Bacillus subtilis und seine Stoffwechselprodukte als Agenzien zur Resistenzinduktion gegen Blattläuse auf Ackerbohne (*Vicia faba*) und Sommerweizen (*Triticum aestivum*).

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***Bacillus subtilis* und seine Stoffwechselprodukte als Agenzien zur Resistenzinduktion gegen Blattläuse auf Ackerbohne (*Vicia faba*) und Sommerweizen (*Triticum aestivum*) - Dissertation Humboldt-Universität Berlin; 32 Tab., 26 Abb.**

Stichworte: Induzierte Resistenz, Ackerbohne, Sommerweizen, *Bacillus subtilis, Aphis fabae, Rhopalosiphum padi*

Zusammenfassung

Bacillus subtilis Stämme vom FZB - FZB24, FZB37 und FZB38 des FZB Biotechnik, Berlin - und ihre Stoffwechselprodukte wurden als Agenzien für eine Resistenzinduktion gegen Schädiger in drei Wirtspflanzen-Erreger-Systemen, *Vicia faba - Uromyces appendiculatus, Vicia faba - Aphis fabae* und *Triticum aestivum - Rhopalosiphum padi* untersucht. Prä-inokulativ wurden Blätter und Saatgut der Pflanzen mit den Testsubstanzen behandelt. Zur Testung gelangten Bakterien-Kulturfiltrat (KF), KF-Zenrifugationsüberstände, die vegetativen Zellen und Sporen der *B. subtilis* Stämme.

Bei lokaler und systemischer Pflanzenbehandlung riefen Bakterien-Kulturfiltrate und KF-Überstände eine Entwicklungshemmung der Uredosporen des Rostpilzes *U. appendiculatus* hervor.

Die Entwicklung der Blattläuse *A. fabae* und *R. padi* auf den Wirtspflanzen wurde mit Hilfe der Lebenstafel-Methode bewertet, wobei die Entwicklungszeit der Tiere (t_D), die Prä-Reproduktionszeit (t_d), die Relative Wachstumsrate (RGR) und die natürliche Wachstumsrate (r_m) ermittelt wurden.

Nach lokaler Applikation der KF-Überstände von *B. subtilis* auf die Wirtspflanzenblätter konnte eine erhebliche Breite antibiotischer (entwicklungshemmender) Wirkungen auf *A. fabae* und *R. padi* beobachtet werden. Die Aphiden benötigten längere Entwicklungs- und Prä-Reproduktionszeiten und gegensätzlich konnte eine geringere Relative (RGR) und natürliche Wachstumsrate (r_m) beobachtet werden.

Diese in den Versuchen festgestellten antibiotischen Wirkungen wurden offensichtlich durch die Pflanze vermittelt. Direkte toxische Effekte der bakteriellen Stoffwechselprodukte auf die Testtiere *A. fabae* und *R. padi* waren nicht erkennbar.

Eine Untersuchung der freien Aminosäuren im Phloemsaft von *Vicia faba* zeigte, daß sich nach Befall von *Aphis fabae* speziell bei den zuvor mit bakteriellem Kulturfiltrat und KF-Überstand behandelten Pflanzen im Vergleich zu der nur mit Wasser behandelten Kontrolle, die Konzentration von neun Aminosäuren änderte. Bei den mit KF-Überstand behandelten Pflanzen blieb auffallend die Konzentration der Aminosäure Serin unverändert, was als ein Hauptgrund für den bei diesen Pflanzen beobachteten antibiotischen Effekt auf die getesteten Aphiden interpretiert wird.

Neben der Induktion antibiotischer Wirkungen ließ sich nach der Pflanzenbehandlung mit *Bacillus subtilis* und seinen Stoffwechselprodukten eine höhere Chlorophyllfluoreszenz und ein besseres Wachstum der Pflanzen feststellen.

Eine Induktion von Resistenz gegenüber *Aphis fabae* und *Rhopalosiphum padi* wird durch Vorbehandlung der Wirtspflanzen mit dem KF-Überstand der *B. subtilis* Stämme FZB24, FZB37 und FZB38 als nutzbar gesehen.

Weiterführende Untersuchungen sollten sich auf das Vorkommen sekundärer Metaboliten orientieren, besonders auf Proteinase inhibierender Proteine, im Phloemsaft mit KF-Überständen von *Bacillus subtilis* behandelter Pflanzen.

Abstract

***Bacillus subtilis* and its metabolites as induced resistance agent against aphids feeding on broad bean (*Vicia faba*) and summer wheat (*Triticum aestivum*)**

Strains of *Bacillus subtilis* FZB (FZB24, FZB37 and FZB38 from FZB Biotechnik Berlin) and its metabolites were investigated for their role in induced resistance in three host–parasite systems, i.e. *Vicia faba* - *Uromyces appendiculatus*, *Vicia faba* - *Aphis fabae* and *Triticum aestivum* - *Rhopalosiphum padi*, following plant foliar and seed treatment. The culture filtrate, supernatant, vegetative cells and spore suspensions of the different strains of *Bacillus subtilis* were examined.

In topical and systemic plant treatment, the culture filtrate and supernatant of *Bacillus subtilis* was shown to inhibit the development of urediospores produced by *Uromyces appendiculatus*. The performance of *Aphis fabae* and *Rhopalosiphum padi* was evaluated using life table tests where the aphids' development time (t_D), pre-reproduction time (t_d), relative growth rate (RGR) and intrinsic rate of natural increase (r_m) were assessed. A wide range of antibiosis effects in *Aphis fabae* and *Rhopalosiphum padi* was observed when the supernatant of *Bacillus subtilis* was used as foliar topical treatment. The tested aphids presented longer development and pre-reproduction time; conversely a lower relative growth rate and intrinsic rate of natural increase was observed.
The antibiosis effect observed in the study was likely mediated *via* the plant. No direct toxicity effect of *Bacillus subtilis* metabolites on *Aphis fabae* and *Rhopalosiphum padi* was observed.

The investigation of the free amino acids of the phloem sap of *V. faba* plants, showed that, following *A. fabae* infestation, the concentration of nine individual amino acids had changed in the supernatant and culture filtrate of *B. subtilis* treated plants compared to the control (water treatment). The concentration of

the amino acid serine concentration remained unchanged in the supernatant induced plants in this study, which was interpreted as the major reason for the observed antibiosis effect on the tested aphids.

Besides the antibiosis, higher chlorophyll fluorescence and enhanced plant growth were evident as a result of plant treatment with *Bacillus subtilis* and its metabolites.

Induced resistance to both *A. fabae* and *R. padi* is suggested when using supernatants (FZB24, FZB37 and FZB38) of *B. subtilis* in topical treatment.

Keywords: Induced resistance, *Bacillus subtilis*, *Aphis fabae*, *Rhopalosiphum padi*

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Abbreviations

A	average mean weight of newly born insect larvae (L_1)
B	adult weight of individual insect
BBA	Biologische Bundesanstalt für Land-und Forstwirtschaft
B50	culture filtrate of a *Bacillus subtilis* strain from IPP Univ. Hannover
CF	culture filtrate
EDTA	ethylenediaminetetraacetic acid
FZB	Biotechnik GmbHBerlin
HPLC	high performance liquid chromatography
INA	2,6 dichloroisonicotinic acid
IPP Univ.	Institut für Pflanzenkrankheiten und Pflanzenschutz, Universität Hannover
JA	jasmonic acid
K	correcting constant
LED	Light Emitting Diode
LM	Landy Medium
Md	number of insect larvae born in a period of time 2 × (td)
OPA	ortho - Phthaldiaaldehyde
PR	pathogenesis-related protein
PI	proteinase inhibitor
RGR	relative growth rate
r.h	relative humidity
r^2	coefficient of correlation
r_m	intrinsic rate of natural increase
SA	salicylic acid
Sp	spores
Sup	supernatant
T_D	development time, time from birth to adult moult
t_d	pre-reproduction time
vc	vegetative cells

1. Introduction

Throughout evolution plants in nature have been subject to constant attack by plant pathogenic microbes, such as fungi and bacteria, as well as by insect pests. As a consequence of this intimate relation between plants and their attackers, a wide range of defence systems against pest and pathogen attacks has evolved in plants (Ryan, 1990; Bowles, 1990; Chessin and Zipf, 1990). Although pathogens and pests have developed systems to overcome these defences, only a very limited number of them succeed in infecting the plants (Collinge et al., 2001). Resistance has, therefore, been continually present in nature. As domestication of plants with partially high productiveness has not been able to preserve natural defence mechanisms, it has not been possible to optimise plant production and yield potential under the influence of biotic and abiotic factors to guarantee quantitative and qualitative yield. In fact, Holt and Birch (1984), describing plant resistance to three aphid species in relation to the taxonomy of the genus *Vicia* and the degree of domestication of the host, concluded that within each taxonomic group, the most domesticated species offered the best conditions for aphid growth. Plant selection based on high yield did not always preserve the genes responsible for plant defence mechanisms (Clarke, 1984). This is why the use of pesticide in plant protection became dominant in the years immediately following the World War II (1939-1945), during which time the chemical industry was at its peak.

The damage of pesticides to the environment (OMS, 1991; Ramade, 1982; Zweig and Aspelin, 1985; Weir, 1986), the steady rise in the costs of new pesticide development, coupled with an exponential increase in the number of insecticide-resistant insect species led to increasing awareness of the value of biological control schemes. In entomology, the development of resistance to many insecticides (Painter, 1958) saw thoughtful entomologists conclude that the search for sources of insect resistance in reducing the populations of insects and the damage caused by them belong in all well-rounded insect control

projects.

Focus on plant health is gaining on the traditional approach of a systematic attack on, and destruction of, diseases and pests. Rather than attempting to achieve one hundred percent protection from pathogen or pest attack, the challenge is discovering the means of securing the plant's health, for example by mobilizing the plant's own defence system against biotic and abiotic stress factors, and thereby, guaranteeing quantitative as well as qualitative yields. Consequently, the search for innovative methods has become a key issue in plant protection sciences.

The notion that plants may be able to develop acquired immunity to infection, following exposure to a pathogen or antigenic substances derived from a pathogen, has existed ever since discovery of the animal immune system in the latter years of the nineteenth century, when it was proven that plant vaccination could lead to changes in the response of tissues to subsequent microbial challenge (Lucas, 1999). Studies of acquired immunity in plants were reported by Ray (1901) and Beauverie (1901). Following the literature review by Chester (1933) and Gaeumann (1951) on the activation of plant defence mechanisms, many publications began to discuss the specific effects of different types of induced resistance.

Induced resistance was subsequently the focus of many experiments. It was obtained in cucumber against *Colletotrichum lagenarium* (Kuc et al., 1975), in tobacco leaves against *Pseudomonas solanacearum* (Squeira and Hill, 1974), as well as against TMV and other viruses in tobacco (Ross, 1961; Loebenstein, 1972). Giebel (1982) intensively researched the mechanisms of induced resistance against nematodes, while Karban (1986) demonstrated that induced resistance against spider mites (*Tetranychus* spp.) was possible in cotton seedlings that either had been mechanically abraded or had primary infections. Agrawal (1998) observed that experimentally induced plants consistently

received less herbivory than untreated ones. The responses of induced resistance in plants, discussed in the literature can be summarized in three main points:

- Building of physical barriers with cell wall modifications and lignification
- Synthesis of chemical barriers marked by production of secondary metabolites, antimicrobial proteins and pathogenesis related proteins (PR proteins)
- Hypersensitive response with programmed cell death

Many studies have been carried out to explain the mechanisms of resistance in plants following pathogen or pest attack. The mechanisms are characterized by *de novo* synthesis of proteins and secondary metabolites, or by the accumulation of already synthesized compounds, together referred to as induced responses (Karban and Baldwin, 1997). Monocotyledon and dicotyledon plants respond to fungal attack with a complex network of defence mechanisms (Dixon and Harrison, 1990), which include the synthesis of polymers, forming physical barriers (cutin, lignin, and callose), antimicrobial metabolites (phytoalexins), and pathogenesis-related proteins (PRs) (Jach et al., 1995).

Pathogenesis-related proteins were initially defined as proteins encoded by the host plant that were induced only in pathological or related situations (Collinge, 2001). Today they have a wider definition, and include proteins induced by wounding and pest attack (van Loon et al., 1994). Induction of these proteins occur both locally and systemically upon pathogen infection, herbivore-attack wounding or under the influence of other stress factors. The biochemical and molecular characteristics of the PRs are classified into 14 families (van Loon and van Strien, 1999) where PR-6 proteins are defined as proteinase inhibitors (PIs) involved in the defence against insects and other herbivores, micro-

organisms, and nematodes (Koiwa et al., 1997; Ryan, 1990).
PIs are the most extensively studied induced defence proteins (Ryan, 1990, 1992) and there is increasing evidence supporting defensive roles for proteinase inhibitors against insects (Ryan, 1990). The applicability of this paradigm to piercing-sucking insects has remained undetermined (Jongsma and Bolter, 1997), because these insects appear to lack endoproteinase activity within their tract (Rahbé et al., 1995) and therefore have not been considered likely targets for PIs. However, Casaretto and Corcuera (1998) noted that after infestation of barley by *Schizaphis graminum* and *Rhopalosiphum padi* the accumulation of chymotrypsin inhibitors was two-fold higher in resistant plants compared to susceptible ones. Adding plant PIs to artificial diet, affects the survival of *Diuraphis noxia*, *S. graminum* and *R. padi* (Tran et al., 1997). Other types of PR proteins intensively studied for their involvement in resistance response include chitinase, β-1,3-glucanase (Dassi et al., 1996) and peroxidase, (Esnault and Chibar, 1997; Gaspar et al., 1982; Moerschabacher, 1992).

Chitinase is cited as the main proteinaceous inhibitor of fungal growth in bean leaves and can be induced by the plant hormone ethylene, or by pathogen attack (Schlumbaum et al., 1986). The activity of chitinase against pests has also been demonstrated; lectin of *Brassica spinescens* and the closely related agglutinin from wheat germ and nettle show significant insecticidal activity when given to *Brevicoryne brassicae* in chemically defined synthetic diets (Cole, 1994). Since chitinase and β-1,3-glucanase are capable of degrading fungal cell walls in vitro (Mauch et al., 1988), these enzymes have received particular examination as possible defence compounds in plants against fungi containing glucan and chitin in their cell walls (Lusso and Kuc, 1996).

In plant defense reaction to pathogens, however, the precise role of peroxidases is not well understood. In vitro essays indicate that their presence with a suitable hydrogen donor and H_2O_2 can produce toxic products (e.g. oxidized phenols)

that are lethal to infecting microbes (Esnault and Chibbar, 1997). Dowd and Lagrimini (1997) reported that a caterpillar species absorbed much less from *Nicotiana* spp. overproducing tobacco anionic peroxidases than the wild type. Additionally, van der Westhuisen et al. (1997), investigating the role of intercellular peroxidase and the chitinase activities of three wheat cultivars [*Tritium aestivum* L. cvs "Tugela DN", "Molopo DN" and "Betta DN"], concluded that these compounds possibly play a role in resistance against the Russian wheat aphid (*D. noxia).*

There is strong evidence that the activities of β –1,3 -glucanase (Ji and Kuc, 1995), chitinase (Irving and Kuc, 1990; Metraux and Boller, 1986; Metraux et al., 1989) and other types of PR proteins correlate with induced resistance. However, Forslund (2000), who predicted β -1,3 -glucanase and chitinase as induced resistance factors in barley *Hordeum vulgare* to *Rhopalosiphum padi,* could not establish such a correlation.

Many other compounds are cited in the literature as induced resistance factors against aphids. In cereals, hydroxamic acids (Niemeyer, 1988), gramine (Kanehisa et al., 1990; Zugina et al., 1988), phenols (Leszczynski et al., 1989) and aconitic acid (Rustamani et al., 1992) have been cited as compounds involved in resistance against different aphid species and could be investigated as natural chemicals that offer possibilities for the control of other insects and pathogens (Corcuera, 1990).

A body of research supports the idea that, in local and systemic induced resistance processes, the mediation of the inducers is necessary for the signal transduction and the expression of specific genes in the induced defence response of the plant. The inducers cited in the literature can be categorized into those of biotic and those of abiotic origins. Biotic inducers are classified into pathogen, apathogen or endophyte type, while abiotic inducers are composed of pure substances or substances of complex nature. Thus, of abiotic inducers,

jasmonic acid (JA) and salicylic acid (SA) are involved in the signalling pathways that allow plants to express induced resistance against pathogens and pests. Jasmonic acid has been shown to have an effect in the signal transduction cascade, mediating inducible plant defence responses against herbivore attack (Enyedi et al., 1992; Sembdner and Parthier, 1993; Mueller et al., 1993). Further, application of JA and its volatile methyl ester jasmonate (MeJA) to plants triggers the synthesis of a number of defence-related secondary metabolites in plant: PIs (Farmer and Ryan, 1990; Koiwa et al., 1997), polyphenol oxidase (POX) (Schaller and Ryan, 1995), thionins (Epple et al., 1997), osmotin (Xu et al., 1994) and alkaloids (Baldwin, 1997; Zhang and Baldwin, 1997). In signal transduction for systemic acquired resistance (SAR), salicylic acid (SA) has been suggested as the signal chemical because its concentration rises dramatically after a pathogen infection (Malamy et al., 1990; Métraux et al., 1990). Raskin (1992) and Hildebrand et al. (1993) have reported that topical application of salicylic acid increases plant resistance to herbivores.

Besides the synthetic inductors, jasmonic acid, salicylic acid (Conti and al. 1996) and the 2,6-dichloroisonicotinic acid (INA) (Metraux et al. 1991; Thieron et al. 1995) or trigonellin (N-Methyl nicotinic acid) (Kraska and Schönbeck, 1993), a variety of other natural inducers are often cited in the literature. Hence avirulent pathogens (Chaudhary et al. 1983, Villich-Meller and Weltzien, 1990; Olivain et al. 1995), plant extracts (Herger et al., 1988), mycorrhiza fungi (Schönbeck and Dehne, 1979; Bargmann and Schönbeck, 1992), rhizobacteria (Defago et al., 1995; van Loon, 1995), and metabolites from bacteria (Maiss, 1987; Steiner, 1990; Steiner and Schönbeck, 1995; Leeman et al., 1995) can serve as induced resistance agents in plants.

Bacillus subtilis, referred to as a Plant Growth Promoting Rhizobacterium (PGPR), has been the subject of investigation in numerous laboratories, because its application is often associated with an increase in plant growth rate. PGPR application to crops or soil for beneficial effects was first reported in

1950s in studies conducted in the former Soviet Union, and later in the West (Zehnder et al., 2001). It was initially thought to enhance crop fertility by increasing the amount of available nitrogen (Cooper, 1959). Additionally, Idris et al. (2002) recently demonstrated that some strains of *B. subtilis* are able to degrade phytate (myo-inositol hexakisphosphate), the major storage compound of plant phosphorus, thus liberating phosphorus for improved plant growth. Besides its PGPR qualities, *B. subtilis* has been reported as having a wider application as a biological control agent, capable of suppressing soilborne pathogens (Dunleavy, 1955; Broadbent et al., 1971; Schippers et al., 1987; Bochow, 1992; Kloepper, 1993; Bochow et al., 1995; Doley S., 1998). Consequently, the role of *Bacillus subtilis* as induced resistance or tolerance agent is receiving considerable investigation.

While the mechanisms that sustain such activity are not well understood, some suggestions have been made. Scheffer (1983) and Voisard et al. (1989) have argued that PGPR strains may activate host defense systems, based on a lack of direct antibiosis of the strains against the pathogens, or on the correlation of biocontrol with plant growth promotion. Direct evidence supporting the hypothesis that PGPR strains, which remain on plant roots, can induce resistance in plants to foliar or systemic pathogens was found in three pathosystems: cucumber and anthracnose (Wei et al., 1991), carnation and *Fusarium* wilt (van Peer et al., 1991), and bean and halo blight (Alstroem, 1991). The mechanisms by which PGPR strains exhibit biological control over soil pathogens include antibiosis through bacterial production of antifungal compounds, competition for ferric iron, competition for infection sites, and production of lytic enzymes (Kloepper, 1993). Some proteins and protein complexes, as well as protease and ammonium play a key role in induced resistance processes in the plant (Bochow, 1998).

The use of culture filtrate of *Bacillus subtilis* as induced resistance agent is gaining interest in many laboratories. It was used to induce resistance against

virus in cucumber and barley (Maiss, 1987). Steiner and Schönbeck (1995) discussed the capacity of *Bacillus subtilis'* culture filtrate to induce resistance in mono cotyledons against plant biotrophic pathogens. Raupach and Kloepper (1996) noted a reduction in cucumber mosaic virus (CMV) infection intensity in cucumber plants following *Bacillus subtilis* application. After treatment with metabolites of *Bacillus subtilis* strain B50, barley was found to be resistant against *Erysiphe graminis* (Schönbeck et al., 1982; Kehlenbeck et al., 1994). It also induced resistance in wheat against *Sitobion avenae* but not against *Rhopalosiphum padi* (Galler et al., 1998).

In this research, three strains of *B. subtilis*, FZB24, FZB37 and FZB38, produced by FZB Biotechnik GmbH, Berlin, were used. While the strains FZB37 and FZB38 are still under investigation, the strain FZB24, tested many times in both in vivo and in vitro tests, has been widely reported as a plant pathogen inhibitor and plant growth stimulator bacterium (Schmiedeknecht, 1993; Schmiedeknecht et al., 1994, 1995, 1996; Bochow, 1995; Fey, 1996; Grosch and Junge, 1996).

Investigation of the role of *Bacillus subtilis* and its metabolites as induced resistance agent against pests has barely begun. While the mechanism by which induced resistance operates is not yet well understood in the majority of host-parasite systems, some researchers, such as Bochow (1995) and Schönbeck et al. (1993) have formulated the mode of action of induced resistance, which can be summarized in the following steps:

- Absence of direct effect of the inducer on the host plant
- Interval of time between application and inoculation
- Synthesis and doping of proteins' activities
- The unspecific character of the inducer
- Systemic character of the inducer
- Treatment efficacy and relationship between treatment dose and efficacy

The absence of direct effect of the inducer on the target pathogen or insect

characterised the fundamental difference between the inducer and conventional pesticides. While classical pesticides are known to act directly on the target organisms, inducers are understood to promote or enhance the host plant's already existing defense system to resist or tolerate any biotic or abiotic stress factor. The various inducer agents identified by the researchers and referred to in the literature are all reported to be active via the plant's own defense mechanisms. Exogenous application of inducers is presumed to not have a direct effect on the target pathogens or insects. The acid, 2,6 dichloroisonicotinic, has no direct effect on bacterial or fungal pathogens, but induces the same set of defense genes that are systemically activated by local pathogen infection (Ward et al., 1991).

