Promotion of resistance of black alder clones (*Alnus glutinosa* (L.) Gaertn.) against *Phytophthora alni* ssp. *alni* by cyclolipopeptide producing bacteria

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Summary

This study demonstrated the promotion of the ability of black alder clones to resist to *Phytophthora alni* ssp. *alni* with the cyclolipopeptide (CLP)-producing *Pseudomonas veronii* isolate PAZ1. The bacterial strain, isolated from an association with *P. alni* ssp. *alni*, and characterised by its production of the secondary metabolites viscosin and massetolides, possessed inhibitory ability against isolates of the oomycete tree pathogen *in vitro*. In plant experiments with alder clones *in vitro*, the treatment with living bacteria showed an earlier start of the rooting process and a promoted root and shoot growth. Compared to non-treated plants, the root system was improved by longer primary roots with abundant secondary roots. The treatment with a methanol extract prepared from strain PAZ1 had no comparable effect. After protective application of strain PAZ1 14 days before the pathogen, the disease incidence was lower and biomass production was higher than in the pathogen control. These results were completed by a greenhouse experiment for a period of 13 months. Bacteria application prior to the pathogen led to reduced *Phytophthora* incidence at two of five clones in the combined treatment.

Key words: *in vitro*, massetolide, *Pseudomonas veronii* PAZ1, tissue culture, viscosin.

Introduction

For about two decades, the aggressive species *Phytophthora alni* (Oomycetes) has been known as a pathogen on alder trees (*Alnus* spp.). The fungus causes lethal root and root collar rot (BRASIER et al., 1995 and 1999). In the beginning of its appearance, the disease has spread very quickly over the European distribution areas of alder species and has recently been found in Alaska (ADAMS et al., 2008). In Germany, the pathogen is present in the most riparian and forest alder stands. Black alder (*A. glutinosa* (L.) Gaertn.) is most frequently affected.

The pathogen can be divided into three subspecies varying in their pathogenicity (IOOS et al., 2006). The subspecies *P. alni* ssp. *alni* is currently the most aggressive type. This type has established itself widely in the Northern German lowlands. The development and

degree of infection proceeds very differently and depends on site conditions, alder provenience, genotype, and tree age (GIBBS et al., 2003, SCHNECK and ZASPEL, 2006). In some localities very high losses have occurred while in others, the disease impact has been relatively weak (WEBBER et al., 2004). The causes of changes in the *P. alni* population and stagnating disease progress which was observed in some areas (CECH, 2001; GIBBS, 2003) are unclear. Specific antagonists could hitherto not be found.

In the course of preliminary investigations of the authors, strains of *P. alni* isolated from alders from natural sites were occasionally associated with specific bacteria which were detectable only on nitrogen-rich media but not on poor media like potato dextrose or oatmeal. The bacteria showed a broad resistance to standard antibiotics. One strain of these accompanying bacteria could be isolated from the hyphal surface oomycete mycelia and was identified as a member of the genus *Pseudomonas* named *Pseudomonas veronii* PAZ1. Although this strain came from a *P. alni* isolate, as a single organism the bacteria developed antagonistic activity *in vitro* against some *P. alni* strains and other fungal pathogens (KRÜGER, 2008).

Bacteria of the genus *Pseudomonas* have been described extensively in the literature as inhibitors of a wide range of plant pathogens and promoters of plant growth (WHIPPS, 2001; HAAS and DEFAGO, 2005; GANESHAN and KUMAR, 2005; HÖFTE and ALTIER, 2010). Furthermore, some species of *Pseudomonas* are involved in bacterial communities of fungal surfaces and frequent inhabitants of mycorrhiza formers, fungal pathogens and saprotrophs (DE BOER et al., 2005; NAZIR et al., 2010).

