Subsequent studies showed that a recessive trait in the non-inducible ecotype affected IR by disrupting ethylene signalling (Ton *et al*., 1999, 2001).

In addition to host specific differences in inducibility and kind of resistance reactions, the virulence mechanisms of pathogens are also variable. Different resistance suppressors (glucans) from different races of *P. infestans* can affect defence responses of potato in a different way (Andreu *et al*., 1998). It could be that the resistance suppressors of the six *P. infestans* isolates used interact differentially with the tomato accessions and their induced resistance mechanisms. This is supported by the fact that host-pathogen interactions were accession and isolate specific (Table 5. 2, Fig. 5. 1).

It has been claimed that unlike race-specific (vertical) resistance or pesticides, induced resistance does not appear to apply selective pressure to pathogen or parasite populations on the basis of any single genetic determinant or specific mode of action, but rather is quantitative because of the cumulative effects of numerous plant defense mechanisms (Sticher *et al*., 1997; van Loon *et al*., 1998). While the race specific effects found in our study appear to contradict these claims, they may not be as important in the field where crops are normally exposed to varying soil conditions and to pathogen populations that are made up of different genotypes. It is therefore likely that a certain level of resistance is normally being induced in the field (Walters, 2009) and that different resistance mechanisms will be acting simultaneously reducing isolate-specific effects. Nevertheless, because of its similarity to horizontal resistance, the effectiveness of induced resistance has the potential to erode over time as the pathogen or parasite population evolves (McDonald & Linde, 2002; Vallad & Goodman, 2004). There is a need for research evaluating the effects of IR on pathogen or parasite populations.

There are many examples where resistance was induced successfully in the field (Reglinski *et al*., 1994; Calonnec *et al.*, 1996; Görlach *et al*., 1996; Morris *et al*., 1998; Zehnder *et al*., 2001; Silva *et al*., 2004). The usefulness of induced resistance in practice and especially for breeding will depend on the one hand on the plant performance under the highly variable conditions found in agricultural practice and on the other hand on how easy it will be to select for inducibility. There is a need to determine if there are environmental conditions that enhance induced resistance to ease selection for this trait. The details concerning effects of single isolates are of great interest and may further help elucidate the mechanisms of induced resistance.

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Figure 5. 1. Area under the disease progress curve (AUDPC) on leaf discs of the 1st, 2nd and 3rd leaf of tomato accessions (a) T10 (b) T54, (c) T61; (d) T72, (e) T74, and (f) T121 either induced with BABA (open bars) or not induced (black bars) (plants were sprayed near run off with a solution of 1 g l^{-1} BABA in demineralised water seven days before inoculation while control plants were sprayed with demineralised water). Challenge inoculations were performed separately with six isolates of *P. infestans*. Leaf age and induction interacted significantly in all cases. Different lower case letters above the bars indicate significant differences within each accession* isolate combination (Tukey-Kramer test, P>0.05). Bars represent ± SD. Data on the figures are the mean of three experiments with six replications each. Data were log-transformed for analysis and back transformed data are presented.

Figure 5. 2. SC (sporulation capacity cm^{-2} *1000) of six isolates of *P. infestans* on leaf discs of the 1st, 2nd and 3rd leaf of tomato accessions (a) T10 (b) T54, (c) T61; (d) T72, (e) T74, and (f) T121 induced with BABA (open bars) or not induced (black bars) (plants were sprayed near run off with a solution of 1 g l^{-1} BABA in demineralised water seven days before inoculation while control plants were sprayed with demineralised water). Challenge inoculations were performed separately with each *P. infestans* isolate. In cases where leaf age and induction interacted significantly, different lower case letters above the bars indicate significant differences within each accession* isolate combination (Tukey-Kramer test, P>0.05). Where the interactions were not significant, different leaf age effects are indicated by upper case letters (Tukey-Kramer test, P>0.05). Effects of BABA treatment were usually significant (linear contrast, $P<0.01$); only insignificant effects of BABA are indicated by ns. Bars represent ± SD. Data for SC are from four replications of the first experiment only. Data were log-transformed for analysis and back transformed data are presented.

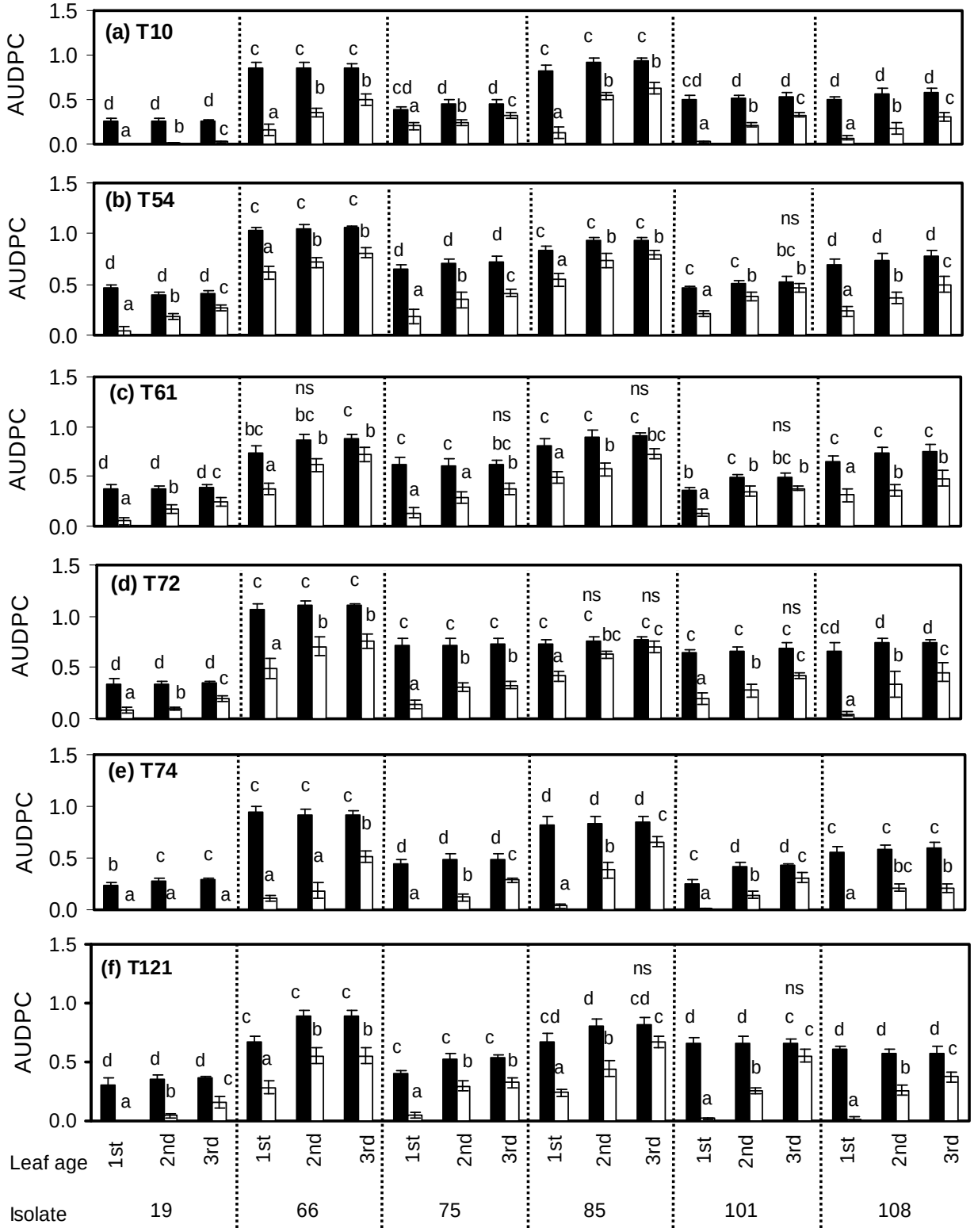
(a) T10
(b) T54
(c) T61
(d) T72
(e) T74
(f) T121
AUDPC
1.5
1.0
0.5
0.0
Leaf age
1st
2nd
3rd
Isolate
19
66
75
85
101
108

Fig. 5. 1

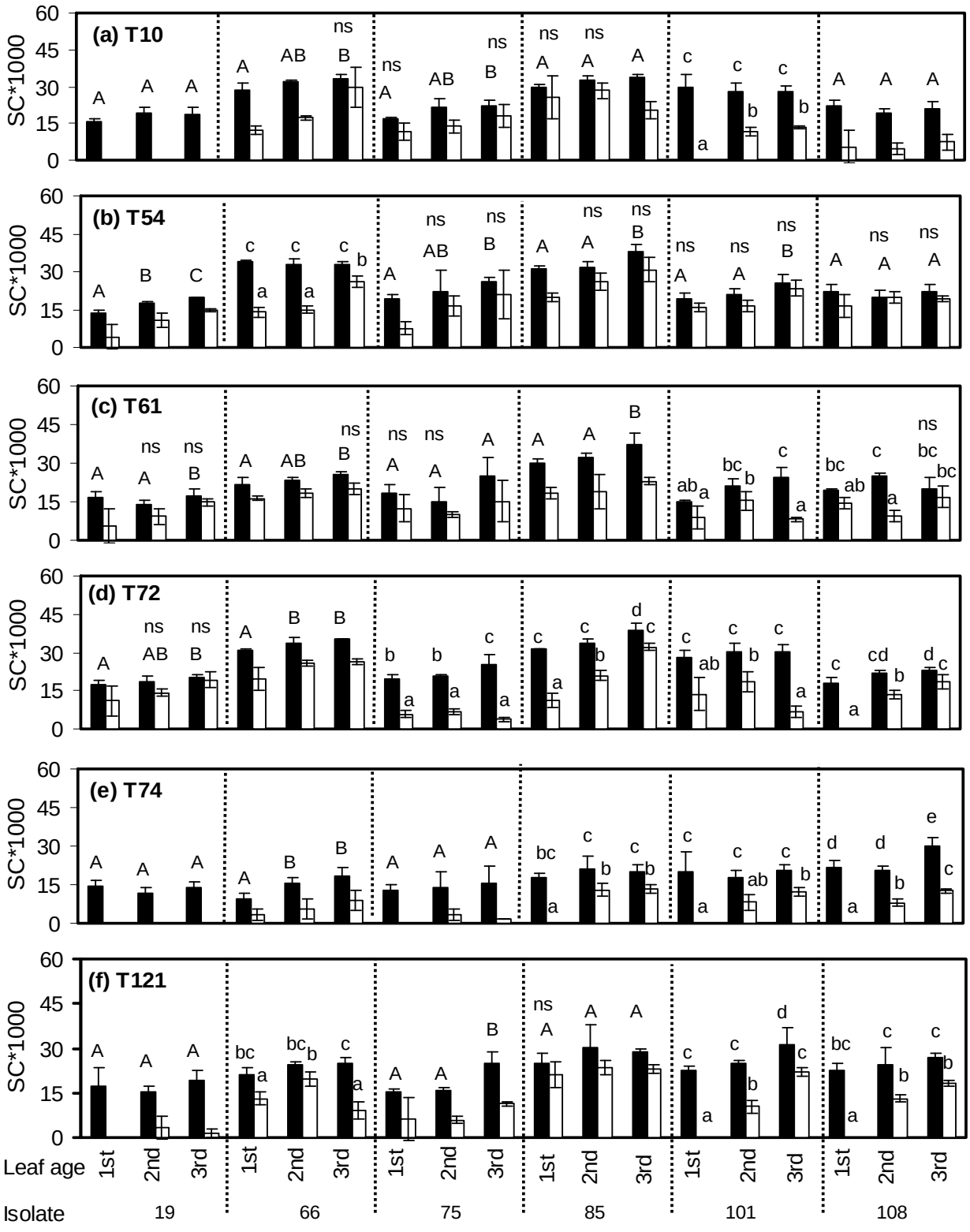
(a) T10
(b) T54
(c) T61
(d) T72
(e) T74
(f) T121
SC*1000
Leaf age
1st
2nd
3rd
Isolate
19
66
75
85
101
108

Fig. 5. 2

Table 5. 1. Origin and codes of tomato accessions used in two trials

Common name	Code	Used in trial[1]	Source
Matina	T3	I	Bingenheim, Germany
Harzfeuer	T9	I	Bingenheim, Germany
Balkonzauber GS	T10	I, II	Erfurter Samen, Germany
Quadro	T11	I	Bingenheim, Germany
Berner Rose	T54	I, II	Bingenheim, Germany
Marmande	T61	I, II	Bingenheim, Germany
Zuckertraube	T72	I, II	Bingenheim, Germany
C1131	T74	I, II	Kathmandu, Nepal
LYC2524	T110	I	IPK, Gatersleben, Germany
Supermarmande	T121	I, II	Thompson & Morgan Ltd, UK
Vollendung	T125	I	IPK, Gatersleben, Germany
Pieralbo	T128	I	INRA, France
Bonny Best	T124b	I	IPK, Gatersleben, Germany

[1] For description of trials I-II see materials and methods

Table 5. 2. Aggressiveness parameters [1] of isolate 75 and isolate 108 on 13 tomato accessions as affected by resistance induction with BABA (plants were sprayed near run off with a solution of 1 g l^{-1} BABA in demineralised water seven days before inoculation while control plants were sprayed with demineralised water)