Given the biochemical characteristics of plant secondary metabolites and the fact that induced resistance relies upon the activation of these, there is a variable interval of time between the application of the inducer agent and the plant biochemical response. In ethylene-treated plants, chitinase activity began to increase after a lag of 6 hours and induced 30-fold within 24 hours (Boller et al., 1983). Induced resistance response in cucumber leaves against *Colletotrichum lagenarium* required 24 hours (Dean and Kuc, 1985). Induction of PR proteins was recorded as early as 25 hours after inoculation and their quantity increased for up to 72 hours following the application of salycylic acid in the form of spray onto the surface of barley leaves (Tamas and Huttova, 1996).

The unspecific character of the above inducer presents an opportunity to obtain induced resistance in different plant species against a range of pest systems. Nevertheless, this quality of the inducer is not valid in every case and researchers have made contradictory observations. For instance, wheat plants treated with *B. subtilis* strain B50 demonstrated resistance to *Sitobion avenae* but not to *R. padi* (Galler et al., 1998).

The systemic character of the inducer is defined as the capacity of the inducer

to transduce its effect from the treated part of the plant to the untreated one and thereby ensure the whole plant is protected. Ross (1961) showed that inoculation of one leaf of a tobacco cultivar possessing local lesion resistance to tomato mosaic virus (TMV) increased the resistance of other leaves on the same plant to TMV, while Staub et al. (1992) reported that tobacco plants treated with two isonicotinic acid derivatives at 200 ppm, by injecting the two lower leaves, were protected systemically against tobacco mosaic virus (TMV), *Pseudomonas syringae* pv. *syringae*, *Tabaci* (Wildfire), and other plant viruses. Induced resistance can be also obtained via local induction where only the inoculated plant parts are protected against subsequent infection. Some compounds known to induce resistance arequoted as acting only locally, not systemically. Ryals et al. (1996) observed that exogenous application of SA will induce resistance only in leaves that are treated with the compound. In both cases, systemic resistance and locally induced resistance, the dose of the inducer agent is reported to not be stochiometric in relation to the plant defense response. Schönbeck et al. (1993) observed that, while the concentration of trigonelline had increased, this did not lead to an increasing efficiency of induced resistance against powdery mildew (*Erysiphe graminis* f. sp. *hordei*) in barley.

Induced resistance mechanisms in host plants against parasites rely on a complex process in which the latent resistance genes of the plant are activated and coupled with reinforcement of the already expressed resistance genes (Schönbeck et al., 1993).

The promotion of plant growth as a result of treatment with *Bacillus subtilis* and its metabolites, and the following change of phytohormonal balance, are understood to play a key role in the preservation of plant health (Bochow et al., 1995; Doley and Bochow, 1996).

The objective of this thesis was to assess whether topically treating *Triticum*

aestivum and *Vicia faba,* respectively mono and dicotyledonous plants, with *Bacillus subtilis* and its metabolites led to induced resistance against *Rhopalosiphum padi* and *Aphis fabae.*

To elucidate the mechanisms of possible induced resistance resulting from the treatments, two physiological tests were conducted. Firstly, the chlorophyll fluorescence of plants pre-treated with *B. subtilis* and its metabolites was measured to assess how the inducers may compensate for the pathological sink created by the feeding activity of aphids. Secondly, qualitative and quantitative assessments of the amino acids were performed.

The methods used to investigate the objectives established in this research were chosen for their reliability in providing clear answers to the complexity of the interaction between the test insects and their host plants.

Bacillus subtilis metabolites were assayed against *Uromyces appendiculatus* on *Vicia faba* to ascertain to what extent they can inhibit the development of these biotrophic fungi. In view of the fact that parallels are often drawn between the mechanism of aphid- plant and pathogen- plant attack (Dreyer and Campbell, 1987), it was strongly hypothesised that the same metabolites could be used in induced resistance tests to aphids in this study.

To assess the performance of individual aphid feeding on pre-treated plants with *B. subtilis* and its metabolites, a life table model test was designed to measure insect growth parameters. A widely used method in assessing plant resistance to aphids (Wojciechowicz-Zytko and van Emdem, 1995) applies the measurement of individual weight gain over the time, the Relative Growth Rate (RGR) (Fischer, 1921; van Emden, 1969) and the intrinsic rate of natural increase (r_m) (Wyatt and White, 1977). Correlation between RGR and r_m values has been completed for some 12-aphid species, including *R. padi* (Kempton et al., 1980; Leather and Dixon, 1984) and *Aphis fabae* (Dixon, 1990; Guldemond

et al., 1998).

Our study assumed that the pre-treated host plants (*Vicia faba* and *Triticum aestivum*) would exhibit resistance characterized either by nonpreference (antixenosis), where the host plant deters aphids from settling and colonising them, or antibiosis, where the increase rate of aphids is restricted (Painter, 1951; Kogan and Ortman, 1978; Dixon, 1987). Host plant resistance is a major strategy in aphids control - if plant resistance increases, this can extend the duration of aphid development, as well as slow down the aphids' reproduction rate (Dreyer and Campbell, 1987). After plants had been exogenously pre-treated with the metabolites of *B. subtilis* and we had observed that this effectively resulted in resistance against aphids, we attempted to elucidate its mechanisms. Acute toxicity and artificial diet tests for the *R. padi* and *A. fabae* were conducted, in order to localize the effect obtained in the induced resistance treatment and to establish whether this effect was a result of a direct contact between the tested aphids and the metabolites, or whether it was mediated via the plant. The results led us to conclude that the acute toxicity and artificial diet tests did not appear to influence the growth parameters of the tested insects and that the induced resistance was most probably due to physiological change following the treatment with *B. subtilis* metabolites.

Two types of physiological tests were then carried out:

Aphid feeding has been reported to induce a pathological sink in their host plant in concurrence with the natural sink. The term “sink” is defined by Lafitte (1984), as the regions of the plant that import photosynthate, whereas 'sources' are organs that export photosynthate. Fouché et al. (1984) and Al-Mousawi et al. (1983) observed cereal crop damage occurrence during feeding when the aphids inject phytotoxic substances into leaves and remove assimilates from leaf vascular tissues.

The first objective was to quantify the changes in the chlorophyll fluorescence

caused by aphid feeding on young seedlings of *Vicia faba* and *Triticum aestivum* plants that had been induced by *B. subtilis* and its metabolites, as well as assess the resistance of those induced plants compared to the control (water-treated plants) by means of aphid biomass analysis.

Secondly, the free amino acids of the *Vicia faba* seedlings were investigated. This was prompted by the frequent citing of primary and secondary compounds as playing important roles in resistance factors to aphids. Primary compounds, e.g. amino acids, may in some part explain the resistance of some oat and barley varieties to *Rhopalosiphum padi* (Tsumuki et al., 1987; Weibull, 1988). Van Emden (1972) could establish, for *Myzus persicae* and *Brevicoryne brassicae,* that amino acids and the allylisothicyanate of the plant analysed by him accounted for a large proportion of observed variability in the mean performance of these aphids. The development of chemically defined diets for aphids (Mittler and Dadd, 1962; Auclair and Cartier, 1963; Griffiths et al., 1975) has made it possible to vary the concentration of single compounds in aphid food in order to test the effect of this variation on aphid growth rate and fecundity. The studies of *V. faba* cultivars by Poehling and Morvan (1984) have indicated that free amino acids are partial determinants of resistance. Weibull (1987) also observed that the relative growth rates of *R. padi* closely followed the phenological change of free amino acids in phloem sap of oat and barley plants as they aged. Srivastava (1987) has proposed three categories of amino acids as important in aphid nutrition. The first category functions as a phagostimulant, the second may possibly have slight inhibitory effect, while the third category has a strong inhibitory effect. When the third category is dominant in the sap, this may decrease feeding and contribute towards the resistance of the plant. Much relevant literature argues that the quantitative concentration of amino acids has less effect than its qualitative variation. Generally, 10 amino acids are considered to be essential in insects' nutrition (Dadd, 1985). Aphids are vicious pest insects; it has even been demonstrated that they manipulate their host plant's nutritional quality by changing amino acid

levels (Sandström et al., 2000). Therefore, the phloem amino acids of *V. faba* plants were measured, in order to pinpoint the extent of influence of *B. subtilis* metabolites on the pre-treated aphid's host plant.

2. Material and methods

2.1. Soil substrate

The soil substrate for plant cultivation in the leaf treatment experiments was a pure commercial substrate “Frühstorfer Einheitserde”, Type P. Part of this soil substrate was sterilised using a steamer machine “STERILO 1k” at 80°C for 12 hours and was used in the seed treatment experiment after cooling.

2.2. Plant material

For the purpose of these experiments, monocotyledon and dicotyledon plants were used.

2.2.1. Broad bean *(Vicia faba*) cv. ‘Hangdown’

Vicia faba seeds were sown in plastic multiple quick pots and, when sprouting, were transferred into 12 cm diameter pots filled with 600 ml of soil substrate. The plants were stored in greenhouse conditions or in a growth chamber, under 16:8 h photoperiod light. The temperature in the greenhouse was 20°C ± 4°C and the relative humidity was between 50-80 %. In the growth chamber, the temperature was 20°C ± 2°C and the relative humidity was between 60-70 %. The plants were regularly watered with tap water and plants of approximately the same size were selected for the planned experiment. All plants used in the experiment unit were aged between two and four weeks.

2.2.2. Summer wheat (*Triticum aestivum*) cv. ‘Nandu’

The wheat seeds were grown under the same conditions as for *Vicia faba* (described above), except that instead of one plant per pot, four plants were grown in each pot.

2.3. Test organism breeding conditions

2.3.1. Fungi

Bean rust (*Uromyces appendiculatus*) - spores of *Uromyces appendiculatus* isolate SWBR I were kindly provided by Prof. Kurt Mendgen, University of Konstanz (Germany). The spores were stored at –20°C to preserve the germination capacity prior to use in the test.

2.3.2. *A. fabae* and *R. padi* breeding conditions

Stock cultures of *A. fabae* and *R. padi* were placedn *Vicia faba* and *Triticum aestivum* plants, respectively and maintained in the growth chambernder 20°C, 60-65 % relative humidity and light/dark regime of 16/8 hours.

The apterous of *A. fabae* and *R. padi* used in all greenhouse experiments were standardised in the following procedure: apterous of *A. fabae* and *R. padi* were caged independently on *Vicia faba* and *Triticum aestivum* plants and after 24 hours, solely the offspring were kept; these offspring were then allowed to continue their development under the conditions described above until adulthood. The standard insects thus obtained were then used in the experiments.

2.4. Clip cages used in aphid testsFehler! Textmarke nicht definiert.

The clip cages used in these experiments were identical to those described by Noble (1958) and slightly modified versions of those used by the Poehling working group in IPP, Univ. Hannover. The border of the Petri dish, with a 35 cm diameter, was brought into contact with an acetone solution for a few seconds, after which a ring of rubber was placed over and fixed to it. This ensured the clip

cage was machine washable without any deterioration of the assembled materials.

Figure 1: Clip cages fixed on test plant leaflets; right and left on *Vicia fabae* and *Triticum aestivum* leaflet, respectively

2.5. *Bacillus subtilis* and its metabolites

Bacillus subtilis strains, FZB24, FZB37 and FZB38 and its metabolites were provided by the laboratory FZB Biotechnik GmbH, Berlin. The culture filtrate of *B. subtilis* isolate B50 used in these experiments was kindly provided by the IPP Univ. Hannover.

2.5.1. Biology and morphology of *Bacillus subtilis* (Ehrenberg) Cohn

According to its morphology and biology, *Bacillus subtilis* belongs to the *Bacillus* species, the *Bacillaceae f*amily and the *Eubateriales* order (Mueller, 1965; Jacob et al., 1981). It was first described by Ehrenberg (1835) and later by Cohn (1872). The bacterium has a stick form and peritric flagella, is gram positive, aerobe, and is a spore-forming bacterium (Schlegel, 1992). The temperature interval of the bacteria is 5-55°C (Sinclair, 1989) with an optimum at 25°C (Gupta and Utkhede, 1986). The pH of the bacteria is between 4.5 and 8.5 with the optimum value around 6-7.5 (Thimann, 1964). Soil is the reservoir of this bacterium, from where it is transferred to various associated environments

including plants and plant materials, foods, animals and marine and freshwater habitats (Priest et al., 1987).

Bacillus subtilis is capable of producing peptide-type antibiotic during its fermentation phases, which are respectively logarithmic, transition and stationary (McKeen et al., 1986). Several strains of *B. subtilis* are cited as producing cyclic lipopeptides, which belong to the family of iturin, fengymycin (Besson et al., 1978; Peypoux, 1980; Mhammedi et al., 1982; Loeffler et al., 1990; Asaka and Shoda, 1996a; Hbid et al., 1996) and surfactin group. The *B. subtilis/amyloliquefaciens* group has been reported to produce IAA and phytase as a phosphorus-mobilizing enzyme essential for plant nutrition. Idris et al. (2002) reported that *B. subtilis* strains, FZB24, FZB42 and FZB45 showed extra cellular phytase activity when cultivated in wheat bran extract, which is known to contain phytate.

2.5.2. *Bacillus subtilis* metabolite productions

After inoculation of *Bacillus subtilis s*pores in the Landy-Medium, a lag phase commences during which the cells increase in mass but do not divide. This is then followed by a phase of exponential growth (also called transition phase) whose physiological state is marked by back-to-back division cycles such that the population doubles in number every generation. During the exponential growth there is no change in average cell mass. Although the cells constantly change in mass as they increase, they then divide rapidly, decreasing in mass. As the rate of growth is exponential, the rate of increase in cell number is initially slow but increases at an ever-faster rate, resulting in the later stages in an explosive increase in cell numbers (Madigan et al., 1997). The exponential phase is followed by the stationary phase classically defined as a physiological point where the rate of cell division equals the rate of cell death; hence viable cell numbers remain constant. The stationary phase usually occurs when cell concentration is so great that an aspect of the environment is no longer able to serve the requirements of exponential growth. Generally, either an essential

nutrient of the culture medium is exhausted or some waste product of the organism builds up in the medium to an inhibitory level and exponential growth ceases (Madigan et al., 1997). The stationary phase is a time of significant physiological change and particularly involves the physiological adaptation of cells to survival through periods of little growth (Abedon, 2001).

During this development period, *B. subtilis* is understood to produce different kinds of metabolites as enzymes and lipopeptide antibiotics (Doley, 1998) which cause important qualitative and quantitative changes in the substance spectrum. During the logarithmic phase, *B. subtilis* has been noted as producing hydrophile oligopeptide antibiotic as bacilysin (Walker and Abraham, 1970; Hilton et al., 1988; Loeffler et al., 1990; Koumoutsi et al., 2004), chlorotetain and rhizocticin (Loeffler et al., 1990), while the stationary phase was characterized by the production of lipopeptide antibiotic as iturin, fengymycin, and surfactin type as the cyclopeptide mycobacillin (Loeffler et al., 1990; Besson, 1994; Ohno et al., 1995).

Figure 2 shows the laboratory principle of production of *B. subtilis* metabolites using Landy – Medium (Table 1).

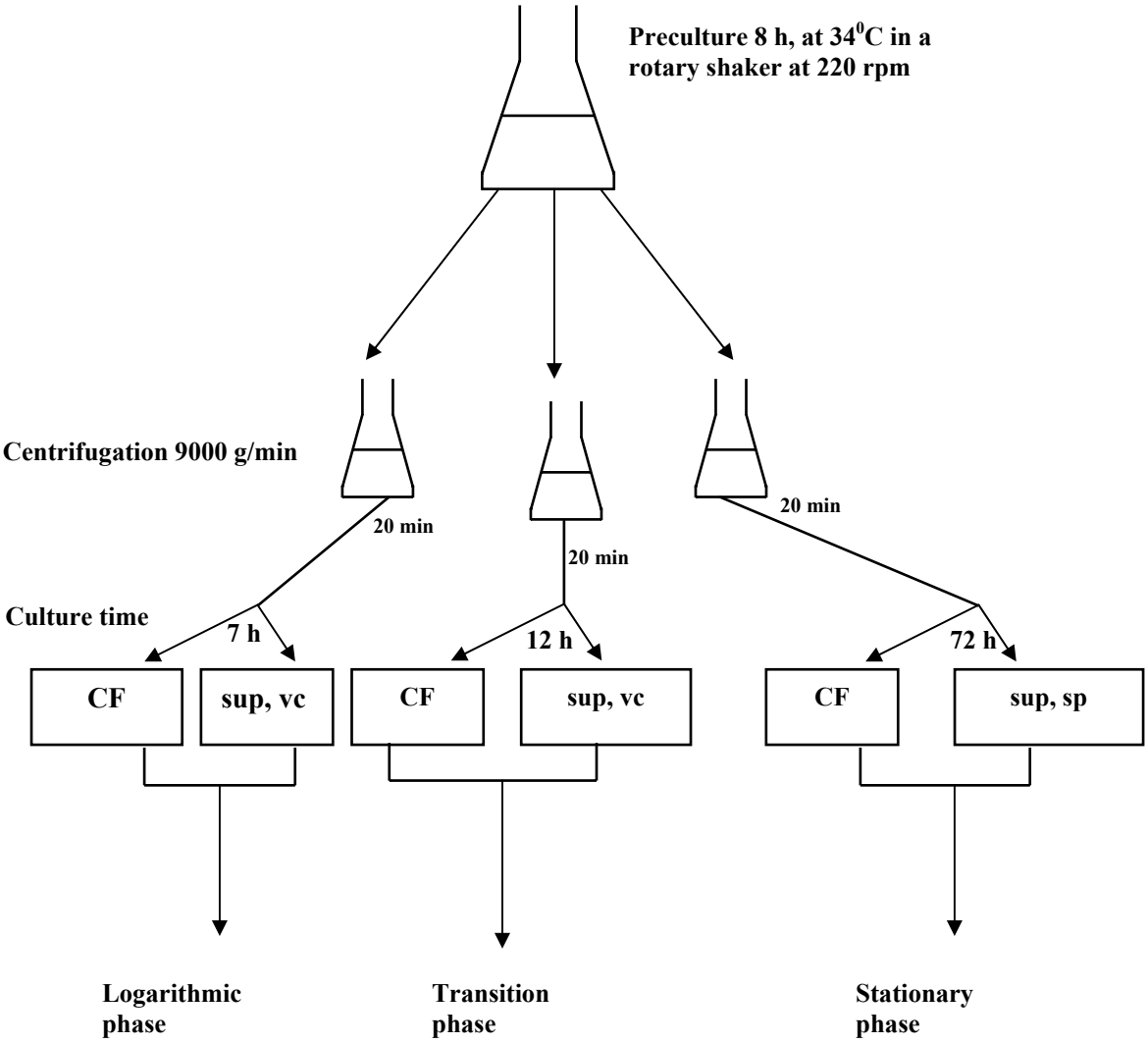

Figure 2: Diagram of the production of *Bacillus subtilis* and its metabolites. CF: Culture Filtrate; sup: supernatant; vc: vegetative cells; sp: spore suspensions

Table 1: Composition of the Landy-Medium (LM) for 1 liter of solution

Glucose*	20	g
$MgSO_4$ (pure)	0.5	g
KCl	0.5	g
Glutamic acid* (pure)	5	g
$MnSO_4 \cdot H_2O$	5	mg
$Fe(SO_4)$	0.1	ml
$CuSO4 \cdot 5H_2O$	0.02	ml

* The marked elements were separately diluted and sterilised.

2.5.2.1. Spore suspensions

The spores used in this experiments were obtained after 72 hours of cultivation, and then washed from the medium by centrifugation at 9000 g/min. Prior to the experiment, the necessary dilution was prepared and the obtained bacterial spore suspensions were used in the activated form by placing them in a warm bath at 60°C for 15 min and then cooling them under laboratory conditions.

2.5.2.2. Culture filtrate

The culture filtrate corresponding to each of the different bacterial growth phases, logarithmic, transition and stationary was obtained by passing the culture supernatant through a Millipore filter (0.2 µm) into sterile flasks. The characteristics of culture filtrate, strain FZB24 of *B. subtilis* in Landy-Medium (Table2) where determined by Beckmann (1995) and FZB Biotechnik GmbH (1995).

Table 2: Characteristics of culture filtrate of strain FZB24 of *Bacillus subtilis* in Landy-Medium (Beckmann, 1995 and FZB Biotechnik GmbH, 1995)

Parameters	log.phase 7hours	trans. phase 12 hours	stat. phase 72 hours
pH-value	5.97	6.61	7.44
Osmotic pressure (mM/kg)	207	136	105
Glutamic acid (mg/ml)	3.27	3.09	0.012
Glucose (g/l)	14.5	13.29	4.2
NH4 (µg/ml)	9.962	6.217	1.162
Protease (µmol/ml)	0.0012	0.009	0.005
Cyclic lipopeptide (g/l)	0.025	0.0293	0.242

2.5.2.3. Supernatant

The supernatant of *Bacillus subtilis* isolate FZB24, FZB37 and FZB38 was used in the transition phase after centrifugation of fermented bacteria.

2.5.2.4. Vegetative cells

After 24 hours of fermentation at 34°C, and following 10 min of centrifugation at 9000 g/min, the vegetative cells were separated from the supernatant, suspended in a physiological salt solution and homogenised.

2.6. Utilisation of the obtained materials in the experiments

2.6.1. Seed pre-treatment with spore suspensions prior to sowing

2.6.1.1. Treatment procedure

The seed materials were first disinfected with sodium hypochlorite (3% active Cl) in aqueous solution for 10 min and rinsed three times with sterile distilled water. The seeds were left to dry and then dipped for 10 min in water, which contained 10^5 cfu/ ml of *B. subtilis* spore suspensions. The control seeds were dipped in distilled water and after a short drying period, they were sown into sterile soil in multiple quick pots and later transplanted into 12 cm diameter pots filled with sterile commercial substrate, as described above. Each pot was watered with the same quantity of distilled water.

