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Control of seed-borne pathogens on legumes by microbial and other alternative seed treatments

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Abstract Greenhouse trials were carried out in order to test the efficacy of different seed treatments as alternatives to chemicals against *Colletotrichum lindemuthianum* cause of anthracnose on bean and *Ascochyta* spp. cause of Ascochyta blights on pea, respectively. Resistance inducers, commercially formulated microorganisms, non-formulated selected strains of different microorganisms (fungi, bacteria and yeasts) and plant extracts were applied as dry or liquid seed treatments on naturally infested seeds. Seedling emergence and disease incidence and/or severity were recorded. Almost all seed treatments turned out to be ineffective in controlling the Ascochyta infections, which is in line with the literature stating that these pathogens are difficult to control. The only alternative treatments that gave some control of *Ascochyta* spp. were thyme oil and a strain of *Clonostachys rosea*. The resistance inducers tested successfully controlled infections of bean by *C. lindemuthianum*. Among the formulated microorganisms, *Bacillus subtilis*-based formulations provided the best protection from anthracnose. Some strains of *Pseudomonas putida*, a disease-suppressive, saprophytic strain of *Fusarium oxysporum* and the mustard powder-based product Tillecur also proved to be effective against bean anthracnose. However, among the resistance inducers as well as among the other groups, certain agents caused a significant reduction of plant emergence. Different alternative seed treatments can therefore be used for the control of *C. lindemuthianum* on bean, while on pea only thyme oil and a strain of *Clonostachys rosea* showed some effectiveness against *Ascochyta* spp.

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Introduction

Bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) are two of the most valuable and broadly cultivated grain legumes in the world. Overall, bean is grown on $34{,}371 \times 10^3$ ha, with a harvested yield of $22{,}660 \times 10^6$ Kg, while pea covers a surface of $6{,}606 \times 10^3$ ha, with a harvested yield of $11{,}286 \times 10^6$ Kg (FAOSTAT 2005). Among the pathogens affecting these species, a major threat is represented by *Colletotrichum lindemuthianum* causing anthracnose on bean (Fig. 1) and by *Ascochyta pisi*, *Mycosphaerella pinodes* (anamorph *Ascochyta pinodes*) and *Phoma medicaginis* var. *pinodella* (in the following commonly referred to as '*Ascochyta* spp.') causing Ascochyta blights on pea (Fig. 2) (Tivoli and Banniza 2007). *Ascochyta* spp. and *C. lindemuthianum* are generally considered to be seed-borne pathogens, and infected seed can be the most important source of inoculum for long distance spread. Infection of seedlings by *C. lindemuthianum* may be both through the seed coat and the cotyledons. *Ascochyta* species penetrate into the inner parts of the seed where they may survive for several years (Tivoli and Banniza 2007). Infections of both pathogens are favoured by high relative humidity and optimal temperature range between 13 and 26°C for *C. lindemuthianum* and between 5°C and 35°C for *Ascochyta* spp.

Fig. 1 Symptoms of anthracnose on a bean seedling resulting from seed-borne infection with *C. lindemuthianum*

Fig. 2 Lesions on pea cotyledons and seedlings caused by seed-borne infection with *Ascochyta* spp.

When fields are grown with bean seeds infested by *C. lindemuthianum*, the yield is reduced because of poor seed germination and seedling vigour. Infected seeds have a lower market value, because their seed spots and blemishes lower the quality rating. Anthracnose is most common and severe on dry bean and snap bean (*P. vulgaris*), but it may also affect lima bean (*P. lunatus*), scarlet runner bean (*P. multiflorus*), mung bean (*P. aureus*), cowpea (*Vigna sinensis*), and broad bean (*Vicia faba*) (Hagedorn and Inglis 1986). Yield losses due to the use of *C. lindemuthianum*-infested seeds ranging from 15% to 32% have been reported from Canada (Conner et al. 2004). Infections of seeds by *Ascochyta* spp. result in slightly depressed, tan lesions with distinctly darkened borders; on leaves and pods the lesions are circular, whereas they elongate on the stem. Infections resulting from diseased seeds are best avoided by using pathogen-free or treated seed. At present, large quantities of seeds are routinely treated with chemical crop protection agents. Certain limitations and environmental disadvantages which have been associated with the use of chemicals as well as the uncertainties about the future availability of fungicides for minor uses (Gullino and Kuijpers 1994) call for the development of alternative methods for seed treatment. However, the present study was also initiated in view of the situation in organic farming where few

