Chapter 6:

Alternative Treatments

Section 1: Compost extracts: leaf bioassays

Section 2: Identification of fungal/bacterial antagonists/plant extracts

Section 3: Identification of antifungal metabolite production/compatibility: leaf assays

Section 4: Optimisation of application frequency/timing/dose rate of microbial/plant extracts

Section 5: Consolidated Report on alternative treatments

Chapter 6: Alternative Treatments

General Introduction

According to the survey in Chapter 2, more than half of the growers did not use copper-based fungicides. The main reasons were that it was not permitted or that it was not necessary but for many, they decided not to use them because they believed it was inconsistent with the principles of organic farming. The remainder did use copper-based fungicides at a range of doses and frequencies and some used alternative treatments. The former were reported to be generally more effective than the latter and for each period of 10 days that foliage life was extended, it was estimated that yield increased by about 5t/ha.

Despite the efficacy of copper-based fungicides, the development of alternative control treatments that are acceptable in organic cropping systems is central to the formulation of effective late blight management control strategies in the future. Fully effective, robust and safe alternatives that can reduce or eliminate the need for copper-based fungicides whilst maintaining crop yields and economic performance would alleviate current problems and expedite the phasing out of the use of copper compounds.

Compost extracts have previously been shown to suppress late blight development when applied to potato leaves. Their exact mode of action has not been determined but direct antifungal, resistance inducing and plant strengthening activities have all been suggested to contribute to the suppressive effects and there may be some beneficial nutrition effects as 'foliar feeds'. Compost extracts can be made on farm and if they gave reliable control would decrease dependency on external inputs such as commercial biological control and plant extract based products. However, little is known about the efficacy of compost extracts made from different composting feedstocks available on organic farms and the influence of the stage of the composting process when the extract is made.

Fungal and bacterial antagonists and plant extracts have been developed over the last 10 years and an increasing number of biological control products for disease control have become available for commercial use. Several of these products and many other antagonists and plant extracts currently under development have proven activity against oomycete fungi which are closely related to *P. infestans*. However, their potential for late blight control has not been determined and these, together with bacterial and fungal strains newly isolated from potato leaves need to be screened. The production of antibiotics or other antifungal metabolites has been the most frequently identified mode of action of antagonistic micro-organisms and plant extracts. Compared with other modes of action, such activity is less desirable, because it is more likely to break down due to resistance development by the pathogen and have side-effects such as toxicity to non-target organisms. Compatibility issues are also involved. For example, sources with antifungal activity may be incompatible with certain (in particular fungal) antagonists in mixed antagonist or plant extract/antagonist preparations. This could reduce or eliminate one or more of the beneficial mechanisms (e.g. competition for phyllosphere nutrients, induction of resistance and hyperparasitism) provided by mixtures compared with a single ingredient.

Application frequency, timing and dose rate of microbial and plant extract based biocontrol treatments have important effects on their efficacy with optimum biocontrol activity at specific concentrations with supra- or sub-optimal concentrations resulting in reduced activity. Application rates and frequencies giving maximum control of blight may also vary between alternative treatments with different modes of action. Furthermore, the persistency of foliar-applied active compounds (chemical fungicides and anatagonists) on leaves varies considerably depending for example on their rainfastness and resistance to breakdown under ultra-violet light.

Section 1: Compost extracts: leaf bioassays

Summary

In Denmark, in detached leaf bioassays all the three types of compost extracts: a mixture of horse manure and field soil in a 2:1 w/w ratio; a mixture of straw and cattle slurry and cattle deep litter controlled potato late blight to varying extents. The efficacies of the extracts were highest when prepared at 52 days after compost initiation and were not significantly different from the copper fungicide control. Extracts made later during the composting period were less effective but sterilisation of the composts at 80 days of age by autoclaving significantly improved the efficacy of one extract indicating that the control was achieved by a chemical rather than biological mechanism.
In the United Kingdom there was no convincing evidence based on results from laboratory based leaf-biossays that extracts made from different compost feedstocks and of different ages suppressed leaflet infection with blight. Observed effects were much smaller and inconsistent than where copper oxychloride was used.
The differences observed between the two sets of experiments – a measure of success in Denmark and disappointing results in the UK demonstrates that successful use of compost extracts as blight control agents is elusive and requires further work to identify those with greatest activity, ensure that these extracts can be prepared consistently and applied at correct timing and frequency and to ensure reproducibility of effects.

Introduction

Compost extracts (also described in the literature as compost teas) have been suggested as an effective method for the control of fungal diseases. Most of the recent work on compost extract development has been carried out in the USA. However, few results have been published in peer reviewed Journals so far. Whilst there is a lack of peer-reviewed published data on the efficacy of compost extracts against late blight in potato, presentations by proponents of compost extracts always stress the importance of using specific organic waste raw materials, standardised composting processes e.g. the use of commercial compost turners and windrow covers and extraction times at specific points of the composting process e.g. immediately after the temperature has peaked in the windrow, when the concentrations of spore-forming bacteria, which are known to produce antifungal compounds are highest. Their exact mode of action has not been determined but direct antifungal, resistance inducing and plant strengthening activities have all been suggested to contribute to the suppressive effects and there may be some beneficial nutrition effects as 'foliar feeds'.

The first stage in the evaluation of compost extracts is to test the efficacy of samples made from composts that can be prepared on-farm from readily available manures and plant materials under standard conditions in the controlled environment of the laboratory on detached potato leaves i.e. bioassays. Promising candidates can then be evaluated under field conditions. The objective of the study reported here was therefore to quantify the activity of extracts of composts made from different raw materials and extracts made at different times during the composting process using an excised leaf bio-assay. Leaf bioassays were made in Denmark in 2001 and the United Kingdom in 2002 for a range of compost types and ages. (The work was delayed in the UK by restrictions associated with the Foot and Mouth Disease outbreak which occurred in 2001). Potentially useful treatments were subsequently tested under field conditions and the results of these experiments are described in Chapter 5: Agronomic Strategies – Foliar sprays and microbial soil inocula.

DENMARK (DK)

Materials and Methods

Preparation of composts

1. Horse manure and field soil (designated 4.1 and 4.2 representing replicates one and two respectively). 60 kg of horse manure were chopped and mixed with 30 kg of field soil from the plough layer of S7 of the Danish Institute of Agricultural Sciences' field station at Foulumgaard.

2. Straw and cattle slurry (designated 5.1 and 5.2 representing replicates one and two respectively): 10.5 kg of chopped spring barley straw were mixed with 21 kg of tap water and then mixed with 56 kg fresh cattle slurry.

3. Cattle deep litter (designated 6.1 and 6.2 representing replicates one and two respectively). 80 kg of cattle deep litter were chopped.

At the beginning of the compost process, samples were from taken from the composts for dry matter determinations and additional chemical analyses. About 70kg of the mixture of ingredients for each compost were placed in separate wooden composting boxes (one cubic metre capacity) on 21 August 2001and temperature sensors were placed in the middle of the composts. The experiment was replicated twice for each compost.

Process control

Temperature, CO_2 and O_2 were measured daily in the middle of the compost using test instruments from Sandberger (Austria). When the temperature exceeded 65 ^{o}C or the CO_2 exceeded 18 %, the compost box was emptied and the compost was replaced into the box again. When CO_2 decreased below 10 %, water was added. The first week after establishment the temperature increased rapidly and the composts were turned accordingly, daily or even twice daily.

Compost extraction

Samples of 6 litres of composts were mixed with tap water up to a total weight of 30 kg at 43, 57 and 81 days after initiation of the compost process. Following seven days of extraction at room temperature, the mixture was filtered through one layer of muslin. The activity of the filtrate was tested against *P. infestans* 48 hours using the detached leaf bioassay.

Detached leaf bioassay

One potato leaf from the variety Sava was placed in oasis in a small plastic box with wet filter paper on the bottom. Five replicates were used per treatment and the two controls (Copper-fungicide and water) were included at each stage of the compost process. The compost extract was applied to the leaf in a spraying cabinet with a spraying boom. After drying, a weak spore solution (100 sporangia/ml) was applied to the leaf with a mist sprayer. Afterwards, the boxes with the detached leaf were placed in a climate chamber (95% relative humidity) with a 12 hours light cycle. Leaves were scored for percent blight until non-treated controls showed 100% leaf blight.

Results

Table 6.1 Percent blight infection of detached potato leaves treated with three different compost extracts at 4, 9 and 5 days after inoculation with *Phytophthora infestans.* Copper-based fungicide was used as a control treatment. At day 80 after the set up of compost extracts, all three extracts were autoclaved to study the biological/chemical effects.

	Days in composting process		
	52	**66**	**80**
Treatment/days after infection	**4 days**	**9 days**	**5 days**
Untreated – uninoculated	0 b	- -	0 d
Untreated	27.0 a	24.4 a	21.4 a
Copper - fungicide	0.8 b	1.1 d	0.6 d
Compost 4.1	3.0 b	9.8 bcd	23.0 a
Compost 4.1 autoclaved	- -	- -	6.2 cd
Compost 4.2	2.2 b	14.4 b	11.0 a-d
Compost 5.1	1.8 b	10.4 bcd	19.0 ab
Compost 5.1 autoclaved	- -	- -	8.0 bcd
Compost 5.2	2.2 b	13.6 b	15.0 abc
Compost 6.1	5.8 b	4.0 cd	12.4 a-d
Compost 6.1 autoclaved	- -	- -	7.0 bcd
Compost 6.2	7.7 b	12.4 bc	5.7 cd
Lsd (P=0.05)	5.6	6.6	8.2

a) Figures with the same letters are not significantly different.

All the compost extracts gave a significant reduction in late blight development in the detached leaf bioassay compared to untreated leaves (Table 6.1). Extracts made 52 days after the initiation of the composting process were as effective as the copper fungicide. The efficacy of the compost extracts declined the later they were prepared in the composting process. The efficacy of the composts was improved by autoclaving at the last compost stage and this was a significant improvement for the compost made from a mixture of horse manure and soil (4.1 vs 4.4).

Conclusion

In the bioassays, all the compost extracts controlled potato late blight to an extent, but overall there were no significant differences between the three compost extracts. Autoclaving i.e. sterilising 80 day old compost did not decrease the efficacy of control of the extracts suggesting that the activity of the compost was chemically- based rather than biological. Compost 6 (cattle deep litter) was chosen to be the basis for the extracts for the tests under field conditions in 2002 and 2003, in both untreated and autoclaved forms and results are described in Chapter 5: Agronomic Strategies – Foliar sprays and microbial soil inocula.

UNITED KINGDOM (UK)

Materials and Methods

Composts were prepared using a Controlled Microbial Composting protocol based on windrows turned with a Sandberger compost turner (Sandberger GmbH, Austria) and application of Topex-covers to maintain uniform water content in the compost. Windrows were turned when CO_2 measurements within the windrow exceeded a specific threshold (approximately 3 times in week one; 2 times in week 2 and 3 and once in weeks 4, 5 and 6; on some occasions the compost was turned again in week 7 and/or 8).
Three types of compost including green-waste (UFAMS, Newcastle City Council), house-hold waste (UFAMS, Newcastle City Council) and composted cattle-manure (Nafferton Farm) were used. All compost extracts were made with a commercial extractor or 'compost tea' maker (*Growing Solutions Inc*., USA).
Extracts were made from composts of increasing ages from young/immature to old/mature. The ages were 2 weeks, 3 months and 12 months for the greenwaste and household-waste compost. For cattle manure, the ages were 2 weeks, one month and 4 months. For each of the composts, the ages of the extracts were designated as being in the early, middle and late stages of the composting process.
There were two separate experiments with identical protocols and in each experiment; two bioassays were performed for each treatment i.e. T1 and T2.

An established leaf bio-assay protocol was followed. Potatoes (variety Nicola) were grown in 15cm diameter pots filled with John Innes No. 3 compost (20 pots each week). Potato leaves were excised from the 4^{th} and 5^{th} youngest leaves at flowering (i.e. at about 6 weeks of age) and used in bio-assays. *Phytophthora* was cultured in Rye and used to prepare suspensions of sporangia for inoculation of the leaves. Leaves were then treated with different extracts (40 µl of compost extract per potato leaflet were applied either 24 hours before or 30 minutes after applying *Phytophthora* suspension (20 µl of 10^5 spores/ml)) and incubated under humidity and temperature conditions conducive for symptom development i.e. in clear plastic boxes on a metal mesh above a wet paper towel and incubated at 15-18°C temperature. The lesion diameters and percentage blight symptoms were assessed at regular 1-2 day intervals until 10 days after inoculation. Lesion development on leaves treated with compost extracts was assessed and compared to untreated leaves and leaves treated with copper fungicide (copper oxychloride) were used as controls.

Results and Discussion

In the first experiment, one month old cattle manure (i.e. 'middle') reduced disease development by about 30-40% compared with the other compost extracts (Figure 6.1); however, this observation was not confirmed in the second experiment (Fig. 6.2). In the second experiment, mature (12 months old or 'late') house-hold waste compost showed 30-45% reduction in late blight although no effect had been evident in the first experiment (Fig.6.2). The achievement of consistent effects is dependent upon producing extracts that are exactly the same from batch to batch from an individual compost feed-stock and this is a key challenge. Even when compost extracts decreased infection of leaves with blight, the effects were very much smaller than with copper oxychloride. Copper oxychloride decreased blight infection by over 80% in every bioassay (Figs. 6.1 and 6.2).

Fig. 6.1 Effect of compost type and extraction time on blight suppression in detached leaf bioassays (first experiment).