2.6.1.2. Reisolation of *Bacillus subtilis* from the roots

For the reisolation of *B. subtilis* from the rhizosphere, 1 g root (fresh weight), according to each variant of the treatment, was taken separately from composite samples of 5 *Vicia faba* plants and 4 wheat plants. The samples were shaken in 99 ml sterile sodium chloride-solution (0.3%) at 220 rpm for 20 min. The dilution series was then prepared and 100 µl from each dilution was mixed with cooled agar in petri plates. The colonies were expressed as the number per gram of roots by measuring the total weight of dry matter from each homogenate. After two days incubation at 25°C the number of *B. subtilis* cfu per g root and substrate dry weight were measured, respectively. Here no attempt was made to differentiate the spores from the vegetative cells in the counting method utilised.

2.6.2. Application of spore suspensions and vegetative cells on the leaves

The spore suspensions 10^7 cfu/ml and vegetative cells 10^7 cfu/ml were applied separately to *Vicia faba* plants, including the leaves, which were completely sprayed using a hand sprayer. After the foliage dried out, the standardised apterae of *A. fabae* were caged on the selected leaves.

2.6.3. Topical treatment of the plant leaves using culture filtrate and the supernatant

The culture filtrate and the supernatant, each a 10 % solution, were sprayed on the plants, including the leaves, using a hand sprayer. After a period of 2-3 days, the standardized insects were caged on the leaves selected for the aphid tests.

2.6.4. Systemic treatment of the plant leaves using culture filtrate and the supernatant

In order to assess the supposed systemic effect of *B. subtilis* metabolites, the two first leaves of *V. faba* plant (counting upwards from the bottom) and the corresponding part of the stem were isolated from the rest of the plant and sprayed with the test solutions. This was to asses whether the metabolites of *B. subtilis* have only a local effect or, in fact, a systemic one, i.e. whether the metabolites could be transported through the other organs of the plant when treatment is limited to a certain part of the plant.

Figure 3: Systemic induced treatment of a *Vicia faba* plant
The three first leaves of *Vicia faba* plant and corresponding stem, marked white in the photo, were isolated and treated with *Bacillus subtilis* metabolites in a systemic test.

2.6.5. Application of supernatants of *Bacillus subtilis* in acute toxicity test

In order to determine whether the supernatants have a direct contact effect on *A. fabae*, an acute toxicity test was conducted. For this purpose, 2 ml of supernatant of *B. subtilis* per treatment was poured over a filter paper placed in a petri plate. The test insects were then gently placed on the wet filter paper for 5 minutes and then removed. In the case of the control insects, water was poured over the filter paper. The insects were then allowed to dry and were then caged onto the untreated plants.

2.7. Rearing Aphis fabae on sterile synthetic diet

Table 3: Composition of synthetic diet (mg/ 100 ml of diet)

Alanine	100	Sucrose	15 000.0
Arginine	270	Ascorbic acid	100.0
Asparagine	550	Thiamin	
Aspartic acid	140	hydrochloride	2.5
Cysteine HCL	40	Riboflavin	0.5
Glutamic	140	Nicotinic acid	10.0
Glutamine	150	Pyridoxin,	
Glycine	80	free base	2.5
Histidine	80	Folic acid	0.5
Isoleucine	80	Ca pantothenate	5.0
Leucine	80	Inositol	50.0
Lysine HCl	120	Choline chloride	50.0
Methionine	40	Biotin	0.1
Phenylalanine	40	KH_2PO_4	500.0
Proline	80	$MgCl.6H_2O$	200.0
Serine	80	Fe sequestrene[a]	1.5
Threonine	140	Zn sequestrene	0.8
Tryptophan	80	Mn sequestrene	0.8
Tyrosine	40	Cu sequestrene	0.4
Valine	80		

Formulation: *p*H adjusted to 7.0 with KOH 1.75 M, and water (distilled-deionised) to make 100 ml of diet.

[a] Sequestrenes are compounds of the metal with sodium EDTA.

2.7.1. Synthetic diet preparation procedure

The composition of the diet, shown in Table 3, accorded with the specifications of Dadd and Krieger (1967). The diet was prepared in volumes of 500 ml, as described by Akey and Beck (1971). The amino acids, vitamins, and salts were added to a sucrose solution of ca. 2/3 of the desired total volume and the mixture was stirred with a counter-rotating mixer for 1-2 hours under a stream of nitrogen gas. The ferric, zinc, and manganese sequestrene were made up as individual stock solutions and added to the diet at the end of the mixing period. The solution was adjusted with 1.75 M KOH to a pH of 7.0, then increased to the

final volume and finally passed through a Millipore filter (0.2 µm) into sterile flasks. The diet was stored in 25 ml quantities at –20°C for up to 1 month.

2.7.2. Feeding apparatus used in sterile feeding test

The experiment cages were composed of a plastic petri plate, 2 rings of equal size, one of which had an incision on the side, and two parafilm layers. Under a laminar box, 2 ml of sterile diet was first poured over one parafilm layer placed over the intact ring. The second layer of parafilm was used to cover the deposited liquid. Care was taken that the liquid did not overflow. Finally, the second, incised ring was stretched over the first ring and the two layers of parafilm, thereby sandwiching the diet between the two pieces of parafilm.

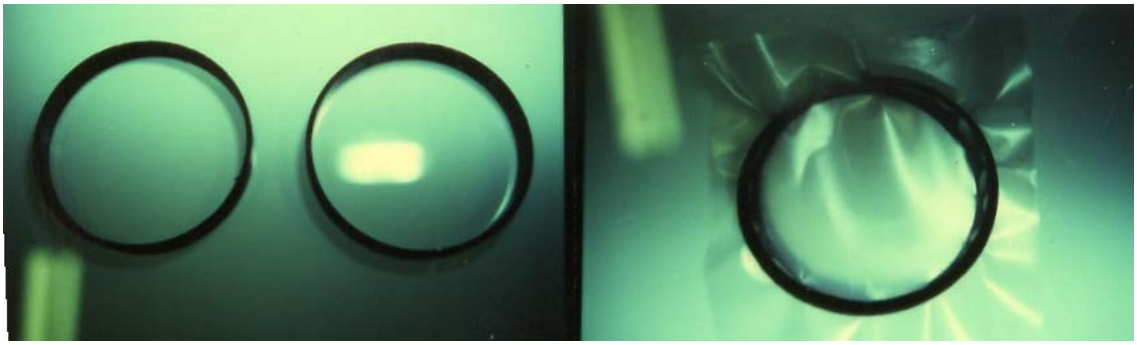

Figure 4: Feeding apparatus for artificial diet. Left photo: two rings with the one ring cut on the side. Right photo: the two layers of parafilm with the feeding liquid sandwiched between them. The ring with the incision is stretched over the intact ring and the two layers of parafilm.

In order to maintain sterile and aseptic conditions during the feeding procedure of the aphids, distilled water was autoclaved for 20 min at 121°C and the parafilm membranes were kept in 70 % ethanol for approximately 24 h, following the methodology of Akey and Beck (1971).

2.8. Artificial infestation of *Vicia faba* with *Uromyces appendiculatus*

Urediospores of *Uromyces appendiculatus* were added to a solution of distilled water containing 0.01 % Tween-20 and stirred with a magnetic stirrer for 5 min. Urediospore concentration was determined using a Bueker haemacytometer and adjusted to 100 000 spores per ml. The obtained suspension was sprayed onto the leaves of two-week-old plants of *Vicia faba* that had been pre-treated 2-3 days prior with *B. subtilis* culture filtrate, supernatants and Landy-Medium. The control plants were pre-treated with water. After the film dried, the plants were incubated at 100% r.h. in the dark. The container was then closed and stored in darkness, which were required conditions for the *Uromyces appendiculatus* to be active. The plants were removed 24 hours later and placed in the growth chamber. After 8-14 days, uredial pustules had developed and were counted.

2.9. Life table tests for *Aphis fabae* and *Rhopalosiphum padi*

To determine the relative growth rate (RGR) and the intrinsic rate of natural increase (r_m), life table model experiments were carried out.

2.9.1. The estimation of Relative Growth Rates of *Aphis fabae* and *Rhopalosiphum padi*

Seedlings of *Vicia faba* and *Triticum aestivum,* when aged 3-4 weeks, received one standardised adult aptera of *Aphis fabae* or *R. padi* respectively, on selected leaves. The aptera was fixed onto the leaves with a clip cage. After 24 hours, 50 new-born larvae (L_1) were collectively weighed to determine the mean weight (A) in mg, according to each variant of the experiment and, of these, a single larva was kept. When the insects performed their development within time (t_D) in days (Fischer, 1921; van Emden, 1969), i.e. the time from birth to adult moult, the individual weight (B) in mg of each insect was again recorded. All

weight measurements in the aphid tests were taken using a fine-balance Sartorius MC5 with readability from 1 µg up 5g.

The three parameters, (A), (B) and (t_D) are used to calculate the RGR, which is the growth per unit weight per unit time of an aphid (Fischer, 1921; van Emden 1969):

$$RGR = \frac{\log_e B - \log_e A}{t_D}$$

The concept of relative growth rate is familiar to plant physiologists. Radford (1967) points out that the use of the RGR does not involve any assumption about the form of the growth curves, and is therefore particularly valuable for comparing different results of different treatments.

2.9.2. The estimation of the intrinsic rates of natural increase (r_m) of *Aphis fabae* and *Rhopalosiphum padi*

The estimation of r_m was carried out according to Wyatt and White (1977). Acordingly, the pre-reproduction time (t_d), corresponding to the day when young larvae in the experimental group were first observed and marked, and the number of larvae born per adult per day were counted during a period of time equalling 2 × (t_d). To avoid double counting, the already counted larvae were immediately removed from the experiment. During this period of time, 2 × (t_d), the recorded number of larvae (Md) served as effective lifetime fecundity, corresponding to the number of larvae born in a generation time (t_d). Based on Md and (t_d), the equation of r_m is postulated theoretically by Dixon (1987) as the rate of increase of a population that has assumed constant age schedule of births and deaths and increasing in number in an unlimited space. The formula is given as follows:

$$r_m = \frac{k \times \log_e(Md)}{t_d}$$

This above equation has been used by several researchers to calculate the r_m value for aphids (Leather and Dixon, 1984; Sandström, 1994; Sandström and Pettersson, 1994; Soraka and Mackay, 1991; Birch and Wratten, 1984; Sotherton and van Emden, 1982). In the formula, k serves as a correcting constant with a value of 0.754 and 0.745 (Frazer, 1972) for *A. fabae* and *R. padi*, respectively. The r_m value derived from this equation is probably more useful in laboratory assessment than in the field. As Carter et al. (1980) pointed out, aphid populations rarely if ever achieve a stable age for distribution and therefore r_m represents a limited value in the field. The experiments mentioned here were conducted in the greenhouse under a controlled environment at 25°C ± 2°C.

To assess the life table of *A. fabae* and *R. padi*, the plant species, *V. fabae* (1 plant per pot) and *T. aestivum* (4 plants per pot) were each arranged in 20 replicates, in random blocks.

2.10. Physiological tests

2.10.1. Chlorophyll fluorescence measurement

The physiological status of green plants can be determined by measuring chlorophyll fluorescence. The level of chlorophyll fluorescence and its spectral distribution depends on a number of factors related to the ability of a plant to perform photosynthesis, which in turn is dependant on adequate plant-growth conditions. Plants stressed by biotic and abiotic factors probably limit chlorophyll

production and exhibit both a lower overall level of fluorescence and a shift in spectral distribution, compared to healthy plants.

To assess this parameter in this study, a fluorimeter PAM-2000 (Heinz Walz GmbH, Effeltrich, Germany) was used to investigate intact selected plant leaves, which had been pre-treated with *B. subtilis* and its metabolites. Water-treated plants served as control. The intact leaf to be measured was inserted into the leaf clip of the fluorimeter. The system then automatically switched on the measuring light and with every saturation pulse the measured data, which was calculated online, was written into a report–file. Fluorescence is excited by very brief but strong light pulses from light-emitting diodes. With the PAM-2000, these pulses are 3 µsec long and repeated at a frequency of 600 or 2000 Herz. The LED light passed through a short filter ($\lambda<670$ nm) and the photodetector was protected by a long-pass filter ($\lambda>700$ nm), as well as a heat-absorbing filter. A highly selective pulse amplification system ignored all signals except the fluorescence excited during the 3 µsec measuring pulses. The photodetector was a PIN-photodiode displaying linear response, while the light intensity changed by factors of more than 10^9. Hence, this measuring system tolerated extreme changes in light intensity (up to several times the intensity of full sunlight) even at light intensities that measured weakly. All yield measurement experiments were performed under cell conditions at 20°C ± 2°C. The laboratory facilities used were provided by Prof. Schmitt J. at the Freie Universität Berlin, FB Pflanzenphysiologie.

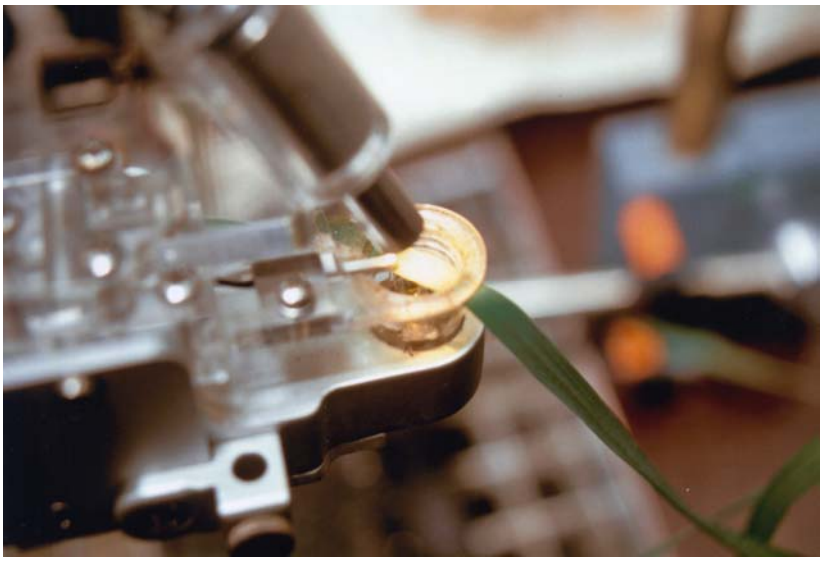

Figure 5: Chlorophyll fluorescence measurement of *Triticum aestivum* leaf, using a fluorimeter PAM-2000

2.10.2. Amino acids investigation

The investigation of amino acids aimed at determining any quantitative and qualitative change following the treatment of *V. faba* plants with *B. subtilis* metabolites.

2.10.2.1. Sample collection

When aged between 3–4 weeks, *Vicia faba* seedlings treated with *B. subtilis* metabolites and control (water-treated) seedlings received on their fourth leaves (counting upwards from the bottom) one clip cage, containing 6 adults of *A. fabae* for each seedling. After a 6-day sucking process, 0.2–0.3 g of the youngest leaves from each seedling were harvested, placed on aluminium sheets and immediately introduced into liquid nitrogen. All samples were collected between 10 a.m and 12 a.m and placed in a freezer at a temperature of –50°C prior to extraction. The experiment was arranged for treated and control plants in two groups: treated *V. faba* plants, with clip cages containing

insects, and parallel plants with empty clip cages. The empty-clip-cage approach sought to eliminate any effect this assemblage may have in the experiment.

2.10.2.2. Amino acids extraction and analysis

The samples were first dry frozen (-20°C, 0.06 mbar, minimum 2 days) and were then thawed to a constant weight, with a deviation of no more than 1 %. The dry material was ground in a mortar; 35 mg of the obtained fine powder was weighed in a 1.5 ml Eppendorf capsule.ch sample had 1000 μl of 80 % ethanol and then 5 μl of internal standard [s-carboxymethyl-l-cysteine] added before being placed in an ultrasonic bath (50-60 KHz, ice cool) for 10 min. The solution was then centrifuged 3 times at 4°C, 6000 g for 10 min each time. During the second and third times, 700 μl of 80% ethanol and 300 μl of 80 % ethanol respectively were added to the debris. Finally, the combined 80 % ethanol supernatant was gathered in a 2 ml Eppendorf capsule. From this, 150 μl was introduced in a millipore ultra-free filter and centrifuged at 4°C, 5000 g for 1–4 hours in order to separate the proteins. Finally the filtered solution was used for the amino acid test.

The amino-acid analysis was performed on the modular Knauer Amino Acid Analyser 830, consisting of 2 HPLC pumps, Type 64, equipped with micro-pump heads, the derivatizer-autosampler, the gradient programmer 50 B, a dynamic mixing chamber, a high temperature oven with temperature control unit, the fluorescence spectromonitor RF 535 or the variable wavelength detector, and the data processor chromatopac C-R6A.

The separation of the derivatised amino acids was performed on Knauer OPA with analytical (250 × 4 mm) or narrow bore (250 × 2 mm) size.

The OPA analytic method (Roth, 1971; Godel et al., 1984; Nakazawa and Arima, 1982; Ashman and Bosserhoff, 1985) was used for the amino-acid analysis.

The amino acids were derivatised automatically using the Knauer Derivatizer-Autosampler at 25°C. The OPA reaction mixture was prepared from 100 μg OPA, 1000 μl methanol, 60 μl mercaptoethanol and 9 ml 1 M boric acid pH 10.4. This mixture was stored frozen at – 20°C and freshly prepared each week.

The amino acids were separated in a gradient from buffer A (90 % 12.5 mm di-sodium hydrogen phosphate pH 6.5 and 10 % methanol) to buffer B (methanol and 3% v/v tetrohydrofuran). The detection was performed using the fluorescence detector at Ex 340 nm, Em 455 nm. Amino-acid standards or analysed samples were diluted in a citrate buffer (19.6 g sodium citrate × 2 H_2O, 20 ml thiodiglycol, 30 ml mercaptoethanol filled with water up to 1000 ml, pH 2.2).

The reaction was performed at 25°C, and the reagent-sample v/v ration was 1:1 (10 μl). OPA and all other reagents were analytical grade from Merck (Darmstadt, Germany).

The above-mentioned acid analysis was performed under the supervision and guidance of Dr. Godt Franz at the Technische Fachhochschule, Berlin.

2.11. Statistical analysis

The statistic analyses were carried out as outlined by Koehler et al. (1984) and Buehl and Zoefel (1998). The various experiments discussed here were repeated more than twice. The numbers of replications are documented below, in Chapter 3. The data of the life tables were tested to determine if assumptions of homogeneity of variances were correct. To test the null hypothesis that *B. subtilis* and its metabolites treatments can negatively influence the growth parameters of *A. fabae* and *R. padi*, SPSS ANOVA Tukey multiple analysis testing was used ($P < 0.05$). In each experiment group, the samples were independently and randomly arranged to minimise systematic error. The statistically significant levels in each group of experiments are marked with different alphabetic letters (see tables and graphics).

3. Results

3.1. Influence of *Bacillus subtilis* metabolites on *Uromyces appendiculatus, Aphis fabae* and *Rhopalosiphum padi*

3.1.1. *Uromyces appendiculatus*

Bacillus subtilis metabolites were assayed against *Uromyces appendiculatus* to gain an indication of the capacity of the isolates used in this research to inhibit the development of the pathogen urediopustules.

In topical and systemic treatment of the host plants (Table 4), the supernatants *of B. subtilis* strains FZB24, FZB37 and FZB38 and the culture filtrate of strains FZB24, FZB37 and FZB38 were able to reduce the number of uredial pustules produced by *Uromyces appendiculatus* compared to the control (water-treated) and the Landy-Medium (LM).

An inhibitive effect was not observed on *U. appendiculatus spores* development for either *V. faba* or the control (water treatment) seedlings in the Landy-Medium treatment.

Table 4: Effect of *Vicia faba* seedlings' pre-treatment with *Bacillus subtilis* metabolites on the number of rust pustules produced by *Uromyces appendiculatus*

Treatment	Topical (number of pustules per leaves)	Systemic (number of pustules per leaves)
Control	965 ± 338.8 a	1111 ± 404.3 a
Landy - Medium	873 ± 325.7 a	822 ± 314.8 a
FZB24 sup	80 ± 33.2 b	275 ± 98.6 b
FZB37 sup	66 ± 23.8 b	341 ± 95.0 b
FZB38 sup	75 ± 26.9 b	262 ± 125.0 b
FZB24 CF		344 ± 118.9 b
FZB37 CF		336 ± 121.5 b
FZB38 CF		417 ± 51.9 b

Values within the same column are means ± standard deviations (n = 10 for FZB24 CF, FZB37 CF, FZB38 CF; n = 5 for control, LM, FZB24 sup, FZB 37 sup, FZB38 sup) and those flanked by different letters are statistically significant (Tukey's test; $p < 0.05$).

Figure 6: *Uromyces appendiculatus* development on pre-treated *Vicia faba* seedlings with *Bacillus subtilis* metabolites. Left: control (water treatment); middle: systemic treatment with supernatant FZB37; right: topical treatment with supernatant FZB37

3.1.2. Influence of pre-treatment of seedlings of *Vicia faba* with culture filtrates of *B. subtilis* FZB24, B50 and Landy-Medium on feeding of *Aphis fabae*

The application of culture filtrate of the *Bacillus subtilis* strain FZB24 obtained from the three different fermentation phases, logarithmic, transition, and stationary, could not reduce the growth parameters of *A. fabae* compared to the control plants treated with tap water (Tables 5, 6). The same result was found after the usage of Landy-Medium and B50 against *A. fabae.* While treatment with culture filtrate of *B. subtilis* at the transition phase treatment showed the lowest r_m, among the treatments, this was not statistically verified (Tables 5, 6).

Table 5: Influence of culture filtrate of *Bacillus subtilis* FZB24 at logarithmic, transition and stationary phases, Landy-Medium and B50 on the growth parameters of *Aphis faba* feeding on *Vicia faba*

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0.036	0.613 ± 0.140 a	6.1 ± 0.3 a	0.461 ± 0.049 a
LM	0.037	0.600 ± 0.152 a	6.1 ± 0.3 a	0.5 00 ± 0.055 a
Log	0.035	0.617 ± 0.120 a	6.1 ± 0.2 a	0.471 ± 0.042 a
Trans	0.038	0.629 ± 0.144 a	6.2 ± 0.4 a	0.453 ± 0.050 a
Stat	0.045	0.631 ± 0.146 a	6.2 ± 0.4 a	0.426 ± 0.049 a
B 50	0.039	0.566 ± 0.134 a	6.2 ± 0.4 a	0.430 ± 0.049 a

Values within the same column are means ± standard deviations (n = 20 for control, LM, log and Trans treatments; n= 12 for Stat.; n= 8 for B50) and those flanked by different letters are statistically significant (Tukey's test; $p < 0.05$). This experiment was performed in Hannover IPP Univ. under greenhouse conditions.
A: Average mean weight of newly born insect larvae (L_1); B: Adult weight of individual insect; t_D: development time, time from birth to adult moult; RGR: Relative growth rate.