Tomato accession	Isolate 75							Isolate 108						
	AUDPC			SC*1000			IE	AUDPC			SC*1000			IE
	Control[2]	Induced[2]	P>F[3]	Control[2]	Induced[2]	P>F[3]	Induced[2]	Control[2]	Induced[2]	P>F[3]	Control[2]	Induced[2]	P>F[3]	Induced[2]
T3	0.36 ab	0.02 ab	***	16.0 a-d	0.0 a	***	0.56 b	0.69 ef	0.18 de	***	23.1 b	17.0 c	*	0.94 c
T9	0.41 a-c	0.05 b	***	13.8 a-c	4.9 ab	***	1.00 c	0.54 a-c	0.07 bc	***	16.0 a	3.1 ab	***	1.00 c
T10	0.38 a-c	0.21 d	***	17.6 b-e	10.4 ab	***	1.00 c	0.51 ab	0.07 c	***	22.1 b	5.5 ab	***	1.00 c
T11	0.34 a	0.00 a	***	11.1 a	0.0 a	***	0.00 a	0.44 a	0.00 a	***	19.4 ab	0.0 a	***	0.00 a
T54	0.65 d	0.18 d	***	19.1 c-e	7.4 ab	***	1.00 c	0.69 ef	0.24 e	***	22.1 b	16.4 c	*	1.00 c
T61	0.62 d	0.14 c	***	18.1 b-e	12.4 b	**	1.00 c	0.65 d-f	0.31 f	***	19.7 ab	14.5 bc	ns	1.00 c
T72	0.71 e	0.14 c	***	19.8 de	5.8 ab	***	1.00 c	0.66 d-f	0.00 a-c	***	18.0 ab	5.5 ab	***	1.00 c
T74	0.44 c	0.00 a	***	12.6 ab	0.0 a	***	0.11 a	0.55 b-d	0.00 a	***	22.0 b	0.0 a	***	0.00 a
T110	0.42 bc	0.00 a	***	19.2 c-e	0.0 a	***	0.11 a	0.62 c-e	0.17 d	***	19.2 ab	0.6 a	***	1.00 c
T121	0.40 a-c	0.05 b	***	15.5 a-d	7.0 ab	***	1.00 c	0.59 b-e	0.01 ab	***	21.3 ab	0.0 a	***	0.83[4] bc
T124b	0.37 a-c	0.00 a	***	16.5 a-d	0.0 a	***	0.06 a	0.56 b-d	0.09 c	***	30.2 c	10.9 ab	***	1.00 c
T125	0.68 de	0.14 c	***	20.4 de	2.8 a	***	1.00 c	0.49 ab	0.10 c	***	21.4 ab	3.7 ab	***	0.56 b

T128	0.43 c	0.00 a	***	22.1 e	0.0 a	***	0.06 a	0.75 f	0.37 f	***	19.3 ab	9.5 ab	**	1.00 c

[1]AUDPC=Area under the disease progress curve (data were log-transformed for analysis, back-transformed data are shown); SC=Sporulation capacity per cm^2 lesion on day five; IE=Infection efficiency for induced treatments, control treatments all sporulated. Data for AUDPC and IE are the mean of three experiments with six replications each while data for SC are from four replications of the first experiment only. Leaf discs were prepared from the youngest fully emerged leaves one week after treatment with BABA (1^{st} leaf).

[2]Numbers within columns followed by different letters are statistically different at $P<0.05$ (SAS Proc Mixed, Tukey-Kramer).

[3]***, ** and *: difference between control and induced treatment were significant at $P<0.001$, $P<0.01$and $P<0.05$, respectively. Non-significant effects are marked by ns ($P>0.05$) (linear contrasts).

[4] IE of 0.83 has SC of 0 because data for SC are from four replications of the first experiment only. See Materials and Methods section for details.

6. Effects of fertilizers and plant strengtheners on the susceptibility of tomatoes to single and mixed isolates of *Phytophthora infestans*

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Abstract

Three organic fertilizers, Horn meal, BioFeed Basis, and Bio-ILSA, and three plant strengtheners Alfalfa extract, PEN, and QUALITY were tested in comparison to chemical fertilizer application and BABA (DL-3-amino-n-butyric acid), respectively for their effects on the reactions of six different tomato varieties against three isolates of *Phytophthora infestans*. Leaf discs were inoculated with 20 µl of a solution of $5*10^4$ sporangia ml^{-1}. Percent diseased leaf area was assessed daily from day four to six. Late blight severity was significantly reduced on plants fertilized with Bio-ILSA and BioFeed Basis as compared to plants fertilized with horn meal and chemical fertilizer. There were no interactions between fertilizers and isolates or fertilizers and varieties. All plant strengtheners significantly reduced susceptibility of all tomato varieties with plant strengthener-isolate and plant strengthener-variety interactions. The reductions in area under the disease progress curve relative to the water control for the different tomato varieties and isolates ranged between 23-78%, 21-77%, 17-66%, and 37-100% for Alfalfa extract, PEN, QUALITY, and BABA, respectively. Similar but somewhat smaller reductions were observed for sporulation capacity. Pathogens usually occur in mixed populations in nature. Therefore, plants treated with plant strengtheners were also challenged with two-way and three-way mixtures of the pathogen isolates. The plant strengtheners were more effective in inducing resistance on plants challenged with isolate mixtures than with single isolates. Thus, BABA performed significantly better than the plant strengtheners in 34 out of 54 (65 %) cases tested, when single isolates were used. When two-way isolate mixtures were used, the percentage was reduced to 45 % (25 out of 54 cases and with the three-way mixtures to 33%, (6 out of 18 cases).

Key words

BABA, induced resistance, isolate specific effects, mixture effects, sporulation capacity

6. 1. Introduction

Late blight, caused by *Phytophthora infestans* (Mont) de Bary is one of the most destructive diseases of tomatoes (*Solanum lycopersicum* L.) affecting organic and conventional tomato production worldwide. Because of a lack of commercial tomato cultivars with sufficient resistance to this disease, organic tomato growers often still rely on copper based fungicides where this is allowed. Copper is a toxic heavy metal that remains in the environment (Brümmer et al. 1986). Its use is therefore banned in many countries and several accreditation agencies will not allow copper usage in organic farming in the future. Hence, alternative strategies for disease control are needed.

Considerable knowledge has accumulated in recent years on the potential use of induced resistance (IR) in plant protection. Especially the mechanisms of induction and potential inducers are being focused on in research. Despite the numerous instances in which induced responses have been demonstrated, they have not found their way into practical plant production and inducibility for resistance is not a trait that breeders currently select for. Before IR can be used in breeding and/ or in production systems, there is a need to determine if and how IR is affected by host and pathogen genotype and by changing environmental conditions. Recently, Sharma *et al.* (in press) showed that tomato varieties vary in inducibility of resistance through the chemical inducer BABA (DL-3-amino-n-butanoic acid). In addition, we showed that the degree of IR is influenced by the pathogen isolate used suggesting that induced resistance reactions may be more specific than commonly thought (e.g. Agrios 2005).

Environmental conditions such as temperature, light, water availability, and nutritional status all may affect the inducibility of resistance. For example, resistance induced by microbial metabolites against powdery mildew on barley was more effective under field conditions than when plants were grown with constant temperature, light and humidity (Falkhof et al. 1988). Hot water treatment of inoculated leaves at 50°C increased the size of Tomato Mosaic Virus (TMV) lesions in Pinto bean leaves (Wu et al. 1969) while in bean cultivar Samsun NN leaves, hot water treatments inhibited development of TMV lesions (Ross & Israel 1970). In the Pinto bean- TMV system, the hot-water treatment selectively inactivates the mechanisms leading to local induced resistance, leaving the

cells around the lesion in a state capable of supporting virus multiplication. In the Samsun NN-TMV system, the hot-water treatment causes severe damage to the cells around the lesion, leading to a rapid collapse of cells making them unable to support additional virus multiplication. In the presence of light, resistance induction in *Arabidopsis thaliana* against bacterial leaf spot caused by *Pseudomonas syringae* pv. *maculicola* through an avirulent strain was successful, while in the dark susceptibility was increased (Zeier et al. 2004). Water stress also has been reported to increase susceptibility to several foliar pathogens (Oerke et al. 1992), while it may enhance resistance to powdery mildew in older leaves of barley (Ayres & Woolacott 1980). The expression of constitutive and induced resistance in *Arabidopsis thaliana* was significantly lower under limiting nitrogen supply (Heil et al. 2000).

In addition to direct effects of the environment on inducibility of resistance, soil management and some organic amendments may affect plant resistance to root as well as foliar plant pathogens (Vallad & Goodman 2004). Disease reductions through compost applications either as extracts to the foliage or as soil amendments may be due to direct antifungal, resistance inducing or plant strengthening effects or indirectly by altering microbial interactions. Thus, compost teas and filtrate solutions of mixtures of compost materials directly affected late blight in potatoes and tomatoes (Brinton et al. 1996; Ghorbani et al. 2005). In contrast, induced resistance was identified as the most likely cause for reductions in the severity of late blight on tomatoes in organically managed soil in comparison to plants given chemical fertilizers (Wang et al. 2000). Several plant or fungus-derived compounds with the potential to reduce the susceptibility of tomatoes to *P infestans* infestation through IR have also been identified (e.g. Quintanilla et al. 2002; Stephan et al. 2005; Thuerig et al. 2006; Unger et al. 2006; Portz et al. 2008).

In organic farming, many different organic fertilizers and supposed plant strengtheners are being made use of for plant production with little systematic knowledge about specific effects of these amendments on plant health. Thus, so far nobody has investigated if and to what extent plant strengtheners interact with management practices such as the use of different types of fertilizers. The objective of this research was to evaluate the effects of several formulated plant strengtheners and biofertilizers and their combination on *P. infestans* severity in tomatoes. The following questions were

addressed: Is the susceptibility of tomato plants affected (i) by the type of fertiliser or (ii) different plant strengtheners and are there variety or isolate specific effects? (iii) How do fertilisers and plant strengtheners interact with variety and isolate? While we have demonstrated isolate specificity of inducibility of resistance (Sharma et al. in press) and this is in itself an important issue that should be studied, pathogen populations in real life typically consist of several to many genotypes or races. Therefore, we also conducted a trial comparing single with mixed isolate inoculations with the aim to determine (iv) if and how the interactions between host genotype, plant strengthener, fertiliser, and isolate are affected by isolate mixtures.

A total of four trials were conducted using leaf discs derived from greenhouse grown young tomato plants raised in a potting mix from organic field soil, yard waste compost and peat. In trial I, the effects of three organic fertilizers were compared to chemical fertilizer for their effects on the susceptibility of six tomato varieties to three pathogen isolates. In trial II, three organic plant strengtheners and BABA were compared for their ability to induce resistance in plants challenged with the same three pathogen isolates and six varieties. In trial III, the interactions of the fertilizers and plant strengtheners were determined for two of the varieties challenged with the three pathogen isolates. There were no interactions between fertilisers, varieties, and isolates and also no interactions between fertilisers and plant strengtheners. In contrast, interactions were highly significant for plant strengtheners, varieties, and isolates. Thus, in trial IV, the effects of BABA and the different plant strengtheners were tested on the six varieties challenged with the three isolates, three two-way and the three-way mixture of the isolates. A preliminary report on the effects of Fertilizers and plant strengtheners has been presented previously (Sharma et al. 2009).

6. 2. Materials and Methods

6. 2. 1. Fertilizers and plant strengtheners used

Two biofertilizers, BioFeed Basis (7.5:2:4 NPK) (AgroBio Products, Wageningen, NL), and Bio-ILSA (12:0:2 NPK) (ILSA Group Arzignano, Vicenza, Italy) were compared with Horn meal (13.7:0:2 NPK) and chemical fertilizer (27:46:40 NPK) application.

BioFeed Basis is a complex mixture of plant proteins derived from seaweed, potatoes, maize, soybean and sesame, enriched with soft ground rock phosphate, potassium sulphate and calcium carbonate (AgroBio Products, Wageningen, NL). Similarly, Bio-ILSA is an organic nitrogen fertilizer manufactured through physical hydrolysis of leather shavings of bovine hides devoid of phosphorus (ILSA Group Arzignano, Vicenza, Italy). Horn meal and chemical fertilizers were purchased commercially. Additional triple super phosphate (46 % P_2O_5) and potassium chloride (40 % K_2O) were used to equilibrate P and K levels for all treatments.

As plant strengtheners BioFeed QUALITY (BFQ) (AgroBio Products, Wageningen, NL), Alfalfa extract (ILSA Group Arzignano, Vicenza, Italy) and PEN (originating from the commercial organic fertilizer, Agrobiosol) (SW-Düngesysteme GmbH, Germany) extract were used. BFQ is a watery multi-compound extract from two types of seaweed: *Ascophyllum nodosum* and *Fucus spp* (AgroBio Products 2007). PEN extract was prepared from dry mycelium of *Penicillium chrysogenum* according to Dong and Cohen (2002): 100 g of dry mycelium (Agrobiosol) was suspended in 1 l of distilled water. The suspension was shaken for 2 h at 100 rpm; stored for 22 h at room temperature; and then briefly agitated and filtered through Whatman No. 1 filter paper. The filtrate was autoclaved for 30 min at 110°C and after cooling; the 10 % PEN extract was stored at 4°C and used within one month. Alfalfa extract is prepared from, *Medicago sativa*. Control treatments included distilled water and the chemical inducer BABA at 1 g l^{-1}.