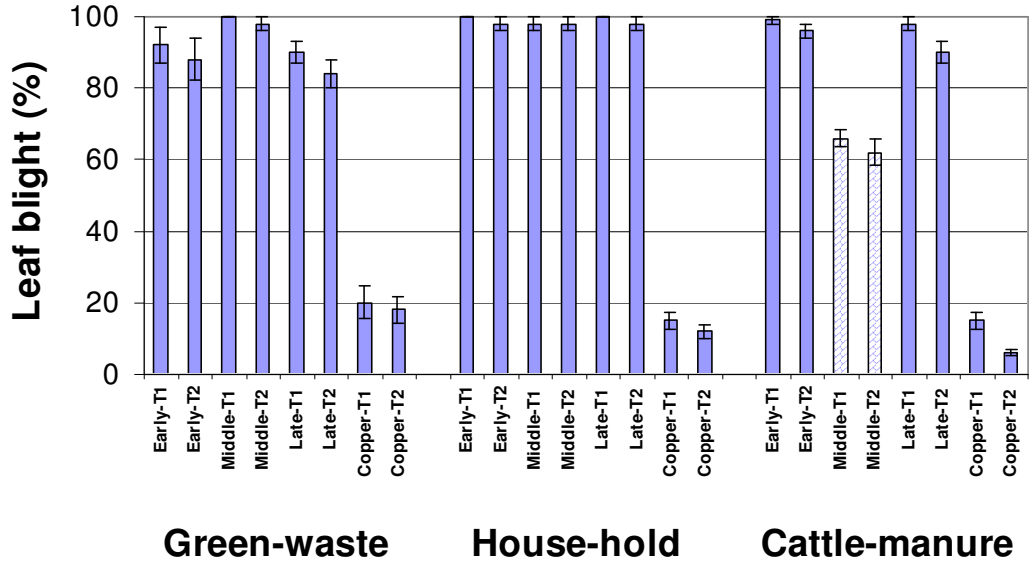
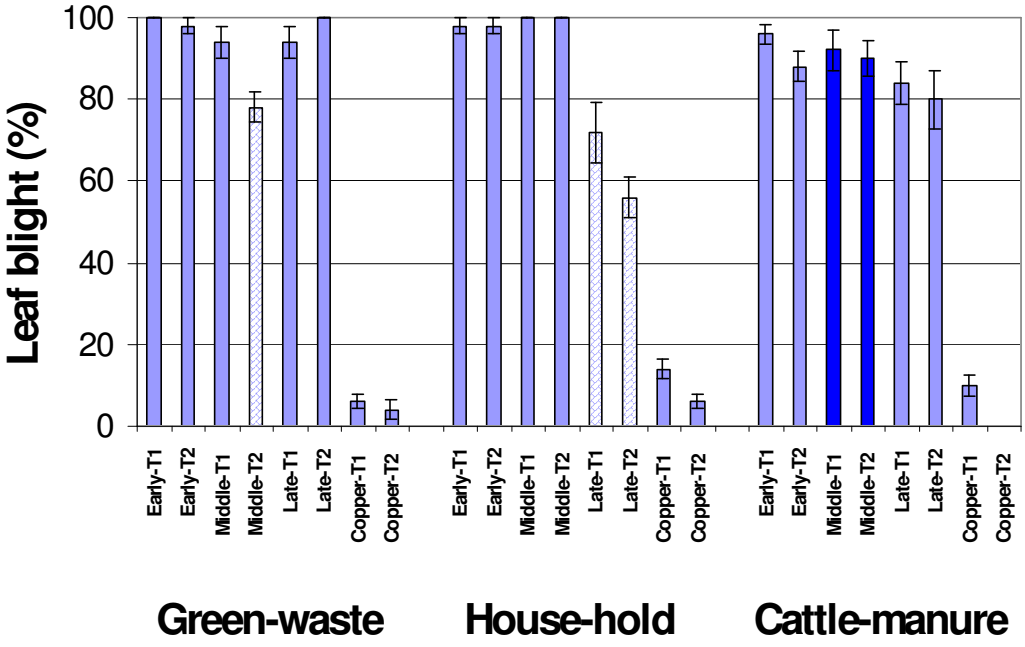


Fig. 6.2 Effect of compost type and extraction time on blight suppression in detached leaf bioassays (second experiment).



None of extracts made from compost (composts made from 3 different raw materials and extracted at different times of the composting process) gave any significant reduction in foliar lesion expansion in the excised leaf bio-assay (individual results not shown). These compost extracts therefore did not appear to have any direct antifungal activity against *Phytophthora infestans*. However, other modes of action may exist that cannot be detected in excised leaf assays. For example, if compost extracts act as elicitors for resistance mechanisms in plants, or improve resistance by supplying micro-nutrients, this may not be detected in excised leaves due to more rapid senescence and inefficient expression of resistance mechanisms in excised leaves. It was therefore decided not to proceed with testing the efficacy of compost extracts in bio-assays under different environmental conditions but to proceed to assess effects of compost extracts on foliar blight and yield under field conditions and the effect of different sprayer technologies on the activity of alternative treatments described in Chapter 5 Section 7: Foliar sprays and microbial soil inocula and Chapter 7: Section 1: Sprayer systems for Cu and novel products.

Section 2: Identification of fungal/bacterial antagonists/plant extracts

Summary

In experiments conducted at BBA (D), two bacterial micro-organisms showed significant effects against *P. infestans* on detached leaves: *Xenorhabdus bovienii* (two phase variants) and *Pseudomonas putida* (I-112) when applied close to the time of infection.
Several other micro-organisms, plant extracts and commercial products had no significant effect against potato late blight in excised leaf bioassays.

Five micro-organisms from potato leaves or from BBA's strain collection which had shown activity against *P. infestans* on detached leaves were taxonomically identified. Among these were three fungi, *Penicillium cyclopium*, *Cladosporium* spec. and *Cladosporium cladosporoides*, and two yeasts, *Bulleromyces albus* and *Debaromyces castellii*.

There are indications that the time period during which the tested copper based fungicides are able to protect potato leaves from infection is possibly dependent from the degree of the biological fitness of the zoospores used for inoculation.

Introduction

This set of experiments was aimed at the screening and identification of potential micro-organisms (MOs), plant extracts (PEs) and commercial products (CPs) showing activity against *Phytophthora infestans in vitro* as well as in detached leaf bioassays. A comprehensive set of MOs, PEs and CPs was tested and as initial *in vitro* experiments and tests on excised leaves had revealed activity of several MOs against *Phytophthora infestans*, identification of these isolates was intensified.

Materials & Methods

Screening of MOs, PEs and CPs by means of excised leaf bioassay

The leaf bioassay procedure was as follows: Potatoes of the cultivar Secura (highly susceptible to *P. infestans*) were cultured for three to four weeks at 17° C with a 16/8 h day/night rhythm. Four leaves of the 5th to 7th branch were collected and placed in 20x20x5cm plastic boxes onto steel wire mesh with soaked filter paper underneath. To record possible resistance inducing or protective effects, both surfaces of the leaves were sprayed with 2.5 ml of a solution of the substance or MO to be tested 24 hours before inoculation of the pathogen. For inoculation a 2.5 ml suspension containing 1x10^5 sporangia/ml was sprayed on the upper leaf side using a mist sprayer. For measuring direct and curative effects, first 2.5 ml of the *P. infestans* suspension (1x10^5 sporangia/ml) were sprayed on the upper side of the leaf. After 60-90 min. a suspension of 2.5 ml of the substance or MO per box was sprayed on the same leaf side.
If not stated otherwise three repetitions (separate experiments) with three replicates (boxes) per treatment were done. Boxes contained four potato leaves each and were incubated at 15° C at a day/night interval of 16/8h. The percentage of diseased leaf area was determined according to JAMES (1971) on the fifth, sixth and seventh day after inoculation with *P. infestans*. In one experiment examination of diseased leaf area was additionally recorded at the eighth and eleventh day after inoculation. From the obtained data the area under disease progress curve (AUDPC) was calculated. For the separation of means the AUDPC values were compared by the Tukey's or Duncan's studentized range test ($p > 0.05$).

The following treatments were tested:

i) microbial isolates (if not stated otherwise cultured in nutrient broth or on nutrient agar; origin of isolates in parentheses):
   Gö857, FZB Eb4 (Georg August University Göttingen, Institute of Plant Pathology and Plant Protection), Gh.B2 (College of Geisenheim), I-112 (*Pseudomonas putida*), II-16/1 (species not known), IV-298a (*Pseudomonas fluorescens*) (latter three: BBA Darmstadt), *Xenorhabdus bovienii* (two isolates/variants cultured on nutrient agar or in TSB, DSMZ, Braunschweig).
   Bacteria were cultured for 7 days (*Xenorhabdus bovienii* for 4 days) in Tryptic soy broth (TSB) and applied as whole culture.
ii) compost and plant extracts (applied concentration in parentheses):
   ready to use compost extracts from BBA and DIAS (partner 6) and two plant extract, *Sphagnum* (2%) and Duckweed (4%)

Plant extracts were prepared by heating de-ionised water containing Tween 80 (0.0125%) to 50 °C and pouring 100 ml over the respective amounts of plant powder. The mixture was stirred for 1 hour without further heating and was then filtered under vacuum. Extracts were used freshly.

iii) commercial products
   B. subtilis MBI600 (1 %), ComCat® (plant strengthener; 0.001 %), BioPlantol® (plant strengthener; 0.2 %), Kendal (plant strengthener; 0.5%)
   The commercial products were added to de-ionised water in the respective concentrations and dissolved by stirring the mixture.

In all experiments de-ionised water and a commercially available copper fungicide (Atempo®, Neudorff, 45 % copper as copper oxychloride) served as controls.

Culturing of *Phytophthora infestans*: *P. infestans* was cultured for about 10-14 days at 15 °C on petri dishes on rye agar containing 0.005% β-Sitosterol and maintained by transferring mycelial plugs from the outer area of the colony head-over on fresh rye agar plates. Fungal

sporangia were washed from the petri dishes with 0.0125 % Tween 80 and adjusted to give a concentration of $1x10^5$ per ml. Before using the suspension for inoculation, it was incubated at 4-5 °C until the zoospores were released to at least 50%.

In vitro testing of different formulations of plant strengthener Kendal®

To test the effect of plant strengthener Kendal® on the growth of *P. infestans* mycelium the product was (after autoclaving) added to rye agar (containing 0.005 % β-Sitosterol) in appropriate amounts to give final concentrations between 0.01 and 1 %. 20 ml each of the mixtures were poured into petri dishes (diameter 85 mm) and after solidification of the medium agar plugs (diameter 7 mm) with *P. infestans* were put upside down in the centre of the petri dishes. After incubation of plates at 15° C in the dark the diameter of the developing mycelium was measured in two perpendicular directions after different time intervals. Plates containing no Kendal® served as control. The experiment was repeated twice with 6 replicates (plates) per repetition for each concentration.

Identification of micro-organisms

Based on previous results further micro-organisms were identified. Fungi were identified by Dr. Helga Nierenberg (BBA Berlin, Institute of Plant Virology, Microbiology and Biological Safety) by morphological determination after cultivation of respective organisms on selective media.
The yeasts were identified by use of the Biolog® technology, a colorimetric assay based on patented Carbon-source "fingerprints": the organism to be identified is cultured on Biolog® universal growth agar medium and then introduced to a variety of preselected carbon sources in a microplate. After incubation the optical density at a defined wavelength is read in a microplate photometer. Results are recorded, saved and automatically compared with memorized fingerprints of a database to select the species with identical or at least very similar fingerprint patterns.

Results & Discussion

Screening of MOs, PEs and CPs by means of excised leaf bioassay

All tested micro-organisms, obtained from other Plant Pathology Units or selected from BBA's strain collection, reduced the infestation with *P. infestans* to some degree in the detached leaf bioassay (Fig. 1.1). But only the *Pseudomonas putida*-isolate I-112 showed a significant effect against *Phytophthora infestans* when applied one hour after inoculation of the pathogen (Fig. 1.1).

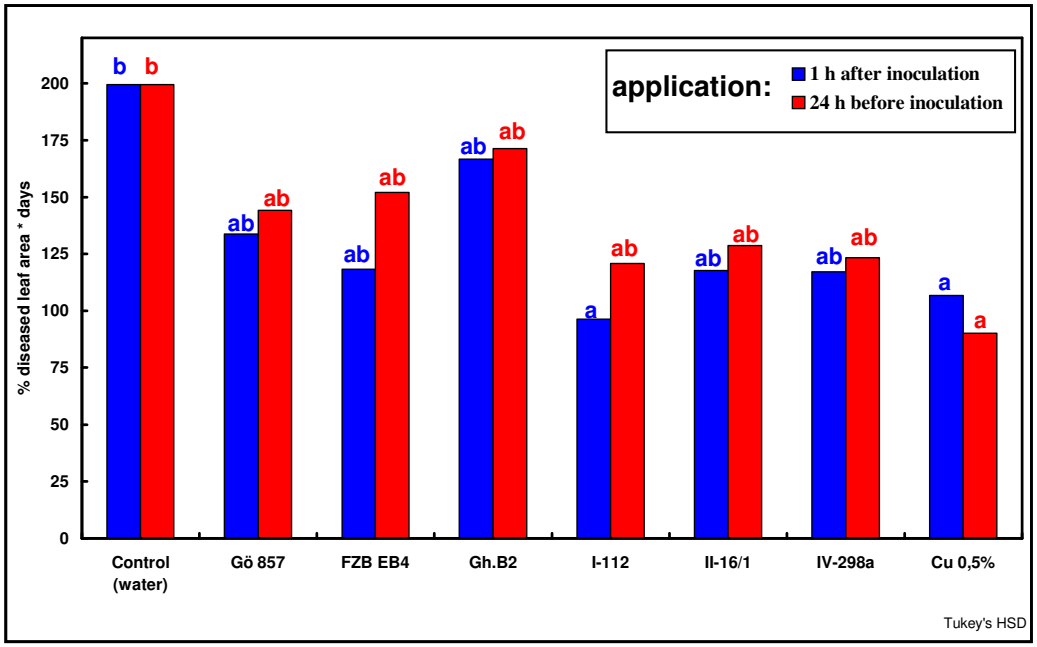


Fig. 1.1: Effectiveness of several bacterial isolates against *P. infestans* on excised potato leaves, expressed as values of the area under disease progress curve (AUDPC). AUDPCs were calculated from examination results from the 5th, 6th and 7th day after inoculation.

Pseudomonas putida is obviously a promising bacterium to control *P. infestans*, as I-112 was - besides isolates 7.1.7 and 1.1.31 (see also section 3 of this Chapter) - the third *P. putida* isolate showing a significant effect against *P. infestans*, especially when applied close to infection. DAAYF et al. (2003) also found that *Pseudomonas putida* was one of the best micro-organisms to control late blight of potato.
Isolate I-112 and additionally isolate IV-298a (*Pseudomonas fluorescens*) which also showed a certain (but not significant) effect against late blight were included in experiments for WP 5.4.

The two most promising micro-organisms tested early in the programme were two isolates of *Xenorhabdus bovienii*, obtained from Dr. Wohlleben, BBA Braunschweig, Institute for Plant Protection in Agriculture and Grassland. Originally the isolates derived from the German Collection of Microorganisms and Cell Cultures (DSMZ). The effect of this bacterium in the detached leaf assay was as good as that of the chemical control (Fig. 1.2) and therefore it was decided to also include this micro-organism in tests conducted under the programme to identify 'Optimisation of application frequency, timing and dose rate of microbial and plant extract-based biocontrol treatments in glasshouse trials described in this Chapter 6: Section 4.