alternative seed treatments are available, especially in the horticultural sector. In EEC regulation 2092/91 the European Union states that the propagation material used in organic agriculture should also be produced according to organic principles. In order to overcome the frequent shortage of organically-produced propagation material, a temporary derogation system was introduced under which the use of conventionally produced propagation material was allowed also in organic farming. In order to make this derogation period as short as possible, new seed sanitation treatments as alternatives for the present use of fungicides are required (Groot et al. 2004).

Compared to cereals, much less testing of alternative seed treatments has been done with vegetables (Koch and Schmitt 2006). A number of bacteria, fungi and yeasts have been reported to have potential for use as biocontrol agents (BCAs) in vegetables (Punja 1997), and some are commercially available (Punja and Utkhede 2003). In general seed treatment is regarded as an efficient method of delivery of microbes. In vegetables, however, to which legumes belong, most field studies with seed application of bacteria or fungi have been conducted to control soilborne pathogens (e.g. Keinath et al. 2000; Xue 2003), and not seedborne pathogens.

Likewise, the majority of experiments dealing with induced plant resistance have been aimed at control of foliar pathogens, and the various inducers employed were in most cases applied to the foliage. There are a few reports describing application of chemical resistance inducers or resistance-inducing rhizobacteria to seeds, but with the aim to control soilborne or airborne pathogens (Benhamou et al. 1994; Elbadry et al. 2006). To our knowledge there are no systematic studies relating to the potential use of resistance-inducing agents against seed-borne pathogens.

The present study was undertaken in order to evaluate the efficacy of different alternative treatments for control of seed-borne infections of bean and pea by *C. lindemuthianum* and *Ascochyta* spp., respectively. The treatments included commercialised and experimental BCAs with documented ability to control diseases in non-vegetable crops, and plant-derived substances and agents known to function as resistance inducers or to have a role in the biochemical events leading to induced resistance.

Materials and methods

Assessment of seed infection

The experiments were performed with one seed lot of bush beans (*P. vulgaris* cv. Hildora) and one of peas (*P. sativum* cv. Jutta). Determination of the degree of seed infection was carried out according to ISTA rules (International Seed Testing Association 2002). Infestation of the bean seed was determined after incubation of surface-sterilised seeds (10 min in sodium hypochlorite, 1% active chlorine) for 7 days at 20°C on potato-dextrose agar (PDA) amended with 0.5 gl^{-1} of ox gall (personal communication by N. Leist, Landwirtschaftliches Technologiezentrum Karlsruhe—Augustenberg).

The degree of infection of the pea seed lot was assessed by the following procedure: 420 seeds were immersed for 10 min in sodium hypochlorite (1% free chlorine), washed with tap water and placed on PDA medium (Merck, Darmstadt, Germany) supplemented with streptomycin (50 mg^{-1}) and rifampicin (10 mg^{-1}) in 14.5 cm Petri dishes at 15 seeds per plate. After 7 days of incubation at 20°C in darkness followed by 4 days under near-ultraviolet (NUV) light, the fungal colonies were visually checked. The two *Ascochyta* species were differentiated based on colony morphology (compare Fig. 3).

Agents tested

The agents tested comprised microorganisms and agents of natural origin. They were divided into three groups, resistance inducers and plant-based products (Table 1), commercial microbial products (Table 2) and experimental microbial preparations (Table 3). A control consisting of seeds treated with sterile distilled water (SDW) (trials with resistance inducers) or of untreated seeds (trials with commercial and experimental microorganisms) was included in each trial. As chemical control, Pomarsol (Bayer Cropscience; 49.0% thiram) was used.