6. 2. 2. Plant material

Six tomato varieties, Matina, Berner Rose, Marmande, Zukertraube (obtained from the heritage seed company Bingenheim, Germany), Balkonzauber (Erfurter Samen, Germany), and Supermarmande (Thompson Morgan Ltd, UK) were used depending upon the trial.

All plants were grown in a greenhouse at 22°C day and 18°C night temperature and a 16/8 h day/night cycle. Ten-day old seedlings were transplanted in 1.3 l pots filled with a potting mixture prepared from organic field soil, yard waste compost (15% by weight) and peat (15% by weight). Fertilizers were added at the time of transplanting (16.5 mg N,

4.4 mg P and 8.8 mg K per pot). Plants were watered daily with 50 ml water to prevent leaching of nutrients. Seven to eight week old plants were used for inoculation.

6. 2. 3. Preparation of pathogen inoculum, inoculations, and assessment

P. infestans isolates 75, 101 and 108 collected locally in 2004 and all virulent on the six tomato varieties were used. The isolates were selected based on their known reactions and interactions with BABA induced resistance on the selected tomato varieties (Sharma et al. in press) *P. infestans* was grown and maintained at 17 °C in Petri dishes on pea agar (125g frozen pea l^{-1} H_2O, 1.5% agar) in the dark. Sporangial suspensions were prepared from 21 day old cultures as described by Sharma *et al.* (in press) and adjusted to a concentration of 5 * 10^4 sporangia ml^{-1}. Tomato leaf discs were prepared using a cork borer (Ø: 18 mm) to standardize the inoculated leaf area, placed on moist filter paper in square shaped Petri plates (10*10 cm^2) and inoculated with a 20 µl drop of the sporangial solution. Inoculated leaf discs were kept in the dark for 24 h at 17 °C and afterwards a 16 h light/ 8 h dark cycle was maintained. Humidity in the Petri plates was maintained by wetting the lids with sterile demineralised water every two days.

Percent diseased leaf area was assessed on day four, five and six (after day six, the controls were fully sporulating). Sporulation capacity (SC) was determined immediately after the final disease assessments on day six by washing the sporangia off the leaf discs as described by Sharma et al. (in press). Data of SC were not obtained for Trial IV (Isolate mixture).

6. 2. 4. Trials conducted

All six tomato varieties and the three *P. infestans* isolates were used in the first two trials. The effects of the four fertilizers were evaluated in trial I while in trial II the effects of the three plant strengtheners were compared to a water control and a BABA treatment in chemically fertilized tomatoes only. From seven days after transplanting, plants were watered weekly five times with 50ml of an aqueous solution of QUALITY, Alfalfa extract and PEN at 4%, 0.1% and 2.5% concentration, respectively; control plants were given water only. The BABA controls were sprayed one week before inoculation to run-off with a solution of 1 g l^{-1} BABA to near run-off.

In trial III, the interactive effects of the fertilizers and the plant strengtheners including BABA with the three isolates were tested on the tomato varieties Balkonzauber and Zukertraube. The application dose and methods of fertilizer and plant strengthener applications were the same as for the first two trials.

For trial IV, isolate mixtures were prepared by mixing isolate solutions of equal sporangial concentrations in equal amounts and inoculating with the mixtures as described above. In addition to the single isolates 75, 101, and 108, there were four isolate mixtures, 75+101, 75+108, 101+108, and 75+101+108, respectively. The experimental setup with respect to varieties and plant strengthener application method and dose was as in Trial II.

Trials I-III all were conducted two times with six replications per treatment per experimental date. Trial IV was conducted only once with six replications. Trials were all conducted between July and September 2008.

6. 2. 5. Data analysis

Area under the disease progress curve (AUDPC) was calculated using the formula of Campbell and Madden (1990) and data were transformed with log (x+1) when necessary to improve the normality and homogeneity of variance. The diseased leaf area (DLA) in cm^2 was calculated from the estimated percentage diseased leaf area. From this, sporulation capacity (SC) was calculated per lesion area.

The combined data from the repeated trials were analysed with the experimental date as a factor to determine any effects or interactions due to experimental repeat. As there were

no effects of the experimental repeat, nor significant interactions between date and the other factors all analyses were performed across experiments resulting in 12 replications per treatment for trials I-III. All experiments were analysed with the GLM procedure of the statistical analysis system version 9.2 (SAS institute, Inc., Cary, NC) as factorial design with interactions. Mean separations were done with Tukey tests ($P<0.05$).

6. 3. Results

The susceptibility of the six varieties to the three isolates and the aggressiveness of the three isolates were very similar with isolate 75 slightly more aggressive than the other two isolates based on AUDPC on the water controls (Fig. 6. 1).

6. 3. 1. Trial I: Fertilizer effects

AUDPC was most affected by fertilizers (F=524, $P<0.01$) followed by isolate (F=95, $P<0.01$) and then by variety (F=12, $P<0.01$). When analysed across experimental runs there was a small interaction between variety and fertilizers (F=2.5, $P<0.01$). This interaction did not exist within experimental run, however and there were no effects of the experimental repeat (F=0.17, $P=0.68$). There were also no significant interactions between the experimental repeat and other factors. Therefore, the variety by fertilizer interaction was ignored as an artefact.

The tomato varieties were significantly more resistant against *P. infestans* when fertilized with BioFeed Basis and Bio-ILSA (AUDPC = 0.59 and 0.64, respectively) than when fertilized with horn meal or chemical fertilizer (AUDPC = 0.89 and 0.94, respectively). The ranges of disease reduction relative to chemical fertilizer application across tomato varieties were 37-55%, 32-48%, and 3-15% for BioFeed Basis, Bio-ILSA and horn meal, respectively.

SC was most affected by isolate (F=167, $P<0.01$) followed by tomato variety (F=47, $P<0.01$) and fertilizer (F=27, $P<0.01$). Sporulation with chemical fertilizer was $41.7*10^3$. All three organic fertilisers reduced SC in comparison to chemical fertilizer independent of isolate and variety used with the effects of BioFeed Basis and BI12 (SC=$35.6* 10^3$ and $37.4* 10^3$, respectively) significantly stronger than those of horn meal (SC=$39.4* 10^3$).

The reductions in SC across the six tomato varieties against the three isolates ranged between 8-36%, 4-21%, and 4-10% relative to chemical fertilizer application when using BioFeed Basis, Bio-ILSA, and horn meal, respectively.

6. 3. 2. Trial II: Plant strengthener effect

The plant strengtheners had the largest effects on AUDPC followed by isolate and tomato variety (Table 6. 1). In contrast to the fertilizer effects, interactions occurred at all levels, however. Again, the effects were the same in both experimental runs without significant effects of experimental repeat.

The three plant strengtheners as well as BABA significantly reduced AUDPC compared to the water control in all cases (Fig. 6. 1). The ranges of reduction in AUDPC relative to the water control across tomato varieties and isolates were 23-78%, 21-77%, 17-66%, and 37-100% for Alfalfa extract, PEN, QUALITY, and BABA, respectively (Table A-6.1A). While usually the effects of BABA were significantly stronger than those of the plant strengtheners this was not the case for Berner Rose, Marmande, and Supermarmande when inoculated with isolate 75. In some other cases one of the three plant strengtheners performed equally well as BABA, e.g. Alfalfa extract on Zuckertraube with isolate 101 or QUALITY on Balkonzauber, Berner Rose, and Supermarmande with isolate 108 (Fig. 6. 1).

Like with AUDPC, the largest effects on SC were also caused by plant strengthener ($F=291$, $P<0.01$) followed by isolates ($F=27$, $P<0.01$) and tomato variety ($F=22$, $P<0.01$). The ranges of reduction in SC relative to the water control across tomato varieties and isolates were 8-55%, 14-52%, 3-52%, and 32-100% for Alfalfa extract, PEN, QUALITY, and BABA, respectively (Table A-6.1B). While there were some significant interactions between the main effects, these occurred only sporadically and with F-values much below the F-values of the main effects. For example, the isolate*variety*plant strengthener interaction was highly significant ($F=1.9$, $P<0.01$), however, the F-value is by one to two orders of magnitude smaller than that of the main effects. While the reduction patterns for AUDPC and SC were more or less similar for BABA on most varieties tested, for Alfalfa extract, PEN and QUALITY there were some distinct deviations (Fig. 6. 2). For example, Alfalfa extract, PEN and BABA did not

differ in their effects on AUDPC of isolate 75 on Berner Rose. However, BABA reduced SC significantly more than the two plant strengtheners. With isolate 101 effects of the three compounds on SC were the same while BABA reduced AUDPC significantly more. Lesion size (i.e. final DLA) and SC*1000 correlated more or less strongly across all inducers and water when separated by tomato varieties and isolates with the highest correlations being observed for isolate 108 (Table 6. 2, Fig. 6. 3). Because AUDPC and final lesion size are closely related, the data on AUDPC and SC shown in Figs. 6. 1 and 6. 2 also illustrate interactions between inducers and resistance components. For example, AUDPC of isolate 101 on Marmande was significantly reduced from 0.95 in the water control to 0.55 when treated with QUALITY. In contrast, SC was statistically not different in the two treatments with 31.9 * 10^3 and 28.2 * 10^3 sporangia cm^{-2} lesion in the water and QUALITY treatments, respectively. On Zuckertraube, the three isolates were not different in AUDPC and SC in the water controls. When treated with Alfalfa extract, disease reduction was significantly greater for isolate 101 than for the other two isolates while SC did not differ among the isolates.

6. 3. 3. Trial III: Interactive effects of fertilizers and plant strengtheners

Overall, AUDPC was a little higher in trial III than in trials I and II. Like in trial I, there were no interactions between isolate and variety with the effects of fertilizers and fertilizer effects were the same as in Trial I. Plant strengthener effects in the chemically fertilized treatments of trial III and II were also alike. Just as before, AUDPC on Zuckertraube treated with Alfalfa extract and BABA and challenged with isolate 101 did not differ (Figure A-6.1). Also, PEN performed significantly better than QUALITY on Balkonzauber inoculated with isolate 101. The same was true with all other fertilisers tested.

The plant strengtheners had the strongest effects on AUDPC (F=3244.21, $P<0.01$), followed by isolate (F=180.88, $P<0.01$), variety (F=165.67, $P<0.01$), and fertilizer (F=34.77, $P<0.01$). The significant interaction between fertilizer and plant strengthener effects with a low F-value (F= 6.91, $P<0.01$) is due to the fact that fertilizer effects were only present in the absence of plant strengtheners (Fig.6. 4A).

Like AUDPC, SC was most affected by plant strengthener (F=1043.75, $P<0.01$) followed by tomato variety (F=220.57, $P<0.01$), fertilizer (F=52.39, $P<0.01$) and isolate (F=8.08, $P=0.0003$). In contrast to AUDPC, the significant effects of the fertilizers BioFeed Basis and Bio-ILSA on SC persisted in the presence of PEN (Fig. 6. 4B) and interactions between variety, isolate, and plant strengthener were significant albeit with low F-values (variety * fertilizer: F=3.34, $P=0.02$; fertilizer * plant strengthener: F=2.97, $P<0.01$; variety * fertilizer * plant strengthener: F=2.19, $P=0.01$).

6. 3. 4. Trial IV: Isolate mixture effect

Like in Trial II, the strongest effect on AUDPC was by plant strengthener, isolate, and then by variety along with a very small plant strengthener*variety*isolate interaction (Table 6. 1). The interactions between the main effects changed somewhat, however, when isolate mixtures were used instead of single isolates. Plant strengthener and isolate effects were stronger in trial IV than in trial II while, in contrast, the plant strengthener*variety*isolate interaction was similar in trial IV with an F-value of 2.7 and 120 df in contrast to an F-value of 4.4 and 40 df in trial II (Table 6. 1).

The isolate mixtures affected the overall susceptibility of the varieties and the degree of resistance induction by the plant strengtheners depending on host genotype (Fig. 6. 5). Disease levels on plants were significantly lower when inoculated with two- and three-way isolate mixtures than with single isolates (Fig. 6. 5). At the same time, the effectiveness of the plant strengtheners increased and the differences between plant strengtheners and BABA decreased with increasing mixture complexity. Thus, BABA performed significantly better than the plant strengtheners in 34 out of 54 (65 %) cases tested, when single isolates were used, in 25 out of 54 (45 %) of the cases, when two way mixtures were used, and in 6 out of 18 (33 %) of the cases when the three way mix was used (Fig. 6.5).

6. 4. Discussion

Two of the three organic fertilizers reduced *P. infestans* severity independent of tomato variety and pathogen isolate. The differences among the fertilizers disappeared to a great

extent when plants were treated with plant strengtheners or BABA. In contrast, while all three plant strengtheners generally were capable of inducing resistance, there were plant strengthener, variety, and isolate specific effects and interactions. Interestingly, the variety specific effects were generally reduced when two-way mixtures of the isolates were used and they disappeared with the three-way mixture. These results warrant more detailed studies of this phenomenon. Trials I-III were highly repeatable and single isolate effects in trial IV were very similar to trial II which suggests that the data are solid.

The lack of interactions between fertilizers and inducers in our study suggests that inducibility of resistance can be studied without too great a concern about the growing substrate used. It would be interesting to compare this to soil-less culture, however, as there are supposedly no or much reduced microbial interactions in the root zone in such systems. The isolate and variety specific interactions with the plant strengtheners need to be considered carefully, in contrast. On the one hand, these can be useful for identifying different mechanisms involved in the resistances induced by the plant strengtheners. On the other hand, studies with single isolates and host genotypes may result in wrong conclusions about the effectiveness of inducers or plant strengtheners in practice.