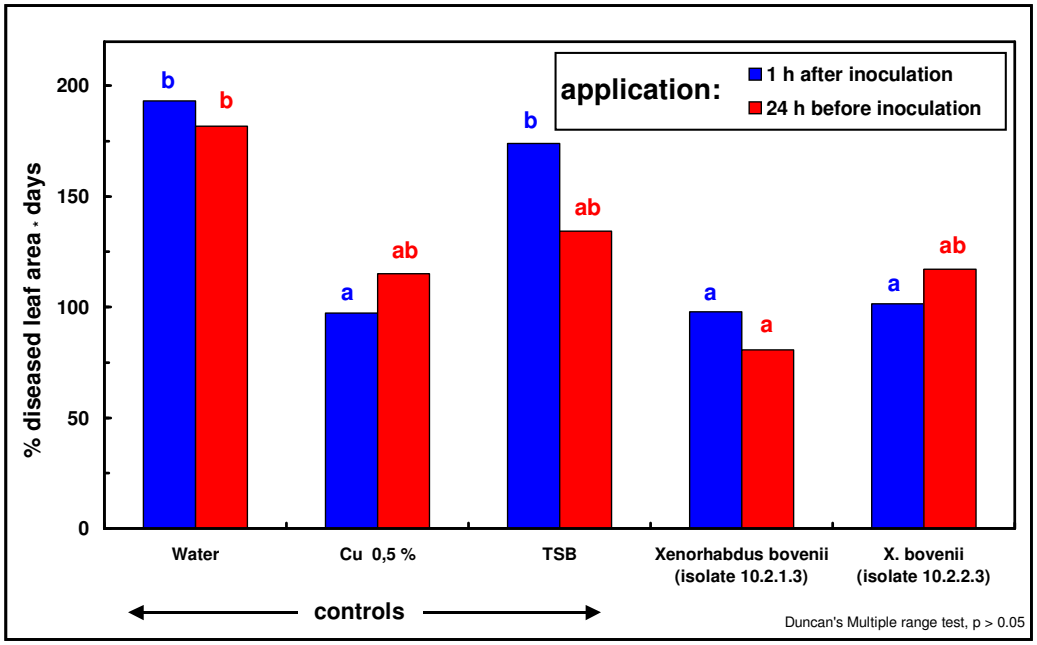


Fig. 1.2: Effectiveness of two isolates of *Xenorhabdus bovienii* against *P. infestans* on excised potato leaves, expressed as values of the area under disease progress curve (AUDPC). AUDPCs were calculated from examination results from the 5th, 6th and 7th day after inoculation. According to the German Collection of Microorganisms and Cell Cultures isolate 10.2.2.3 is the secondary form (phase II) of isolate 10.2.1.3, which is indicated as primary form (phase I variant).

Xenorhabdus bovienii (Enterobacteriaceae) is a gram negative bacterium that forms entomopathogenic symbiosis with soil nematodes. Most species of *Xenorhabdus* exist as (at least) two variants that differ in physiological and biochemical characteristics (AKHURST 1980; BOEMARE and AKHURST 1988). For several years it has been known that especially phase one variants of *Xenorhabdus* spec. produce (among others) antibiotic and antimycotic compounds (AKHURST 1982, CHEN et al. 1994). Pure cultures of the phase two variant, which is formed when the bacteria are maintained under stationary phase conditions in the laboratory, often lack or possess only reduced levels of these metabolites (BOEMARE and AKHURST 1988; FORST et al. 1997). Consequently, most authors report that only the primary form of *Xenorhabdus* spp. inhibits the growth of micro-organisms, but not the secondary form (AKHURST 1982; CHEN et al. 1994). NG and WEBSTER (1997) successfully applied ethyl acetate extracts from the culture filtrate of *Xenorhabdus bovienii* to control *Phytophthora infestans* in both *in vitro* and *ad planta* tests. The experiments were solely conducted with extracts from phase one variants. In our detached leaf bioassay, cultures from both phase variants showed a nearly identical efficacy against *Phytophthora infestans* when applied close to inoculation. Treatment 24 hours before inoculation resulted in a weaker efficacy of the phase two variant (Fig 1.2, isolate 10.2.2.3).
So far, we only recognized differences between the two isolates/variants concerning their yellow pigmentation (Fig. 1.3). As the primary form of *Xenorhabdus* spec. cultures is sometimes unstable (AKHURST 1982) and the two phase variants may spontaneously change from one to the other (FORST et al. 1997), *in vitro* tests were done to (re-)determine the respective phases of the two isolates/variants.

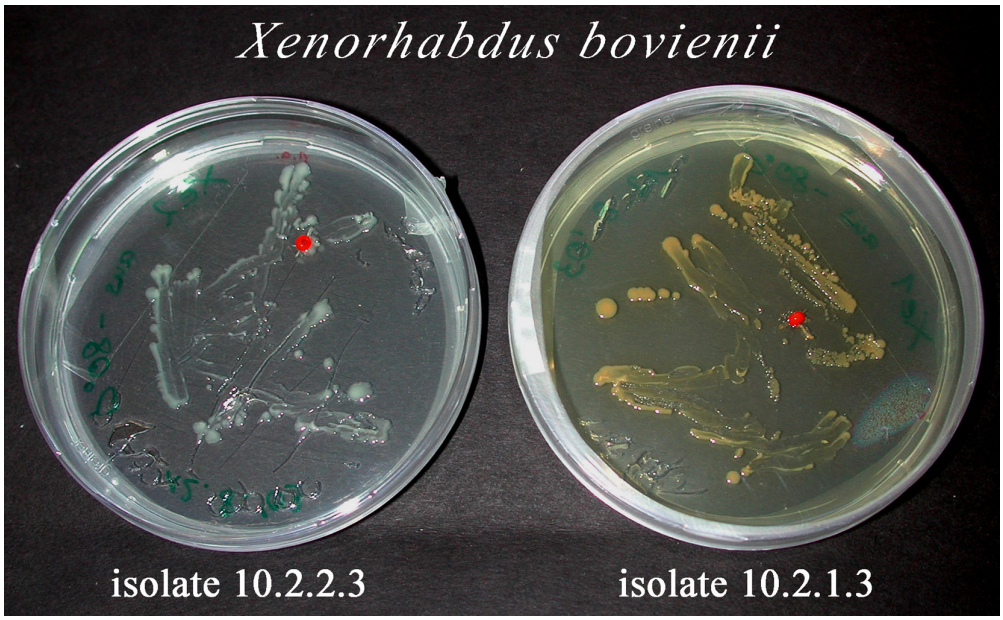


Fig. 1.3: Photograph of the two isolates of *Xenorhabdus bovienii* used in the detached leaf bioassay, cultured on nutrient agar. According to the German Collection of Microorganisms and Cell Cultures (DSMZ) isolate 10.2.2.3 is the secondary form (phase II variant) of isolate 10.2.1.3, which is indicated as primary form (phase I variant). (Red dots = beads from stock cultures)

The following products or preparations that were tested during the reporting period, showed no effect against *Phytophthora infestans* in detached leaf bioassays (two repetitions each, data not shown):

- *Sphagnum* extract
- Duckweed extract
- Compost extract from BBA
- Compost extract from partner 6 (DIAS)
- MBI600 (*B. subtilis*)
- ComCat® (Plant strengthener)
- BioPlantol® (Plant strengthener)

As an example Fig. 1.4 shows leaves representative for disease expression in one of these experiments.

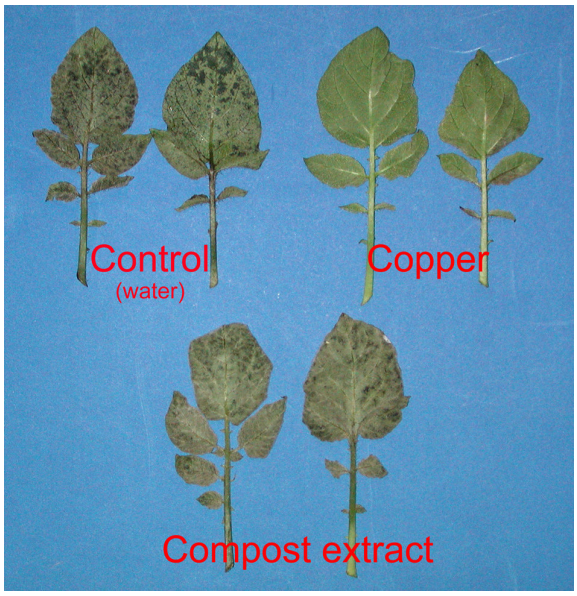


Fig. 1.4: Photograph showing leaves from a detached leaf bioassay. Leaves were treated with a compost extract derived from partner 6 (DIAS). Control and Copper = treatment with deionised water or 0.5 % Atempo® respectively.

Comparison of different copper fungicides

As the effectiveness of the chemical control "Atempo®" (Neudorff) sometimes dropped to an insufficient level, we compared the performance of the batch used so far with two other chemical fungicides, one containing 45 % copper as copper oxychloride (as Atempo®) and the other based on 30 % copper as copper hydroxide. No differences in the efficacy between the Atempo® batch and the other chemical fungicides could be detected in the standard excised leaf bioassay. All three products showed a sufficient efficacy in this experiment (Fig. 1.5), indicating that the Atempo® batch had not lost its efficiency. Atempo® therefore was further on used in all following experiments as control.
The fungicide containing copper as copper hydroxide (Cuprozin®) was slightly more effective than the other two chemicals based on copper oxychloride.

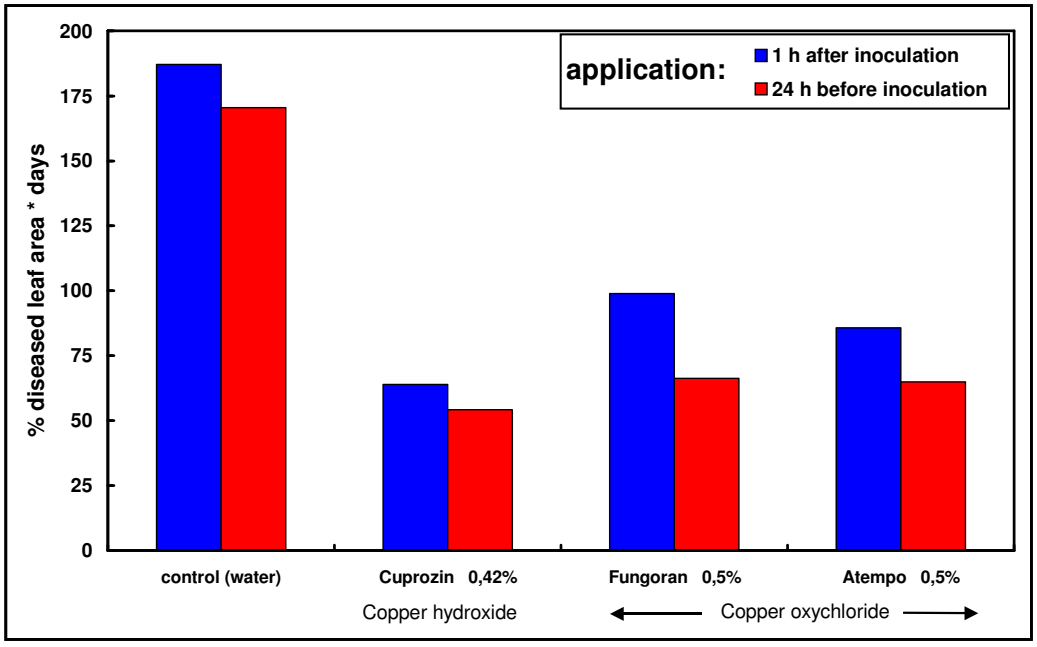


Fig. 1.5: Effectiveness of three copper based fungicides against *P. infestans* on excised potato leaves, expressed as values of the area under disease progress curve (AUDPC). AUDPCs were calculated from examination results from the 5th, 6th and 7th day after inoculation.

Two of the repetitions of the "copper experiment" were additionally examined at the 8th and 11th day after inoculation (Fig 1.6).

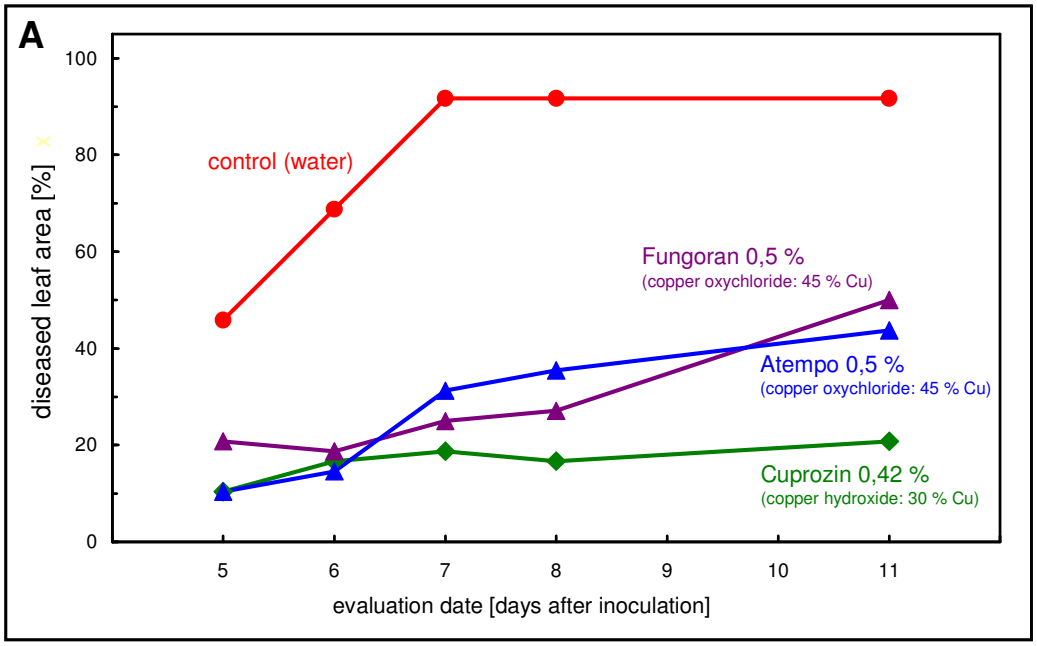


Fig. 1.6. A (see next page for fig. 1.6. B and legend)

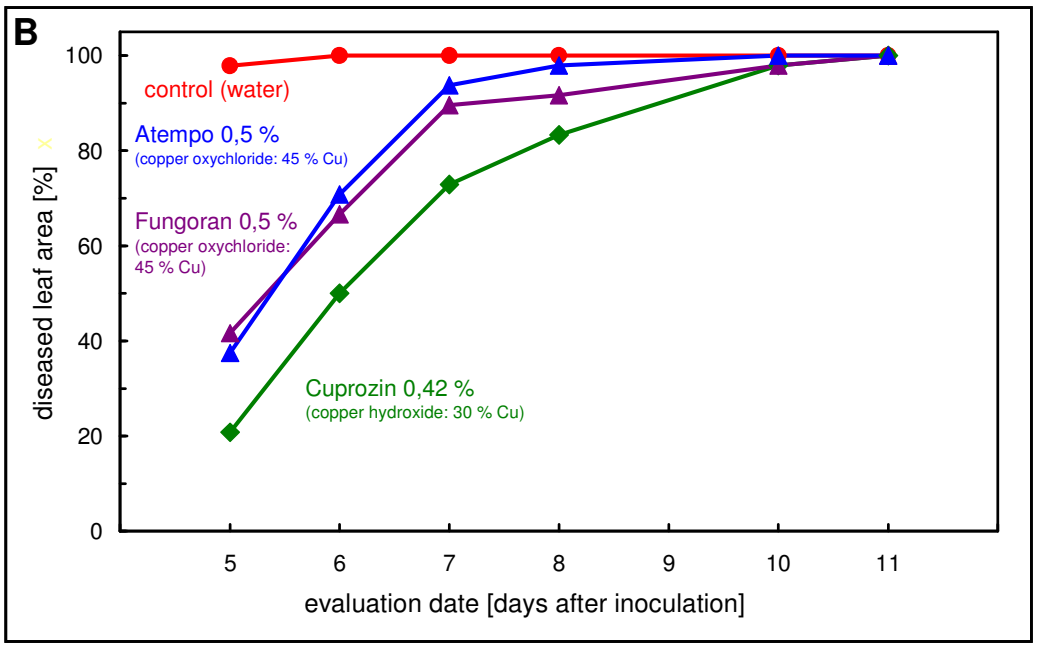


Fig 1.6: Time course of infection with *Phytophthora infestans* on excised potato leaves after treatment of leaves with different chemical fungicides. Treatments were done 1 hour after inoculation **A.** Leaves inoculated with less mobile zoospores. **B.** Leaves inoculated with more viable zoospores.