The investigation of secondary metabolite formation of *P. veronii* PAZ1 by methods of liquid chromatography and mass spectrometry as well as Nuclear Magnetic Resonance (NMR) revealed a range of cyclic lipopeptides (CLPs), among others the main CLP viscosin and isomers of massetolides (KRÜGER, 2008). The CLP compounds produced by micro-organisms such as *Pseudomonas* species may serve different and multiple purposes, for example play a role in antagonistic interactions with other organisms and share functions like antimicrobial activity, biofilm formation, and motility (LAYCOCK et al., 1991; NIELSEN, 2002; DE SOUZA et al., 2003; RAALJMAKERS et al., 2006 and 2010). The massetolide A-producing strain *P. fluorescens* SS101 was effective in preventing infection of tomatoes by *Phytophthora infestans* leading to a significant reduction in the expansion of late blight (TRAN et al., 2007). Former studies had shown that surfactant-producing *Pseudomonas* iso-

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lates possessed lytic activity against zoospores of multiple oomycete pathogens like *Pythium* and *Phytophthora* species (DE SOUZA et al., 2003).

In the present work, results of the effect of the CLP-producing strain *Pseudomonas veronii* PAZ1 on the growth of black alder clone plants as well as on disease development after co-inoculation with a pathogenic *P. alni* ssp. *alni* strain are demonstrated.

Material and Methods

The investigation was carried out in two steps, in axenic culture *in vitro* and *in vivo* under greenhouse conditions.

Establishing and propagation of alder clone plants

Black alder seeds were harvested from trees with differently pronounced disease symptoms growing in the marshland region of the river Oder. Seed batches No. 4 and 8 derived from outwardly healthy trees, whereas No. 5 and 12 were picked from strongly affected ones. The seeds were sown in the greenhouse, and after 6 to 12 months the seedlings were cut up to shoot tips and nodal segments. After surface disinfection with 0.25% mercury chloride and subsequent rinsing in deionised water, the explants were transferred to tissue culture conditions. The clone establishment was successful on modified Woody Plant Medium (WPM, Duchefa Biochemie BV, The Netherlands, according to LLOYD and MCCOWN, 1980) supplemented with 3.5 µM zeatin as growth regulator and solidified by 0.8% Phytoagar in Erlenmeyer flasks (100 ml) were filled with 25 ml nutrient medium and covered with aluminium foil before autoclaving at 121°C (pressure: 105 kPa) for 20 min. Shoot cultures were grown at 20 to 23°C with an irradiance of 27.5 to 28 µmol m⁻² s⁻¹ supplied by warm-white fluorescent tubes (day:night = 16:8 hours). Once per month, elongated shoots were cut up and shoot tips and nodal segments were transferred to fresh nutrient medium. For production of complete plants, 15–20 mm-long shoots were rooted on WPM with half concentrated macro and micro nutrients, 0.49 µM IBA and 0.27 µM NAA. After two weeks, rooted plantlets were transferred to commercial propagation soil containing peat, perlite, clay and a low amount of fertiliser (“Einheitserde, Typ VM”) and were grown under high humidity in the greenhouse (plastic tunnels with mist irrigation). After successive hardening, the black alder clone plants were ready for inoculation experiments in the greenhouse.

Propagation of *P. alni*

Isolates of *P. alni* ssp. *alni*, obtained from damaged trees of alder stands in Eastern Germany (marshland region of river “Oder”) in 2007–2008, were grown on carrot agar (CA: carrot pieces 5%, agar-agar 1.5% per 1000 ml deionized water, pH 6.0) at 20°C for 20 days. For this study, the strains AL5, WU1, and WU7 from three different trees of two separate stands were used.

Propagation of *Pseudomonas veronii* PAZ1

The bacteria strain, originally isolated from hyphae of a *P. alni* ssp. *alni* culture in 2008, designated to

Pseudomonas veronii PAZ1, was grown on M1 agar (malt extract 1%, yeast extract 0.4%, glucose 0.4%, agar-agar 1.5%, pH 7.0) for 6 to 7 days. Bacterial suspensions for plant treatment were prepared with cells scraped from plates and adjusted with sterile tap water to 10⁸ cells per ml.

The ITS1/4 region of 16S rDNA of the strain *Pseudomonas veronii* PAZ1 used in this work, was sequenced (GATC Konstanz, Germany) and the resulting sequence with a total length of 1392 bp was deposited in GenBank under the accession number KP858504.