The effects of fertilizers were more apparent for AUDPC than for SC (Fig. 6. 4). When comparing different aggressiveness parameters of *P. infestans* AUDPC proved to be a robust measure of isolate performance in epidemics (Fry 1978). SC, in contrast, is subject to restrictive assumptions and many measurement errors and thus a less reliable parameter. However, SC determines the amount of secondary inoculum produced and thus the number of potential future lesions making it an important epidemiological parameter.

For induced resistance to take place plants have to take up the inducer and the appropriate resistance reactions to a given pathogen have to be triggered. Differences among cultivars in their response to various inducers could be due to an array of differences along the path from resistance inducer uptake to the delivery of the resistance response: the pathogen recognition mechanisms, the resistance mechanisms available, or the delivery of the resistance response. A variety of defence mechanisms are activated in tomato plants induced by BABA, including physical barriers and biochemical changes leading to resistance against *P. infestans* (Cohen 2002). For PEN, induction of early defense-related

compounds such as ethylene, peroxide, increase of peroxidase enzymes and intercellular acidification in tomatoes against *P. infestans* have been found (Thuerig et al. 2006; Unger et al. 2006). The seaweed extract in the plant strengthener QUALITY might have had biostimulant activities which induced resistance in tomatoes. Several compounds, including seaweeds have been shown to have biostimulant activity acting as positive plant growth regulators or as metabolic enhancers (Miller 1990). Similarly, Ertani et al. (2009) found biostimulant and hormonal activity, especially gibberellins, in Alfalfa extract.

Different isolates may also have different inducing effects as they may possess different resistance suppressors and/or virulence factors (Andreu et al. 1998). Depending on isolate different resistance mechanisms may thus be triggered. This could have led to the interactions between the three *P. infestans* isolates with the tomato varieties and resistance induction by different plant strengtheners. Mixing isolates may lead to the simultaneous triggering of several resistance mechanisms, leading to an overall lower susceptibility as found in trial IV (Fig. 6. 5). While avirulent isolates have been shown to be potent resistance inducers (e.g. Enkerli et al. 1993; Martinelli et al. 1993; Calonnec et al. 1996; Yan et al. 2002), our data suggest that not only avirulence but also other genetic differences among isolates of similar aggressiveness may induce resistance when co-inoculated.

Host-pathogen interactions start at the moment a spore lands on the host. We only measured AUDPC and SC in detail in this study after applying a mean of 100 sporangia per 20µl drop of inoculum per leaf disc. This led to 100% infection efficiency in the water controls. The uneven levels of correlation between SC and final diseased leaf area or AUDPC of the isolates on the varieties demonstrate that the varieties and plant strengtheners may vary in their effects on lesion expansion and SC.

Resistance induction may also reduce germination and infection efficiency, however (Kochman & Brown 1975; Martinelli et al. 1993; Calonnec et al. 1996; Jeun et al. 2000; Yan et al. 2002). In a preliminary trial, activation (i.e. germination with or without further development) and establishment (i.e. hyphal development after penetration) of sporangia was investigated microscopically with histochemical aniline blue staining on Balkonzauber and Super Marmande either pre-treated with BABA or not and challenged

with single or mixed isolates in four replications. In Super Marmande, BABA treatment had no effect on spore activation or establishment. Also, while there was variation, there were no statistically significant differences among the single isolates. When inoculated with a mixture of isolates 75 and 108, however, spore activation was significantly increased above the mean activation of the single isolates in the water controls (mixture: 63%, mean of isolates: 34%) while establishment was considerably reduced (mixture: 5%, mean of isolates: 19%, P=0.06) (Data not shown).

In Balkonzauber, no significant effects were seen in spore activation (Fig. 6. 6 A, B) while multiple interactions could be observed with respect to establishment of infections, however (Fig. 6. 6 C, D). Percent establishment of isolate 101 was significantly higher in BABA treated plants than in water treated plants, while the reverse was true for isolate 108. In the controls, the mixture of 75 and 101 established significantly more infections (30%) than the mean of the single isolates (8%) (Fig. 6. 6 C). In contrast, when induced with BABA all mixtures established fewer infections than the means of the respective single isolates. This was statistically significant in the mixture of isolate 101 and 108 (Fig. 6. 6 D). Thus, like AUDPC and SC, isolate germination and penetration may also be affected in a variety specific way in response to induction treatment or mixed inoculation indicating that different resistance mechanisms may be involved in the different variety-isolate interactions. There are many opportunities for these to be expressed as variation in the efficiency of recognition and signal transduction from original recognition through to delivery of the response. Which of these processes is most important or shows most variation is not known. Very low sporangial concentrations, e.g. through spray inoculation of leaves, would need to be used to study these interactions in detail.

Besides pointing to possibilities to study resistance induction mechanisms in detail we have found that if applied in a commercial type of setup the plant strengtheners and fertilizers we used in this study can indeed reduce the susceptibility of greenhouse grown and field grown tomatoes to *P. infestans* (Schulte-Geldermann 2008). The results of the mixed inoculations are of particular interest in practice where mixed inoculum is the rule rather than the exception. On the one hand, this means that most likely plants are already induced in the field as suggested by Walters (2009) and impressively demonstrated by Calonnec et al. (1996) for *Puccinia striiformis* on wheat. This also provides evidence that

diversity on the pathogen side may be of advantage especially in a scenario where resistance is incomplete as in the case for the tomato *P. infestans* pathosystem. This is in line with the suggestion by Mundt (2002) who pointed out that diversified host populations will support more diverse pathogen populations and that pathogen diversity is positively related to the disease control by the mixture.

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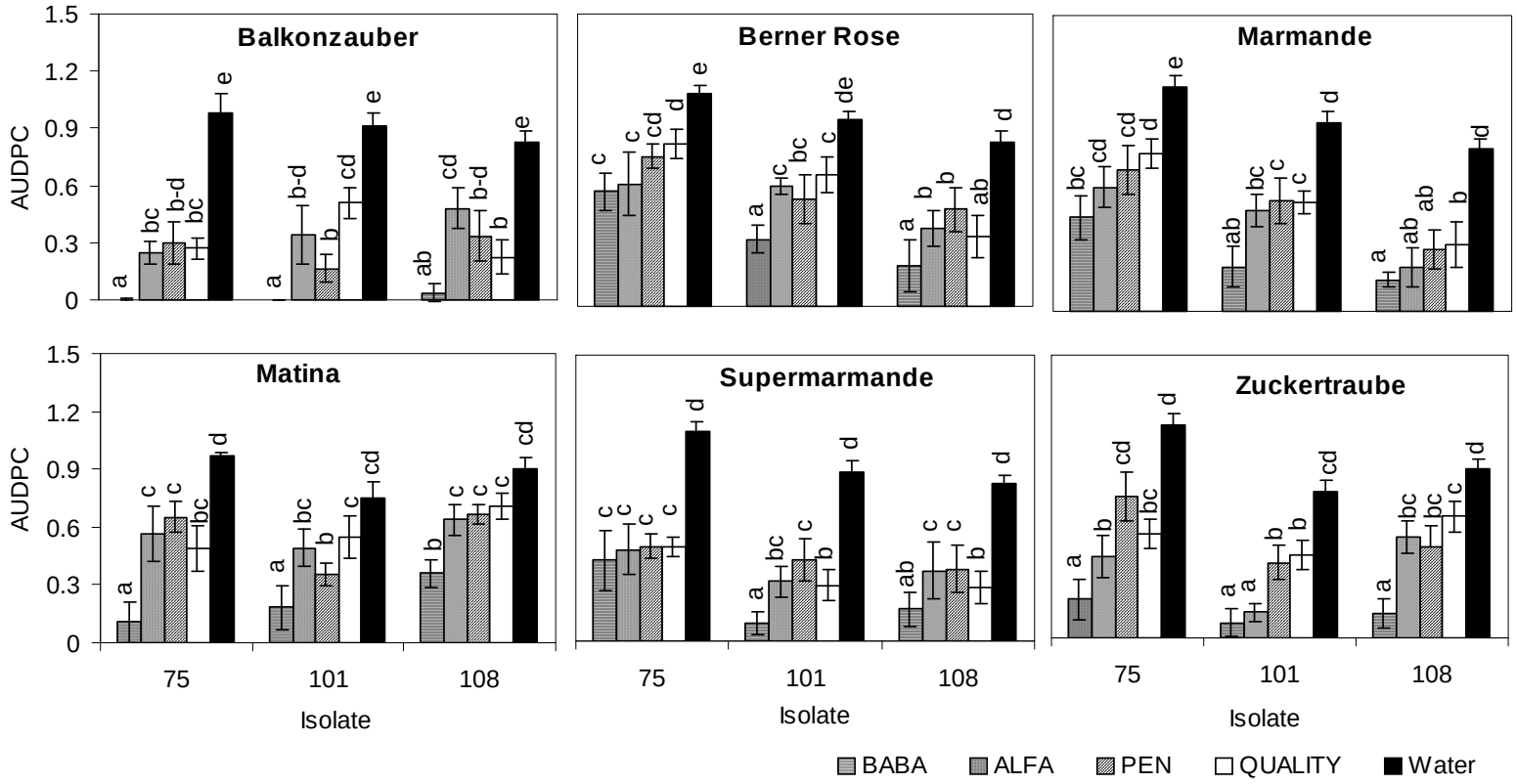

Figure 6. 1. Effects of the plant strengtheners Alfalfa extract (ALFA), PEN, and BioFeed QUALITY in comparison to a water control and chemical induction through BABA on the area under the disease progress curve (AUDPC) (back-transformed data) of six tomato varieties challenged with three isolates of *P. infestans*. The presented values are the means ±SD of two experiments with six replications each. Within each figure bars marked with different letters are significantly different (P≤0.05, Tukey test).

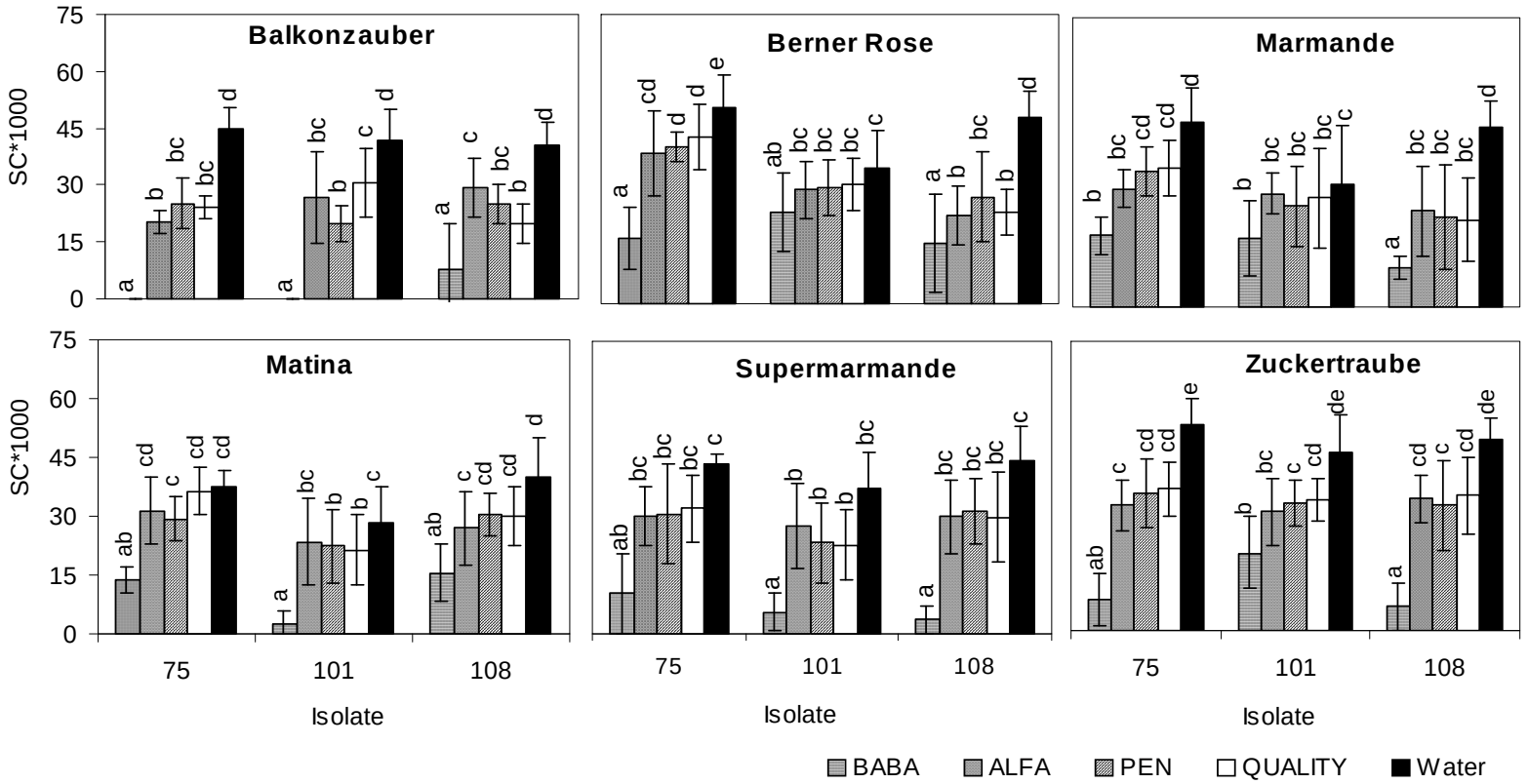

Figure 6. 2. Effects of the plant strengtheners Alfalfa extract (ALFA), PEN, and BioFeed QUALITY in comparison to a water control and chemical induction through BABA on the Sporulation capacity cm^{-2} lesion on day six after inoculation (SC*1000) of six tomato varieties challenged with three isolates of *P. infestans*. The presented values are the means of two experiments ±SD with six replications each. Within each figure bars marked with different letters are significantly different ($P \leq 0.05$, Tukey test).