Although the fungal cultures had about the same age and preparation of zoospore suspensions followed the standard protocol, the microscopical examination of the inocula revealed differences in the mobilty of the released zoospores. This could be the reason for the different time course of the disease in the two experiments
(Fig. 1.6 A/B): Leaves inoculated with more mobile zoospores (Fig 1.6 B), showed already at the first day of examination a distinctly higher infestation than those inoculated with “slower” zoospores (Fig 1.6 A). These were all still in a relatively good condition at this day (Fig. 1.6 A; 5th day): In this repetition (Fig. 1.6A) the degree of infestation reached higher levels not before the 10th day after inoculation. Aparently this had an effect on the protection provided by the copper fungicides: Leaves inoculated with less mobile zoospores were longer protected by the copper fungicides and development of the disease was delayed.
The effect of the shift also became visible from the progress of disease in the water control. Inoculation with more mobile zoospores resulted in nearly totally diseased leaves already at the first evaluation date (Fig. 1.6 B), while in the other repetition (Fig. 1.6 A) the infection level of leaves was only about 45 % at this time, increasing over the next days. Maximum infection was reached not before the 7th day.
Taken together the results indicate that the time course of disease as well as the duration of protection by the tested copper based fungicides was possibly depending on the degree of the biological fitness of the zoospores. This may explain the differences in performance of the copper fungicide observed previously. It must also be expected that the differences in fitness of the zoospores also affected the level of control by the biological treatments.

Comparison of two different formulations of the plant strengthener Kendal®

All results obtained in experiments with the plant strengthener Kendal® (including those described in Section 4 of this Chapter) indicated a strong direct effect of this agent on *Phytophthora infestans*, which is very unusual for plant strengtheners. Therefore *in vitro* experiments with *P. infestans* plugs on rye agar containing the product in different concentrations were started. All concentrations tested (≥ 0.05 %) showed a total inhibition of fungal growth (results not shown), confirming the assumption of a very strong direct effect. While the *in vitro* experiments were still running, BBA was informed by the German distributor of Kendal®, that the product unfortunately had been delivered in a wrong formulation by the Italian manufacturer (Valagro® S. p. A.). A comparison of the wrong with the correct formulation revealed that the latter had no effect on *Phytophthora infestans* neither *in vitro* (Fig. 1.7) nor in detached leaf assays (Fig. 1.8).

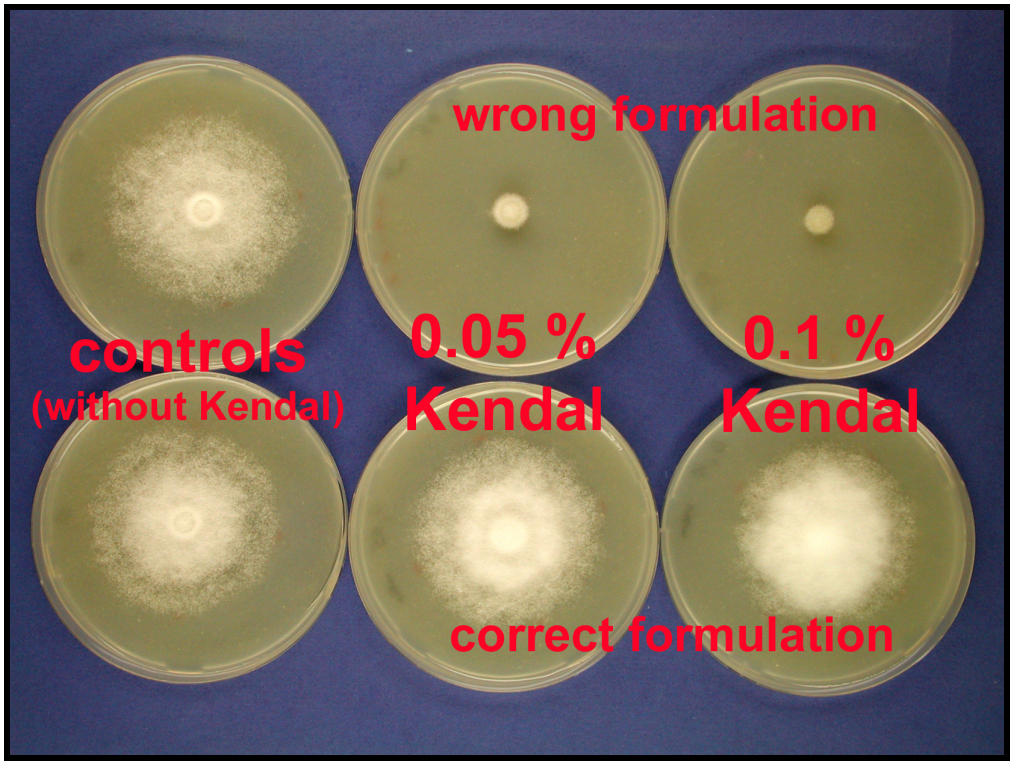


Fig. 1.7: Growth of Phytophthora infestans on rye agar containing 0.05 and 0.1 % Kendal® in two formulations each. Left: Control plates both containing no Kendal® in medium. Photograph was taken 8 days after beginning of the experiment.

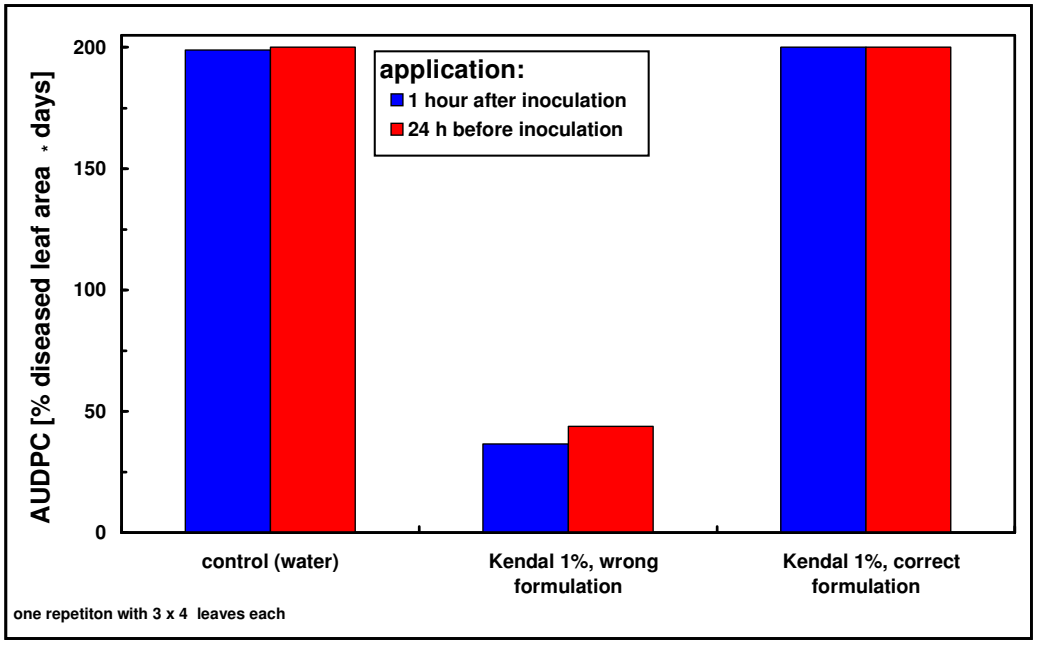


Fig. 1.8: Effectiveness of two formulation variants of the plant strengthener Kendal® against *P. infestans* on excised potato leaves, expressed as area under disease progress curve (AUDPC). AUDPCs were calculated from examination results from the 5th, 6th and 7th day after inoculation.

The batch with the "wrong" formulation of Kendal® was actually intended to be sent to the US. Different from the "correct" formulation it contains phosphonates, a chemical group known to have fungicidal effects against oomycetes.

Identification of potential microbial antagonists

Fungi and yeasts that were identified during the investigations are listed in table 1.1.

Table 1.1: Micro-organisms identified during the investigations.

isolate no.	species	method of identification	
		microscopy/morphology	Biolog*
3.6.3.	*Penicillium cyclopium*	X	
4.6.4.	*Cladosporium* spec.**	X	
4.6.9.	*C. cladosporoides*	X	
4.5.4.	*Bulleromyces albus*		X (88)
4.5.12.	*Debaromyces castellii*		X (100)

* values in paranthesis = probability index
** identification up to species level not possible and too elaborate respectively

Conclusions

As effects were promising in detached leaf bioassays, isolates I-112 (*Pseudo-monas putida*) and the two isolates (variants) of *Xenorhabdus bovienii* were further tested in experiments described in Section 4 and I-112 was also included in combination experiments described in Section 3.
It was also decided that tests on the influence of the kind of inoculation (e. g. droplets vs. spraying) on efficacy of MOs on *P. infestans* would also be examined.

ADDENDUM

Following completion of the work on this part of the programme some additional excised leaf biossays were conducted:

Preliminary results showed that

- 1:4 dilutions of a culture filtrate from *Xenorhabdus bovienii* (isolate 10.2.1.3, phase variant I) were as effective as the undiluted filtrate. Furthermore the efficacy of the filtrate did not drop, when it was stored for about 14 days at 4° C and then used again in an excised leaf bioassay.
- A new commercial product (company Proagro Ltd.), a combination of Copper oxychloride and a "protein", gave good results. Compared to the standard product Atempo®, the amount of Copperoxychloride is about 40fold lower in the new product.

Section 3: Identification of fungal/bacterial metabolite production/compatibility: leaf biossays

Summary

Biocontrol efficacy may be improved by Biological Control Agents (BCA) combinations of suitably compatible organisms. Most isolates in this study were found to be compatible. Studies to determine the potential for integrated use with Elot-Vis® revealed that *Bacillus pumilus* (1.1.9) and *Pseudomonas putida* (7.1.7 and 1.1.31) were compatible with Elot-Vis® at concentrations of up to 9.5, 7.6 and 3.6% respectively.

As regards single treatments applied at the same time as *Phytophthora infestans*, isolates 1.1.9, 1.1.13 and Elot-Vis® (20%) all completely prevented disease development in detached leaf assays (King Edward).

Isolate 1.1.9 was the most effective at controlling *P. infestans* on organic potato leaves (Claret and Pink Fir Apple) even with a high endogenous level of disease present.

Introduction

Compatibility tests between plant extracts and isolated organisms were done first *in vitro*, so as to determine their negative/compatible parameters and therefore their potential for use in mixed treatments. The effects of different concentrations of *S. canadensis* and *R. rhabarbarum* on growth and development of isolates were also recorded. Single and combination treatments of isolates and plant extracts were tested in leaf bioassays.

Materials and Methods

A. Compatibility of plant extracts (*Solidago canadensis* and *Rheum rhabarbum*) with the 10 selected isolates

Filtered suspensions of 1% and 5% of each of *S. canadensis* and *R. rhabarbarum* in Tween 80 (0.0125%) were used to prepare 0-0.05% and 0-0.25% gradient plates by adding the appropriate volume of such suspensions to molten NA or MEA. Such gradient plates were inoculated with a 100 µl drop of liquid cultures of isolates, spread over the surface of the plate. Plates were incubated at 21 ºC for 3 d before enumeration of colonies.

Determination of inhibitory concentrations of Elot-Vis ® using Gradient plates

10 and 20% suspensions of Elot-Vis® were used to prepare gradient plates of various concentrations. Such gradient plates were inoculated with a 100 µl drop of liquid cultures of isolates, spread over the surface of the plate. Plates were incubated at 21 ºC for 3 d before enumeration of colonies. *In vitro* compatibility tests between isolates and Elot-Vis® were then made.

Pairs of microbial isolates were tested at four different application times (applied at the same time, at 24hr intervals, at 72hr intervals and at 7 d intervals) and incubated at 21ºC. Some isolates [*B. pumilus* (1.1.9), *P. putida* (7.1.7), *P. putida* (I1121) and *P. fluorescens* (IV298a)] were also tested at 37ºC. Different application times were used to evaluate if an isolate which had already developed would affect the initial growth of another. Assessment of the growth of isolates inoculated together was classified as compatible or as showing inhibition. Results were compiled and are shown in Table 2.1.

B. Screening isolates for antagonism *in planta* (leaf assays)

The treatments used in this experiment were: bacterial species identified as being antagonistic *to P. infestans*; two fungi and one yeast which had shown *in vitro* activity against *P. infestans*; three plant extracts *S. canadensis* and *R. rhabarbarum* and Elot-vis® and isolates I1121 and IV298a.

For all detached leaf bioassays, leaves were aseptically removed from potato plants and placed on top of plastic mesh above moist tissue in plastic boxes.

Treatments applied mixed with *P. infestans*

Two detached leaves from 4 week old potato plants (cv. 'King Edward') were placed, upperside up, in a plastic inoculation box. One ml of Elot-Vis ® (10% or 20% concentration) and 1 ml of the test isolate (1.1.9, 1.1.13, 1.1.31, 7.1.7, 1.2.11, 3.6.3, 4.6.9 or 4.5.12) were mixed with 1 ml of *P. infestans* (1 x 10^7 zoospores/ml). Such suspension was used to inoculate leaves (1-3 x 10µl drops per leaf). A negative (sterile distilled water, SDW) and positive (*P. infestans* only) control were used. They were incubated (15°C and dark) for 7 days. Disease was assessed on the 5^{th} to 7^{th} day post inoculation, according to JAMES (1971)

(class 1: no infection; class 2: only small necrotic spots visible; class 3: very few aerial spots; class 4: many aerial spots and class 5: aerial spots on 50% or more of the leaf) and the area under the disease progress curve (AUDPC) was calculated.