Extraction of secondary metabolites from PAZ1

Pseudomonas veronii PAZ1 was grown on M1 agar for 10 days. Afterwards, an extraction with methanol followed, including a threefold ultrasonic treatment, each for 30 minutes. The methanol was removed with a rotary evaporator and the mixture was dried *via* lyophilisation. The metabolites were resolved with methanol and dried after centrifugation. For a complete extraction, the residue was extracted with ethyl acetate, dried and merged with the methanol extract. For separation of the CLP fraction, the dried methanol extract was first dissolved in 10 ml H₂O and applied on two solid phase extraction cartridges (Oasis HLB, Waters). The residue was dissolved in 5 ml methanol, diluted 3:1 with H₂O and also applied. The CLP fraction was eluted with pure methanol.

Plant experiments under axenic conditions

Glass tubes (95 mm long with 28 mm diameter) were filled with 7.5 ml of modified half-concentrated WPM and closed with aluminium foil. A part of the water for medium formulation (20%) was substituted by carrot water which was prepared by cooking 50 g carrot pieces in 200 ml water for 20 min. After removing the carrot pieces, the liquid was used to fill up the medium to one litre. As gelling agent, 1.2% Phytoagar was used. Alder shoots from shoot propagation *in vitro* with a length of 10 to 20 mm were transplanted into tubes, and cultivated at 20°C for 8 weeks under the light conditions described above for tissue culture.

Pathogenicity experiment 1 *in vitro*

The influence of *P. alni* and of living *Pseudomonas veronii* PAZ1, as well as its secondary metabolite extract, applied solely and in combination on growth and vitality of alder clone plants was examined.

Inoculation of alder shoots with the pathogen in axenic culture was done by placing a small agar plug (2 x 2 mm) overgrown with mycelium of the isolate AL5 directly beside the shoot base of shoot tips harvested from tissue culture shortly before. For inoculation of axenically growing plants, the shoots of the latter were dipped into the suspension (preparation see above) before transfer to the test medium.

Enrichment of plant medium by metabolite extract was applied by adding 3 ml/L to the warm CA medium (48 to 50°C). One control medium was prepared with the same amount of methanol added to the warm agar for the comparison of secondary metabolite extract of

bacteria and one control with untreated shoots was included. The co-inoculation with the pathogen was performed at the same time. Four alder clones were tested (12/55, 8/65, 5/7, and 4/5). The first number of the clone label is referring to the mother tree the seeds were harvested from. Each variant contained 25 ramets.

Pathogenicity experiment 2 *in vitro*

Shoots from three alder clones (8/65, 5/7, and 4/5) were co-cultivated with living bacteria of *P. veronii* PAZ1 on CA medium for two time periods (7 days and 14 days, respectively) before inoculation with the pathogen using

P. alni agar plugs was executed as described above. Each treatment respectively control variant comprised 25 ramets.

The total observation time was eight weeks for both *in vitro* experiments.

Root development and disease symptoms were evaluated after four weeks (experiment 1) and three weeks (experiment 2), respectively for the first time, and then weekly. Root formation was evaluated by a 5-class scale (1 = no roots, 2 = roots not longer than 2 mm, 3 = roots not longer than 10 mm, 4 = formation of primary roots longer than 10 mm, 5 = formation of primary roots

Table 1. – Experiment 1: Fresh weight (FW) of shoots and roots and shoot development of alder clones *in vitro* after 8 weeks cultivation time following treatments with *Pseudomonas veronii* PAZ1, *Phytophthora alni*, methanolic metabolite extract of *P. veronii* PAZ1 alone or in combination with *P. alni* and influence of the treatments on rooting quality (values indicated with the same letter are not significantly different according to the Tukey-test, $p < 0.05$).