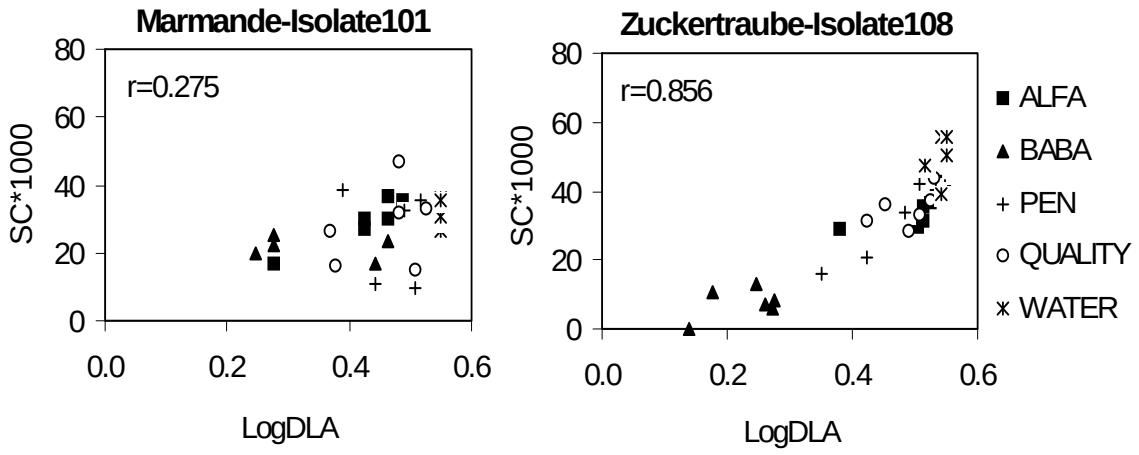

Figure 6. 3. Correlation between diseased leaf area (LogDLA) and Sporulation capacity (SC*1000) per cm^2 lesion on day six after inoculation on Marmande (Pearson correlation r =0.275, P=0.0364) and on Zuckertraube (r =0.856, P<0.01) treated with water, the plant strengtheners Alfalfa extract (ALFA), PEN, or BioFeed QUALITY or BABA and challenged with *P. infestans* isolates 101 or 108, respectively.

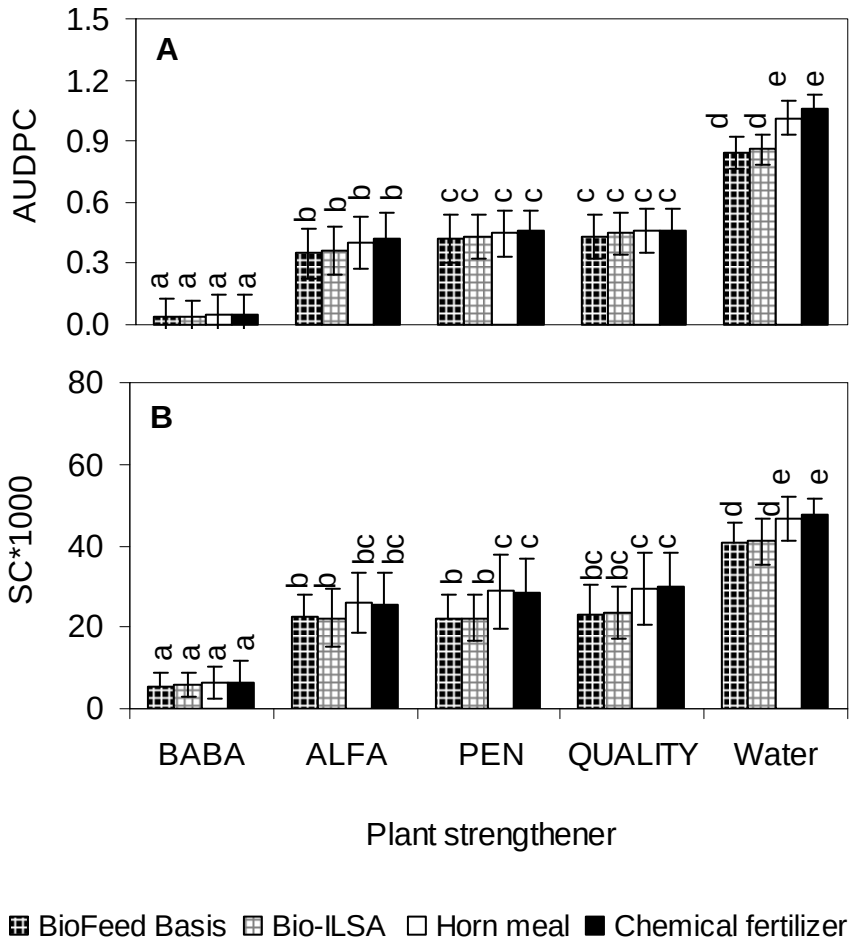

Figure 6. 4. Interactive effects of fertilizers and the plant strengtheners Alfalfa extract (ALFA), PEN, and BioFeed QUALITY on area under disease progress curve (AUDPC) (back-transformed data) on the tomato varieties Balkonzauber and Zukertraube challenged with three *P. infestans* isolates. The presented values are the means across isolates of two experiments with six replications each. Bars represent SD. Significant differences are marked with different letters above the bars (P=0.05, Tukey test).

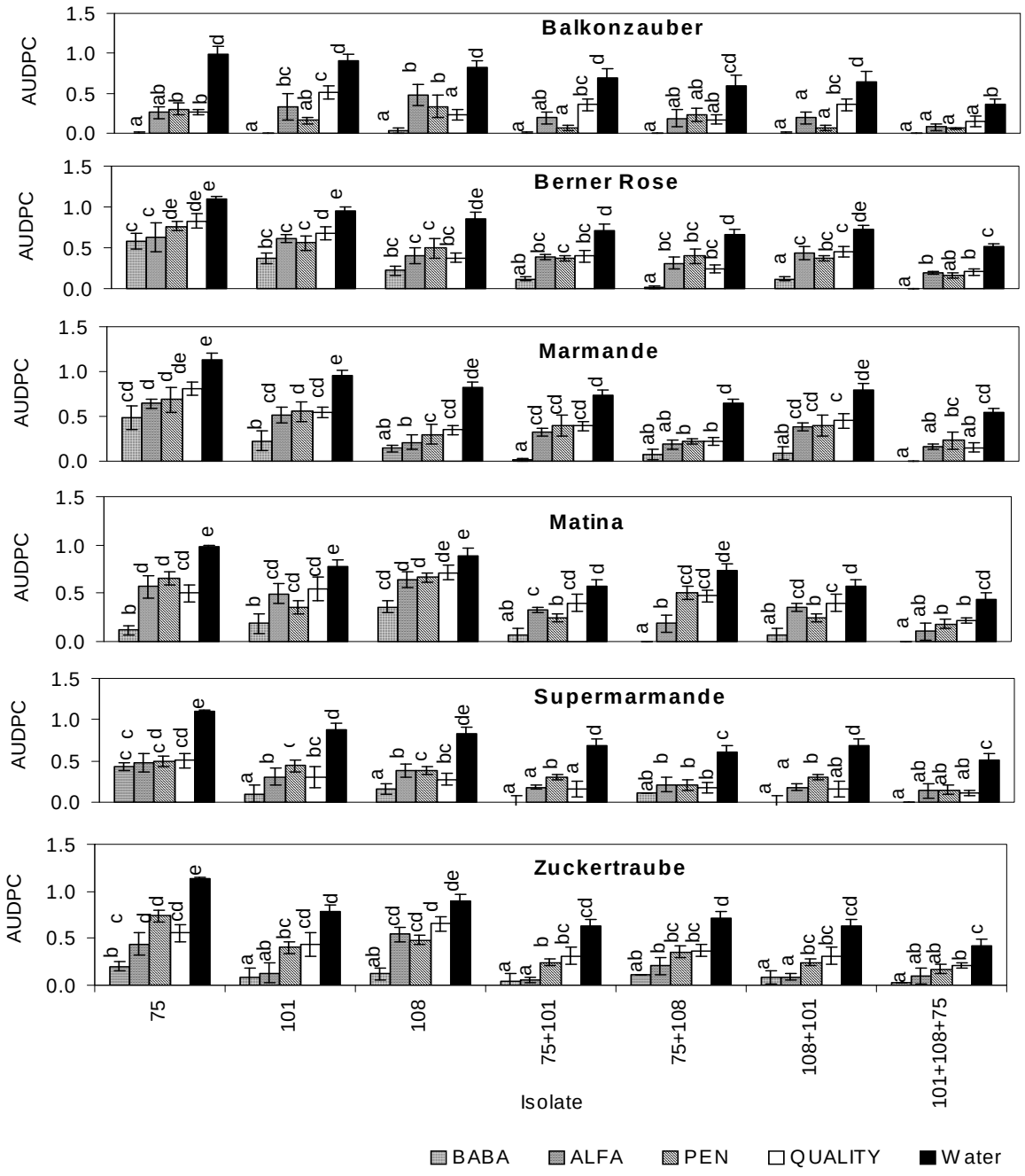

Figure 6. 5. AUDPC (Area under the disease progress curve) (back-transformed data) of *P. infestans* on six tomato varieties when treated with water, the chemical inducer BABA, the plant strengtheners Alfalfa extract (ALFA), PEN, or BioFeed QUALITY and then challenged either with single isolates, two-way or a three-way mixture. Error bars represent the standard deviation. The presented values are based on one experiment with six replications. Significant differences in AUDPC are marked with different letters above the bars ($P \leq 0.05$, Tukey test).

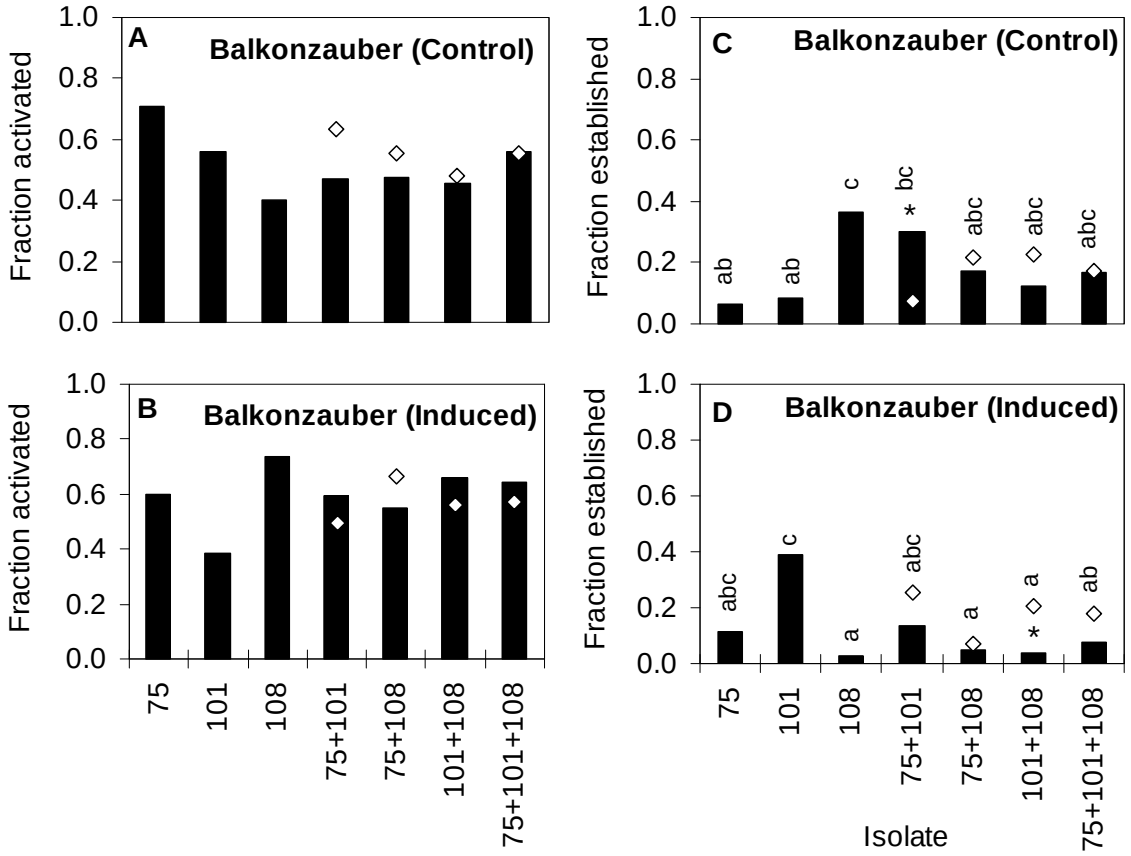

Figure 6. 6. (A, B) Percent of spores that were activated but did not develop further and (C, D) percentage established infections (i.e. hyphal development after penetration) in the tomato variety Balkonzauber either treated with water (Control, A, C) or with BABA (Induced, B, D) one week before challenge inoculation with *P. infestans*. Inoculations were performed with three single isolates (75, 101, or 108) or all possible isolate mixtures. For the isolate mixtures the diamonds show the expected values (i.e. the mean of the respective single isolates). Different letters above the bars indicate that they differ significantly (Tukey-Kramer Test, $P<0.05$) (Comparisons apply across both graphs c and d). * indicates that a mixture is significantly different from the mean of the expected value (linear contrast, $P<0.05$) (data were Arcsine square root transformed for analysis, back-transformed data are shown).