Organic potato leaves - single treatments

Potato leaves of the cultivars Claret and Pink Fir Apple were freshly harvested on the morning of the experiment from an organic grower (Bridgefoot organic farm, Newmachar). A 20 µl drop of the isolate (1.1.9, 1.1.13, 1.1.31, 7.1.7, 1.2.11, IV298a or I1121) was placed on each of two leaves, placed upperside upwards in a plastic box. These were incubated at 15°C for 2-3 hours after which time the residual inoculum was absorbed using tissue paper and a drop (20 µl) of *P. infestans* was placed in the same inoculation spot. Negative (SDW) and positive (*P. infestans* only) controls were also prepared and disease was assessed on the 6^{th} and 7^{th} days post inoculation.

COMBINATION TREATMENTS

Potato leaves were sprayed with 5ml of *Solidago canadensis* (1%); *Rheum rhabarbarum* (1%); combination of *S. canadensis* (1%) and 1.1.9 and a combination of *R. rhabarbarum* and 7.1.7. Combinations were prepared in a 1:1 ratio. The plastic boxes were covered for 30 minutes before removing the lids and allowing the leaf surfaces to dry. Two hours later, 1-3 drops of *P. infestans* (4 x 10^6 sporangia/ml) were placed on each leaf using a Pasteur pipette. A negative (SDW) and a positive (*P. infestans* only) control were used. All plastic boxes containing leaves were incubated (15°C and dark) for 7d. Disease assessment was made 5^{th}, 6^{th} and 7^{th} d post inoculation.

Isolates applied 24 hours before *P. infestans*

This experiment was identical to that described above except that treatments were applied 24 hours before addition of 1-3 drops of *P. infestans* (1 x 10^5 sporangia/ml).

PETRI DISH BIOASSAYS

Petri dish experiments were performed using sterile Petri dishes containing filter paper (Whatman, 9cm) moistened with SDW. One leaf was placed in each Petri dish, upperside up.

Treatments applied 24 hours before *P. infestans*

Leaves from 4 week old potato plants (cv. 'King Edward') were inoculated with 1-3 drops of *P. infestans* (1 x 10^5 zoospores/ml) using a Pasteur pipette and incubated (15°C and dark) for 24 hours. After this time, the residual inoculum was absorbed with tissue paper and 1-3 drops of either Elot-Vis ® (10 or 20% concentration) or the isolates (1.1.9, 1.1.13, 1.1.31, 7.1.7, 1.2.11, 3.6.3, 4.6.9 or 4.5.12) was placed on the same inoculation spot. A negative (SDW) and positive (*P. infestans* only) control were also used. Petri dishes were incubated (15°C and darkness) and disease assessment was made on the 7^{th} day post inoculation.

COMBINATION EXPERIMENTS

Isolates applied 2-3 hours before *P. infestans*

This experiment was essentially the same as those performed in plastic boxes

Isolates applied 24 hours before *P. infestans*

This experiment was essentially the same as those performed in plastic boxes. In addition, isolates 1.1.13, IV298a and I1121 were also used.

WHOLE PLANT EXPERIMENTS

Leaves from 4 week old potato plants (cv. 'King Edward') were inoculated with mixed suspensions of isolates (1.1.9, 1.1.13, 1.1.31, 7.1.7 and 1.2.11) mixed with *P. infestans* (1.2 x10^7 zoospores/ml). Mixed suspensions were prepared by mixing 1.5 ml *P. infestans* (1.2 x 10^7 zoospores/ml) with 1.5 ml of the isolate and 1 ml SDW. A negative (SDW) and positive (*P. infestans* only) control were also used. Leaves were sprayed with 4 ml of the relevant treatment. Plants were left uncovered for 30-60 minutes at room temperature until the leaf surface was dry before covering them with plastic bags which were taped to the pot to prevent air flow and placed in a tray with water creating 100% relative humidity environment. The plants were then placed in a growth chamber with 16 hour photoperiod (8 hours darkness) at 15°C and disease was assessed 7 days post inoculation.

Treatments applied 2 hours before *P. infestans*

Leaves from 4 week old potatoes (cv. 'Cara') were sprayed with 6 ml of either isolate 1.1.9 or 7.1.7 and allowed to dry at room temperature. After 2 hours, a cylindrical plastic mesh was placed round each plant before spraying with 6 ml of *P. infestans* (1 x 10^5 sporangia/ml). The plants were then covered with plastic bags to prevent air flow and placed in a tray with water to create 100% relative humidity conditions. The plants were then placed in a growth chamber (15°C; 16 hours light/ 8 hours dark) and assessed at 5, 6 and 7 d post inoculation. A positive control of SDW and negative control of Copper (0.5%) were also used.

Treatments applied 24 hours before *P. infestans*

This experiment as performed as per treatment application 2 hours in advance of inoculation with *P. infestans*, except that treatments were applied 24 hours in advance. A combination Rhubarb (5%) with 7.1.7 and a combination of Solidago (5%) with 1.1.9 were used. Single applications of each treatment were also used. The plants were then placed in a growth chamber (15°C; 16 hours light/ 8 hours dark) and assessed at 5, 6 and 7 d post inoculation. A positive control of SDW and negative control of Copper (0.5%) were also used.

Results and Discussion

A. Compatibility of plant extracts (*Solidago canadensis* and *Rheum rhabarbum*) with the 10 selected isolates

The microbial isolates showed either no inhibition (compatible) or inhibition of growth when tested against each other (as indicated in Table 2.1). The assessment was made by visual observations of plates at the tested application times.

The isolates that showed incompatibility by inhibiting or being inhibited by other isolate(s) were: *Bacillus sphaericus* (1.1.13) with *B. licheniformis* (1.2.11); *B. licheniformis* with *B. pumilus* (1.1.9). On this basis these isolates were designated as not compatible and were not selected for combination studies. Most of the other isolates appeared to be compatible or cooperative when grown together allowing their potential use as combination treatments in future work.

Table 2.1.: *In vitro* compatibility between isolates at 4 different application times (at the same time, 24hrs, 72hrs and 7 days interval). The isolates are presented as inhibited and inhibitor.

	inhibited											
inhibitor	1.1.3	1.1.9	1.1.13	1.1.31	7.1.7	1.2.11	1.2.21	3.6.3	4.6.9	4.5.12	I1121	IV298a
1.1.3												
1.1.9												
1.1.13												
1.1.31												
7.1.7												
1.2.11												
1.2.21												
3.6.3												
4.6.9												
4.5.12												
I1121												
IV298a												

Legend: Compatible Inhibition

Quantitative analysis of concentration of Elot-Vis® inhibitory to isolates

Gradient plate experiments were set up with *Bacillus licheniformis* (1.2.11), *Pseudomonas putida* (isolates 7.1.7 and 1.1.31), *Bacillus sphaericus* (1.1.13) and *Bacillus pumilus* (1.1.9). Gradient plates with concentrations 0-5%, 0-10% and 0-20% Elot-Vis® were made and results are shown in Figure 2.1.

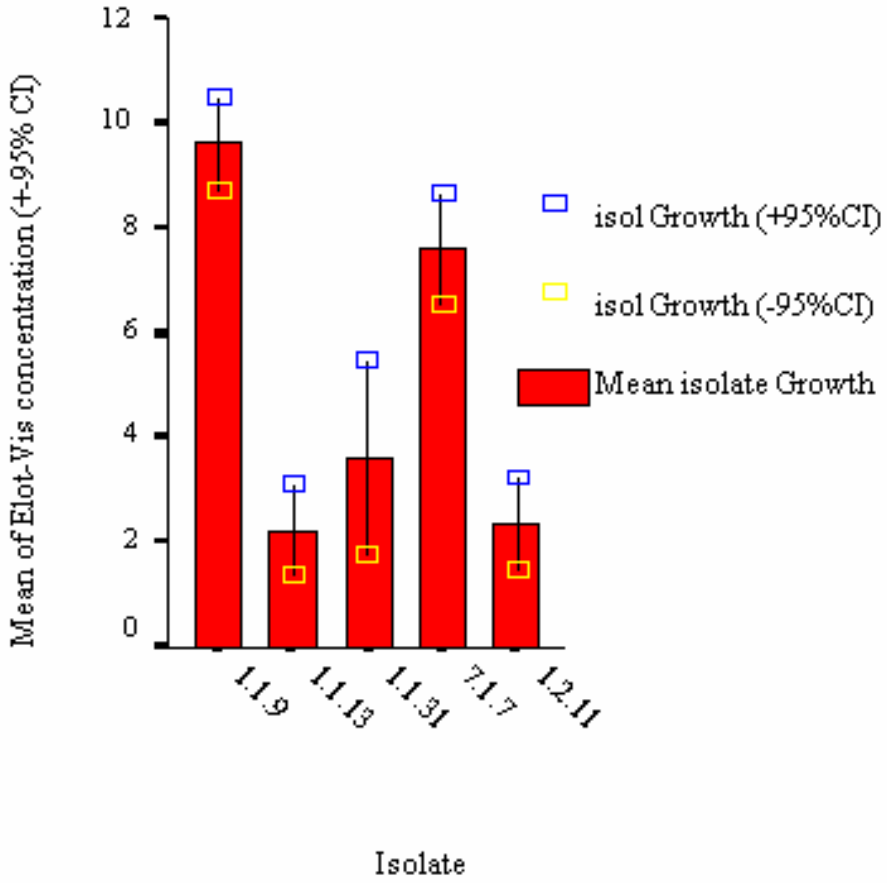


Figure 2.1.: Highest concentration up to which selected isolates are compatible with Elot-Vis® (0-20% gradient plates)

Bacillus pumilus (1.1.9) and both *Pseudomonas putida* isolates (7.1.7 and 1.1.31) grew well on Elot-Vis® gradient plates. These three isolates were shown to be capable of growth at up to 9.5, 7.6 and 3.6% Elot-Vis® concentration, respectively. *B. sphaericus* (1.1.13) and *B. licheniformis* (1.2.11) were able to grow at a concentration of around 2%. No isolates were able to grow at 20% Elot-Vis® concentration.

B. Screening isolates for antagonism *in planta* (leaf assays)

Treatments mixed with *P. infestans*

Results of the *in planta* antagonism experiments of bacterial isolates *B. pumilus* (1.1.9), *B. sphaericus* (1.1.13), *P. putida* (1.1.31), *P. putida* (7.1.7), *B. licheniformis* (1.2.11), the yeast 4.5.12 and plant strengthener Elot-Vis®, obtained on the 5th, 6th and 7th observation day were analysed and are presented in Figure 2.2.

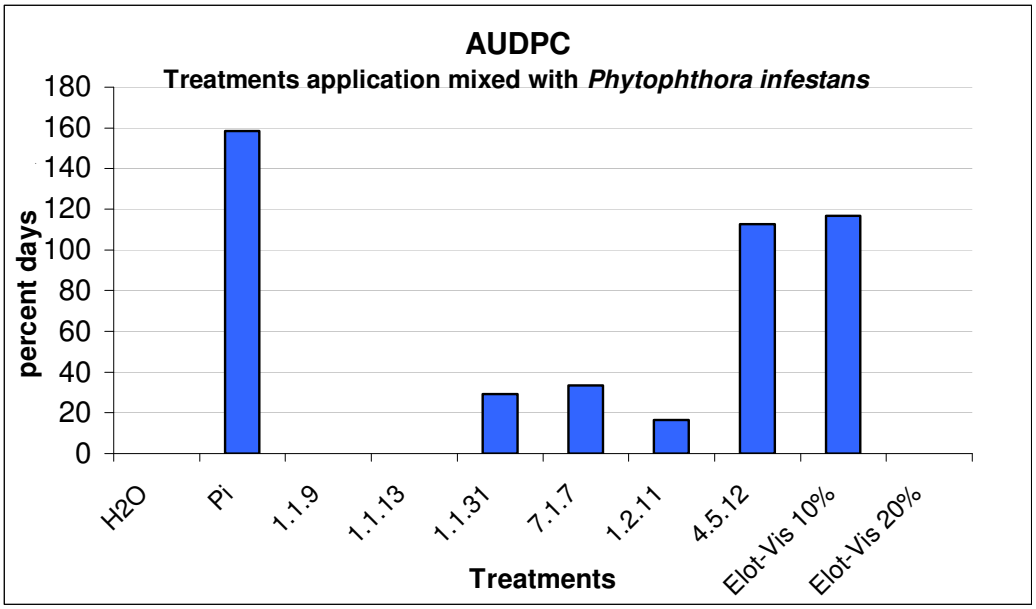


Figure. 2.2.: Area under the disease progress curve for each treatment applied mixed with *P. infestans*.

Results showed that when treatments were applied mixed with *P. infestans*, *B. pumilus* (1.1.9), *B. licheniformis* (1.1.13) and Elot-Vis 20% controlled the disease completely.

Organic potato leaves – single treatments

Tests were performed on potato leaves sampled from an organic grower to give some indication of the potential for biocontrol for the organic grower. The results of *in planta* antagonism of isolates [*B. pumilus* (1.1.9), *B. licheniformis* (1.1.13), *P. putida* (1.1.31), *P. putida* (7.1.7), *B. licheniformis* (1.2.11), *P. fluorescens* (IV298a) and *P. putida* (I1121)] applied 3 hours before inoculation of *P. infestans* on two different organic potato varieties, Claret and Pink Fir Apple, supplied by an organic grower (Bridgefoot organic farm, Newmachar) were analysed and are presented as AUDPC in Figure 2.3.

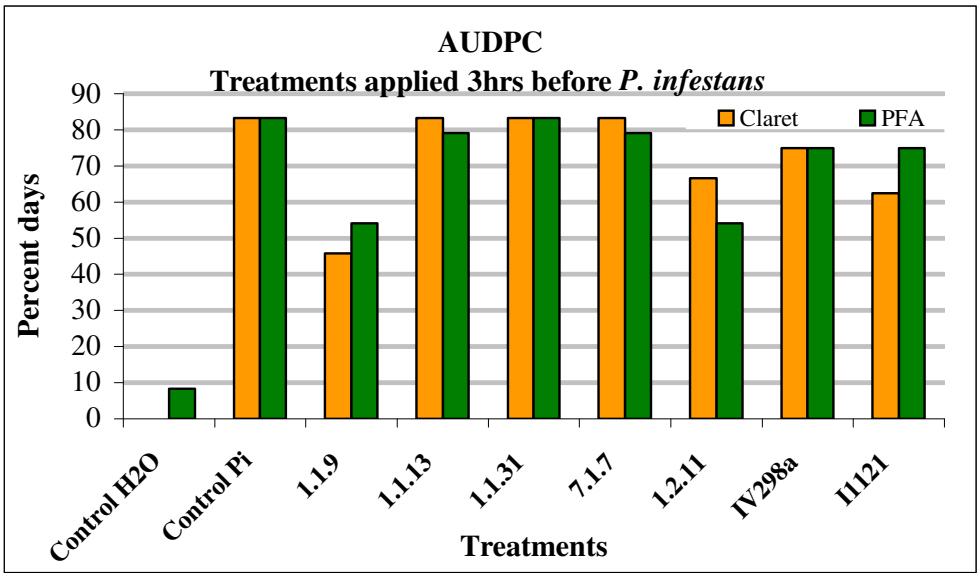


Figure 2.3: Area under the disease progress curve for each treatment applied 3 hours before *P. infestans* on organically grown potato leaves cv. 'Claret' or Pink Fur Apple' (PFA).