Table 6: Influence of culture filtrate of *Bacillus subtilis* FZB24 at logarithmic, transition and stationary phases, Landy-Medium and B50 on the growth parameters of *Aphis faba* feeding on *Vicia faba*

Treatment	Md	t_d (days)	r_m
Control	44.4 ± 9.6 a	7.6 ± 0.7 a	0.391 ± 0.028 a
LM	47.5 ± 9.5 b	7.7 ± 0.7 a	0.389 ± 0.031 a
Log	49.9 ± 9.9 b	7.5 ± 0.5 a	0.395 ± 0.034 a
Trans	44.0 ± 10.9 a	7.5 ± 0.5 a	0.377 ± 0.03 a
Stat	52.9 ± 10.1 b	7.8 ± 0.6 a	0.396 ± 0.024 a
B 50	51.2 ± 11.2 b	7.4 ± 0.5 a	0.400 ± 0.03 a

Values within the same column are means ± standard deviations (n = 20 for control, LM, log and trans treatments; n= 12 for Stat.; n= 8 for B50) and those flanked by different letters are statistically significant (Tukey's test; $p < 0.05$). This experiment was performed in Hannover IPP Univ. under greenhouse conditions.
Md: Number of insect larvae born in a period of time 2 × (t_d); t_d: pre-reproduction time; r_m: Intrinsic rate of natural increase

3.1.3. Influence of pre-treatment of seedlings of *Triticum aestivum with culture filtrate of B. subtilis strains* FZB24, FZB37 and FZB38 on feeding of *Rhopalosiphum padi*

The application of culture filtrate of *B. subtilis* strains, FZB24, FZB37 and FZB38 onto the leaves of summer wheat (*Triticum aestivum*) did not significantly influence the growth parameters of *R. padi* (Tables 7, 8). Though a slight negative effect on the growth parameters of *R. padi* was observed, this was not statistically verified.here were indications that in the treatments, the development time and the pre-reproduction time are longer, in contrast to the RGR and the r_m, which are shorter, when compared to the control (water treatment) (Tables 7, 8).

Table 7: Influence of culture filtrate of three strains (FZB24, FZB37, FZB38) of *Bacillus subtilis* at transition phase on the growth parameters of *Rhopalosiphum padi* feeding on *Triticum aestivum*

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0.040	0.638 ± 0.130 a	6.0 ± 0.0 a	0.460 ± 0.033 a
FZB 24 CF	0.040	0.660 ± 0.137 a	6.2 ± 0.5 a	0.455 ± 0.045 a
FZB 37 CF	0.037	0.608 ± 0.12 a	6.3 ± 0.7 a	0.447 ± 0.050 a
FZB 38 CF	0.038	0.612 ± 0.097 a	6.2 ± 0.4 a	0.453 ± 0.035 a

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20).
A: Average mean weight of newly born insect larvae (L_1); B: Adult weight of individual insect; t_D: development time, time from birth to adult moult; RGR: Relative growth rate

Table 8: Influence of culture filtrate of three strains (FZB24, FZB37, FZB38) of *Bacillus subtilis* at transition phase on the growth parameters of *Rhopalosiphum padi* feeding on *Triticum aestivum*

Treatment	Md	t_d (days)	r_m
Control	39.9 ± 7.1 a	6.8 ± 0.6 a	0.413 ± 0.039 a
FZB 24 CF	41.3 ± 10.4 a	6.8 ± 0.4 a	0.409 ± 0.032 a
FZB 37 CF	39.8 ± 11.4 a	7.0 ± 0.3 a	0.394 ± 0.045 a
FZB 38 CF	41.8 ± 110 a	6.8 ± 0.4 a	0.410 ± 0.049 a

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20).
Md: Number of insect larvae born in a period of time 2 × (t_d); t_d: pre-reproduction time; r_m: Intrinsic rate of natural increase

3.1.4. Influence of pre-treatment of *Vicia faba seedlings with culture filtrate* of *Bacillus subtilis strains* FZB24, FZB37, FZB38, and supernatant of B. *subtilis strain* FZB37 on *Aphis fabae* feeding

The application of culture filtrate of *B. subtilis* strains FZB24, FZB37, FZB38 and supernatant of strain FZB37 revealed that only the supernatant of strain FZB37 on *V. faba* was able to significantly influence the development of *A. fabae*. The growth parameters of *A. fabae*, t_D and t_d are longer, whereas the RGR and the r_m, were found to be lower in the FZB37 supernatant treatment compared to the control (water-treated) and the culture filtrate treatment (Tables 9, 10). Thus the RGR and t_d values in the FZB37 supernatant treatment were statistically different to the control and culture filtrate FZB24, FZB37 and FZB38 treatments (Table 9). The r_m value was found to be statistically different from FZB 38 CF and control but not from FZB 24 CF and FZB 37 CF treatments (Table 10). Following these observations, further experiments were performed to test the supernatants of other strains of *B. subtilis.*

Table 9: Influence of culture filtrate of three strains (FZB24, FZB3, FZB38) and supernatant FZB37 sup of *Bacillus subtilis* at transition phase on the growth parameters of *Aphis fabae* feeding on *Vicia faba*

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0.051	0.878 ± 0.129 a	6.0 ± 0.0 a	0.472 ± 0.026 a
FZB 24 CF	0.046	0.886 ± 0.124 a	6.1 ± 0.2 a	0.486 ± 0.027 a
FZB 37 CF	0.046	0.846 ± 0.191 a	6.1 ± 0.3 a	0.476 ± 0.047 a
FZB 38 CF	0.049	0.798 ± 0.142 a	6.2 ± 0.4 a	0.454 ± 0.048 a
FZB 37 sup	0.047	0.772 ± 0.233 a	7.2 ± 0.4 b	0.385 ± 0.053 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20).
A: Average mean weight of newly born insect larvae (L_1); B: Weight of adult of individual insect; t_D: development time, time from birth to adult moult; RGR: Relative Growth Rate

Table 10: Influence of culture filtrate of three strains (FZB24, FZB3, FZB38) and supernatant FZB37 sup of *Bacillus subtilis* at transition phase on the growth parameters of *Aphis fabae* feeding on *Vicia faba*

Treatment	Md	t_d (days)	r_m
Control	50.6 ± 9.2 a	7.2 ± 0.4 a	0.413 ± 0.023 a
FZB 24 CF	44.3 ± 11.0 a	7.4 ± 0.5 a	0.386 ± 0.038 b
FZB 37 CF	43.6 ± 8.8 a	7.5 ± 0.6 a	0.380 ± 0.037 b
FZB 38 CF	44.4 ± 11.9 a	7.3 ± 0.4 a	0.392 ± 0.044 a
FZB 37 sup	44.7 ± 11.1 a	8.5 ± 0.7 b	0.336 ± 0.034 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20).
Md: Number of insect larvae born in a period of time 2 × (t_d); t_d: pre-reproduction time; r_m: Intrinsic rate of natural increase

3.1.5. Influence of pre-treatment of seedlings of *Vicia faba and Triticum aestivum* with supernatants of *Bacillus subtilis* strains FZB24, FZB37 and FZB38 on feeding of *Aphis fabae* and *Rhopalosiphum padi*

The pre-treatment of *V. faba* and *T. aestivum* plants with supernatants of the three strains FZB24, FZB37 and FZB38 of *B. subtilis* clearly showed that the growth parameters of *A. fabae* and *R. padi* feeding on corresponding host plants were significantly affected (Tables 11, 12, 13, 14). The insects referred to presented longer t_D and t_d, as opposed to a lower RGR and r_m (Tables 11, 12, 13, 14). The effects of the supernatants of *B. subtilis* constitute a typical case of induced resistance in *Vicia faba* and *Triticum aestivum* plants.

While the supernatants of the different strains of *B. subtilis* (FZB24, FZB37 and FZB38) used in this research could influence growth parameters of *A. fabae* and *R. padi*, no statistical difference among the three strains was observed (Tables 11, 12, 13, 14).

Table 11: Influence of supernatants from *Bacillus subtilis* FZB24, FZB37 and FZB38 on growth parameters of *Aphis fabae* feeding on *Vicia faba*

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0.050	0.823 ± 0.177 a	6.0± 0.0 a	0.463 ± 0.066 a
FZB 24 sup	0.049	0.612 ± 0.177 b	6.7 ± 0.8 b	0.377 ± 0.066 b
FZB 37 sup	0.045	0.594 ± 0.169 b	6.6 ± 0.6 b	0.391 ± 0.068 b
FZB 38 sup	0.053	0.679 ± 0.157 b	6.6 ± 0.5 b	0.388 ± 0.055 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20).
A: Average mean weight of newly born insect larvae (L_1); B: Weight of adult of individual insect; t_D: development time, time from birth to adult moult; RGR: Relative growth rate

Table 12: Influence of supernatants from *Bacillus subtilis* FZB24, FZB37 and FZB38 on *Aphis fabae* feeding on *Vicia faba*

Treatment	Md	t_d (days)	r_m
Control	55.4 ± 6.9 a	7.0 ± 0.0 a	0.432 ± 0.013 a
FZB 24 sup	52.5 ± 11.7 a	7.7 ± 0.7 b	0.389 ± 0.043 b
FZB 37 sup	46.6 ± 13.9 a	7.6 ± 0.6 b	0.383 ± 0.058 b
FZB 38 sup	49.2 ± 10.6 a	7.6 ± 0.5 b	0.386 ± 0.039 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20).
Md: Number of insect larvae born in a period of time 2 × (t_d); td: pre-reproduction time; r_m: Intrinsic rate of natural increase

Table 13: Influence of supernatants from *Bacillus subtilis* FZB24, FZB37 and FZB38 on *Rhopalosiphum padi* feeding on *Triticum aestivum*

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0,038	0.516 ± 0.092 a	5.9 ± 0.4 a	0.444 ± 0.037 a
FZB 24 sup	0.037	0.393 ± 0.084 b	6.0 ± 0.0 a	0.390 ± 0.036 b
FZB 37 sup	0.035	0.364 ± 0.103 b	6.0 ± 0.0 a	0.385 ± 0.056 b
FZB 38 sup	0.041	0.446 ± 0.107 a	6.0 ± 0.0 a	0.393 ± 0.044 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20 for control, FZB24 sup and FZB37 sup; n = 15 for FZB38 sup).
A: Average mean weight of newly born insect larvae (L_1); B: Adult weight of individual insect; t_D: development time, time from birth to adult moult, RGR: Relative Growth Rate

Table 14: Influence of supernatants from *Bacillus subtilis* FZB24, FZB37 and FZB38 on *Rhopalosiphum padi* feeding on *Triticum aestivum*

Treatment	Md	t_d (days)	r_m
Control	38.8 ± 6.6 a	6.8 ± 0.4 a	0.400 ± 0.028 a
FZB 24 sup	30.1 ± 6.1 b	7.1 ± 0.2 a	0.358 ± 0.024 b
FZB 37 sup	29.6 ± 13.2 b	7.5 ± 0.7 b	0.331 ± 0.068 b
FZB 38 sup	32.9 ± 7.1 a	7.4± 0.6 b	0.351 ± 0.035 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20 for control, FZB24 sup and FZB37 sup; n = 15 for FZB38 sup). Md: Number of insect larvae born in a period of time 2 × (t_d); td: pre-reproduction time; r_m: Intrinsic rate of natural increase

Of all three tested supernatants of *B. subtilis* (Figures 7, 8, 9, 11, 12, 13, 14), the supernatant of strain FZB38 in the test on *A. fabae* was found to present the

best correlation between RGR and r_m values (Figure 10). In the *R. padi* test, we observed only a weak correlation between RGR and r_m values for the three strains of *B. subtilis* (Figures 12, 13, 14). In the same test, the control (water-treated) showed a relative higher correlation between the RGR and r_m (Figure 11).

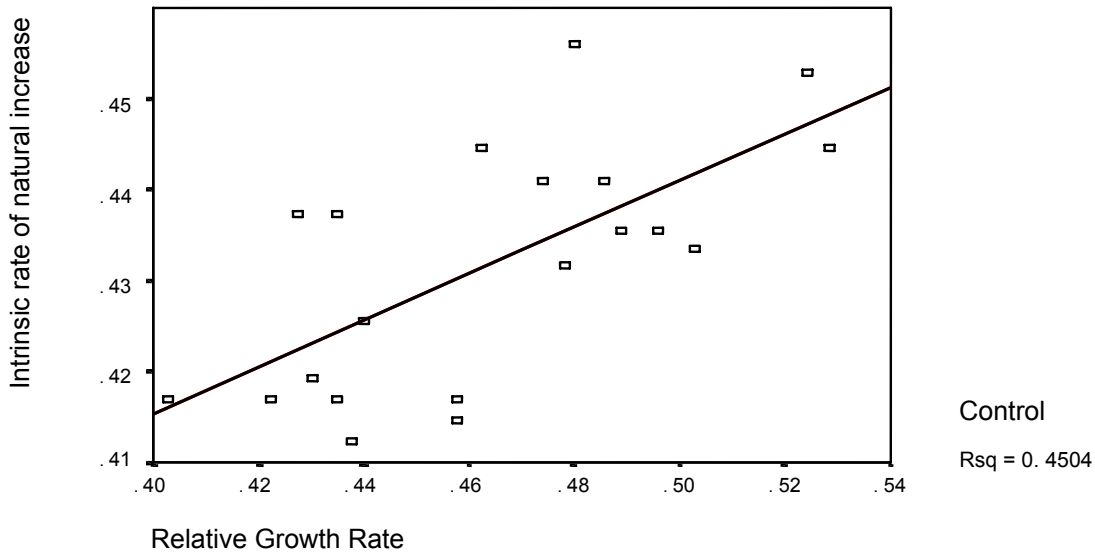

Figure 7: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with water (control), $r^2 = 0.4504$

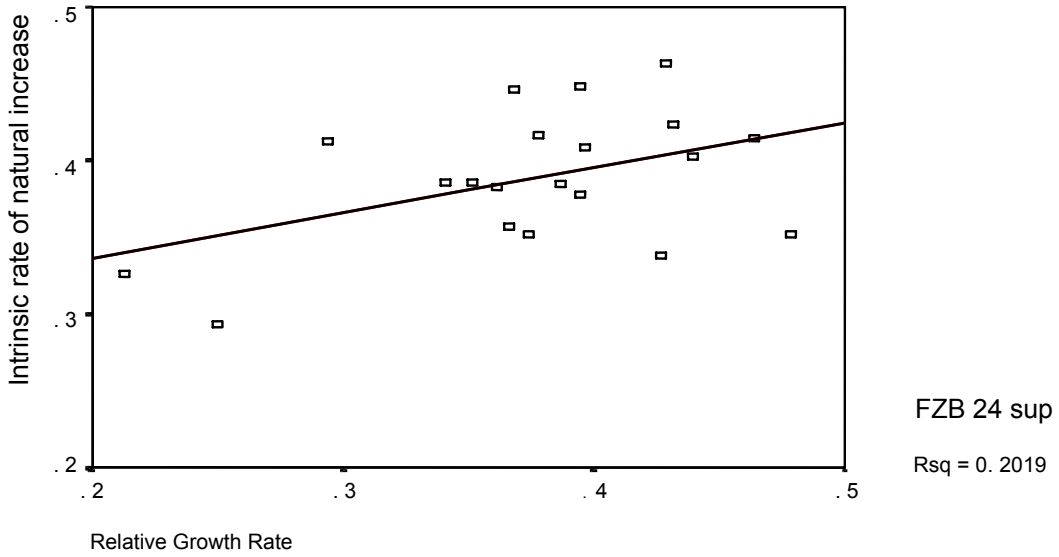

Figure 8: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with supernatant of *Bacillus subtilis* strain (FZB24), $r^2 = 0.2019$

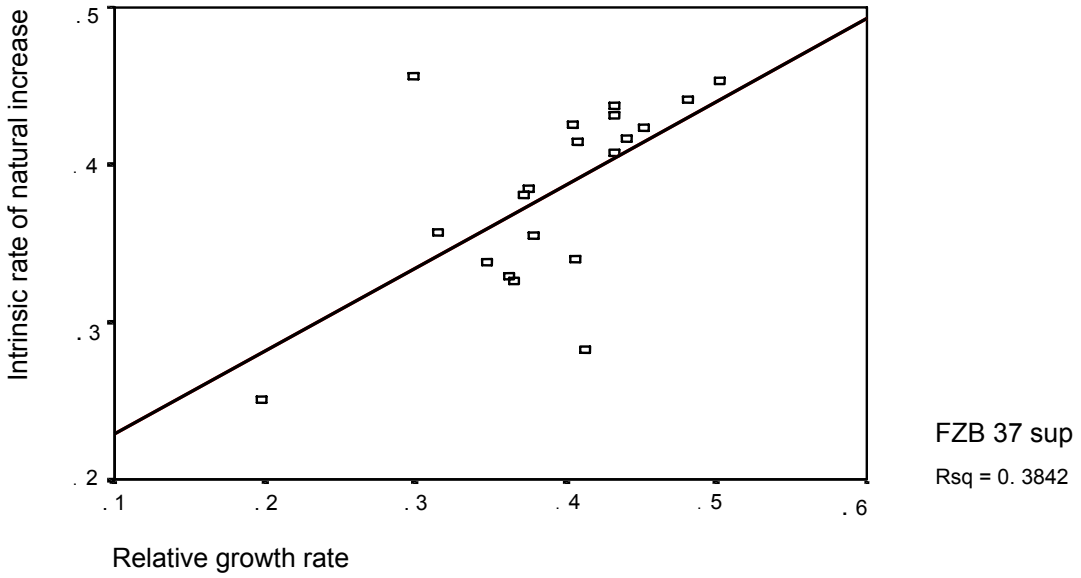

Figure 9: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with supernatant of *Bacillus subtilis* strain (FZB37), $r^2 = 0.3842$

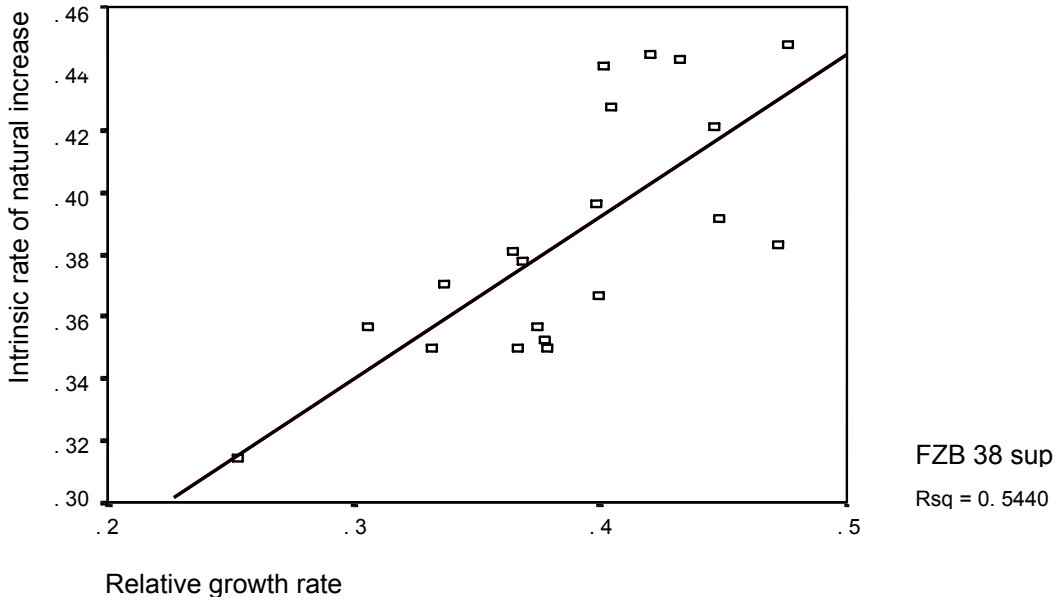

Figure 10: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with supernatant of *Bacillus subtilis* strain (FZB 38), $r^2 = 0.5440$

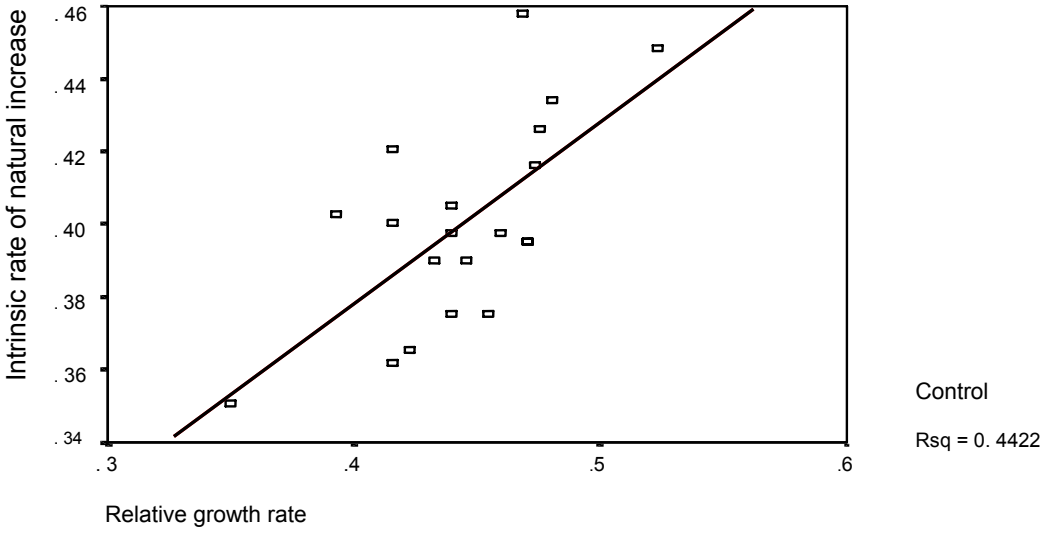

Figure 11: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Rhopalosiphum padi* feeding on *Triticum aestivum* treated with water (control), $r^2 = 0.4422$