Table 6. 1. Analysis of variance for area under the disease progress cure (AUDPC) in trials II and IV for the effects of the plant strengtheners Alfalfa extract (ALFA), PEN, or BioFeed QUALITY compared to water and the chemical inducer BABA when challenged with three isolates in trial II or the three isolates and four isolate mixtures in trial IV.

Source[1]	Cumulative disease from day four to six (AUDPC)					
	Trial II			Trial IV		
	DF	F Value	P Value	DF	F Value	P Value
Isolate (Iso)	2	97.3	0.0001	6	287.9	0.0001
Tomato variety (Var)	5	68.0	0.0001	5	75.3	0.0001
Plant strengthener (PS)	4	627.5	0.0001	4	1099.6	0.0001
Iso* Var	10	34.4	0.0001	30	12.3	0.0001
Iso*PS	8	4.5	0.0001	24	2.9	0.0001
Var*PS	20	8.8	0.0001	20	10.0	0.0001
Iso*Var*PS	40	4.4	0.0001	120	2.7	0.0001

[1]Source of variation. See materials and methods for detailed information

Table 6. 2. Pearson correlation (r) between LogDLA and SC*1000 of three *P. infestans* isolates on six tomato varieties treated with water, the chemical inducer BABA, the plant strengtheners Alfalfa extract (ALFA), PEN, or BioFeed QUALITY (Trial II)

Tomato variety	LogDLA vs. SC*100		
	Isolate 75	Isolate 101	Isolate 108
Balkonzauber	r=0.775 (**[1])	r=0.679 (**)	r=0.578 (**)
Supermarmande	r=0.344 (**)	r=0.695 (**)	r=0.685 (**)
Matina	r=0.389 (**)	r=0.639 (**)	r=0.777 (**)
Berner Rose	r=0.518 (**)	r=0.608 (**)	r=0.760 (**)
Marmande	r=0.649 (**)	r=0.275 (*)	r=0.736 (**)
Zuckertraube	r=0.845 (**)	r=0.562 (**)	r=0.856 (**)

[1]** and * inside the parenthesis indicate Pearson correlation (r) between LogDLA and SC*1000 of three *P. infestans* isolates on six tomato varieties treated with water, the chemical inducer BABA, the plant strengtheners Alfalfa extract (ALFA), PEN, or BioFeed QUALITY were significant at P<0.01and P<0.05, respectively.

7. General discussion

Interest in induced resistance (IR) has increased dramatically in the last 30 years because of the prospect of broad spectrum disease control. However, numerous controlled and uncontrolled experiments on IR have resulted in unsatisfactory results (see Vallad & Goodman 2004; Walters et al. 2005a for review).

As the literature review shows much knowledge has been accumulated about the mechanisms of resistance induction. Considering especially that many authors describe IR as generally race-non specific (Sticher et al. 1997; Van Loon et al. 1998) it appears strange that no systematic tests have been conducted about this question previously. Similarly, little attention has been paid to the factors that are likely to influence the effectiveness of IR in the field.

Using multiple host and pathogen genotypes, organic amendments and plant strengtheners in a vast number of combinations the research described in this thesis has yielded several new results that may be of use to breeders as well as directly in practical agriculture.

Considering the multitude of mechanisms involved in IR it was to be expected that it should be variety and inducer specific. For the same reason it is also not overly surprising that isolate specific effects were found in IR of tomatoes against *P. infestans*. The fact that different compounds not only vary in the degree of resistance induced but are also host-genotype and isolate specific puts into question if breeding for inducibility will be a useful route to take as it could make varieties dependent on specific inducers only.

Details concerning effects of single isolates are of great interest and may further help elucidate the mechanisms of IR. Also, with induction being isolate specific this suggests that, contrary to the commonly stated belief (e.g. Agrios, 2005) pathogens should be able to adapt to IR. However, it needs to be kept in mind that in the field normally many different pathogen genotypes are present at the same time. Even the use of very simple 2-way and 3-way isolate mixtures with isolates that were virulent already resulted in a great reduction of variety, isolate, and inducer specific effects. This suggests that the isolate specificity may not be important in the field but rather that overall performance of

inducers might be enhanced. However, these results will need to be confirmed in repeated experiments with different types of isolate mixtures also including avirulent isolates.

In this context it is also important to determine how long induction will remain effective. While the leaf disc tests were useful to obtain standardised and repeatable results the reactions of the plants could be followed ran for five to six days only. Using whole plants and isolate mixtures may result in overall better induction for longer as the mixtures themselves appear to increase plant resistance. On the long run, it would be worthwhile to measure the effect of isolate mixtures in field experiments using whole plants. However, doing such experiments well in the field is very difficult because of pathogen migration within the field and from outside sources (Shattock 1976).

Combining different inducers might be an interesting option in practice to enhance plant performance. However, care has to be taken to avoid negative effects on plants. For example, Bion (BTH) reduces diseases caused by a broad spectrum of pathogens across a diverse range of crops (see Vallad & Goodman 2004 for review). However, the efficacy of IR induced by Bion depends on a number of variables, such as the dose and frequency of Bion, host genotype as well as the growth stage of the plant. Thus, Bion treatment even was found to increase severity of late leaf spot pathogen, caused by *Cercosporidium personatum* in peanuts (Zhang et al. 2001). In addition, Bion reduced shoot fresh weight in sunflower (Prats et al. 2002), suppressed growth of tobacco and cauliflower (Csinos et al. 2001; Ziadi et al. 2001) and reduced shoot growth and leaf enlargement in cowpea (Latunde-Dada & Lucas 2001). Romerio et al. (2001) found that the use of Bion severely affected the growth of pepper plants resulting in reduced yields.

There is a short lag period following treatment with an inducer for induced resistance to be expressed (Walters et al. 2005a). Following the lag period, there are several possible outcomes in terms of resistance expression like (i) defences are triggered and there is no further change in defences following pathogen challenge, (ii) defences are triggered and there is a further increase in these defences or the activation of a different set of defences following pathogen challenge, and (iii) defense mechanisms are not expressed until pathogen challenge has occurred, i.e. the plants are only primed (Walters et al. 2005b). Direct induction of defences is likely to be more costly than priming, especially in the absence of disease. Thus, Van Hulten et al. (2006) found that priming involved fewer

costs than direct induction of defences and, indeed, was beneficial in terms of the plant growth rate and fitness under disease pressure. Priming appears therefore to have clear ecological benefits and would also represent a promising approach for crop protection.

As well Alfalfa extract as QUALITY have had overall positive or neutral effects on yields of tomatoes in experiments in a commercial greenhouse set-up and/ or in a field experiment (Schulte Geldermann 2008). In contrast, foliar application of PEN has also resulted in phytotoxicity especially on grapes (Thuerig et al. 2006). However, when applied to the soil as was done in the experiments reported here no such effects were observed on tomatoes. PEN is derived from a commercial organic fertiliser (AgroBiosol) which is made of antibiotic free residues from Penicillin production (SW-Düngesysteme GmbH, Germany) and it is well known among organic farmers that this fertiliser has beneficial effects in increasing plant resistance.

Various studies of Baider & Cohen (2003), Ryley et al. (2003), Liljeroth et al. (2010) have shown additive/synergistic effect of inducers in combination with fungicides against pathogens under controlled and field conditions. Therefore induced resistance should not be considered as 'one approach that fits all' (Walters et al. 2005a) but can be part of integrated pest management that can fit into crop protection programs. There it also will be important to consider the timing of application and frequency of application, and the appropriate fungicide dosage.

The substitution of traditionally used fungicides such as copper based products and sulphur has been a major focus of organic agriculture in the recent past (Speiser et al. 2000). The result with the most immediate practical implications especially for organic farmers is therefore the confirmation that the three plant strengtheners tested all are effective resistance inducers for tomatoes against *P .infestans* and can be applied via the soil. In addition, the two complex organic fertilizers (but not horn meal) may also increase the overall resistance of tomatoes to *P. infestans*. This is in line with other studies where organic soil amendments exhibited resistance to foliar as well as soil borne pathogens (see Vallad & Goodman 2004 for review). This is good news for organic farmers who have to make use of inducers that are allowed in organic farming. The two commercial compounds Bion and Messenger cannot be used in organic farming as the active ingredient of Bion is the synthetic compound BTH and Messenger contains a

harpin obtained from genetically modified bacteria. Combining moderately resistant plants with a combination of plant strengtheners with the best possible growing conditions / organic fertilisation regimes including biologically highly active and disease suppressive composts, e.g., might thus increase the overall resistance within the growing system considerably.

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Appendices

Appendix I: Figures

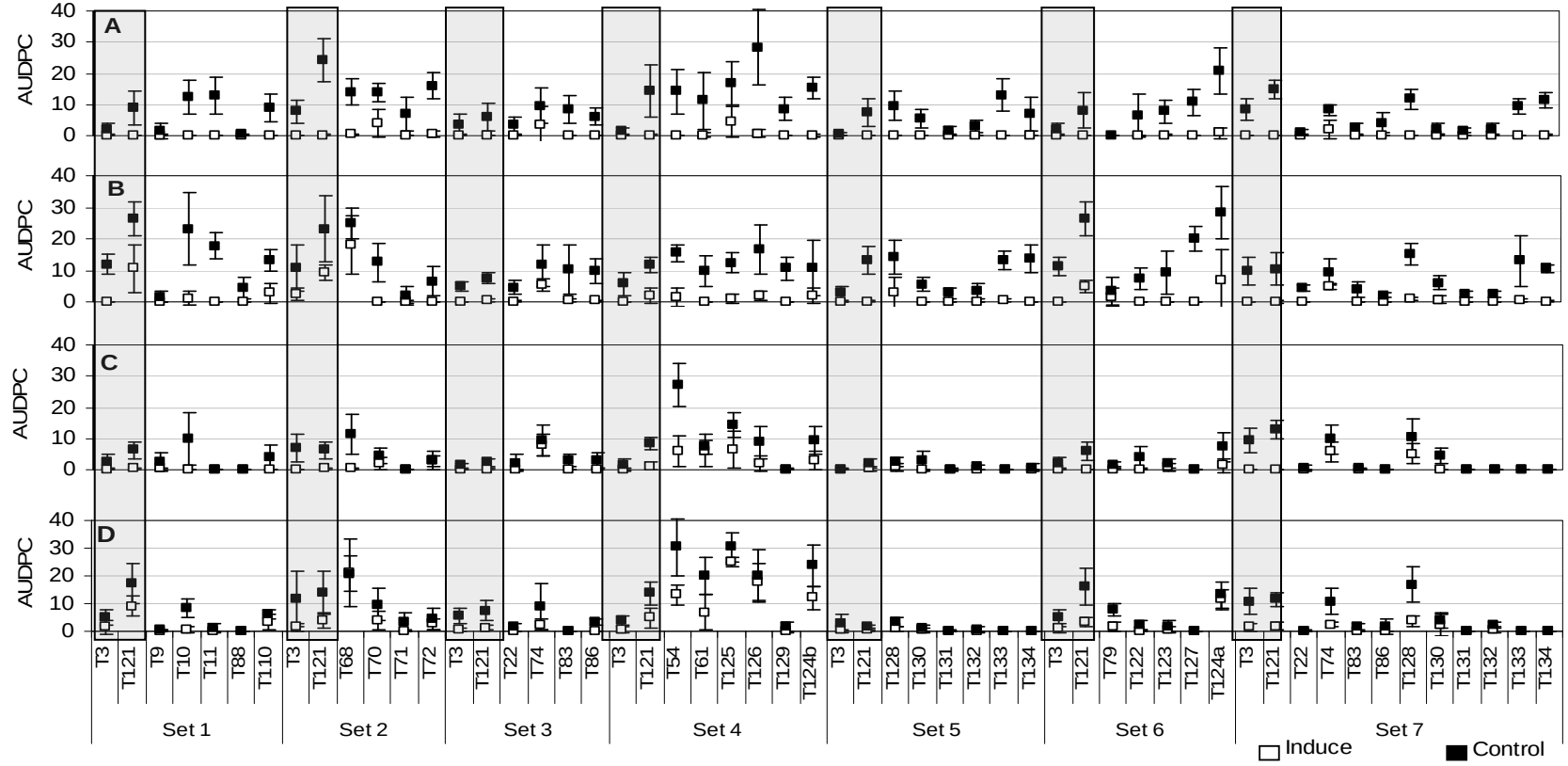

Figure A-4. 1. Area under the disease progress curve (AUDPC) on detached leaflets of 32 tomato accessions when induced with BABA (white) or not induced (black) (A) for isolate 108 on 1st leaf (B) isolate 108 on 2nd leaf (C) for isolate 101 on 1st leaf and (D) isolate 101 on 2nd leaf (untransformed data). Vertical numbers on the x-axis represent tomato accessions (see Table 4.1 for names of accessions). There were altogether seven sets (dates) of inoculation. In each set Supermarmande (T121) and Matina (T3) were included (shaded). Isolate 101 did not infect the controls successfully in set 5 and many of the accession in set 3 were resistant. The accessions of set 3 and 5 were therefore repeated in set 7**.**