Combination treatments

Combination treatments between bacterial isolates and plant extracts were chosen based on the results of *in vitro* compatibility tests. Combinations tested (on organically grown leaves) were: *S. canadensis* (1%) with *B. pumilus (*1.1.9) and *R. rhabarbarum* (1%) with *P. putida* (7.1.7).

Treatment application 2hrs and 24hrs before *P. infestans* inoculation

B. pumilus (1.1.9) and *P. putida* (7.1.7) were mixed with plant extracts *S. canadensis* and *R. rhabarbarum*. The results of *in planta* screening on Claret and Pink Fir Apple cultivars of single and combination treatments when applied 2hrs and 24hrs before *P. infestans* inoculation are presented as AUDPC in Figures 2.4 (cv. 'Claret') and 2.5 (cv. 'Pink Fir Apple'). Figure 2.4 shows that isolate 1.1.9 gives almost complete control compared to Pi infected and Water Control (no Pi treatment) in Claret. The two combination treatments also gave better disease control than the other single treatments. Isolate 1.1.9 gave best disease control in Pink Fir Apple similar, although not as good as, that observed in Claret. The only combination treatment to show respectable disease control was 1.1.9 with Solidago (1%) and only when applied 2 hours before *P. infestans*.

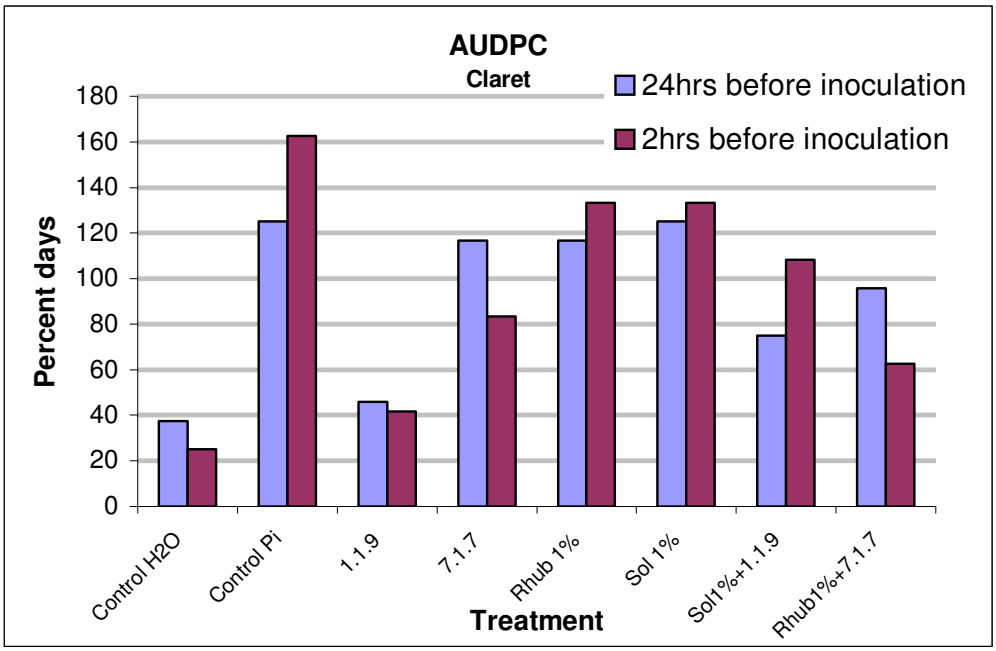


Figure 2.4.: Area under the disease progress curve for each treatment applied 2 or 24 hours before *P. infestans* on organically grown potato leaves cv. 'Claret'.

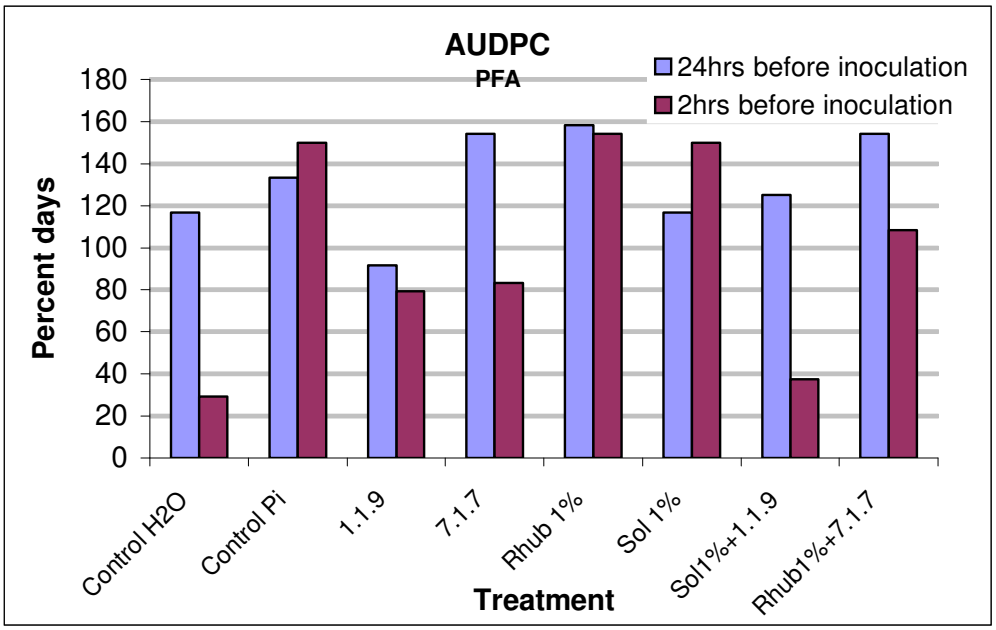


Figure 2.5.: Area under the disease progress curve for each treatment applied 2 or 24 hours before *P. infestans* on organically grown potato leaves cv. 'Pink Fir Apple' (PFA).

Petri dish bioassays

Single Treatments

Treatments mixed with *Phytophthora infestans*

In planta experiments in Petri dishes were prepared to test isolates *B. pumilus* (1.1.9), *B. sphaericus* (1.1.13), *P. putida* (1.1.31), *P. putida* (7.1.7), *B. licheniformis* (1.2.11), the fungi *P. cyclopium* (3.6.3) and *C. cladosporioides* (4.6.9), the yeast 4.5.12 and plant strengthener Elot-Vis against *P. infestans* obtained on the 5th, 6th and 7th observation day were analysed and are presented as AUDPC in Figure 2.6.

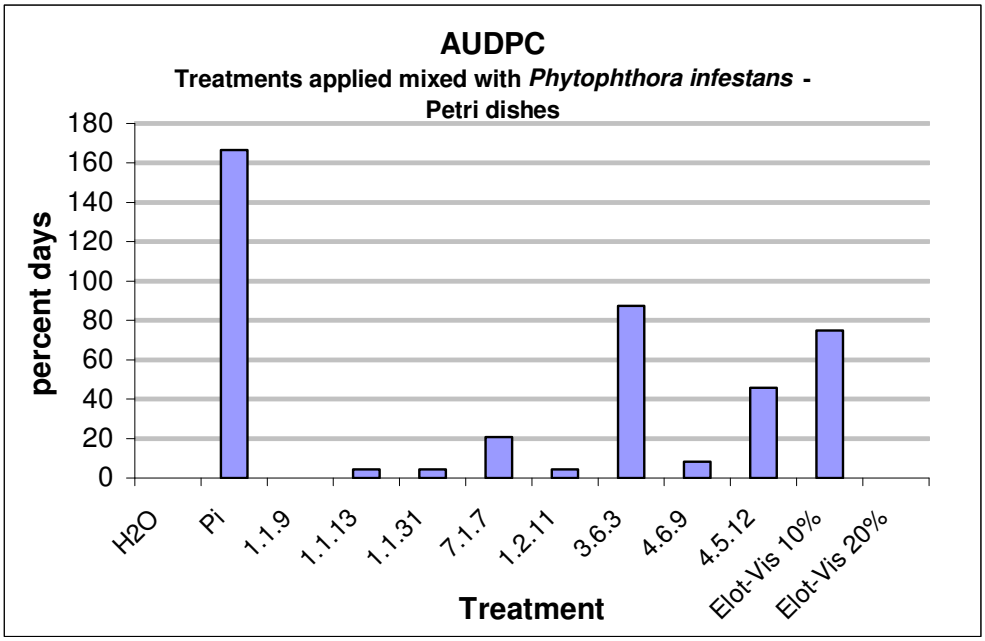


Figure 2.6.: AUDPC obtained when single treatments were applied mixed with *P. infestans* using detached potato leaves cv. 'King Edward' in Petri dishes.

Single treatments with 1.1.9, 1.1.13, 1.1.31, 7.1.7, 1.2.11, 4.6.9 and Elot-Vis®(20%) were especially effective whereas 4.5.12 and Elot-Vis® (10%) were less effective.

Treatments application 24hrs after *Phytophthora infestans* inoculation

Combination Treatments

Combinations tested using detached leaves of Claret and PFA organic varieties in Petri dishes were: *S. canadensis* (1%) with *B. pumilus* (1.1.9) and *R. rhabarbarum* (1%) with *P. putida* (7.1.7).

Treatment application 2 hours before *Phytophthora infestans* inoculation

Results of screening for antagonistic effect of isolates *B. pumilus* (1.1.9), *P. putida* (7.1.7), plant extracts *S. canadensis* (1%) and *R. rhabarbarum* (1%) and their combinations, when applied 2 hours before pathogen inoculation, in Petri dishes, were analysed and presented as AUDPC, in Figure 2.7.

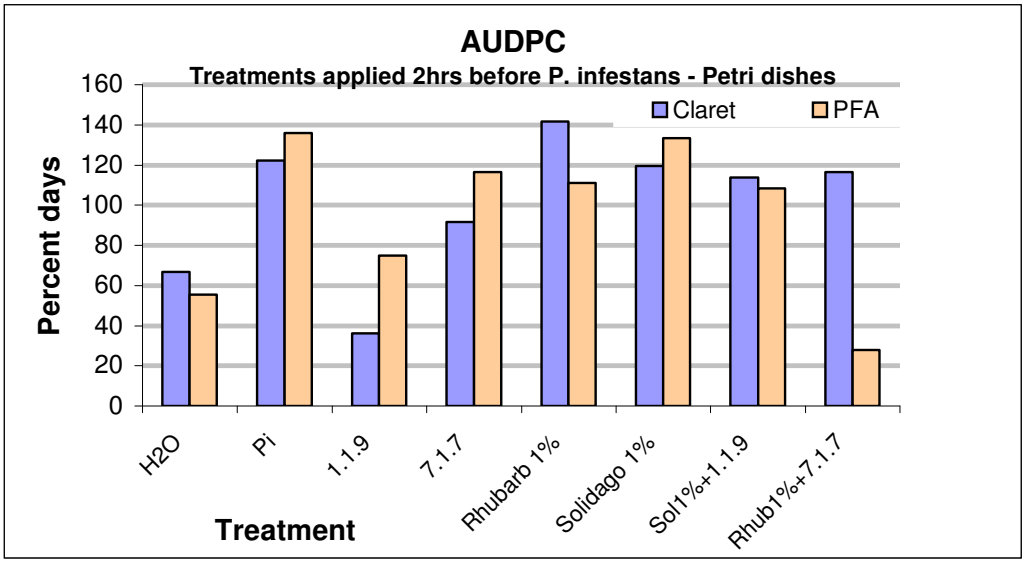


Figure 2.7.: Area under the disease progress curve obtained when single and combination treatments were applied 2hrs before *P. infestans* on detached leaves of organically grown potatoes cv. 'Claret' and 'Pink Fir Apple' in Petri dishes.

It was noted that these leaves from the organic grower later on in the season showed endogenous levels of *P. infestans* disease even in the Water Controls. However, 1.1.9 singly with Claret and PFA, and Rhubarb 1%+7.1.7 in combination with PFA gave reasonably high levels of disease control.

Treatments application 24hrs before *Phytophthora infestans* inoculation

B. pumilus (1.1.9) and *P. putida* (7.1.7) were applied mixed with plant extracts *S. canadensis* (1%) and *R. rhabarbarum*. *P. putida* (I1121) and *P. fluorescens* (IV298a) were also used. The results of single and combination treatments applied to leaves of Claret and Pink Fir Apple potato cultivars 24hrs before *P. infestans* inoculation are presented as AUDPC in Figure 2.8. In this case when leaves with high endogenous levels of *P. infestans* were allowed to incubate under favourable disease development conditions for a period of 24 hours, prior to the introduction of biocontrol agents, very little disease control was obtained. Presumably the endogenous *P. infestans* levels are already too high an inoculum load for the biocontrol agents to cope.

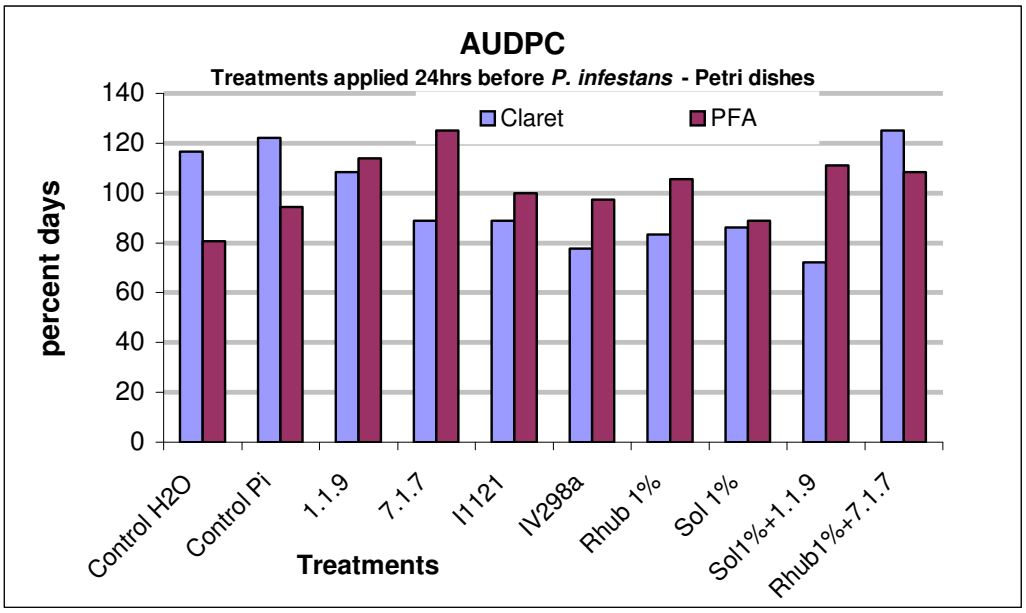


Figure 2.8.: Area under the disease progress curve for each treatment applied 24 hours before *P. infestans* on organically grown potato leaves cv. 'Claret' or 'Pink Fur Apple' (PFA) in Petri dishes.