Clone	Treatment No.	Treatment	Shoot FW (mg)	Root FW (mg)	Shoot length (mm)	No. of plants with long main roots (score 4-5; n=25)
12/55	1	Control (untreated)	59 b	42 ab	14.8 bcd	10
	2	Control (Methanol)	59 b	61 a	14.1 cd	21
	3	<i>Pseudomonas</i> PAZ1	75 a	63 a	15.8 abc	18
	4	<i>P. alni</i>	27 c	0 c	13.2 d	0
	5	<i>Pseudomonas</i> PAZ1 extract	55 b	21 bc	17.1 a	9
	6	<i>Pseudomonas</i> PAZ1 extract + <i>P. alni</i>	31 c	0 c	16.6 ab	0
8/65	1	Control (untreated)	52 b	14 b	17.9 c	14
	2	Control (Methanol)	39 c	39 c	19.2 bc	0
	3	<i>Pseudomonas</i> PAZ1	72 a	51 a	22.8 a	16
	4	<i>P. alni</i>	25 d	0 c	21.4 ab	0
	5	<i>Pseudomonas</i> PAZ1 extract	52 b	6 c	20.6 ab	0
	6	<i>Pseudomonas</i> PAZ1 extract + <i>P. alni</i>	29 d	0 c	21.8 ab	0
5/7	1	Control (untreated)	59 b	7 b	17.5 bc	10
	2	Control (Methanol)	58 b	4 b	17.6 bc	6
	3	<i>Pseudomonas</i> PAZ1	100 a	57 a	20.3 a	21
	4	<i>P. alni</i>	35 b	0 b	17.0 c	0
	5	<i>Pseudomonas</i> PAZ1 extract	65 b	7 b	19.3 ab	6
	6	<i>Pseudomonas</i> PAZ1 extract + <i>P. alni</i>	34 b	0 b	18.8 abc	0
4/5	1	Control (untreated)	50 c	11 bc	14.4 c	7
	2	Control (Methanol)	62 b	9 bc	16.0 bc	18
	3	<i>Pseudomonas</i> PAZ1	90 a	73 a	17.7 ab	25
	4	<i>P. alni</i>	37 d	0 c	19.7 a	0
	5	<i>Pseudomonas</i> PAZ1 extract	62 b	13 b	19.9 a	16
	6	<i>Pseudomonas</i> PAZ1 extract + <i>P. alni</i>	39 d	0 c	18.4 ab	0

Table 2. – Experiment 1: Comparison of the distribution of rooting scores of alder clones after 8 weeks cultivation time; data indicating p-values of Wilcoxon rank sum test; for treatments see Table 1.

Clone	Amount of rooted plants			
	Comparison between treatments			
	1 vs. 3	1 vs. 4	2 vs. 5	2 vs. 6
12/55	0.0434	<0.0001	0.0002	<0.0001
8/65	<0.0001	<0.0001	0.3773	0.0002
5/7	0.2822	<0.0001	0.8489	<0.0001
4/5	0.0063	<0.0001	0.5845	<0.0001

longer than 10 mm with secondary roots and partly formation of dark root cortex). The damage caused by *P. alni* was evaluated using a 4-class scale (1 = dead shoots or leaves with severe necroses, 2 = brown discolorations and necroses on most leaves, 3 = dark discolorations and small necroses, 4 = green plants). After eight weeks, shoot length and fresh weight of shoots and roots were assessed.

Plant experiment in the greenhouse

The effect of *P. veronii* PAZ1 alone and together with *P. alni* was studied in a greenhouse experiment with potted alder clone plants. Rooted plants of the five clones 4/12, 4/60, 8/25, 8/65, and 12/55 were transferred from tissue culture to single pots with 350 g soil (commercial growth substrate mixed with sand, 4:1) in May 2009. After cultivation under high humidity for 2 weeks, they were gradually hardened. Seventeen days after transfer of plantlets from tissue culture, bacteria suspension was prepared as described above. Two ml of a suspension of 10^9 cells ml⁻¹ were applied around the shoot base.

The application of *P. alni* by zoospores was carried out in August 2009. The preparation of inoculum suspension was carried out using a zoospore mixture of three isolates AL5, WU1 and WU7. The overgrown plates (CA) were covered with tap water which was exchanged daily. Three leaves of alder plants from tissue culture were added per plate. Sporangia with motile zoospores were produced at 15°C after 6 to 8 days. Because the sporangia production of the three used *P. alni* strains was approximately equal, the zoospore suspension was mixed in the same ratio and was adjusted to 10^6 spores

per ml. Three ml were applied directly to the shoot base of alder plants.