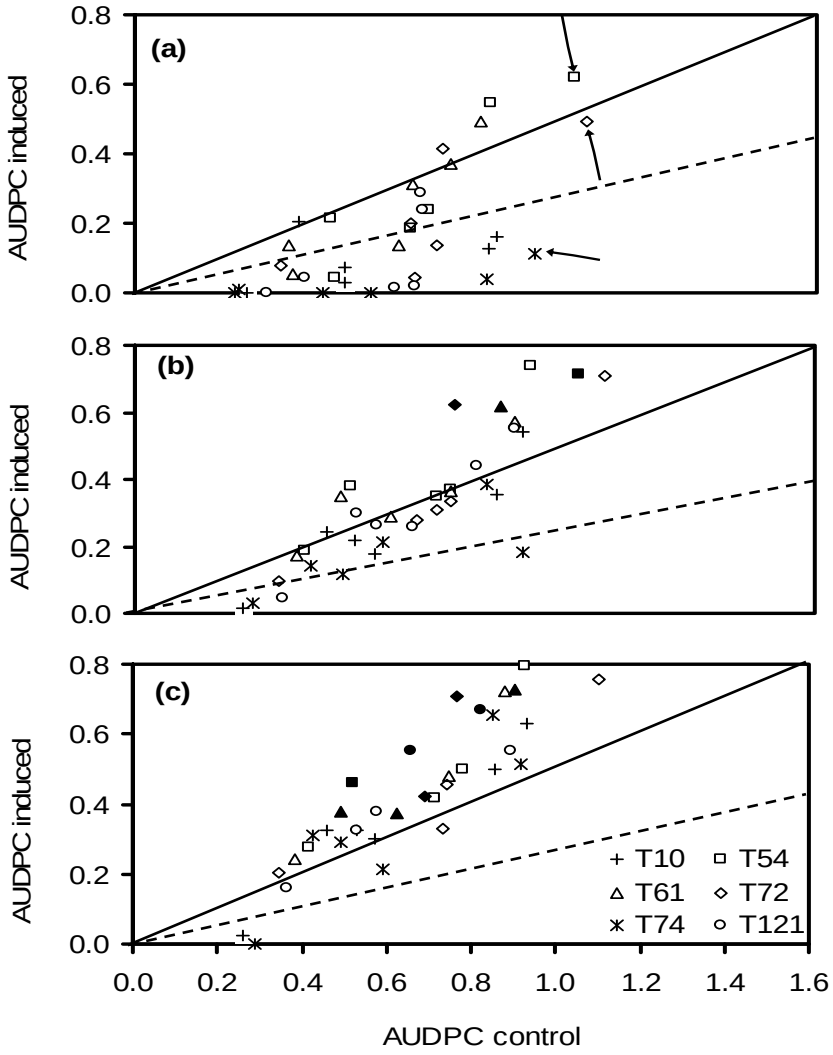

Figure A-5. 1. AUDPC (back-transformed data) on six tomato accessions not induced (x-axis) or induced (y-axis) with BABA and challenged with six different *P. infestans* isolates on (a) the 1st leaf, (b) the 2nd leaf, and (c) the 3rd leaf. The solid diagonal line indicates 50%, the dashed line 75% disease reduction, respectively. Filled symbols indicate that AUDPC on the induced leaves was not significantly different from the controls (Linear contrast, $P<0.001$). The three arrows in figure (a) indicate three tomato accessions T54, T72 and T74 which are of the same susceptibility to isolate 85 when not induced but differ in levels of induction. Data on the figures are the mean of three experiments with six replications each.

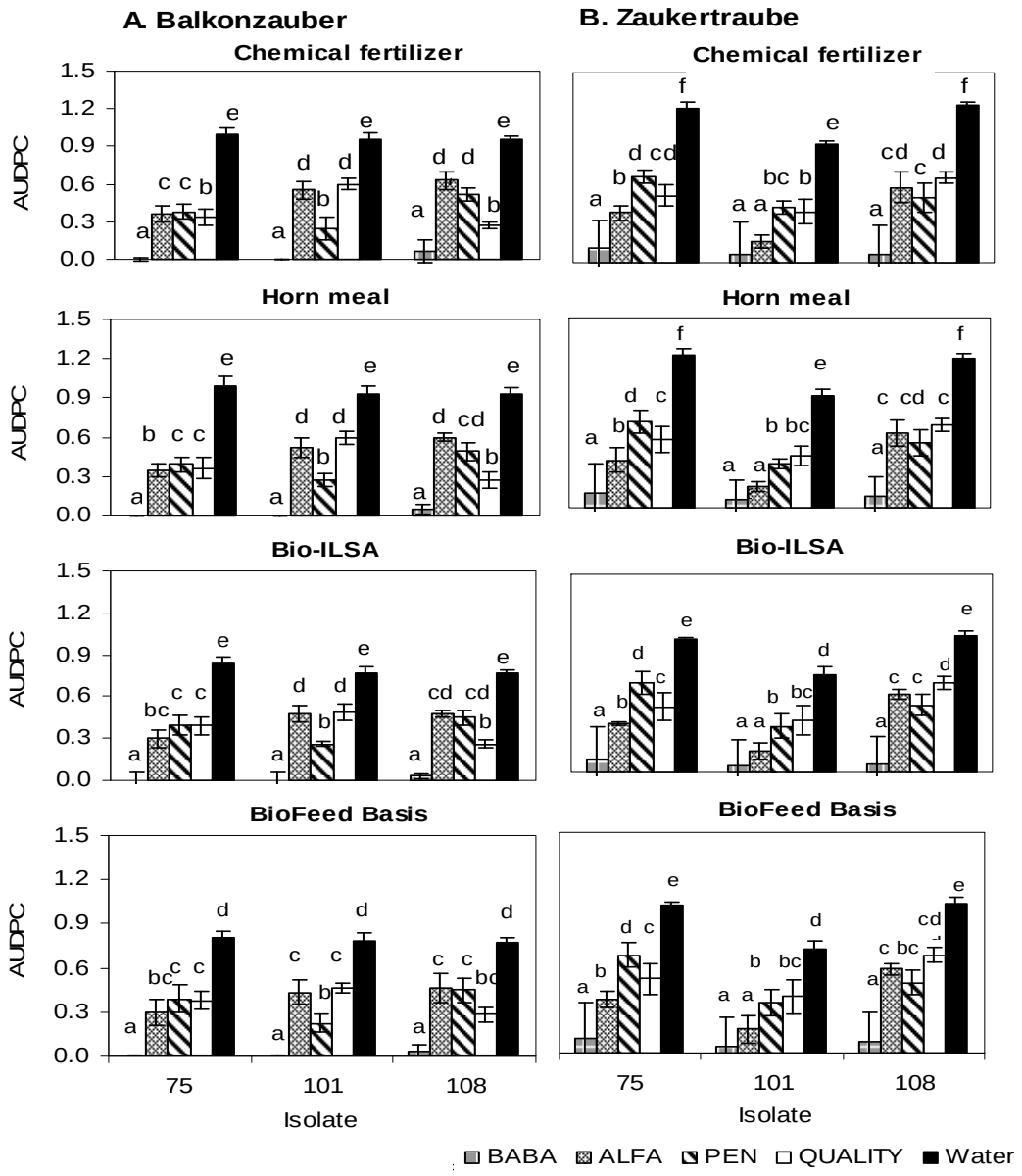

Figure A-6. 1. AUDPC (Area under the disease progress curve) (back-transformed data) of three isolates on Tomato accession (A) Balkonzauber and (B) Zukertraube across chemical fertilizer, Horn meal, Bio-ILSA 12 and BioFeed Basis with and with out plant strengtheners (control). The presented value is the mean of two experiments with six replications each. Bars represent ± SD. Significant differences are marked with different letters above the bars ($P \leq 0.05$, LS means, Tukey test).

Appendix II: Tables

Table A-3. 1. ANOVA of inoculation of different isolates of late blight for the whole plant (leaf infection)[1]

Source of Variance	Experiment 1			Experiment 2			Experiment 3		Experiment 4	
	DF	F value	Pr > F	DF [3]	F value	Pr > F	F value	Pr > F	F value	Pr > F
Variety	1	1.3	0.327	1	0.5	0.467	1.1	0.309	0.1	0.706
Isolate	2	17.4	**<0.001**[4]	1	23.4	**<0.001**	34.2	**<0.001**	0.4	0.536
PS[2]	3	0.4	0.742	3	3.9	**0.012**	3.9	**0.011**	2.5	0.068
Variety *Isolate	2	0.4	0.697	1	0.8	0.389	0.1	0.847	0.5	0.473
Variety *P.S	3	0.3	0.801	3	0.3	0.806	2.7	0.053	0.5	0.715
Isolate*PS	6	1.1	0.399	3	1.1	0.374	1.8	0.155	0.6	0.644
Variety *Isolate*PS	6	0.4	0.864	3	0.7	0.572	0.6	0.612	1.1	0.376
Error	70			80						
Total	93			95						
	R^2 = 0.398 CV = 69.9			R^2 = 0.346 CV =68.4			R^2 = 0.437 CV =89.2		R^2 = 0.154 CV =79.3	

[1] See Table A-3.3 for data. Data were log-transformed before analysis.

[2] PS= Plant Strengtheners

[3] Degree of freedom (DF) for all source of variance is same for experiment two, three and four

[4] Bold numbers indicate that effects were significant at P<0.05

Table A-3. 2. ANOVA of inoculation of different isolates of late blight for detached leaves[1]

Source of Variance	Experiment 1			Experiment 2			Experiment 3		Experiment 4	
	DF[3]	F value	Pr > F	DF [3]	F value	Pr > F	F value	Pr > F	F value	Pr > F
Variety	1	17.2	**<0.001**	1	8.7	**0.004**	0.1	0.753	7.7	**0.007**
Isolate	2	63.0	**<0.001**	1	154.6	**<0.001**	185.3	**<0.001**	232.2	**<0.001**
PS[2]	3	0.7	0.556	3	9.7	**<0.001**	17.7	**<0.001**	35.1	**<0.001**
Variety *Isolate	2	3.5	**0.035**	1	9.3	**0.004**	0.5	0.486	7.3	**0.008**
Variety *P.S	3	0.2	0.925	3	1.2	0.310	1.1	0.343	0.4	0.756
Isolate*PS	6	0.2	0.964	3	1.9	0.145	2.7	0.050	0.3	0.802
Variety *Isolate*PS	6	0.1	0.994	3	0.8	0.508	0.6	0.637	0.6	0.592
Error	70			80						
Total	93			95						
		R^2 =0.688 CV =30.7			R^2 = 0.726 CV = 21.6		R^2 = 0.759 CV = 13.5		R^2 = 0.816 CV = 12.6	

[1] See Table A-3.3 for data. Data were log-transformed before analysis.

[2] PS= Plant Strengtheners

[3] Degree of freedom (DF) for all source of variance is same for experiment two, three and four

[4] Bold numbers indicate that effects were significant at P<0.05

Table A-3. 3. Effects of the isolate, variety and plant strengtheners on AUDPC of the late blight of tomato on leaf infection of the whole plant and detached leaf inoculation. The numbers are the means of four replications each in experiment 1, while in experiment 2, 3, and 4, there were six replications and only two isolates were used

Source		Whole plant inoculation (leaf infection)				Detached leaf inoculation			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Variety	Celsior (C)	45.4	48.3	36.8	63.8	290.2	240.2	253.1	256.5
	Cerise Rot (CR)	52.4	53.5	44.3	67.9	221.1	273.7	250.9	238.9
Isolate	108.04	78.0 a1	68.1 a	62.2 a	69.1	362.3	327.4	299.2 a	296.2
	48/58	35.4 b	33.7 b	18.9 b	62.5	143.3	186.5	204.9 b	199.2
	72/69.2	33.2 b				261.2			
Plant Strengthener	Water	50.7	70.8 a	60.8 a	90.7 a	239.5	296.5 a	289.1 a	281.5 a
	Fungend	46.5	38.1 b	26.3 b	59.3 b	257.2	212 b	220.4 c	193.1 c
	BF enzyme	54.3	49 b	34.9 b	53.7 b	254.1	267.5 a	240.3 b	258.0 b
	Ausma	44.1	45.9 b	40.5 ab	59.8 b	271.7	251.7 a	258.4 b	257.0 b
Variety*isolate	Celsior*108.04	91.0	62.51	57.7	63.3	368.3 a	293.7 b	302.7	296.4 a
	Celsior*48/58	32.9	34.3	15.9	64.4	188.2 b	186.7 c	203.6	216.6 b
	Celsior*72/69.2	38.3				316.3 a			
	Cerise Rot*108.04	102.4	73.9	66.7	75.1	358.3 a	361.1 a	295.6	296.0 a
	Cerise Rot*48/58	44.4	33.3	22.0	60.7	98.4 c	186.2 c	206.2	181.7 c
	Cerise Rot*72/69.2	36.8				206.1 b			