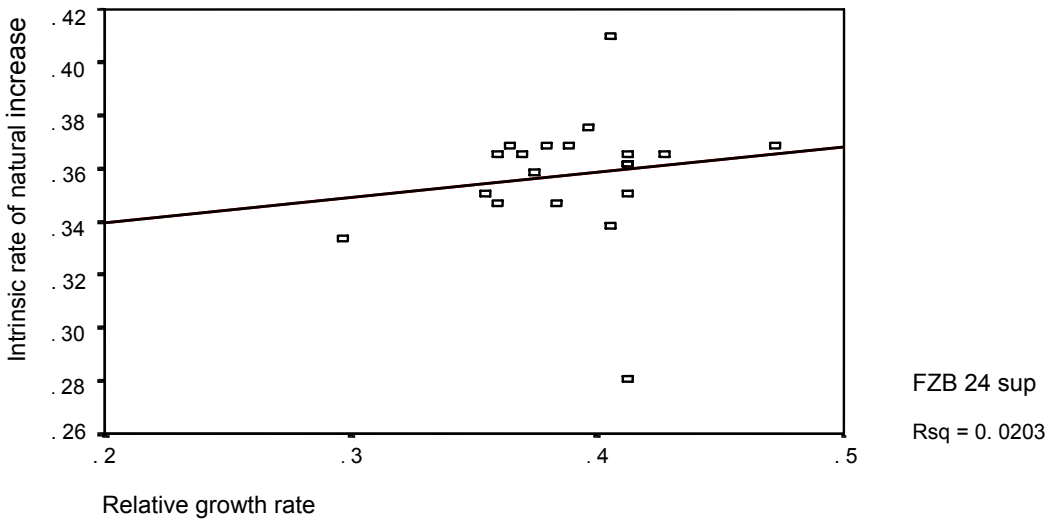

Figure 12: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Rhopalosiphum padi* feeding on *Triticum aestivum* treated with supernatant of *Bacillus subtilis* strain (FZB24), $r^2 = 0.0203$

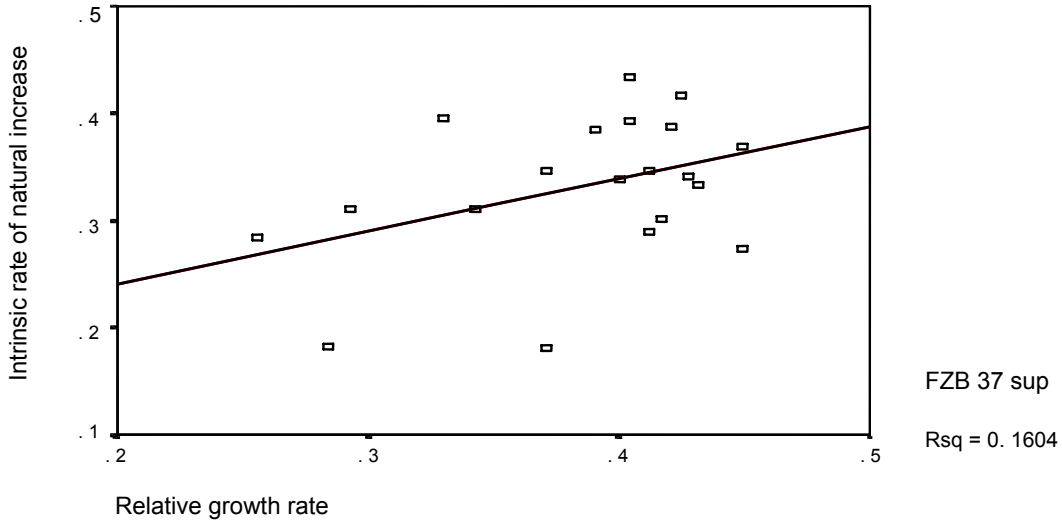

Figure 13: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Rhopalosiphum padi* feeding on *Triticum aestivum* treated with supernatant of *Bacillus subtilis* strain (FZB37), $r^2 = 0.1604$

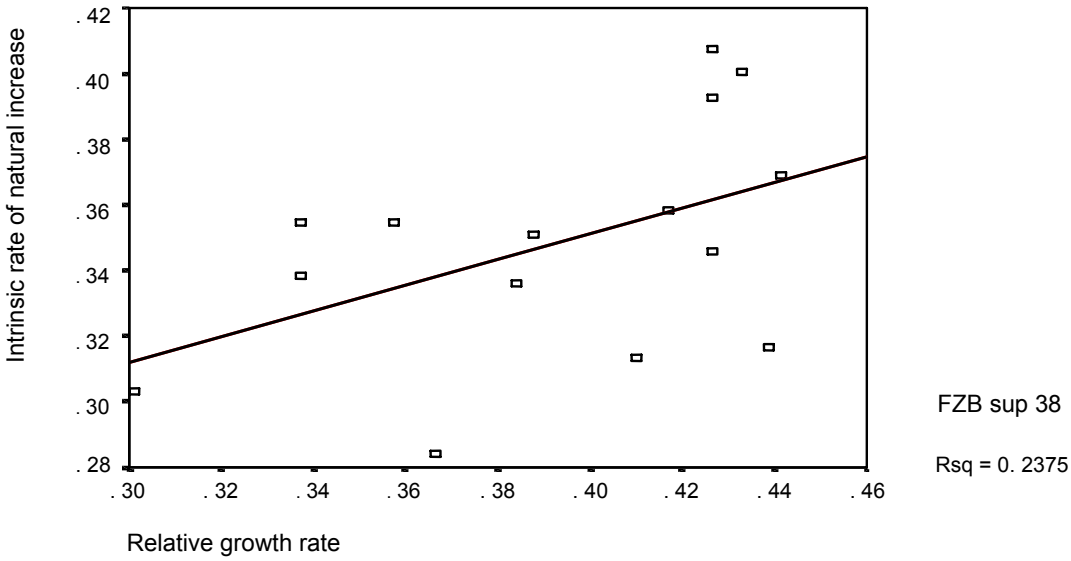

Figure 14: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Rhopalosiphum padi* feeding on *Triticum aestivum* treated with supernatant of *Bacillus subtilis* strain (FZB38), $r^2 = 0.2375$

3.1.6. Influence of pre-treatment of *Vicia faba* foliar with spore suspensions of *Bacillus subtilis* strains FZB24, FZB37 and FZB38 on feeding of *Aphis fabae*, compared to the influence of the bacteria supernatants

As it was unclear whether the obtained induced resistance effects were specific to the supernatant, the spore suspensions of the strains FZB24, FZB37 and FZB38 of *B. subtilis* were directly compared to the supernatants of strains FZB24 and FZB38 of *B. subtilis*.

As shown in Tables 15 and 16, the spore suspensions of the mentioned strains of *B. subtilis* could not reduce the growth parameters of *A. fabae* - the supernatants alone were able to achieve this. In supernatants of *B. subtilis* treatments, *A. fabae* and *T. aestivum* presented reduced adult weight, longer t_D and t_d compared to the spore suspension treatments. The number of insect larvae born to *A. fabae* and *T. aestivum* in a time period of × (t_d), Md, was found

to be very low in supernatant treatments compared to the spore suspension treatments. The same observation was made for the values of RGR and r_m (Tables 15, 16).

Table 15: Influence of spore suspensions of three strains (FZB24, FZB37, FZB38) of *Bacillus subtilis* applied onto the foliage of *Vicia faba* seedlings compared to supernatant from strains, FZB24 and FZB38 of *Bacillus subtilis*. The growth parameters of *Aphis fabae* were assessed.

Treatment	A (mg)	B (m)g	t_D (days)	RGR
Control	0,036	0.627 ± 0.048 a	6.2 ± 0.4 a	0.464 ± 0.029 a
FZB24 sps	0.039	0.634 ± 0.066 a	6.4 ± 0.5 a	0.441 ± 0.037 a
FZB37 sps	0.036	0.611 ± 0.058 a	6.1 ± 0.5 a	0.430 ± 0.039 a
FZB38 sps	0.043	0.654 ± 0.081 a	6.2 ± 0.4 a	0.439 ± 0.030 a
FZB24 sup	0.036	0.449 ± 0.122 b	6.9 ± 0.4 b	0.363 ± 0.042 b
FZB38 sup	0.033	0.416 ± 0.119 b	6.8 ± 0.4 b	0.371 ± 0.065 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20). This experiment was performed at theBA under greenhouse conditions.
A: Average mean weight of newly born insect larvae (L_1); B: Weight of adult of individual insect; t_D: development time, time from birth to adult moult; RGR: Relative growth rate

Table 16: Influence of spore suspensions of three strains (FZB 24, FZB 37, FZB 38) of *Bacillus subtilis* applied onto the foliage of *Vicia faba* seedlings compared to supernatant from strains, FZB 24 and FZB 38 of *Bacillus subtilis*. The growth parameters of Aphis fabae were assessed.

Treatment	Md	t_d (days)	r_m
Control	44.6 ± 6.9 a	6.8 ± 0.4 a	0.406 ± 0.027 a
FZB 24 sps	44.0 ± 7.1 a	7.0 ± 0.5 a	0.396 ± 0.023 a
FZB 37 sps	44.1 ± 7.7 a	7.0 ± 0.5 a	0.391 ± 0.033 a
FZB 38 sps	47.1 ± 9.5 a	7.2 ± 0.4 a	0.402 ± 0.030 a
FZB 24 sup	29.15 ± 6.9 b	7.8 ± 0.4 b	0.324 ± 0.032 b
FZB 38 sup	30.7 ± 9.0 b	7.5 ± 0.6 b	0.330 ± 0.400 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test, $p < 0.05$; n = 20). This experiment was performed at the BBA under greenhouse conditions.
Md: Number of insect larvae born in a period of time 2 × (t_d); td: pre-reproduction time; r_m: Intrinsic rate of natural increase

In this experiment the following correlations (Figures 15, 16, 17, 18) were found between the RGR and r_m values for each treatment, showing that those insects with bigger RGR also have higher r_m. The correlation was verified for the control (water-treated), with more than 68 % of the test insects (Figure 15), the spore suspensions of *B. subtilis* strain FZB 37, more than 74 % (Figure 16) and supernatant of *B. subtilis* strain FZB38, approximately 42 % (Figure 18). The spore suspensions of *B. subtilis* strain FZB38 showed a weak correlation with an approximate estimate of only 24 % of the tested insects (Figure 17).

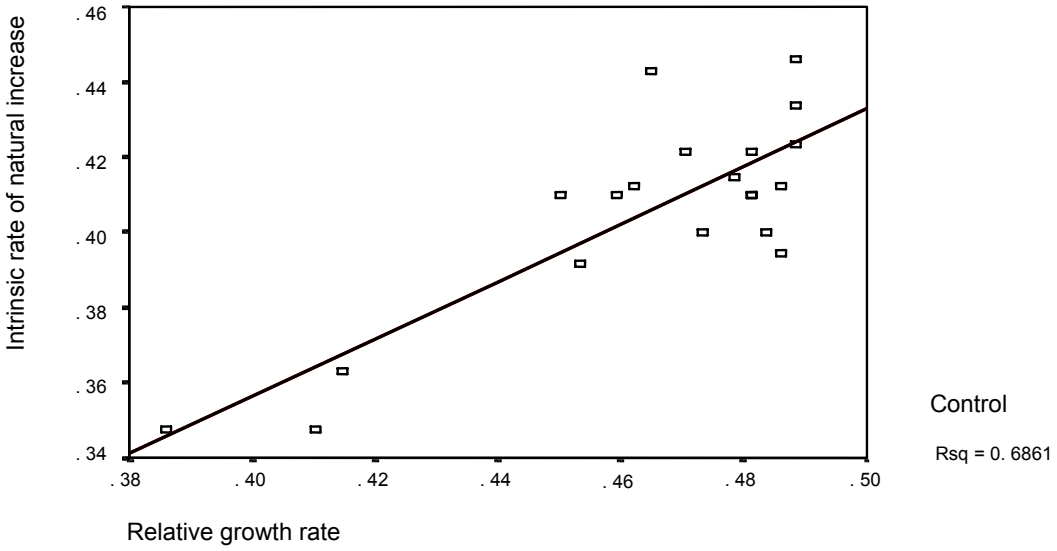

Figure 15: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with water (Control), $r^2 = 0.6861$

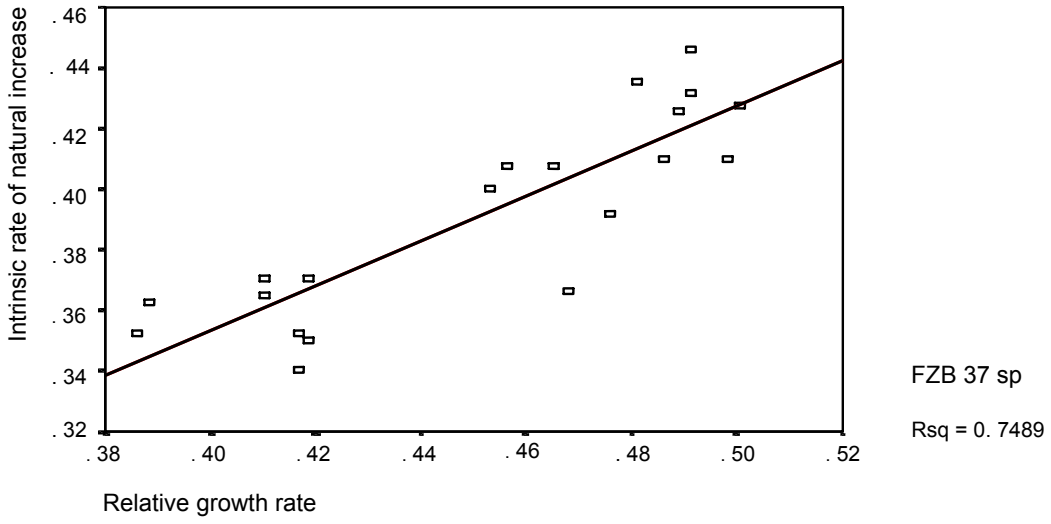

Figure 16: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with spore suspensions of *Bacillus subtilis* strain (FZB37), $r^2 = 0.7489$

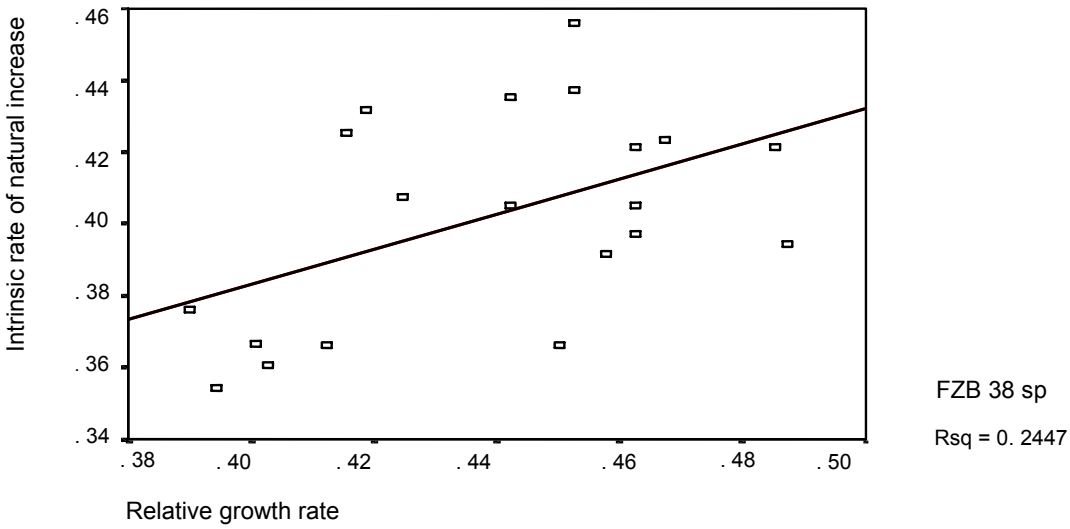

Figure 17: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with spore suspensions of *Bacillus subtilis* strain (FZB38), $r^2 = 0.2447$

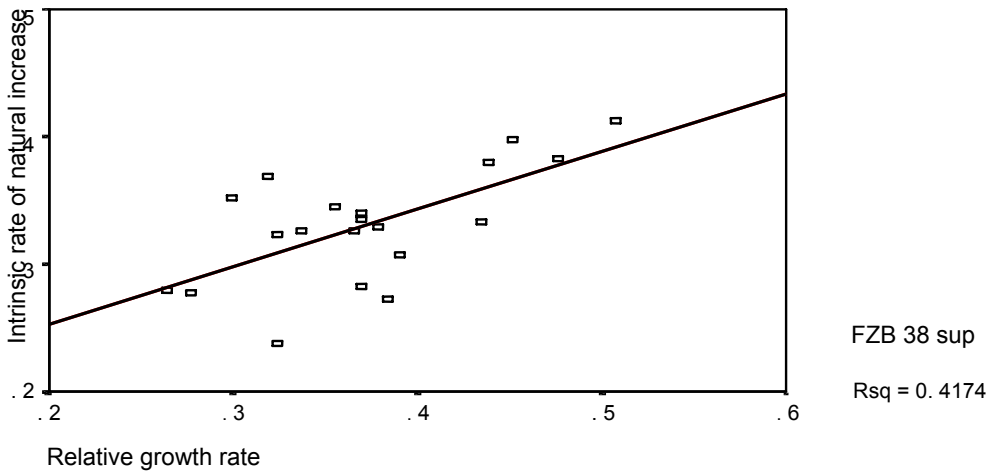

Figure 18: Intrinsic rate of natural increase (r_m) in relation to relative growth rate (RGR) for *Aphis fabae* feeding on *Vicia faba* treated with supernatant of *Bacillus subtilis* strain (FZB 38), $r^2 = 0.4174$

3.1.7. Influence of pre-treatment of *Vicia faba* seedlings with vegetative cells of *Bacillus subtilis* strains FZB24, FZB37 and FZB38 on feeding of *Aphis fabae*

Host plant foliage pre-treatment with vegetative cells of *B. subtilis* strain FZB24, FZB37 and FZB38 was unable to significantly influence the growth parameters of *A. fabae* feeding on *V. faba* (Tables 17, 18). However, following this treatment we could observe longer t_D and t_d, as opposed to slightly diminished r_m. However, this result was not statistically verified.

Table 17: Influence of vegetative cells from *Bacillus subtilis* strain FZB24, FZB37 and FZB38 at transition phase on *Aphis fabae* feeding on *Vicia faba*

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0.037	0.630 ± 0.120 a	6.0 ± 0.0 a	0.469 ± 0.032 a
FZB 24 vc	0.034	0.609 ± 0.127 a	6.2 ± 0.4 a	0.464 ± 0.055 a
FZB 37 vc	0.036	0.576 ± 0.117 b	6.1 ± 0.2 a	0.455 ± 0.039 a
FZB 38 vc	0.038	0.635 ± 0.090 a	6.1 ± 0.2 a	0.465 ± 0.029 a

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test; n = 20; $p < 0.05$).
A: Average mean weight of newly born insect larvae (L_1); B: Weight of adult individual insect; t_D: development time, time from birth to adult moult; RGR: Relative growth rate

Table 18: Effect of *Vicia faba* plant pre-treatment with vegetative cells from *Bacillus subtilis* strain FZB24, FZB37 and FZB38 at transition phase on *Aphis fabae*

Treatment	Md	t_d (days)	r_m
Control	48.6 ± 7.9 a	7.0 ± 0.0 a	0.417 ± 0.017 a
FZB 24 vc	48.8 ± 8.8 a	7.2 ± 0.4 a	0.407 ± 0.030 a
FZB 37 vc	44.9 ± 7.1 a	7.1 ± 0.2 a	0.406 ± 0.019 a
FZB 38 vc	49.0 ± 7.2 a	7.1 ± 0.2 a	0.415 ± 0.020 a

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test; n = 20 $p < 0.05$).
Md: Number of insect larvae born in a period of time 2 × (t_d); t_d: pre-reproduction time; r_m: Intrinsic rate of natural increase

3.1.8. Direct exposure of *Aphis fabae* and *Rhopalosiphum padi* to supernatants of *B. subtilis strains* FZB24, FZB37 and FZB38

Based on the test results obtained from the pre-treated plant foliage on *A. fabae* and *R. padi*, it was necessary to continue the investigations in order to establish the causes of the positive activity found in supernatant treatments against *A. fabae* and *R. padi* feeding on their respective host plants (*V. faba* and *T. aestivum*). Thus, it was decided to expose the insects directly to the supernatants to exclude any effect from direct contact. This acute toxicity test with supernatants of *B. subtilis* was compared to the treatments (Tables 19, 20, 21, 22), in which the plants were topically pre-treated with the supernatant of *B. subtilis* strain (FZB37), and the aphids thereafter caged on the selected leaves for feeding.

The acute toxicity test showed that the growth parameters of the *A. fabae* and *R. padi* (Tables 19, 20, 21, 22) were not affected, in contrast to the plant foliage pre-treatment, which showed a significant change.

The antibiosis effect observed with supernatant treatments to *A. fabae and R. padi* are likely to be mediated via the plant, and not caused by direct contact between the test insects and the metabolites of *B. subtilis*.