Conclusions

1. Some single treatments (eg. Elot-Vis® (20%), isolates 1.1.9 and 1.1.13) showed strong disease control in detached leaf assays using King Edward potato leaves.
2. Compatibility studies showed the only incompatible combinations to be *Bacillus sphaericus* (1.1.13) with *B. licheniformis* (1.2.11) and *B. licheniformis* with *B. pumilus* (1.1.9).
3. *Bacillus pumilus* (1.1.9) and *Pseudomonas putida* (7.1.7 and 1.1.31) were compatible with Elot-Vis® at concentrations of up to 9.5, 7.6 and 3.6% respectively.
4. Biocontrol efficacy of the combinations Rhubarb (1%) + 7.1.7 with PFA gave reasonably high levels of disease control as did Solidago (1%) + 1.1.9 (when applied 2 hours before *P. infestans*). Whereas with Claret, the best combination was again with Rhubarb (1%) and 7.1.7. although at a lower level of efficacy.

ADDENDUM

Supplementary investigations conducted after the main programme of experiments had finished :

- The effects of culture duration (14d, 7d, 5d, 2d and overnight cultures) and concentration (200%, 100%, 50%, 25%, 12.5%, 10% and 6.25%) on efficacy of isolates 1.1.9 (*Bacillus pumilus*) and 7.1.7 (*Pseudomonas putida*) was investigated in detached leaf bioassays with *Phytophthora infestans*.Culture duration: The most effective concentrations were different on the three evaluation dates (6, 7 and 8 days post-inoculation). The AUDPC suggested that overall isolate 7.1.7 was more effective than isolate 1.9.9 and that 7 day culture lengths were optimal for both isolates.Treatment concentration: Isolate 7.1.7 revealed a steady increase in efficacy with increasing concentration. In contrast, isolate 1.1.9 showed a moderate efficacy at concentrations higher 50 % and no effect at lower concentrations. As in the culture duration experiment, isolate 1.1.9 was overall less effective than 7.1.7.

- It appears that culture length had little impact on efficacy of isolates 1.1.9 and 7.1.7. For isolate 7.1.7 there seems to be a linear relationship between treatment concentration and efficacy, while isolate 1.1.9 in this respect indicated a threshold reaction. Generally, 7.1.7 was more effective for control of *Phytophthora infestans* on excised potato leaves.

Section 4: Optimisation of application frequency/timing/dose rate of microbial/plant extracts

Summary

In greenhouse tests on potted plants rhubarb extract in a concentration of 5 % showed a significant effect against late blight when applied two days before inoculation, indicating an indirect effect on infestation of potato plants with *Phytophthora infestans*.
Two bacterial antagonists (I-112, IV298a) did not control late blight any longer under greenhouse conditions.

Xenorhabdus bovienii (phase I variant) and the commercial product Serenade® (*Bacillus subtilis* preparation) in a concentration of 5 % showed good effects against late blight in greenhouse tests and under "field" conditions (comparable to chemical control). Effects were best when the treatments were applied close to inoculation, indicating a direct effect of the two treatments.

Especially for treatment with *X. bovienii* an accumulating effect seems to play a role when repeated applications are carried out, resulting in a better, more stable control of *Phytophthora infestans*.

Introduction

'Semi-field' trials and one "field" trial were conducted with potted plants in order to test the performance and durability of the most promising alternative treatments under climatical conditions are close to practice. The performance of four bacterial isolates, one plant extract, and two commercial products were examined (partly in different concentrations). According to the results from experiments described in Section 2 0f this Chapter 6 'Identification of fungal/bacterial antagonists/plant extracts', application of the respective MOs, PEs or CPs was carried out at different days before and/or the day of inoculation.
Alternative agents were supplied to FAL-Reckenholze for further evaluation (see Chapter 6).

Materials & Methods

Durability of alternative control agents under semi-field and field conditions

The experimental design for semi-field trials was as follows: Potatoes of the cultivar Secura (highly susceptible to *P. infestans*) were cultured for about three weeks at 17° C with a 16/8 h day/night rhythm. Pots containing at least 2 potato stalks were selected, maximum number of stalks was 4. Plants were put into an open greenhouse (Fig. 3. 1.) and treated with the respective agents or cultures of MOs with a mist sprayer until run-off. During incubation of plants, basic weather data were recorded. After the desired incubation time, leaves were randomly taken from treated plants and placed in 20x20x5cm plastic boxes onto steel wire mesh with soaked filter paper underneath (4 leaves per box each). Inoculation and incubation of leaves after inoculation were carried out as described under 1. 3. 1. Assessment of the infected leaf area was done at the 7th day after inoculation according to JAMES 1971.
For testing of selected treatments under "field" conditions potted plants were grown and selected as described above and put on a field near BBA for one week. Plants were treated 3 times at two day intervals (i. e. treatments were at day 1, 3 and 5) as described above. Leaves from all treatments were collected daily, put into plastic boxes (4 per box) and inoculated and incubated as described above. Evaluation of the experiment was done 6 days after inoculation.

In all experiments de-ionised water and a commercially available copper fungicide (Atempo®, Neudorff) served as controls. Three independent experiments with two replicates (boxes) per repetition were done for each treatment in case of the semi-field trials, whereas the experiment under field conditions was only repeated once with 2 boxes per treatment. For the separation of means the AUDPC values were compared by Turkey's studentized range test ($p > 0.05$), or the standard deviation of replicates or repetitions respectively was calculated.

Fig. 3.1.: Open greenhouse with potted potato plants during incubation after treatment with alternative control agents.

Treatments were based on the results of experiments described in Sections 2 and 3 of this Chapter. In the semi-field trials (i. e. open greenhouse), bacterial isolates I-112 (*Pseudomonas putida*), II-298a (*Pseudomonas fluorescens*), *Xenorhabdus bovienii* 10.2.1.3. and 10.2.2.3., *Rheum rhabarbarum*-extract and Kendal® (unfortunately wrong formulation, see section 1.4.3.) were examined in one concentration each at 6 different application dates (in relation to the date of inoculation).
Furthermore, plant strengtheners Kendal® (wrong formulation; see section 1.4.3) and Serenade® as well as extracts from *Rheum rhabarbarum* were tested in three different

concentrations each at two different application dates each (in relation to the date of inoculation).
In the experiment in which treatments and incubations were done under "field" conditions, Serenade®, *Xenorhabdus bovienii* 10.2.1.3. and Kendal® (wrong formulation, see above) were tested in one concentration each for their efficacy against *P. infestans*.

Bacteria were cultured for 7 days (I-112, II-298a) and 4 days (*Xenorhabdus bovienii*) respectively in Tryptic soy broth (TSB) on a rotary shaker and applied as whole culture. The *Rheum* extracts were prepared by heating de-ionised water containing Tween 80 (0,0125%) to 50 °C and pouring 100 ml over the respective amounts of dried and grinded leaves. The mixtures were stirred for 1 hour without further heating and were then filtered under vacuum. Extracts were used freshly. The products were added to de-ionised water in the respective concentrations and dissolved by stirring the mixture.
As controls, a copper treatment, a water control and - where necessary - a control consisting of pure culture medium only were applied.

Culturing of *Phytophthora infestans*: *P. infestans* was cultured for about 10-14 days at 15 °C on petri dishes on rye agar containing 0.005% β-Sitosterol and maintained by transferring mycelial plugs from the outer area of the colony head-over on fresh rye agar plates. Fungal sporangia were washed from the petri dishes with 0.0125 % Tween 80 and adjusted to give a concentration of $1x10^5$ per ml. Before using the suspension for inoculation, it was incubated at 4-5 °C until the zoospores were released to at least 50%.

Supply of partner FAL- Reckenholz with alternative treatments

Rhubarb leaves were collected from a field of a commercial grower in Schifferstadt, Germany and cut-up. *Solidago* plants were collected from areas close to BBA Darmstadt. Complete plants, including flowers and seeds, were collected and cut up. The leaves or plants were dried in a "Hygrex-Labortrockner" under mild conditions so that no fermentation but fast dryness was achieved. Dried material was ground to powder and sent to FAL.
Micro-organisms (cultured on nutrient agar in petri dishes) as well as sufficient amounts of commercial products were sent to FAL – Reckenholz (See Chapter 7)

Results & Discussion

Performance of different treatments under semi-field conditions

Experiments for described in this section on 'Optimisation of application frequency/timing/dose rate of microbial/plant extracts'were mainly performed during the very hot summer of 2003. Probably due to the very high temperatures (maximum 42° C), and partly direct sunlight on treated plants during all experiments in the open greenhouse, there was no effect of bacterial isolates I-112 and IV-298a against *Phytophthora infestans* (data not shown). As previous bioassays indicated a direct effect on the pathogen (i. e. necessity of living bacteria), the inefficiency is probably due to a rapid dying of bacteria under these adverse environmental conditions.
Experiments using the two *Xenorhabdus bovienii* isolates as treatments were conducted under the same unfavourable climatic conditions. Nevertheless, at least isolate 10.2.1.3 was still able to reduce late blight infection by about 40-60 % when applied 4-1 day(s) before or at the day of inoculation (Fig. 3.2). The effect(s) of this isolate were significant at 2 and 1 day(s) before and at the day of inoculation with *P. infestans* (statistics not shown in fig. 3.2). Treatment one day before or at the day of inoculation even resulted in a clearly better control of late blight by isolate 10.2.1.3 than the copper fungicide did. Isolate 10.2.2.3 was clearly less effective than 10.2.1.3, which could be due to reduced levels of antibiotics produced by this variant (phase II, e. g. BOEMARE and AKHURST 1988, compare section 1.4.1). It is not clear why both

isolates showed very similar good effects against late blight under controlled conditions (Fig. 1.2) but revealed remarkable differences in the greenhouse test (Fig. 3.2). Possibly, this is due to phase changes of isolate 10.2.2.3 during culturing (compare section 1.4.1) or production of different (amounts of) antimycotic metabolites of the two variants.

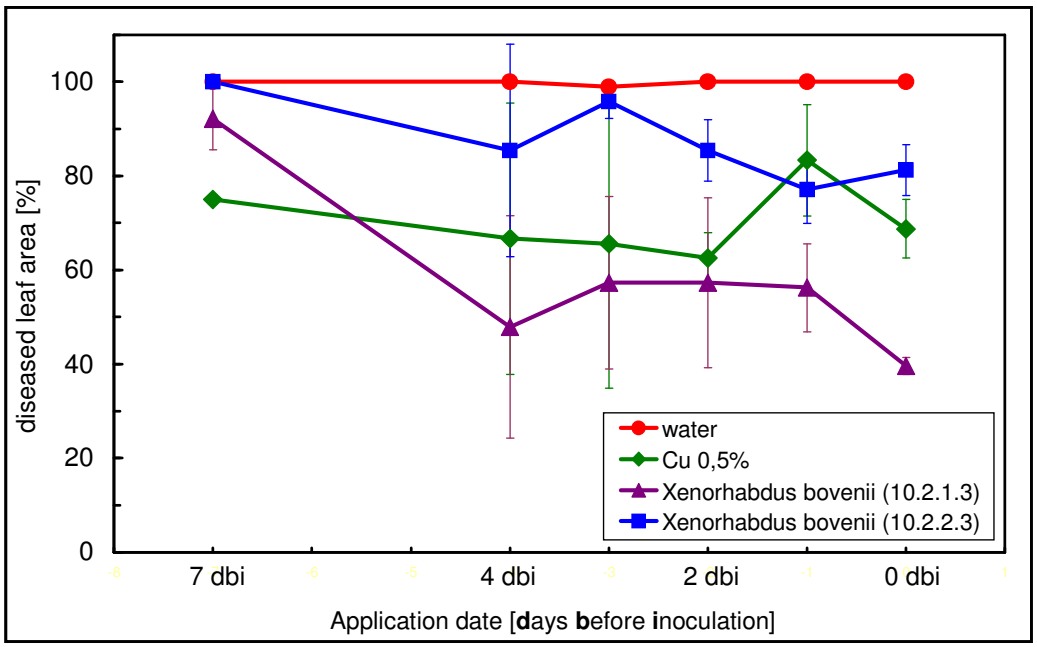
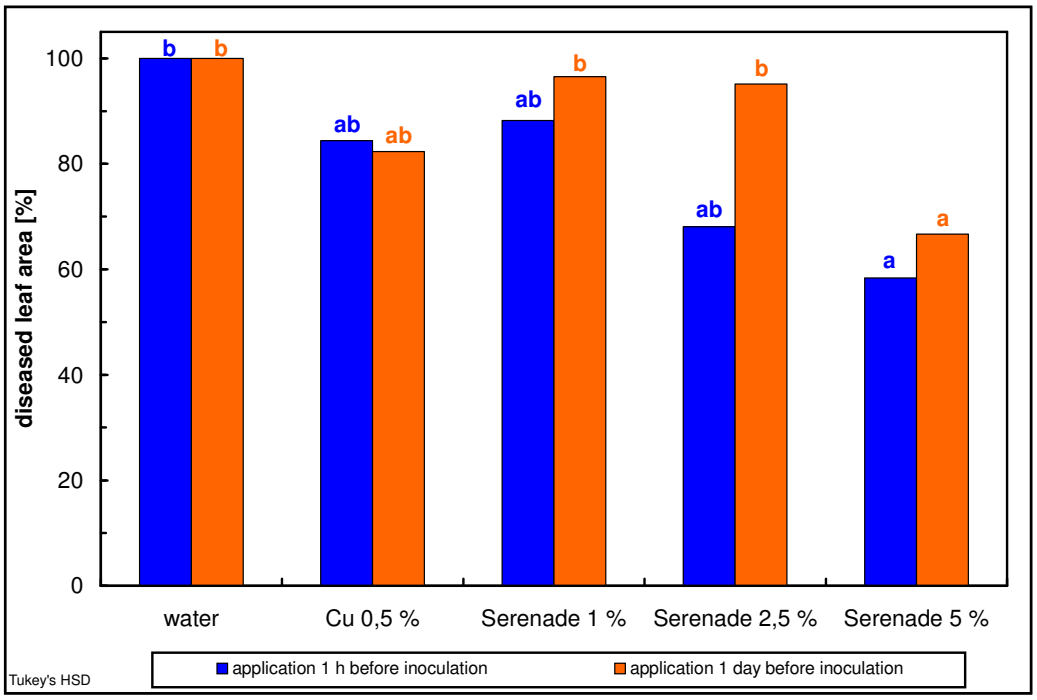


Fig. 3.2: Infection of potato leaves after treatment of potted plants with two *X. bovienii* isolates and different incubation periods in an open greenhouse. Inoculation with *P. infestans* and subsequent incubation of excised leaves were conducted under optimum condition for infection (see "Materials and methods" for details). water / Cu = controls, i. e. leaves were treated with water or Atempo® respectively.