A total of 30 replications was used for each of both treatments. The plants were cultivated in the greenhouse for 13 months. Pots treated by *P. alni* were separated from the pots without pathogen treatment to prevent the transfer of zoospores by moisture droplets. Temperature and light conditions followed the natural course as closely as possible. During winter months, the greenhouse temperature was kept above zero. At the end of September 2009, shoot length was measured for the first time. The experiment was finished in June 2010 with an assessment of growth traits (shoot length, weight of shoot and root system) and an evaluation of disease incidence which was scored visually by counting the number of plants with necrotic lesions on shoots caused by *P. alni*.

Statistics

All data resulting from plant growth experiments were analysed statistically using the SAS 9.2 software (SAS Institute Cary, NC, USA). Interactions between treatments, plant weight and shoot length were analysed by ANOVA followed by Tukey test with $P < 0.05$. A comparison of rooting frequencies and extent of disease incidence between the samples were tested by Wilcoxon rank sum test at the threshold of $P = 0.05$.

Results

Axenic plant experiments

Results of tests under *in vitro* conditions showed distinct differences of shoot and root growth between the

Table 3. – Experiment 2: Fresh weight (FW) of shoots and roots and shoot length of alder clones *in vitro* after 8 weeks cultivation time following treatments with *Pseudomonas veronii* PAZ1 alone and in combination with *Phytophthora alni* at two inoculation dates and influence of the treatments on rooting and disease severity (values indicated with the same letter are not significantly different according to the Tukey-test, $p < 0.05$).

Clone	Treatment No.	Treatment	Shoot FW (mg)	Root FW (mg)	Shoot length (mm)	No. of plants with long main roots (score 4-5; n=25)	No. of plants with severe symptoms on shoots (score 1-2; n=25)
8/65	1	Control (untreated)	92 a	16 b	20.9 a	25	-
	2	<i>Pseudomonas</i> PAZ1	98 a	22 a	18.9 a	23	-
	3	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i> added 7 days after bacteria inoculation	43 c	10 c	19.2 a	13	22
	4	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i> added 14 days after bacteria inoculation	71 b	15 b	18.9 a	20	12
5/7	1	Control (untreated)	81 b	9 c	17.9 c	16	-
	2	<i>Pseudomonas</i> PAZ1	119 a	19 a	22.8 a	25	-
	3	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i> added 7 days after bacteria inoculation	64 b	12 bc	21.2 ab	25	18
	4	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i> added 14 days after bacteria inoculation	79 b	16 ab	19.7 bc	25	15
4/5	1	Control (untreated)	88 b	13 b	19.7 bc	24	-
	2	<i>Pseudomonas</i> PAZ1	135 a	36 a	22.1 a	25	-
	3	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i> added 7 days after bacteria inoculation	59 c	13 b	18.0 c	24	19
	4	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i> added 14 days after bacteria inoculation	86 b	20 b	21.5 ab	24	10

Table 4. – Experiment 2: Comparison of rooting frequencies and of severe disease symptoms (score 1-2) on shoots after 8 weeks cultivation time; data indicating p-values of Wilcoxon rank sum test; for treatments see Table 2.

Clone	Amount of rooted plants						Severe symptoms of <i>P. alni</i> on shoots
	Comparison between treatments						
	1 vs. 2	1 vs. 3	1 vs. 4	2 vs. 3	2 vs. 4	3 vs. 4	
8/65	0.0514	<0.0001	0.0184	0.0013	0.6153	0.0107	<0.0001
5/7	0.0001	0.0010	<0.0001	0.2743	0.3020	0.0437	0.2404
4/5	0.0296	0.0058	0.0131	0.0044	0.0771	0.0886	0.0179

alder clones and the effects of clone, treatment and clone x treatment interaction were statistically significant ($p < 0.001$). The best shoot growth was observed for clone 5/7 (Experiment 1) and 4/5 (Experiment 2), respectively, and the best root growth for clone 12/55 (Experiment 1) and 4/5 (Experiment 2), respectively (Tables 1 to 4).

The treatment of non-rooted shoots with living bacteria of *P. veronii* PAZ1 resulted in increased root and shoot growth in the majority of clones. The clone 12/55 possessed a high spontaneous rooting ability but the rooting was further supported by the bacteria treatment. The shoot length was forced as well for some clones. Compared to untreated control plants, the root systems had significantly higher fresh weights with a higher portion of long primary roots with abundant secondary roots. This effect was confirmed in the two independent experiments *in vitro*.