Table 19: Effect of direct exposure of *Aphis fabae* to supernatant of *Bacillus subtilis* FZB24, FZB37, and FZB38

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0.033	0.610 ± 0.111 a	6.2 ± 0.4 a	0.470 ± 0.048 a
FZB 24 sup	0.038	0.673 ± 0.157 a	6.3 ± 0.5 a	0.459 ± 0.062 a
FZB 37 sup	0.038	0.680 ± 0.060 a	6.7 ± 0.5 a	0.432 ± 0.036 a
FZB 38 sup	0.037	0.609 ± 0.119 a	6.4 ± 0.5 a	0.438 ± 0.049 a
FZB 37 sup (foliage treatment)	0.040	0.491 ± 0.122 b	6.6 ± 0.7 a	0.382 ± 0.071 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test; n = 10; $p < 0.05$).
A: Average mean weight of newly born insect larvae (L_1); B: Weight of adult individual insect; t_D: development time, time from birth to adult moult; RGR: Relative growth rate

Table 20: Effect of direct exposure of *Aphis fabae* to supernatant of *Bacillus subtilis* FZB24, FZB37, and FZB38

Treatment	Md	t_d (days)	r_m
Control	49.1 ± 13.4 a	7.2 ± 0.4 a	0.406 ± 0.039 a
FZB 24 sup	46.4 ± 10.7 a	7.4 ± 0.5 a	0.390 ± 0.038 a
FZB 37 sup	41.7 ± 10.9 a	7.7 ± 0.5 a	0.381 ± 0.035 a
FZB 38 sup	41.7 ± 12.0 a	7.4 ± 0.5 a	0.378 ± 0.044 a
FZB 37 sup (foliage treatment)	38.4 ± 18.8 a	7.4 ± 0.7 a	0.362 ± 0.073 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test; n = 10; $p < 0.05$).
Md: Number of insect larvae born in a period of time 2 × (td); t_d: pre-reproduction time; r_m: Intrinsic rate of natural increase

Table 21: Effect of direct exposure of *Rhopalosiphum padi* to the supernatant of *Bacillus subtilis* strains FZB24, FZB37 and FZB38 on its growth parameters

Treatment	A (mg)	B (mg)	t_D (days)	RGR
Control	0.040	0.500 ± 0.100 a	5.9 ± 0.4 a	0.436 ± 0.042 a
FZB 24 sup	0.040	0.605 ± 0.128 a	6.2 ± 0.5 a	0.440 ± 0.048 a
FZB 37 sup	0.037	0.588 ± 0.132 a	6.3 ± 0.7 a	0.439 ± 0.052 a
FZB 38 sup	0.039	0.565 ± 0.110 a	6.0 ± 0.0 a	0.443 ± 0.032 a
FZB 37 sup (foliage treatment)	0.034	0.359 ± 0.100 b	6.0 ± 0.0 a	0.384 ± 0.054 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test; n = 10; $p < 0.05$).
A: Average mean weight of newly born insect larvae (L_1); B: Weight of adult of individual insect; t_D: development time, time from birth to adult moult; RGR: Relative Growth Rate

Table 22: Effect of direct exposure of *Rhopalosiphum padi* to the supernatant of *Bacillus subtilis* strains FZB24, FZB37 and FZB38 on its growth parameters

Treatment	Md	t_d (days)	r_m
Control	37.9 ± 9.6 a	6.5 ± 1.5 a	0.382 ± 0.089 a
FZB 24 sup	40.1 ± 12.9 a	6.8 ± 0.4 a	0.405 ± 0.041 a
FZB 37 sup	40.2 ± 13.6 a	7.0 ± 0.3 a	0.393 ± 0.044 a
FZB 38 sup	40.7 ± 7.9 a	7.2 ± 0.5 a	0.391 ± 0.034 a
FZB 37 sup (foliage treatment)	33.3 ± 6.7 a	7.5 ± 0.6 a	0.350 ± 0.03 b

Values within the same column are mean ± standard deviations and those flanked by different letters are statistically significant (Tukey's test; n = 10; $p < 0.05$).
Md: Number of insect larvae born in a period of time 2 × (t_d); t_d: pre-reproduction time; r_m: Intrinsic rate of natural increase

3.1.9. Effect of culture filtrates from *Bacillus subtilis* strains FZB24, FZB37 and FZB38 added to artificial diet of *Aphis fabae*

While the acute toxicity test discussed above provided strong evidence that the tested supernatants of *B. subtilis* are only active against *A. fabae* and *R. padi* via the plant, further tests were conducted to confirm this role of *B. subtilis* metabolites when added to artificial diet and submitted to *A. fabae*. This test was conducted in order to ascertain what form of influence the culture filtrate would have on the feeding of *A. fabae*.

Culture filtrates from *B. subtilis* FZB24, FZB37 and FZB38 were incorporated in the artificial diet for *A. fabae* and the relative growth rates of the feeding insects were assessed. As shown in Table 23, the measured RGRs of the insects were not affected. It is likely that the culture filtrate of *B. subtilis* has no direct effect on *A. fabae*.

Table 23: Incorporation of culture filtrate of *Bacillus subtilis* strains FZB24, FZB37, and FZB38 into artificial diet of *Aphis fabae* and assessment of its growth parameters

Treatment	A (mg)	B (mg)	t_D	RGR
100% diet	0.035	0.461	8	0.322
0.1% of water	0.035	0.461	8	0.322
1% of water	0.036	0.464	8	0.319
0.1% FZB 24 CF	0.037	0.466	8	0.317
1% FZB 24 CF	0.037	0.464	8	0.316
0.1% FZB 37 CF	0.037	0.456	8	0.314
1% FZB 37 CF	0.038	0.456	8	0.311
0.1% FZB 38 CF	0.038	0.470	8	0.314
1% FZB 38 CF	0.037	0.456	8	0.314

A: Average mean weight of newly born insect larvae (L_1); B: Adult weight of individual insect; t_D: development time, time from birth to adult moult; RGR: Relative Growth Rate

Figure 19: *Aphis fabae* feeding on artificial diet

3.2. Physiological tests

3.2.1. Chlorophyll measurement and aphid biomass assessment

3.2.1.1. Leaf treatment experiments

The chlorophyll fluorescence of *Vicia faba* plants was measured on the fourth leaves while *A. fabae* were caged on the third leaves. In the case of the *T. aestivum* plant, the measurement was taken on the third leaf while *R. padi* were caged on the second leaf. It was consequently found in both cases (*V. faba* and *T. aestivum*) that the chlorophyll fluorescence was higher in supernatant pre-treated plants with aphids caged on them, but this was not statistically verified (Tables 24, 26). The corresponding fresh weight and dry weight of the *A. fabae*

and *R. padi* measured were also found to be lower compared to the control (water treatment). This was statistically verified in the case of *A. fabae* dry weight but not for *R. padi* (Tables 25, 27).

Table 24: Influence of supernatant of *Bacillus subtilis* strain FZB37 on the chlorophyll fluorescence of *Vicia faba* leaves. The measurement was obtained on the fourth leaves of the plant while on the third leaves either 4 adults of *A. fabae* were fixed or an empty clip cage was fixed per plant.

Number of caged insects	Chlorophyll fluorescence yield	
	Control	FZB37 sup
0	0.605 ± 0.038 a	0.598 ± 0.043 a
4	0.582 ± 0.040 a	0.610 ± 0.038 a

Mean values ± standard deviations (n=8) on the same line, followed by the same letters are not significantly different (Tukey's test; $p < 0.05$).

Table 25: Influence of supernatant of *Bacillus subtilis* strain FZB37 on the fresh weight and dry weight of *Aphis fabae* after 6 days sucking process on *Vicia faba*

Test variant	Fresh weight in (mg)	Dry weight in (mg)
Control	11.229 ± 9.208 a	3.122 ± 2.327 a
FZB37 sup	10.655 ± 8.634 a	2.284 ± 1.773 b

Mean values ± standard deviations (n=8) in the columns followed by various letters are statistically significant. (t - test; $p < 0.05$).

Table 26: Influence of supernatant of *Bacillus subtilis* strain FZB37 on the chlorophyll fluorescence of *Triticum aestivum* leaves. The measurements were obtained on the tertiary leaves while on the secondary leaves either 4 adults of *R. padi* or an empty clip cage were caged onto each plant.

Numbers of caged insects	Chlorophyll fluorescence yield	
	Control	FZB37 sup
0	0.440 ± 0.031 a	0.506 ± 0.054 a
4	0.432 ± 0.078 a	0.487 ± 0.049 a

Mean values ± standard deviations (n=8) on the same line followed by different letters are statistically significant (Tukey's test; $p < 0.05$).

Table 27: Influence of supernatant of *Bacillus subtilis* strain FZB37 on the fresh weight and dry weight of *Rhopalosiphum padi* after 6 days sucking process on *Triticum aestivum*

Test variant	Fresh weight in (mg)	Dry weight in (mg)
Control	4.794 ± 1.318 a	1.586 ± 0.511 a
FZB37 sup	4.160 ± 1.093 a	1.130 ± 0.561 a

Mean values ± standard deviations (n=8) in the columns followed by the same letters are not significantly different (t - test; $p < 0.05$).

3.2.1.2. Seed treatment experiments

After measuring the influence of supernatants of *B. subtilis* FZB37 on the chlorophyll fluorescence of pre-treated *V. faba* and *T. aestivum,* on which *A. fabae* and *R. padi* were respectively fixed for feeding, it was decided to measure the effect of seed treatment with spore suspensions of *B. subtilis* on the host plants of *A. fabae* and *R. padi*.

Seedlings of *V. faba* and *T. aestivum,* grown in sterile soil from seeds pre-treated with spore suspension of *B. subtilis* showed better growth (Figures 20, 21) and chlorophyll fluorescence yields. The higher value of chlorophyll

fluorescence was statistically verified for *T. aestivum* seedlings but not for *V. faba* (Tables 28, 30). The fresh weight and dry weight of aphids feeding on such plants were negatively affected compared to the control (water-treatment) (Tables 29, 31), but these values were not statistically verified.

Table 28: Influence of seed pre-treatment with spore suspensions of *Bacillus subtilis* FZB37, which were sown into sterile soil substrate on the chlorophyll fluorescence of *Vicia faba* leaves. The measurement was obtained on the fourth leaves while on the third leaves either 4 adults of *Aphis fabae* or an empty clip cage were fixed onto each plant.

Number of caged insects	Chlorophyll fluorescence yield	
	Control	FZB37 sps
0	0.481 ± 0.043 a	0.514 ± 0.042 a
4	0.464 ± 0.062 a	0.498 ± 0.048 a

Mean values ± standard deviations (n=8) on the same line followed by different letters are statistically significant (Tukey's test; $p < 0.05$).

Table 29: Influence of seed pre-treatment with *Bacillus subtilis* FZ37 spore suspensions on the fresh weight and dry weight of *Aphis fabae* feeding on *Vicia faba* sown in sterile soil

Test variant	Fresh weight in (mg)	Dry weight in (mg)
Control	52.7 ± 19.6 a	14.1 ± 5.9 a
FZB37 sps	46.3 ± 16.8 a	13.4 ± 4.7 a

Mean values ± standard deviations (n=8) in the columns followed by different letters are statistically significant (Tukey's test; $p < 0.05$).

Table 30: Influence of seed pre-treatment with *Bacillus subtilis* FZB37 spore suspensions, which were sown into sterile soil substrate on the chlorophyll fluorescence of *Triticum aestivum* leaves. The measurement was taken on the third leaves while on the second leaves either 4 adults of *Rhopalosiphum padi* or an empty clip cage were fixed on each plant.

Number of caged insects	Chlorophyll fluorescence yield	
	Control	FZB37 sps
0	0.448 ± 0.049 a	0.456 ± 0.056 a
4	0.370 ± 0.063 a	0.504 ± 0.036 b

Mean values ± standard deviations (n=8) on the same line followed by different letters are statistically significant (Tukey's test; $p < 0.05$)

Table 31: Influence of the pre-treatment of seeds with *Bacillus subtilis* FZB37 spore suspensions, which were sown into sterile soil, on the fresh weight and dry weight of *Rhopalosiphum padi* feeding on summer wheat (*Triticum aestivum*)

Test variant	Fresh weight in (mg)	Dry weight in (mg)
Control	7.3 ± 3.8 a	3.2 ± 1.7 a
FZB37 sps	5.2 ± 2.8 a	2.2 ± 1.3 a

Mean values ± standard deviations (n=8) in the columns followed by different letters are statistically significant (t - test; $p < 0.05$).

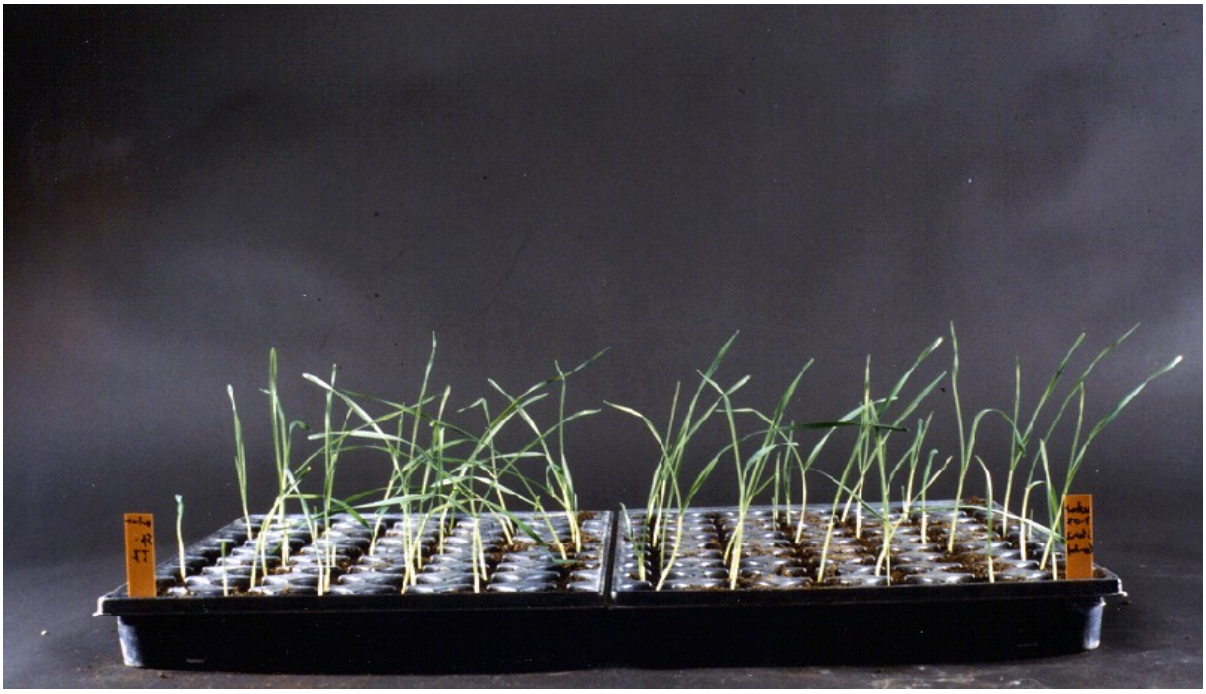

Figure 20: Summer wheat seedlings (*Triticum aestivum*). Left: seed treated with water. Right: seed pre-treated with spore suspensions of *Bacillus subtilis*

Figure 21: Broad bean (*Vicia faba*) seedlings. Left: seed pre-treated with sterile water. Right: seed pre-treated with spore suspensions of *Bacillus subtilis*

3.2.2. Amino acids from phloem

Total Concentration of amino acids in the phloem sap of *Vicia faba*

The total concentration of free amino acids increased following the leaf treatments with the supernatant and culture filtrate of *B. subtilis* strain FZB37, as compared to the control (water-treatment) (Figure 22).

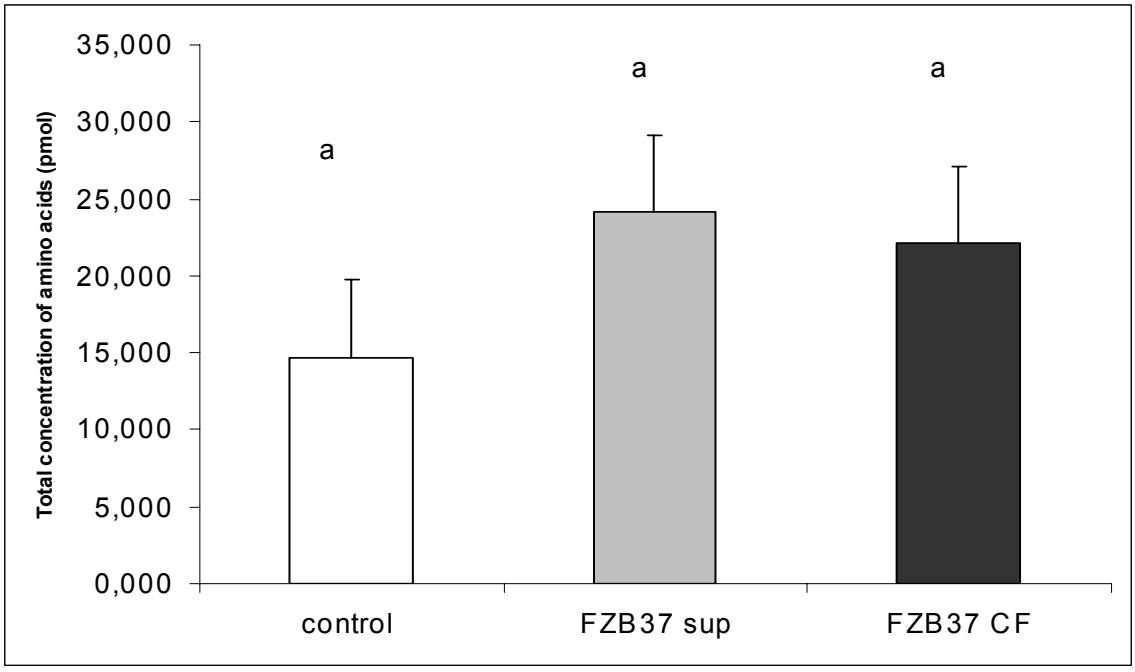

Figure 22: Total concentration of amino acids (pmol) following topical treatment with *Bacillus subtilis* metabolites of *Vicia faba* foliage, without *Aphis fabae* infestation. Bars with lines are mean ± standard deviations and those followed by different letters are statistically significant (Tukey's test; $p < 0.05$). Control: plant leaves treated with water; FZB37 sup: plant leaves treated with supernatant of *Bacillus subtilis* strain FZB37; FZB37 CF: plant leaves treated with culture filtrate of *Bacillus subtilis* strain FZB37

Following *A. fabae* infestation, the total concentration of free amino acids in control and culture filtrate treatments increased (Figure 23), whereas in plants treated with supernatant of *B. subtilis* strain FZB37 no major variation was observed. For a statistical overview of this observation, compare Figure 22 and Figure 23.

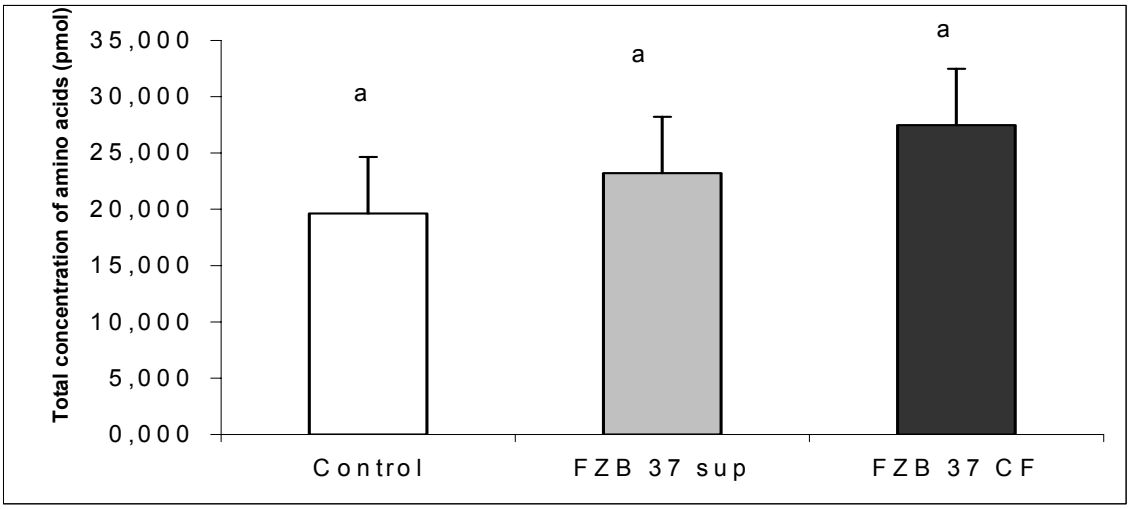

Figure 23: Total concentration of amino acids (pmol) following topical treatment with *Bacillus subtilis* metabolites of *Vicia faba* foliage with *Aphis fabae* infestation
Control: Plant leaves treated with water; FZB37 sup: Plant leaves treated with supernatants of *Bacillus subtilis* strain FZB37; FZB37CF: plant leaves treated with culture filtrate of *Bacillus subtilis* strain FZB37.
Bars with lines are mean ± standard deviations and those followed by different letters are statistically significant (Tukey's test; $p < 0.05$).

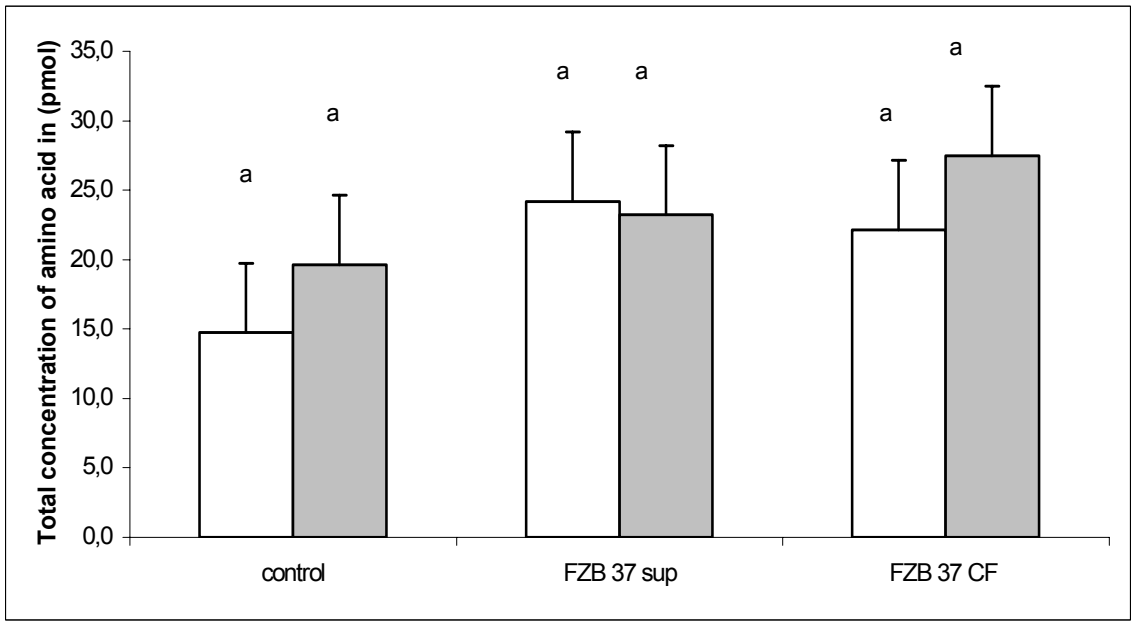

Figure 24: Total concentration of amino acids (pmol) in phloem sap of *Vicia faba*. In the same group of bars, the open bars represent cases with 0 insects caged on the plant leaves, while the shaded bars show treatments with 6 insects caged on the selected plant leaves.
Bars with lines are mean ± standard deviations and groups of bars followed by different letters are statistically significant (Tukey's test; $p < 0.05$).