Under the same adverse environmental conditions for the treatments (see above), also the commercial product Serenade® (based on *B. subtilis*) and extracts from *Rheum rhabarbarum* were less effective than in previous experiments under controlled conditions (Fig. 3.3). Nevertheless, at a concentration of 5 % Serenade® showed a significant effect against *P. infestans*, both when applied 1 hour and 1 day before inoculation of leaves with the pathogen (Fig. 3.3. A). Control of late blight by Serenade® was (slightly) better when the treatment was done very close to inoculation, which confirms results from previous experiments under controlled conditions, that the metabolites of the bacterium have a direct effect on *P. infestans*.
As in the case of Serenade®, extracts from *R. rhabarbarum* were only effective against late blight in the greenhouse tests at the highest concentration tested (5 %) (Fig. 3.3. B). A significant effect could only be recorded when the extract was applied 2 days before inoculation with *P. infestans* but not one day before inoculation (Fig. 3.3. B). This finding supports previous results, that the extracts from rhubarb have a protective, indirect effect against late blight.



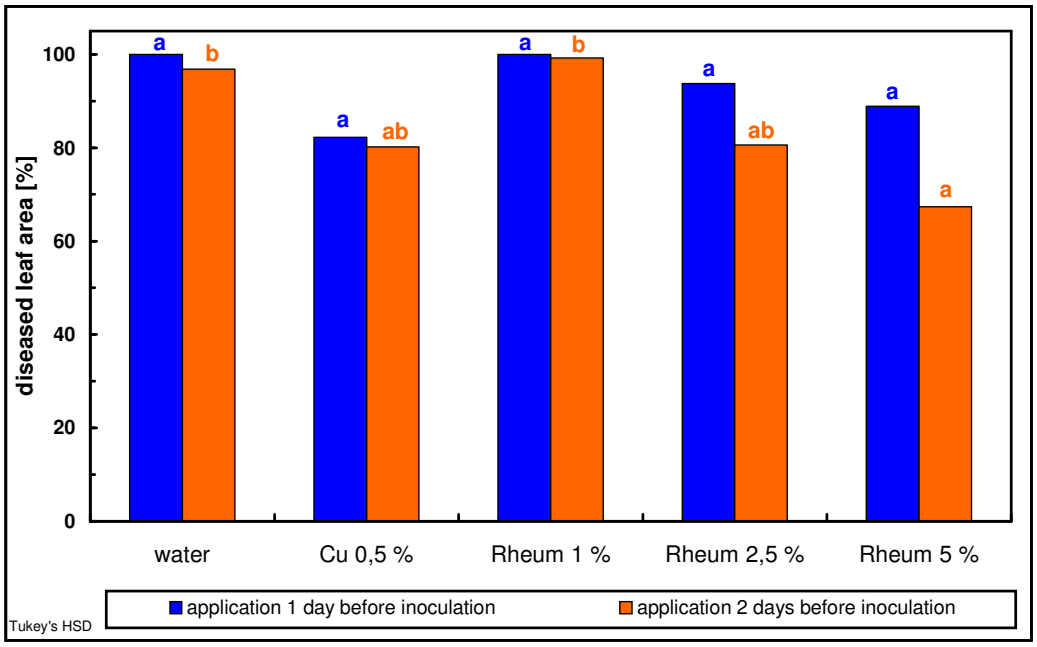


Fig. 3.3.: Infection of potato leaves by *P. infestans* after treatment of potted plants with 3 different concentrations of **A.** Serenade® and **B.** *R. rhabarbarum* extract in an open greenhouse. For every treatment and concentration two incubation periods each were tested. Inoculation with pathogen and subsequent incubation of excised leaves were conducted under optimal conditions for infection (see "Materials and methods" for details). water / Cu = controls, i. e. leaves were treated with water or Atempo® respectively.

The same experiments as shown in figure 3.2 (monitoring over 8 days) and figure 3.3 (3 concentrations at two application dates each) were conducted with the plant strengthener Kendal® (results not shown). However these experiments were all made with the wrong formulation (see section 1.4.3).

Concerning the poor performance of the chemical control Atempo® see end of next section.

Performance of different treatments under "field" conditions

Also in the experiments with potted plants on a field the infestation levels with *P. infestans* were overall relatively high for all treatments (including the copper control) (Fig. 3.4). This is very probably due to the fact that - similar to the experiments in the open greenhouse - the incubation of the applied products and the bacterium took place on the field under relatively difficult conditions (sunny during nearly the whole time, relatively high temperature) while inoculation of leaves with the pathogen and subsequent incubation was done under optimum conditions for infection by *P. infestans* (worst case scenario). However, both tested treatments, Serenade® and *X. bovienii*, were as effective as the chemical control.

As mentioned previously Serenade® is more effective against late blight when applicated close to inoculation with *P. infestans*. This was also the case in the "field" trial at the beginning of the experiment (Fig 3.4. A; 1st to 3rd day). Infestation was low at the days of application and relatively high at the second day, when Serenade® was not applied. At the next days of the experiment this effect seemed to be superposed by the repeated application: with leaves from the 4th to 6th day the infestation remained on a relatively low level and did not increase anymore at days when no application was done.
The increase of infestation towards the 8th day (Fig. 3.4. A) is probably due to a wash off of treatments from the leaves (heavy rainfall on 7th day) and/or because no further application was done at the 7th day.

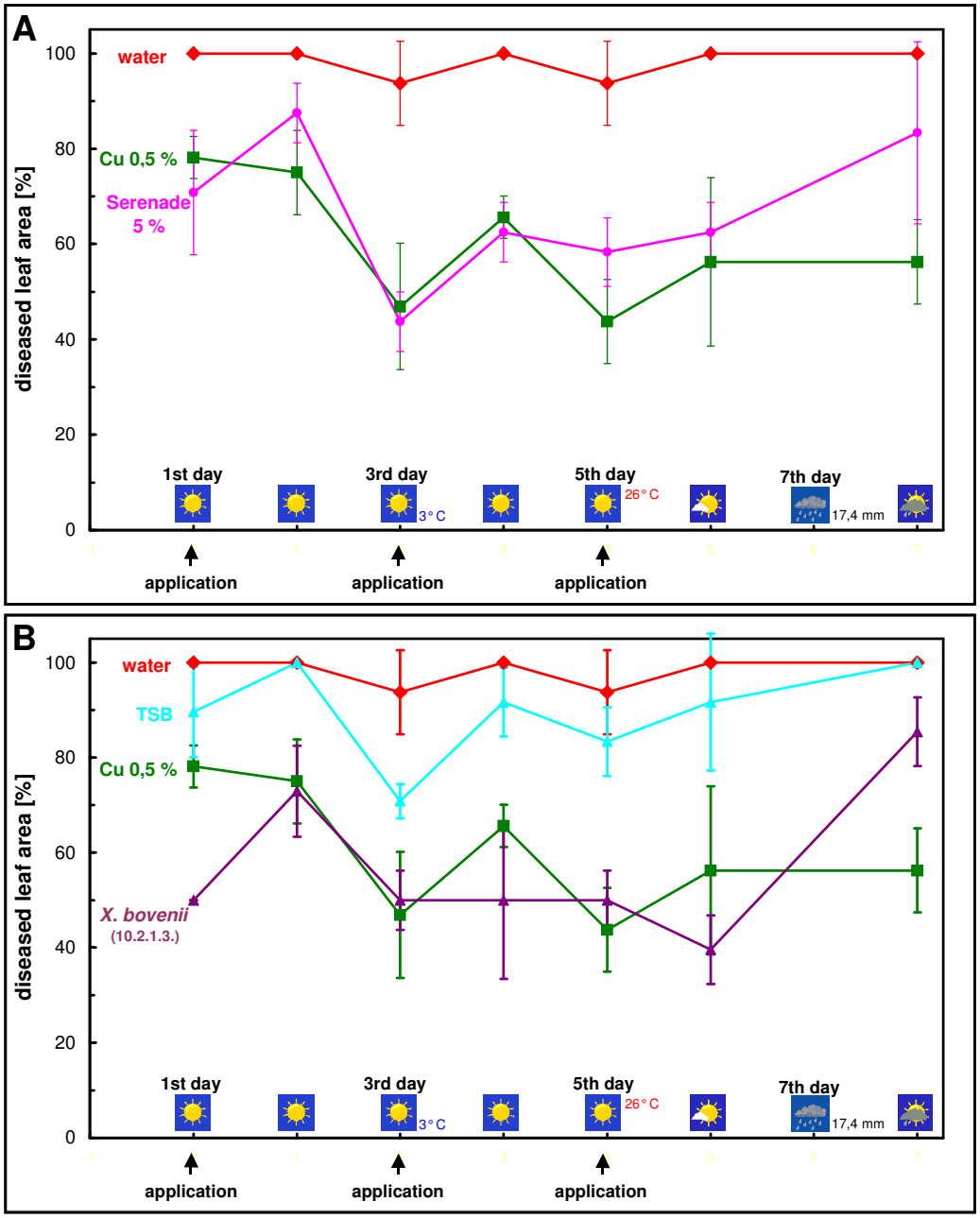

A
diseased leaf area [%]
100
80
60
40
20
0
water
Cu 0,5 %
Serenade
5 %
1st day
3rd day
5th day
7th day
3° C
26° C
17,4 mm
application
application
application
B
diseased leaf area [%]
water
TSB
Cu 0,5 %
X. bovenii
(10.2.1.3.)
1st day
3rd day
5th day
7th day
3° C
26° C
17,4 mm
application
application
application

Fig. 3.4: Efficiency of **A.** Serenade® and **B.** *Xenorhabdus bovienii* (isolate 10.2.1.3) against *Phytophthora infestans*. Treatments and subsequent incubation of potted plants were done on a field, inoculation of leaves and subsequent incubation under controlled conditions (see "Material and methods" for details). water / Cu = controls, i. e. leaves were treated with water or Atempo® respectively, TSB = pure culture medium as additional control for *X. bovienii*. NB: All results were obtained from one experiment, i. e. course of water and copper control are identical in figs. 3.4. A and 3.4. B. Graphs were only plotted seperately due to better clearness.

Similar results were found for plants treated repeatedly with *Xenorhabdus bovienii* (Fig. 3.4. B). At the first day of the monitoring (first application) infestation of leaves by *P. infestans* was quite low, but increased towards the second day (no application). But from the third day on infestation remained relatively low, even at days where no application was done.
Thus, especially for treatment with *X. bovienii* an accumulating effect of the treatments seems to play a role when repeated applications are carried out.
Leaves treated with pure TSB medium as a control partly showed a slight effect against late blight, which is already known (WOHLLEBEN, personal communication). But overall infestation levels were at all days clearly higher than after application of the bacterial culture.
As the effect of the bacterial culture (isolate 10.2.1.3) was good under the predominant conditions, the effective metabolites of the *X. bovienii* isolate are obviously also stable under exposure to solar radiation.
As in the case of Serenade® a relative strong increase of infestation towards the 8th day (Fig. 3.4. B) was also recorded for plants treated with *X. bovienii*. The reasons are probably the same as mentioned for Serenade® (see above).

The above described experiment, treatment of potted plants under field conditions (fig. 3.4), was also done with the plant strengthener Kendal®, but unfortunately with the wrong formulation. The results (not shown) again revealed a strong direct effect on *P. infestans*, probably due to phosphonates (see section 1.4.3).

The efficacy of the applied copper fungicide was unsatisfactory in all experiments described in this section and also in some of the experiments described in section 2. The reason for this phenomenon is not clear although attempts were made to clarify the problem (see section 1.4.2.). As described above (section 1.4.2.) no loss of efficacy of the Atempo® batch used in these experiments was responsible for its poor performance, as efficacy in control experiments was comparable to other chemical fungicides (Fig. 1.5). Therefore, Atempo® was also used in the greenhouse tests and in the "field" trial to evaluate the efficacy of alternative treatments.
As mentioned above, the experiments were - partly by chance - performed under worst case conditions: As in the case of the alternative treatments even the copper control is possibly not able to sufficiently protect the potato plants when on the one hand climatic conditions are very unfavourable for treatments and on the other hand inoculum pressure is high and environmental conditions are at optimum for infection.

Conclusions

1. Although on the one hand environmental conditions for treatments were very stringent and on the other hand conditions for infection by *P. infestans* were optimal, one MO (*Xenorhabdus bovienii*), one PE (*Rheum rhabarbarum*) and one CP (Serenade®) revealed remarkable effects against late blight.
2. Especially for *X. bovienii* and the *Rheum* extract experiments on supporting the treatments by stabilizing formulations, e. g. wetting agents, or a combination with other effective treatments could probably increase their efficacy.

ADDENDUM

- Supplementary*, In vitro* experiments on the mode of action of *X. bovienii* showed that metabolites emitted into the culture filtrate seem to be the effective components. Culture filtrates of both phase variants of *X. bovienii* strongly inhibited zoospore release of *P. infestans*. But only phase variant I also had a clear inhibiting influence on growth of *P. infestans* mycelium on rye agar. The additional influence of the culture filtrate of *X. bovienii* phase variant I on growth of *P. infestans* could be responsible for the higher efficay of this isolate in the excised leaf bioassay.

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Section 5: Consolidated Report on Alternative Treatments

Please refer to Chapter 7: Application and Formulation Technology, Section 3: Consolidated report on Alternative Treatments and Application & Formulation Technology which considers the range of both laboratory and experiments with related themes:

Chapter 5: Agronomic Strategies, Section 7: Foliar sprays and microbial soil inocula
Chapter 6: Alternative treatments, Section 1: Compost extracts: leaf biossay
Chapter 6: Alternative treatments Section 2: Identification of fungal/bacterial antagonists/plant extracts
Chapter 6: Alternative treatments Section 3: Identification of antifungal metabolite production/compatibility: leaf assays
Chapter 6: Alternative treatments Section 4: Optimisation of application frequency/timing/dose rate of microbial/plant extracts (glasshouse)
Chapter 7: Application and Formulation Technology, Section 1: Sprayer systems for Cu and novel products
Chapter 7: Application and Formulation Technology, Section 2: Improved formulation and comparison with existing anti-fungal treatments