The treatment with bacteria initiated an earlier start of root formation which could be observed clearly in both experiments (Figure 1 and 2). The development of long main roots was completed 2 to 3 weeks earlier than at control plants. Addition of the secondary metabolite extract of PAZ1 to the growth medium (Experiment 1) had no effect on shoot biomass formation. It caused a weak inhibition of root formation, whereby a weak increase of shoot length was recorded (Table 1). The application of the pathogen alone prevented the rooting completely. Most plantlets died or developed necroses on shoots with sporangia formation. The combination of *Pseudomonas* extract with *P. alni* did not prevent the harmful influence of the pathogen on alder shoots.

In contrast, the results of the treatment with living bacteria and time-delayed *P. alni* inoculation indicated that *P. veronii* PAZ1 was able to partly or completely

Figure 1. – Experiment 1: Rooting course of plants from four alder clones after treatment with living *Pseudomonas veronii* PAZ1 or with extract of the bacteria during 7 weeks (number of plants with scored root structure from 4–5).

Figure 2. – Experiment 2: Rooting course of three alder clones after treatment with living *Pseudomonas veronii* PAZ1 alone and in combination with *P. alni* during 8 weeks (number of plants with scored root structure from 4–5).

Table 5. – Number of alder clone plants with disease symptoms caused by *Phytophthora alni* in the greenhouse experiment after 13 months: Comparison of treatments by *P. alni* alone and in combination with *Pseudomonas veronii* PAZ1 (n=30).

Clone	Treatment	No. of living plants	No. of plants with disease symptoms	Significance (Wilcoxon rank sum test, p-value)
12/55	<i>P. alni</i>	30	20	0.1942
	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i>	30	15	
4/12	<i>P. alni</i>	27	20	0.0007
	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i>	26	7	
4/60	<i>P. alni</i>	25	18	<0.0001
	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i>	30	7	
8/25	<i>P. alni</i>	25	14	0.8446
	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i>	30	16	
8/65	<i>P. alni</i>	30	11	0.5870
	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i>	30	9	
Total of all clones	<i>P. alni</i>	137	83	<0.0001
	<i>Pseudomonas</i> PAZ1 + <i>P. alni</i>	146	49	

compensate the harmful effects of the pathogen on biomass production and number of diseased alder plants with severe symptoms (Tables 3 and 4). The effect of the treatment was significant at two of the three tested clones only, when the bacteria were applied two weeks before the pathogen.

Greenhouse experiment

As already was noted in the axenic plant experiments, significant growth differences of shoot biomass and length between alder genotypes were also determined in the greenhouse experiment. Compared to the most vigorous clone 8/65, the clone 4/60 showed a below average growth, and possessed only 61% of shoot length and 37% of shoot biomass after the cultivation period of one year.

In contrast to the experiments *in vitro*, differences of shoot length and biomass between the treatments could not be observed under non-sterile conditions in soil, neither after 16 weeks, nor after 13 months (data not

shown). However, after the combined inoculation with bacteria and *P. alni* in the greenhouse, the number of plants with necrotic lesions on shoots was significantly reduced in two of five alder clones, also in the total number of alder clone plants (Table 5).

Discussion

This work presents results on the effect of a selected bacteria strain on growth and susceptibility against the serious oomycete pathogen *P. alni* on alder clones. The micropropagation technique allowed fast preparation of black alder clone material to evaluate the plant response in inoculation tests with the strain *P. veronii* PAZ1 originally living in association with the pathogen. The strain attracted attention because of its production of secondary metabolites, e.g. CLPs, with known and unknown structures (KRÜGER, 2008), which had shown an antibiotic activity against strains of the fungus-like pathogen *P. alni* ssp. *alni* *in vitro* (data not shown).

CLP-producing *Pseudomonas* strains were found in many natural habitats, water reservoirs, plant cultivation systems, and also in and on fungal hyphae (RAAIJMAKERS, 2010; MUSCH and ALATOSSAVA, 2002). The frequency of CLP-producing strains compared to the total *Pseudomonas* population is strongly alternating and amounted, for example, in Dutch agricultural soils to 1–5% (DE SOUZA et al., 2003). The functions of CLPs in natural habitats have been described as antagonistic towards other microorganisms and beneficial for the mobility to new niches (RAAIJMAKERS et al., 2006).