As indicated in Figure 24, treatment with supernatant of *B. subtilis* FZB37 caused no variation in the total concentration of amino acid when compared to the same group of open bars (0 insects caged on treated plant leaves) and shaded bars (6 insects caged on treated plant leaves). In contrast, there is a noticable difference in total concentration of amino acid between the two groups of bars in the control and culture filtrate treatments. This observation suggests a qualitative difference between the culture filtrate and the supernatant of *B. subtilis* strain FZB37. Feeding activity of *A. fabae* mobilizes more amino acids in the case of water and culture filtrate treated plants.

Individual amino acids from phloem sap of *Vicia faba*.

In Figure 25 with no insect feeding on the pre-treated plants, changes were observed for the concentration of only three individual amino acids, histidine, alanine and methionine, in the control, FZB sup 37 and FZB CF 37 treatments.

In contrast with *A. fabae* feeding on pre-treated plants, a marked change occurred in individual amino acids and was found to be significant for nine of them, as indicated in Figure 26. The histidine level is higher in FZB37 CF treatment than the control and FZB37 sup treatments. Glycine and threonine concentrations in FZB37 CF treatment were found to be higher than in FZB37 sup treatment, but were not different from the control (water treatment). Alanine was found to be in a higher concentration in the FZB37 sup treatment than the control but not different from that in the FZB37 CF. Tyrosine level in FZB37 sup treatment was higher than control treatment but not different from FZB37 CF treatment. Leucine and lysine were both present in a higher concentration in the FZB37 CF treatment, when compared to the control and FZB 37 sup treatments. Serine and asparagine concentrations in FZB37 sup treatment were lower when compared to the control and FZB37 CF treatment. Comparing Figure 25 and Figure 26, we can observe that the concentration of serine did not change for

FZB37 sup treatment, as opposed to the control and FZB37 CF treatments, suggesting that the supernatant is capable of protecting the integrity of this amino acid, vital for insect growth in general and particularly for, in this case, *A. fabae*.

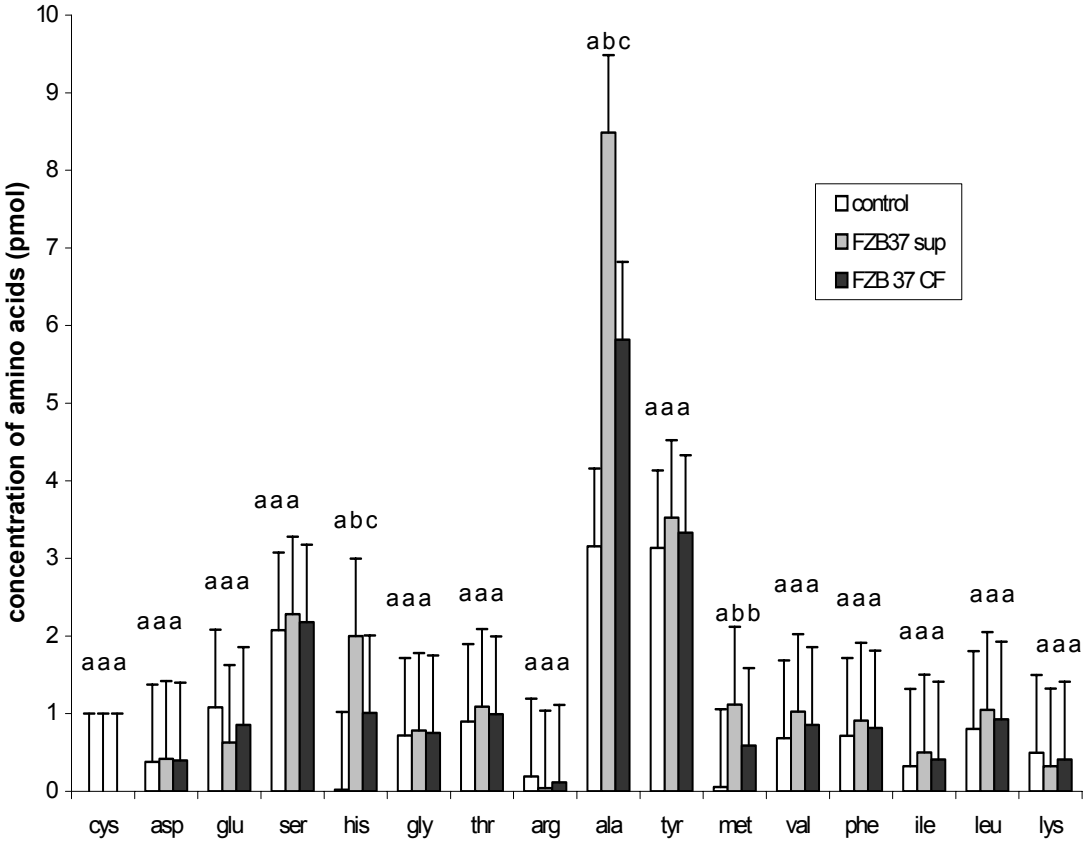

Figure 25: Phloem concentration of amino acids (pmol) from pre-treated and untreated *Vicia faba* plants, without *Aphis fabae* infestation. Bars with lines are means ± S.E. Groups of bars followed by different letters are statistically significant (Tukey's test; $p < 0.05$). Alanine (ala), arginine (arg), asparagine (asp), cysteine (cys), glutamine (glu), glycine (gly), histidine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), serine (ser), threonine (thr), tyrosine (tyr), valine (val)

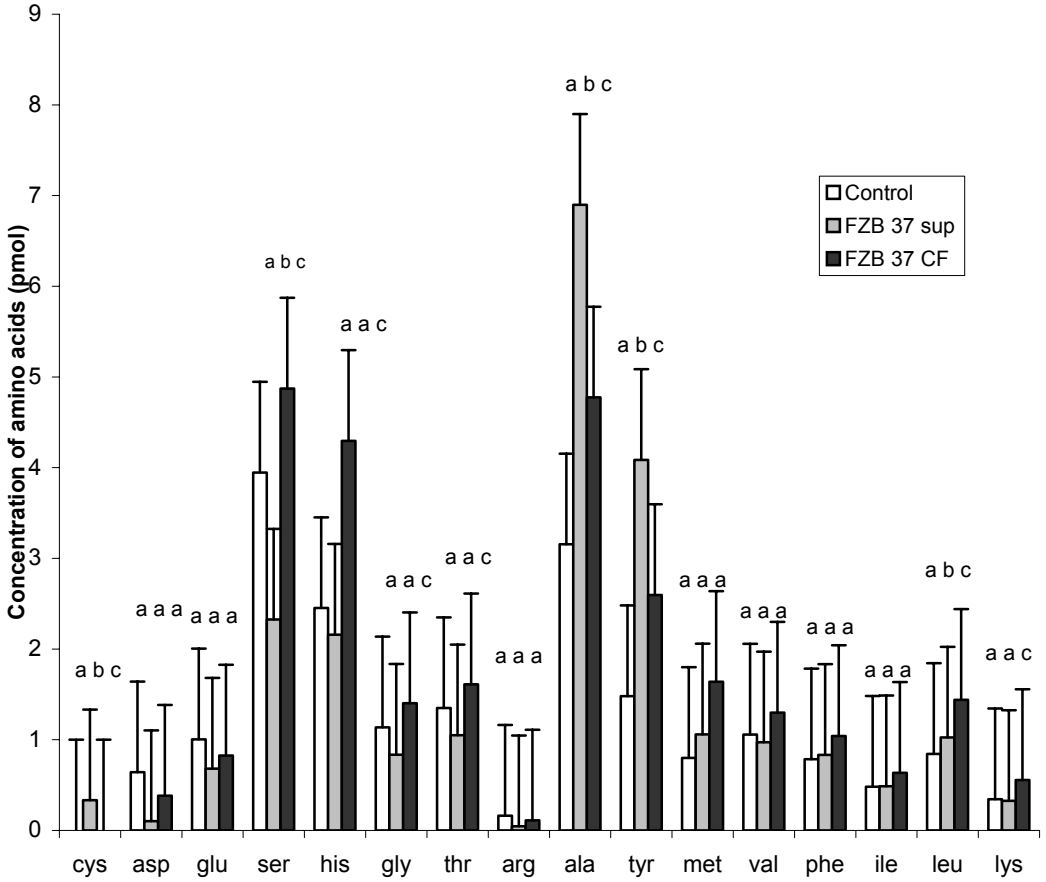

Figure 26: Phloem concentration of amino acids (pmol) from pre-treated and untreated *Vicia faba* plants on which 6 adults of *Aphis fabae* had been caged for 6 days. Bars with lines are means ± standard deviations. Groups of bars followed by different letters are statistically significant (Tukey's test, $p < 0.05$). Alanine (ala), arginine (arg), asparagine (asp), cysteine (cys), glutamine (glu), glycine (gly), histidine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), serine (ser), threonine (thr), tyrosine (tyr), valine (val)

3.3. Reisolation of spores of *B. subtilis* from sterile soil substrate cultivated with *Vicia faba* and *Triticum aestivum*

As demonstrated by Thonart et al. (1994), *B. subtilis* can develop better if inoculated in sterile soil passing from 10^5 cells/g to 10^8 cells/g of soil. This was confirmed by the results of our experiment, in which spores of *B. subtilis* strain FZB 37, with which *V. faba* and *T. aestivum* seedlings had been innoculated at a concentration of 10^5 cfu/ml and cultivated in the soil, were reisolated and

counted. In both plant systems higher colonies of organisms resembling *B. subtilis* were found (Table 32).

Table 32: Number of spores of *Bacillus subtilis* strain (FZB37) reisolated from the soil substrate and root material of *Vicia faba* and *Triticum aestivum* seedlings expressed as [10^8 per g dry weight of roots/ substrate]. For both plant species, *Vicia faba* and *Triticum aestivum*, 4 corresponding insects or an empty clip cage were caged on their selected leaves.
FZB37 sp: *Bacillus subtilis* strain FZB37 spore suspensions; titer: 10^5 cfu/ml

Seed Treatment	*Vicia faba*		*Triticum aestivum*	
	Rhizosphere	Soil substrate	Rhizosphere	Soil substrate
FZB37 sp (Treatment with 4 insects)	150	142	118	108
FZB37 sp (Treatment without insect)	140	133	123	113
Distilled water (Treatment with 4 insects)	0	0	0	0
Distilled water (Treatment without insect)	0	0	0	0

4. Discussion

The here presented results show that in all the studies, induced resistance was possible in both the studied host plants, summer wheat (*Triticum aestivum*) and broad bean (*Vicia faba*), where the supernatant of *B. subtilis* was used. This effect was not caused by direct toxicity of the supernatant to the insects. This was demonstrated in the acute toxicity test, where aphids were directly exposed to the supernatants. The culture filtrates of *B. subtilis* were used in the artificial diets to observe whether the relative growth rate of *A. fabae* would be affected. Obviously it was not possible to introduce the supernatant into the artificial feeding test since the diet was maintained as sterile. While the chlorophyll fluorescence measurement in the treated plant leaf experiments showed better trends, it was not significantly affected. Conversely, introducing the spore suspensions via seed treatment into sterile soil, *T. aestivum* seedlings showed significant higher chlorophyll fluorescence compared to the control (water treatment). In order to understand all the observations noted here and the established induced resistance against *A. fabae* and *R. padi*, the discussion will focus on the following points:

- The patterns of resistance to *A. fabae* and *R. padi* following the treatment with supernatants of B. subtilis strains, FZB24, FZB37 and FZB38
- The feeding habits of the aphids
- The role of amino acids in aphid nutrition and resistance patterns
- The chlorophyll fluorescence measurement and positive trend due to *B. subtilis* and its metabolites
- Unspecific character of the inducer
- The future of induced resistance in aphid control

4.1. The patterns of resistance in different host—parasite systems

Vicia faba - Uromyces appendiculatus

Before testing *B. subtilis* and its metabolites against aphids, it was important to establish the effect of our strains against a biotrophic pathogen. The use of *Uromyces appendiculatus* here confirmed that the culture filtrate and supernatant of *B. subtilis* strains FZB24, FZB37, and FZB38 can reduce the development of urediospores. Other researchers have already reported that *B. subtilis* and its metabolites play such a role. The culture supernatant and other metabolites of *B. subtilis* have been found to have a higher inhibitory effect on the number of *Uromyces phaseoli* pustules of more than 90% (Baker et al., 1983; Mizubuti et al., 1995), as well as on *Uromyces appendiculatus* (Baker et al., 1985; Bettiol et al., 1992). Although the mode of action is not completely understood it is suggested that an inhibitory metabolite or a toxic substance that suppressed conidial germination is involved. Since our experiment showed that systemic treatment with culture filtrate and supernatants of *B. subtilis* strains FZB24, FZB37, and FZB38 is effective in reducing urediospores of *Uromyces appendiculatus*, one can assuredly state that the inhibitory effect is mediated *via* the plant system itself. Any toxic effect due to direct contact between the urediospores themselves and the bacteria metabolites can be excluded. The effect can be explained by a physiological change in the plant sap that prevents *Uromyces appendiculatus* from developing normally. As we did not further investigate the causes of this inhibitory effect, we have to rely on existing research, which stipulates that, despite the differences in food uptake between *Uromyces appendiculatus*, a biotrophic fungus, and aphids, that is, phloem sucking insects, both require the same form of nutrient, based on the mobilisation of amino acids on their host plants.

Vicia faba – Aphis fabae* and *Triticum aestivum- Rhopalosiphum padi

Aphids are economically significant pests of arable plants. Of approximately 4,000 species and closely related genera in the world, about 250 are serious pests. Aphids feed by sucking plant sap which results in direct plant damage by reducing the plant's resources (Dreyer and Campbell, 1987); they can cause disease in the plant by disrupting the tissues, injecting toxins, and by transmitting viral diseases (Dixon, 1987; 1978; Pickett et al., 1992). It is estimated that 60 % of all plant viruses are spread by aphids (Schwarz, 1985). Unlike many other insects, aphids can reproduce without mating (parthenogenesis) and, given their high reproductive rates and short maturation times, explosive increases in aphid populations can occur in very short periods (Dixon, 1987; 1978). The above-mentioned characteristics demonstrate that the aphid is a serious pest and a limiting factor in crop production. Today most aphid control methods depend heavily upon the use of chemicals and, despite early success stories, numerous aphid species have developed resistance to aphicides (Devonshire, 1989). Thus looking for a new approach to aphid control is of great interest to agriculture.

The experiments discussed here were able to establish induced resistance to *A. fabae* and *R. padi* in greenhouse conditions. In both studied insects, *A. fabae* and *R. padi*, the characteristics of the resistance were similar. Aphids feeding on supernatant of *B. subtilis* strains FZB24, FZB37 and FZB38 induced plants had longer development times (t_D) and pre-reproduction (t_d) times, with accordingly lower relative growth rates (RGR) and intrinsic rates of natural increase (r_m). This kind of resistance to aphids, when growth and development of the insect are negatively affected, is usually termed as antibiosis. It has been reported by other researchers as well as utilized in aphid population dynamic study. Birch and Holt (1984), studying the sources of resistance to aphids in *Faba* bean and its wild relatives, found that longer aphid development times and lower reproductive rates were the key factors involved in antibiosis. The maximum

possible rate of population increase that occurs when there are no biotic restraints on the aphids is the intrinsic rate of natural increase (Birch, 1948). Large individuals of *A. fabae* are reported to be more fecund and have a higher reproductive rate than small individuals (Dixon and Wratten, 1971; Taylor, 1975; Dixon and Dharma, 1980a) and therefore those factors that affect growth and development and thus govern adult size also primarily determine reproductive rate and fecundity (Dixon and Dharma, 1980b).

Our research found that the supernatant of *B. subtilis* strain FZB24, FZB37 and FZB38 limit the increase of *A. fabae* and *R. padi,* whose performance was affected when feeding on pre-treated host plants.

The description of antibiosis correlates with our observations of the performance of *A. fabae* and *R. padi* following the pre-treatment of their host plants with supernatants of *B. subtilis* strains FZB24, FZB37 and FZB38. As defined by Painter (1951), antibiosis takes place when a plant becomes resistant by exerting an adverse influence on the growth and survival of the insect. These effects are characterized by the reduced survival of the insects or reduced weight and size with longer time taken to complete the lifecycle, as well as a reduced growth rate and reduced fecundity. This type of resistance is different from antixenosis or nonpreference in which a host plant displays a degree of resistance by exerting an adverse effect on the insect's behavior (Beck, 1965).

Beck (1965) points out that a distinction must be drawn between resistance to feeding and resistance that acts by interfering with the physiological processes underlying growth, metamorphosis, and reproduction, since such physiological effects may be caused by metabolic inhibitors in the plant tissues, or by the plant's failure to provide either specific nutrients or the nutrient balance required by the insect. Additionally, these two types of resistance, "nonpreference" and "antibiosis" can coexist since they are above all empirical values. We cannot easily distinguish between these two values, as we did not investigate the

feeding behavior of the aphids with the use of electronic monitoring test methods. Investigating the role of hydroxamic acids in resistance to *R. padi* on cereal, Givovich et al. (1992) reported that a high mesophyll concentration of hydroxamic acids may provide a level of feeding deterrence during stylet penetration, while a phloem sap concentration may also provide deterrence combined with antibiosis. The causes of resistance to aphids can be multiple and each aspect of the aphid's relation to its host plant when feeding on plants with induced resistance is of interest. Electronic monitoring of aphid feeding has been mostly used to investigate host plant resistance or suitability (Adams et al., 1982; Haniotakis et al., 1978; Kennedy et al., 1978; Montllor et al., 1990; Shanks and Chase, 1976). However, as quoted by Montllor (1991), behavior does not necessarily differ between good and poor hosts, though some correlation between behavioural characteristics and performance parameters might be expected.

While the supernatants of *B. subtilis* strains FZB24, FZB37, and FZB38 were able to induce resistance against *A. fabae* and *R. padi* (Tables 11, 12, 13, 14), the use of other metabolites as culture filtrate, vegetative cells and spore suspensions of the same bacteria and strains presented different results.

Indeed, induced resistance after foliar treatment with culture filtrate, spore suspensions and vegetative cells of *B. subtilis* was not possible (Tables 5, 6, 7, 8, 9, 10, 15, 16, 17, 18). On the basis of the life table of *A. fabae* and *R. padi*, the growth parameters seem to be negatively affected in some cases; however this was not statistically confirmed. Available research has confirmed the inhibitive effect on plant pathogens from *B. subtilis* culture filtrate. Doley (1998) reported an increased of β-1, 3 glucanase activity in tomato seedlings treated with culture filtrate at the logarithmic and transition phases of *B. subtilis* strain (FZB24). While β-1, 3 glucanase was cited in induced resistance cases, this was not verified in our research. The introduction of culture filtrate in the artificial diet also did not show any influence on the relative growth rate of *A. fabae*. The

results observed here show that there is a qualitative difference between the supernatant and the culture filtrate of *B. subtilis*.

B50 culture filtrate of another strain of *B. subtilis* also could not induce resistance to *A. fabae* in our experiment (Tables 5, 6). This culture filtrate was, however, found to induce resistance in wheat plants against *Sitobion avenae* but not against *R. padi* (Galler et al., 1998). An explanation could be the qualitative differences between the culture filtrates of these two different strains of *B. subtilis*. It has been demonstrated that the antagonistic qualities of *B. subtilis* are strain dependant (Schönbeck et al., 1971; Broadbent et al., 1971; Swinburne et al., 1975; Krezel and Leszczynska, 1978; Utkhede, 1984; Tschen and Kuo, 1985; Huber et al., 1987; Hiraoka et al., 1992; Orihara et al., 1995; Asaka and Shoda, 1996).

4.2. The feeding habits of the aphids

Aphids as sap suckers are known to be highly sensitive to changes in the plant hormonal balance. Chatters and Schlehuber (1951) observed that the cell walls of the phloem cells and their contents were stained differently in those on which aphids were feeding, than in control tissues, which suggests that changes occur in the chemical composition of infested tissue. Aphids' distribution between and on the host plants is largely determined by variations in the quality of phloem (Dixon, 1985), and aphids that reach the phloem of resistant cultivars of their host plant tend to cease feeding shortly after the phloem is penetrated (Nielson and Don, 1974). While it is mentioned that some aphid species may prefer feeding on mesophyll tissue (Lowe, 1967), the primary food of aphids remains the phloem sap (Montllor, 1991). The process of host selection by aphids is characterized by a series of steps, involving a range of cues. Klingauf (1987) divided this process of host selection into the following: attraction, testing of the plant surface, penetration and testing the phloem.

The phloem long-distance translocation system of plants appears to function both as a nutrient delivery system and as an information superhighway (Zimmermann, 1960; Eschrich and Heyser, 1975). The central role of the phloem in the translocation of nutrients has long been recognized. Aphids feeding on its host plant are understood to secrete substances into plants that rapidly induce changes in growth and translocation. Many of these changes in plant metabolism are to the aphid's advantage (Dixon, 1975). Wittmann (1995) observed that, following *R. padi* infestation, the saccharose and glutamic acid content of wheat seedling leaves were found to be reduced. He concluded that the reduction of amino acids concentration in infested wheat seedlings was mainly due to the feeding habits of *R. padi,* in which a pathological sink was established in its host plant. Other researchers have come to the same conclusion on the question of aphid feeding. Thus Hawkins et al. (1987) pointed out that *Acyrthosiphon pisum* and *Aphis craccivora* damage to legume plants can independently induce a new sink-source relation to their advantage. We believe the role of the supernatants of *B. subtilis* strains (FZB24, FZB37 and FZB38), used in research as induced resistance, has been to react against such manipulation and preserve the plant integrity. Correspondingly, it was possible to show that *A. fabae* and *R. padi* could not use *V. fabae* and *T. aestivum* respectively to their advantage since the growth parameters of these aphids were largely influenced by the treatments with supernatants of *B. subtilis*. Even though behavioural studies were not conducted in this research to evaluate further resistance mechanisms of supernatant-induced plants to aphids, we would presume that such tests would demonstrate variation between treated and untreated plants. An electronic monitoring technique used by Dreyer et al. (1987) to measure aphid probing showed that initially, the length of probing time required to reach the phloem on an aphid-resistant line is longer than as on a corresponding susceptible line. Galler (2001) made the same observation, reporting that *S. avenae* feeding on B50 induced plants needed a longer period of time to reach the phloem compared to S. avenae from non-induced plants.