[1] Numbers within the group followed by different letters are significantly different at P<0.05, t-test LSD (whole plant inoculation) and P ≤0.05, LS means (detached leaf inoculation)

Table A-3. 4. Repeated measures analysis of the effects of the isolate, variety and plant strengtheners on % DLA over time on whole plants

Source of Variance	Experiment 1			Experiment 2			Experiment 3		Experiment 4	
	DF	F value	Pr > F	DF[1]	F value	Pr > F	F value	Pr > F	F value	Pr > F
Variety	1	1.2	0.270	1	0.4	0.552	0.1	0.814	0.1	0.731
Isolate	2	16.6	**0.001**	1	19.9	**0.001**	19.8	**0.001**	0.6	0.451
PS[2]	3	0.3	0.841	3	3.2	**0.029**	3.1	**0.031**	2.2	0.112
Variety *Isolate	2	0.4	0.679	1	0.8	0.384	0.1	0.749	0.5	0.477
Variety *PS	3	0.3	0.847	3	0.3	0.861	2.1	0.107	0.3	0.819
Isolate*PS	6	1.3	0.283	3	1.1	0.346	1.5	0.214	0.5	0.705
Variety *Isolate*PS	6	0.4	0.853	3	0.6	0.612	0.5	0.702	0.9	0.451
Time	5	85.9	**0.001**	5	75	**0.001**	39.9	**0.001**	57.2	**0.001**
Time* Variety	5	1.6	0.171	5	0.4	0.869	0.4	0.839	0.3	0.933
Time*Isolate	10	5.5	**0.001**	5	6.7	**0.001**	4.3	**<0.001**	1.8	0.112
Time*PS	15	0.9	0.554	15	1	0.478	1.7	0.051	1.1	0.395
Time* Variety *Isolate	10	0.4	0.941	5	0.7	0.655	1.6	0.156	0.3	0.924
Time* Variety *PS	15	0.1	1.000	15	0.4	0.976	1	0.448	0.2	0.999
Time* Variety *PS	30	1.2	0.207	15	0.8	0.702	0.8	0.639	0.2	0.999
Time* Variety *Isolate*PS	30	0.4	0.999	15	0.6	0.914	0.8	0.683	0.6	0.892

[1] Degree of freedom (DF) for all source of variance is same for experiment 2-4

[2] PS- Plant Strengtheners

[3] Bold numbers indicate that effects were significant at P<0.05

Table A-3. 5. Repeated measures analysis of the effects of the isolate, variety and plant strengtheners on % DLA over time on detached leaflets

Source of Variance	Experiment 1			Experiment 2			Experiment 3		Experiment 4	
	DF	F Value	Pr > F	DF[1]	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Variety	1	21.9	**0.000**	1	2.2	0.666	0.2	0.666	6.7	**0.011**
Isolate	2	61.1	**0.001**	1	148.1	**0.001**	156.7	**0.001**	200.4	**0.001**
PS[2]	3	0.5	0.686	3	10.1	**0.001**	14.9	**0.001**	35.2	**0.001**
Variety *Isolate	2	6.3	**0.003**	1	3.7	0.527	0.4	0.527	6.3	**0.014**
Variety *PS	3	0.4	0.782	3	2.3	0.412	1.1	0.412	0.6	0.645
Isolate*PS	6	0.3	0.937	3	1.8	**0.018**	3.6	**0.018**	0.5	0.689
Variety *Isolate*PS	6	0.2	0.985	3	1.2	0.631	0.6	0.631	0.5	0.708
Time	4	326.6	**0.001**	4	796	**0.001**	1751.6	**0.001**	1349.9	**0.001**
Time* Variety	4	4.2	**0.004**	4	5.9	**0.001**	7.1	**0.001**	4.4	**0.001**
Time*Isolate	8	14.4	**0.001**	4	15.5	**0.001**	66.7	**0.001**	57.2	**0.001**
Time*PS	12	0.3	0.995	12	4.4	**0.001**	4.5	**0.001**	5.6	**0.001**
Time* Variety *Isolate	8	6.1	**0.001**	4	2.1	0.098	0.9	0.472	3.2	**0.015**
Time* Variety *PS	12	0.7	0.792	12	1.1	0.332	2.2	**0.025**	0.7	0.718
Time* Variety *PS	24	0.8	0.686	12	3.1	**0.000**	1.9	**0.029**	3.7	**<0.001**
Time* Variety *Isolate*PS	24	0.3	0.999	12	1.2	0.278	1.2	0.309	0.6	0.806

[1] Degree of freedom (DF) for all source of variance is same for experiment two, three and four

[2] PS- Plant Strengtheners

[3] Bold numbers indicate that effects were significant at P<0.05

Table A-4. 1. Analysis of variance (ANOVA) for the effects of accessions and BABA compared to control on 1st and 2nd leaf age against two *P. infestans* isolates 108 and 101

Isolate	Effect	1st leaf			2nd leaf		
		DF	F Value	Pr > F	DF	F Value	Pr > F
108.04	Accession (Acc.)	30	11.8	<0.001	31	12.7	<0.001
	BABA vs water (Treat.)	1	757.1	<0.001	1	600.6	<0.001
	Acc.*Treat.	30	9.8	<0.001	31	5.4	<0.001
101.04	Accession (Acc.)	26	13.3	<0.001	25	34.6	<0.001
	BABA vs water (Treat.)	1	148.4	<0.001	1	105.7	<0.001
	Acc.*Treat.	26	5.3	<0.001	25	3.25	<0.001

Table A-4. 2. Analysis of variance (ANOVA) of the main effects (isolate, leaf age, Accession, Treatment) and their interaction

Source of Variance	DF	F Value	Pr > F
Isolate	1	26.8	<0.001
Leaf age (LA)	1	12.8	0.000
Isolate*LA	1	3.3	**0.070**[1]
Accession (Acc)	31	47.4	<0.001
Isolate*Acc	26	11.4	<0.001
LA*Acc	31	4.7	<0.001
Isolate*LA*Acc	24	2.7	<0.001
Treatment (Treat)	1	1159.3	<0.001
Isolate*Treat	1	66.5	<0.001
LA*Treat	1	13.8	0.000
Isolate*LA*Treat	1	0.8	**0.357**
Acc *Treat	31	11.9	<0.001
Isolate*Acc*Treat	26	4.0	<0.001
LA*Acc*Treat	31	4.2	<0.001
Isolate*LA*Acc*Treat	24	1.8	0.012

[1]Bold numbers indicate that effects were not significant at P<0.05

Table A-5. 1. Analysis of variance table for effects of the experimental repeat (date) and interactions between date and other factors on area under the disease progress curve (AUDPC) of *P. infestans* in trials I and II (leaf disc experiments).

Source[1]	Trial I[2]			Trial II		
	DF[3]	F Value	Pr > F	DF[3]	F Value	Pr > F
Date	2	0.87	*0.417*	2	0.19	*0.823*
Date*Isolate (Iso)	2	1.47	*0.232*	9	1.30	*0.232*
Date*Accession (Acc)	24	0.37	*0.998*	10	0.65	*0.775*
Date*Iso*Acc	24	0.18	*1.000*	45	0.93	*0.611*
Date*Treatment (Treat)	2	1.32	*0.268*	2	0.45	*0.637*
Date*Iso*Treat	2	1.01	*0.365*	9	0.18	*0.996*
Date*Acc*Treat	24	0.29	*1.000*	10	0.72	*0.709*
Date*Iso*Acc*Treat	24	0.20	*1.000*	45	0.84	*0.775*
Date*Leaf age (LA)				4	1.52	*0.193*
Date*LA*Iso				18	1.00	*0.459*
Date*LA*Acc				20	0.57	*0.932*
Date*LA*Iso*Acc				90	0.62	*0.998*
Date*LA*Treat				4	1.28	*0.276*
Date*LA*Iso*Treat				18	0.63	*0.875*
Date*LA*Acc*Treat				20	0.40	*0.992*
Date*LA*Iso*Acc*Treat				90	0.70	*0.987*

[1]Only the date and its interaction with other factors of the experiments are presented in this table in oder to simplify the table

[2] Trial I had three factors (2 treatments, 2 isolates, 13 tomato accessions), trial II had 4 factors (2 treatments, 6 isolates, 3 leaf ages, 6 accessions). See Materials and Methods section for details.

[3]Degree of freedom. Trial I: Total DF=935 and error DF=780, Trial II: Total DF=3671 and error DF=3060

Table A-5. 2. (A) AUDPC (area under the disease progress curve) (log-transformed data), (B) SC (sporulation capacity per cm^2 *1000), and (C) IE (infection efficiency) of six isolates on six tomato accessions depending on leaf age without (control) and after induction with BABA. The range of protection through induction across all accessions is given. Data for AUDPC and IE are the mean of three experiments with six replications each. Data for SC are from four replications of the first experimental run only.

Isolate	Leaf age 1st			Leaf age 2nd			Leaf age 3rd		
	Control	Induced	Range of %Prot.[1]	Control	Induced	Range of %Prot.	Control	Induced	Range of %Prot.
A. AUDPC									
19	0.95	0.07	80-100	0.95	0.22	57-100	0.98	0.41	39-100
66	3.36	1.04	50-92	3.68	1.72	36-86	3.69	2.22	23-53
75	1.72	0.31	52-100	1.92	0.75	49-80	1.96	0.98	34-64
85	2.83	0.93	43-97	3.18	1.79	22-65	3.24	2.41	19-41
101	1.51	0.26	60-98	1.74	0.75	30-70	1.78	1.22	15-47
108	2.02	0.31	59-100	2.24	0.82	59-74	2.29	1.17	42-71
B. SC									
19	15.81	3.40	38-100	16.01	6.21	23-100	18.21	8.41	6-100
66	24.42	13.12	26-65	27.03	16.91	20-65	28.42	20.10	11-63
75	17.03	7.23	32-100	18.02	9.32	26-77	23.13	11.82	19-90
85	27.42	16.04	14-100	30.31	21.80	14-42	32.71	23.71	17-40
101	22.51	6.41	18-100	23.81	13.40	22-59	26.60	14.31	9-78
108	21.00	6.12	26-100	21.82	11.41	0-77	23.93	15.52	14-65
C. IE									
19	1.00	0.48	0-100	1.00	0.83	0-100	1.00	0.83	0-100
66	1.00	0.99	0-6	1.00	0.99	0-6	1.00	1.00	0
75	1.00	0.85	0-89	1.00	1.00	0	1.00	1.00	0
85	1.00	1.00	0	1.00	1.00	0	1.00	1.00	0
101	1.00	0.96	0-25	1.00	1.00	0	1.00	1.00	0
108	1.00	0.76	0-100	1.00	0.99	0-6	1.00	1.00	0-6

[1]Percent protection (%Prot.) was calculated with the following formula: (1-induced/control)*100

Table A-6. 1. Mean effects of plant strengtheners across six tomato varieties inoculated with three isolates of *P. infestans* on the (A) AUDPC (Area under the disease progress curve) (log-transformed data) and (B) SC (Sporulation capacity per cm^2 *1000). The range of protection through induction among the varieties is given. Data for AUDPC and SC are the means of two experiments with six replications each

Plant strengtheners (PS)	Isolate 75			Isolate 101			Isolate 108		
	Absolute value	%Protection[1] Range	Mean	Absolute value	%Protection Range	Mean	Absolute value	%Protection Range	Mean
A. AUDPC									
Alfa extract	0.40	34-67	45	0.33	29-78	47	0.36	23-67	42
BABA	0.25	37-100	66	0.14	56-100	78	0.16	52-94	74
PEN	0.47	23-62	35	0.34	34-77	46	0.36	21-55	41
Quality	0.45	18-65	39	0.4	22-60	35	0.35	17-66	44
Water	0.73			0.63			0.62		
B. SC*1000									
Alfa extract	30.6	16-55	34.0	27.9	8-36	24.1	28.0	28-53	37.7
BABA	11.3	61-100	75.7	11.6	32-100	68.4	9.9	61-92	77.9
PEN	32.8	20-44	29.3	25.8	14-52	29.7	28.4	23-50	36.8
Quality	34.8	3-46	25.0	27.9	12-39	24.0	26.9	25-52	40.2
Water	46.4			36.8			44.9		

[1]Percent protection (%Prot.) was calculated with the following formula:

(1-PS/Water)*100

List of publications

Sharma, K., Butz, A.F., Finckh, M.R., 2010. Effects of host and pathogen genotypes on inducibility of resistance in tomato (*Solanum lycopersicum* L.) to *Phytophthora infestans*. Plant Pathology (in press) DOI: 10.1111/j.1365-3059.2010.02341.x.

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Finckh, M. R., Butz, A.F., Schulte-Geldermann, E., Bruns, C., **Sharma, K.** 2009. Genetic variation in inducibility of resistance in tomatoes against *Phytophthora infestans* and the influence of biofertilizers and plant strengtheners. In: Proceedings of BioExploit/Eucarpia workshop on "The role of Marker Assisted Selection in breeding varieties for organic agriculture", 25-27 February, 2009, Wageningen, The Netherlands. S. 51.

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Affidavit

The contents and subject matter of this thesis are the original work conducted by the author, except where otherwise acknowledged. None of the work has been previously submitted either in whole or in part, for a higher degree at this or any other institute.

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