The results of the present study indicate that the bacterial strain *P. veronii* PAZ1, when applied to alder clone plants, operated in two directions. The plant tests under axenic conditions demonstrated a clear growth-promoting effect by the living bacteria, especially an increased formation of root biomass followed by enhanced shoot growth. A growth-promoting effect by the secondary metabolite fraction extracted from cultures of *Pseudomonas* strain PAZ1 was not detected. The stimulating effect of strain PAZ1 on alder growth may be caused by phytohormone production (IAA, cytokinines, GA) and improved plant nutrition (solubilization of phosphates, associative nitrogen fixation, increased iron uptake). These are two important mechanisms by which plant growth promoting *Pseudomonas* strains can directly affect the plant development (PODILE and KISHORE, 2006). However, in complex environments under non-sterile soil conditions, it seems very difficult to ensure the growth promoting activity of inoculated bacteria over longer periods. In contrast to the experiments *in vitro*, it cannot be stated that the inoculation with *P. veronii* PAZ1 had a measurable impact on growth of alder clone plants in the greenhouse experiment.

Nevertheless, the bacteria had a suppressive effect against *P. alni* on alder clones *in vitro* and *in vivo* if they were given time advantage. This head-start seems to be necessary for the successful propagation of the inoculant and for colonizing the plant. The effect of disease suppression was still apparent after one year under semi-controlled greenhouse conditions. The observed effects are obviously, besides other factors, related to the production of viscosin by strain PAZ1. Viscosin is a CLP produced by pseudomonads. It possesses biosurfactant-like and antimicrobial properties, and has been successfully tested against the root pathogenic microorganisms *Pythium ultimum*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (NIELSEN et al., 1999; ANDERSEN et al., 2003; LE et al., 2012). Moreover, a direct action of viscosin against oomycetes was detected by inhibition of zoospores motility and promoted lysis (DE BRUIJN et al., 2007). Biosurfactants play a role in the distribution of microorganisms on plant surfaces as well as in plants by modifying the motility and ability for biofilm formation of the microorganisms. *Pseudomonas*-derived surfactants can contribute to biological activity on plant and root surfaces by promoting the availability and uptake of nutrients and microbial metabolites. Antibiotic activity of biosurfactants against pathogenic microbes may have an impact on inter- and intraspecific competition during plant colonization, self-defence and on pathogenesis (D'EAS et al., 2010).

Another metabolite from lipopeptide producing members of *Pseudomonas* with confirmed antibiotic activity is massetolide A as it was reported by RAAIJMAKERS et al. (2010). Compounds related to massetolide A, such as the isomers of massetolide E, G, and H, have been found during the investigations of KRÜGER (2008) in *P. veronii* PAZ1. A massetolide A-producing strain, as well as the purified compound, inhibited *P. infestans* on tomato plants which not only prevented infection of leaves, but also significantly reduced the expansion of existing infections as well as the formation of sporangia (TRAN et al., 2007). It was also shown that a massetolide A – deficient mutant had a weak suppressive effect against the target pathogen. This indicated an increased systemic resistance of tomato plants elicited by the applied bacteria. Further studies showed a concentration-dependent direct impact of massetolide A on oomycete pathogens. The effects included encystment of zoospores and autoaggregation at low concentrations and disruption of membranes of the zoospores within a few minutes at higher concentrations (DE SOUZA et al., 2003; VAN DE MORTEL et al., 2009). The massetolides A to H have been detected in marine members of *Pseudomonas* which exhibited *in vitro* antimicrobial activity against species of *Mycobacterium* (GERARD et al., 1997). It can be supposed that the massetolides E, G, and H from *P. veronii* PAZ1 detected by KRÜGER (2008) also possess an inhibitory activity against fungi and oomycetes and, together with the antibiotically active main CLP viscosin, operate similarly as described for massetolide A. Further studies are necessary to investigate the specific properties of the identified single CLPs, especially those from the massetolides E, G, and H, their antibiotic activity, and their influence on the interaction between the bacteria and pathogenic strains of *P. alni* on alder plants.

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