Following foliar treatment with supernatant of *B. subtilis* FZB37, the chlorophyll fluorescence in both plant systems demonstrated a better yield (Tables 24, 26). The fresh weight and dry weight of aphids were reduced; this, however, was found to be statistically significant for *A. fabae* but not for *R. padi* (Tables 25, 27). One explanation could be the small number of replications used in the experiments.

The seed pre-treatment with spore suspensions of *B. subtilis* strain FZB37 showed a positive trend with a better chlorophyll-fluorescence level measured in the plant leaves and a diminished fresh weight and dry weight of *A. fabae* and *R. padi* feeding on respective host plants (Tables 28, 29, 30, 31).

4.3. The role of amino acids in aphid nutrition

Total concentration of free amino acids in phloem sap

The increase in the total concentration of free amino acids in supernatant and culture filtrate treated plants was not statistically verified (Figures 23, 24) pre- and post-infestation with *A. fabae*. The higher level of amino acids found in *V. faba* seedlings (Figure 23) following the supernatant treatment could be attributed to the growth-stimulating qualities of the inducers used in this research on the plants. It has frequently been observed that the total concentration of free amino acids in the plant does not necessarily correlate with resistance to aphids. Sandström and Pettersson (1994), studying the resistance of *Acyrthosiphon pisum* on pea genotypes, concluded that the total concentration of amino acids in the phloem sap was not responsible for the differences in the performance of the aphid. The higher level of total concentration of free amino acids in the treated plants cannot influence the change in individual amino acids since this variation was noticeably significant once the *A. fabae* started to feed. The individual variation of amino acids here observed could be explained by the feeding behaviour of *A. fabae*, which acts as a sink in mobilising the nutrient from plant source organ (Figures 25, 26).

Concentration of individual amino acids in phloem sap

Several researchers have studied the amino acids required for general insect and particularly aphid growth. Many different amino acids, including arginine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, thrytophan and valine, have been referred to as essential for insects (Dadd, 1985). *Acyrtosiphon pisum* requires the presence of arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine for growth (Retnakaran and Beck, 1968) and Turner showed that *Aphis gossypii* required sulphur containing amino acids and cysteine. Dadd and Krieger (1968) in their basal diet for *Myzus persicae* mentioned the doubtlessly important role of serine for growth. They substituted eight of the amino acids included in standard diet with large amounts of serine or alanine and concluded that, in the case of alanine, the lowest level provided the best growth, whereas with serine, double concentration was optimal and enabled aphids to achieve weights only slightly inferior to those achieved on the standard diet. This obviously speaks for the importance of serine in aphids' nutrition. Essentially eight amino acids are considered as important for the development of *A. fabae* when reared on synthetic diets (Leckstein and Llewellyn, 1973). Thus the two authors found that after the individual omission of alanine, histidine, methionine, proline or serine, *A. fabae* diet intake was lower than that of the complete diet. The omission of cysteine, phenylalaline, or tyrosine failed to reduce diet intake. Other researchers have categorized the amino acids involved in aphids feeding. Weibull (1987) observed that the variation in amino acids composition alone was sufficient to create separate levels of resistance, expressed in as differences in population growth between cultivars.

In *A. fabae* treatments with supernatant and culture filtrate of *B. subtilis* strain FZB37 the concentration of individual phloem amino acids of *V. faba* changed, as indicated in Figure 25. Among the essential amino acids for *A. fabae* to which we have referred, the serine concentration in supernatant treatment, which does

not vary when comparing Figures 25 and 26, was found to be significantly lower when compared to the control (water treatment) and the culture filtrate of *B. subtilis* strain FZB37.

The fact that the concentration of serine did not change in supernatant FZB37 treated plants could suggest that the integrity of this amino acid was preserved in the phloem sap following the treatment. As reported by Ryan (1990) most animals require proteolysis to degrade and use the component amino acids of the proteins they consume. The unchanged concentration of serine in FZB37 sup induced plants in our study could be interpreted as a failure of the serine protease produced by *A. fabae* to degrade the plant protein and liberate enough serine for the aphids' growth, development and reproduction. Wolfson and Murdock (1990) discuss the importance of serine in insect nutrition, finding that insects mainly use one or a combination of serine, cysteine and aspartic acid as major digestive proteolytic enzymes. Furthermore, it is said that inhibitors of these enzymes are produced by plants, and presumably modulate the growth and development of pests by attenuating protein degradation (Koiwa et al., 1997). At the same time aphids are said to be able to replace the missing amino acids in its diet supply, probably via the intracellular symbionts (Srivastava et al., 1984). Indeed, omission of the usual ten essential amino acids from the diets of larvae of *M. persicae,* derived from mothers treated with antibiotics, resulted in a considerable reduction in growth (Mittler, 1971). It is even suggested that aphid symbionts may synthesize the missing amino acids. While our research did not quantify to what extent serine was supplied to the *A. fabae,* it did demonstrate that this aphid did not overcome the negative effects linked to the host plant pre-treated with supernatant of *B. subtilis* strain FZB37.

Proteinase inhibitors (PIs) have often been associated with resistance against pathogens (Peng and Black, 1976; Roby et al., 1987) and herbivores (Urwin et al., 1995; Johnson et al., 1989) in relevant literature. In experiments attempting to identify new resistance compounds against aphids, PIs are cited as potential

and interesting materials. Five plant proteinases were tested for the ability to control *Diuraphis noxia*, *Schizaphis graminum*, and *R. padi*. Thus it was found that though the effectiveness of the inhibitors varied, the proteinase inhibitors from potato were potentially effective proteins for the control of these species (Tran et al., 1997). More specifically, serine proteinase inhibitors were cited as the main factor in inducible resistance of tomato foliage to lepidopteran larvae (Johnson et al., 1995; Stout et al., 1996). Proteinase inhibitors were also said to be detrimental to the growth and development of insects from a variety of genera including *Heliothis*, *Spodoptera*, *Diabiotica* and *Tribolium*. A body of research has cited serine proteinase inhibitors as one of the inducible defensive mechanisms that plants have evolved to inhibit the digestive enzymes of piercing-sucking insects and microorganisms (Green and Ryan, 1972; Ryan, 1978; Hilder et al., 1987; Johnson et al., 1989).

Although our research did not investigate the phloem sap for PI proteins, it is tempting to speculate that serine proteinase inhibitors could be found in supernatant FZB37 induced plants as the concentration of serine has remained the same in supernatant FZB37 induced plants as shown in Figure 24.

In the interaction plant-insect, two main strategies seem to be involved. On one hand, the plant has to evolve mechanisms to protect the operational integrity of the phloem, which contains the flow of nutrients and information necessary for further growth and development, and on the other, the insect looks for the same nutrient through proteolysis, using enzyme proteases, trying to mobilise amino acids for its growth, development, and reproduction. In this competition for the nutrient, the inducer functions as a group of signalling molecules, capable of activating the expression of genes, which can help the plant to maintain or even boost its defense integrity. Piercing-sucking insects, whose primary nutrition is gained through uncontrolled access to the phloem sap, pose a dangerous challenge to the integrity of the phloem system. It is interesting to note that numerous plant PIs have been demonstrated to modify plant-arthropod

interactions, *via* their role as digestibility reducers, toxins, or modifiers of feeding behavior (Broadway et al., 1986; Duffey and Stout, 1996). The applicability of PIs in the defense response of plants to piercing-sucking insects remains questionable though, since these insects appear to lack endoproteinase activity within their digestive tract (Rhabé et al., 1995). However researchers have reported the presence of proteases in aphid alimentary tract and saliva (Bramstedt, 1948; Srivastava and Auclair, 1963; von Dehn, 1961; Klingauf, 1987). Van Emden (1966) studied the factors of resistance in plants to *Myzus persicae* and concluded that this aphid benefits in particular from the products of proteolysis in the plant. Many researchers have attached importance to the availability of amino acids to the aphids' feeding site since this determines growth and reproduction.

Poehling and Morvan (1984) and Poehling and Doerfer (1984) reported that only small amounts of amides and amino acids are likely to be translocated from other tissues to the feeding sites of aphid colonies and that this increase is mainly the result of synthesis and/or lysis of leaf proteins. This observation supports our findings that the infestation of *A. fabae* introduced a change in the concentration of individual amino acids (Figure 23). The remarkable aspect of our results is that the concentration of serine in supernatant of *B. subtilis* strain FZB37 induced plants remained the same prior to and post *A. fabae* infestation. Other individual amino acids, besides serine, may have played a role in the observed antibiosis effect but research has not yet been conducted to determine to what extent this may be the case. Other individual amino acids whose concentrations changed after supernatant treatment compared to the control (water treatment) were cysteine, alanine, tyrosine, and leucine.

Weismann and Halanda (1968) attributed the development of *A. fabae* on spindle, *Euonymus europaeus*, to the presence in high concentration of asparagine and arginine, which accords with our findings. The asparagine level in our research was very low after supernatant treatment, when compared to culture filtrate and control treatments. The higher concentration of alanine,

reported as phagostimulant, observed here in the supernatant of *B. subtilis* strain FZB37 treatment, when compared to the control and culture filtrate of *B. subtilis* strain FZB37 treatments, could not have overturned the antibiosis effect inflicted by the inducer to *A. fabae*.

4.4. Chlorophyll fluorescence measurement and aphids' fresh weight and dry weight assessment

Foliar treatment

Chlorophyll fluorescence is very useful in the study of the effects of biotic and abiotic stress on plants since photosynthesis is often reduced in plants experiencing adverse conditions, such as water deficit, temperature, nutrient deficiency, polluting agents, attack by pathogens and insects (Fracheboud, 1999). In our study the chlorophyll fluorescence yield in treated *V. faba* and *T. aestivum* plants with supernatant of *B. subtilis* strain FZB37 and infested with *A. fabae* and *R. padi* respectively, was found to be higher, though not statiscally significant (Tables 24, 26), compared to the control (water treated plants). The reduced aphids' fresh weight and dry weight (Tables 25, 27) in supernatant treatments could be an indication that the tested insects' biology has been affected as statistically verified in the case of *A. fabae* treatment (Table 25).

Aphid feeding is often associated with a creation of a pathological sink, which concurs with the plant natural sink and reduces infected plants' photosynthesis capacity to a low level. In our study, after six days of sucking activity, *A. fabae* and *R. padi* feeding on plants treated with *B. subtilis* metabolites failed to fully reorganize their host plants' nutrient for their own nutritional needs. Wang et al. (2004) who used photosynthetic-rate measurement to assess resistance of wheat plants through aphid and plant biomass analysis reported that the biomass of *D. noxia* feeding on susceptible wheat plant was significantly higher than the one of *D. noxia* from resistant wheat lines. Similar observation was

made in our research. Due to the small number of replications used in the experiments, it was not possible to confirm this result statistically.

Seed treatment

Seed pre-treatment with spore suspensions showed a higher and significant chlorophyll-fluorescence yield *of T. aestivum* seedlings infested with *R. padi* (Table 30). Fresh weight and dry weight (Table 31) of *R. padi* caged on treated plants were diminished compared to the control plants (water treatment). The biology of the *R. padi* was negatively affected as it has failed to establish a pathological sink detrimental to the plants pre-treated with spore suspensions of *B. subtilis*. The negative effect of aphid attacks on plants was reported by Mallott and Davy (1978), who stated that two weeks of sucking activity by *R. padi* led to a diminished vegetative growth of barley as well as a reduced photosynthesis capacity. In his research, Witmann observed that fresh mass of leaves from induced plants that were submitted to aphid attacks had higher weight compared to untreated plants. In field tests potato and cotton seeds pre-treated with *B. subtilis* spore suspensions have demonstrated better yields compared to control plants treated with conventional fertilizers (Yao and Bochow, 2002; 2003).

Relevant literature reports, in addition to the positive effect of better growth attributed to spores suspensions of *B. subtilis*, some cases of foliar disease and insect control via seed treatment with induced resistance agents. Field experiments in cucumber demonstrated that plants grown from seed treated with PGPR sustained significantly lower populations of cucumber beetles, *Diabrotica undecimpunctata howardi* and *Acalymma vittatum*, as well as a lowered incidence of bacterial wilt disease when compared with nontreated control plants and plants sprayed weekly with the insecticide esfenvalerate (Zehnder et al., 1997). The findings of these researchers also demonstrate that PGPR reduce the triterpenoid beetle-feeding stimulant cucurbitacin. The direct benefit of

PGPR to crops is manifold. Press et al. (1996) demonstrated the capacity of some genera of PGPR bacteria to induce systemic disease resistance in crops by producing salicylic acid. In our experiment, the higher chlorophyll-fluorescence level could be attributed to the PGPR qualities of *B. subtilis*, which have already been discussed in numerous other studies (Doley and Bochow, 1996).

4.5. Unspecific character of the inducer

Interestingly, it was found in our research that the supernatants of the three different strains of *B. subtilis* (FZB24, FZB37 and FZB38) used could induce resistance in both *V. faba* and *T. aestivum* to *A. fabae* and *R. padi* respectively. The inducer could also reduce the development of urediospores produced by *Uromyces appendiculatus.* This supports the hypothesis of the unspecific character of the inducer postulated in the introduction and concurs with the findings of others. B50-treated wheat and barley have been demonstrated to be resistant against *Erysiphe graminis* (Schönbeck et al., 1982; Kehlenbeck et al., 1994). Foliar jasmonic acid applied at concentrations not causing toxicity significantly reduced the performance *of Tetranychus pacificus* as well as the root-feeding grape phylloxera (*Viteus vitifoliae*) in grapevines (Omer et al., 2000). By inoculating tobacco leaves with tobacco mosaic virus, reproduction of *Myzus persicae* was reduced by 13 % and the growth rate of *Manduca sexta* was reduced by 27 % on the treated plants and resistance to viral, bacterial and pathogens was increased (McIntyre et al., 1980). In the same vein, Agrawal (1998) also reported that induced resistance observed by him was not species-specific and affected earwigs, aphids, grasshoppers, flea beetles, and lepidopteran larvae.

However a cross-protection function of the inducer is not always the case. Thus Galler et al. (1998) reported that B50-treated wheat is resistant against *Sitobion avenae* but not against *Rhopalosiphum padi*. Specificity in induced resistance

can be distinguished in two ways. Firstly, by a range of organisms being affected by a given response trigger by the inducer, or secondly, by the capacity of the induced plant to generate distinct chemical responses to different types of damage. If a given inducer possesses these two major qualities, it is very probable that this inducer's field of activity is larger. In our study our inducer demonstrated its capacity to challenge a biotrophic pathogen and two different species of aphids. Nevertheless, further studies are necessary to determine the type of secondary metabolites produced by the treated plants infested with the pathogens and pests, as plant response to pathogens or pests can be type-specific. Specificity elicitation showed that aphid feeding induced peroxidase and lipoygenase activities but not polyphenol oxidase (catechol oxidase) and proteinase inhibitor activities, as opposed to *Helicoverpa zea* whose feeding induces polyphenol oxidase and proteinase inhibitors but not those of peroxidase (Stout et al., 1998). Furthermore, Agrawal (2000) observed that specialist and generalist herbivores may also respond differently to induced plant responses and in some cases induced phytochemicals that inhibit feeding by some herbivores are feeding stimulants or toxins sequestered by specialists (Agrawal and Karban, 1999). Carroll and Hoffman (1980) here showed that induced cucurbitacins in *Cucurbita moschata* attracted some herbivores while inhibiting feeding by others. However, it is also emphasised in the literature that this interreaction between insect response and plant secondary compounds is complex. Hence it should be noted that although specialist herbivores may be attracted to plants with high levels of secondary compounds (Bowers, 1992), they may still be susceptible to the toxic effects of the phytochemicals (Adler et al., 1995). Thus the investigation of plants secondary metabolites is of great interest for better performance of *B. subtilis* metabolite induced plants.

4.6. The future of induced resistance in aphids' control

Attempts to reduce crop damage have included selective breeding for resistance, but often many different genes control resistance traits, making it difficult or even impossible to genetically select a desired attribute. Decreased crop yields are also commonly encountered in resistance strains. Accordingly, there exists a strong need for compositions and processes to improve the resistance of plants under attack by herbivores (Ryan, 1995). It would be of great interest to study qualitatively the supernatant and the culture filtrate of *B. subtilis* in order to identify the main substance(s) in the supernatant, responsible for the transduction of signals necessary for the activation of the plant defense system. Once such information is available, the supernatant can perform better and be used as a stable bioproduct. This constitutes an important step towards the production of the supernatant of *B. subtilis* as a commercially-available induced resistance agent.

5. Conclusion

The objectives of this thesis were to establish the possible role of *Bacillus subtilis* and its metabolites as an induced resistance agent against *Aphis fabae* and *R. padi*. Furthermore, it was our goal to unveil some mechanisms which sustain these induced resistance effects in aphid host plants. The general conclusions derived from this work can be summarized as follows:

Induced resistance to both *A. fabae* and *R. padi* was possible when using supernatants (FZB24, FZB37 and FZB38) of *B. subtilis* in topical treatment.

The supernatants and culture filtrate of *B. subtilis* (FZB24, FZB37 FZB38) were able to inhibit development of urediospores of *Uromyces appendiculatus* in both topical and systemic treatments.

Neither the culture filtrate, the spore suspensions nor the vegetative cells of *B. subtilis* strains, FZB24, FZB37 and FZB38 were able to significantly influence the growth parameters of *A. fabae* and *R. padi*.

B50 treatment was not effective in diminishing *A. fabae* growth parameters.

Landy-medium treatment also did not affect the growth parameters of *A. fabae* and *R. padi*. This suggests that the induced resistance effects obtained with supernatants of *B. subtilis* strains FZB24, FZB37 and FZB38 cannot at this stage be attributed to the growth medium of the bacteria.

No antibiosis effect against *A. fabae* and *R. padi* was observed in the acute toxicity test, where the supernatants of *B. subtilis* strains FZB24, FZB37 and FZB38 were used. When culture filtrates of the same strains of *B. subtilis* were added to artificial diet, the relative growth rate of *A. fabae* was also not significantly affected.

In our acute toxicity and artificial feeding tests, we concluded that the antibiosis effects on *A. fabae* and *R. padi* were not due to any contact effect, but mediated only via the plants pre-treated with supernatants of *B. subtilis* strains, FZB24, FZB 37 and FZB38.

Furthermore, in order to explain the mechanisms of induced resistance, we can conclude that the positive trend of the chlorophyll fluorescence yield in supernatants-induced plants constitutes evidence that change occurred in the plants due to treatments with *B. subtilis* and its metabolites, which had a further influence on the tested aphids' biomass. The biomass of *A. fabae* and *R. padi* feeding on plants treated with *B. subtilis* and its metabolites was reduced but was not found to be statistically significant, probably due to the small number of replications in the experiment.

B. subtilis spores suspensions introduced into sterile soil had a positive effect on chlorophyll fluorescence. The latter parameter has been found to be higher and statistically significant in *Triticum aestivum* seedlings after its seeds have been pre-treated with spore suspensions of *B. subtilis* strain FZB37. The fresh weight and dry weight of the *R. padi* feeding on such treated host plants were found to be negatively affected compared to the control treatment (water treated plant seed). Chlorophyll fluorescence was found also higher in *Vicia faba* seedlings treated in the same way as *T. aestivum,* but was not statistically significant.

The free amino acid in the phloem sap of *Vicia faba* plants was demonstrated in the investigation to have quantitatively and qualitatively changed.

We could not observe a significant change in the concentration of the so-called 10 essential amino acids in aphids' nutrition in our study.

The total concentration of the amino acid was higher in supernatant and culture filtrate of *B. subtilis* strain FZB37 treated plants compared to the control (water treatment).

The concentration changed qualitatively in nine of the individual amino acids, cysteine, serine, histidine, glycine, threonine, alanine, tyrosine, leucine and lysine. Of this group, serine was found not to have altered for supernatant treated plants, as opposed to the control and culture filtrate of *B. subtilis* strain FZB37 treatments.

Serine is presumed to play the key role in the antibiosis effect observed in our research, as this amino acid is understood to influence the growth and development of some insects, including aphids.

6. Future perspectives

In light of the results achieved by this investigation of *B. subtilis* and its metabolites as induced resistance agent to *Aphis fabae* and *Rhopalosiphum padi,* the following areas need to be explored:

To prove the role of serine in antibiosis mechanisms to the tested insects, a test should be conducted to attest the presence of secondary metabolites, especially proteinase inhibitors proteins in the phloem sap of the plant after it has been treated with the supernatants of *B. subtilis* strains FZB 24, FZB 37 and FZB 38. The PIs proteins belong to the inhibitors proteins family that is capable of inhibiting the proteinases production by aphids, in order to decompose the plant proteins and liberate enough serine for the insect nutrition.

Furthermore, it should be investigated if the PIs found in the supernatant induced plants kill or block development and reproduction of aphids when added to their diet.

Identifying the PIs involved in induced resistance could serve as a useful compound, leading to the development of chemical control strategies and may also provide a valuable tool in the identification of cellular receptors involved in initiating the resistance.

Furthermore, we could show in our research that *B. subtilis* growth was better in sterile soil substrate, thus this offers the opportunity to treat seed material with *B. subtilis* FZB 37 as well as spraying the plant leaves with a solution of supernatant. Hence this full-bacterialized plant could be tested against *A. fabae* and *R. padi* opening a new perspective for biological plant protection means.

7. Literature

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9. Declaration

I, the author, declare that the work in this thesis was carried out in accordance with the regulations of the Humboldt University of Berlin (Germany).

The work is original except where indicated by special reference in the text and the dissertation has not been presented to any other University for examination either in Germany or in other countries.

Vinaman Yao