Resistance inducers and plant-based products

All agents, except Tillecur and thyme oil were prepared in SDW at the concentrations indicated in Table 1. The solutions were stirred until the agents had dissolved and subsequently diluted when neces-

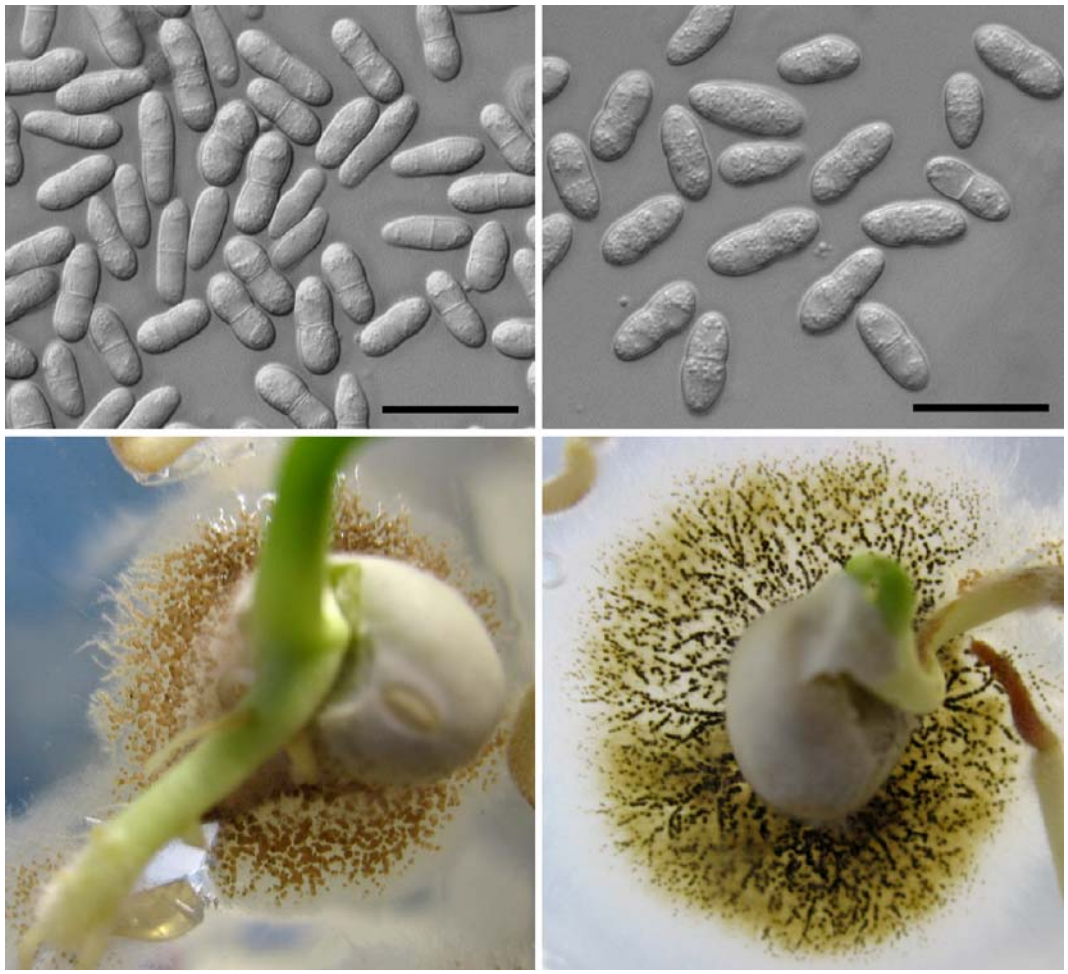

Fig. 3 Spores and colonies of *Ascochyta pisi* (*left*) and *A. pinodes* (*right*). *Bar*=15 μm

Table 1 Resistance inducers and plant-based products used

Product	Active ingredient(s)	Reference	Producer/supplier	Concentration used
Bion 50 WG	50% acibenzolar S-methyl (BTH)	Deepak et al. (2006)	Syngenta, Basel, Switzerland	0.1 mg l^{-1}
Chitoplant	Chitosan	Benhamou et al. (1994)	ChiPro GmbH, Bremen, Germany	0.5%
Salicylic acid	–[a]	Mauch-Mani and Metraux (1998)	Sigma (S 5922)	10 mg l^{-1}
Jasmonic acid	–	Pozo et al. (2004)	Sigma (J 2500)	1 mg l^{-1}
Comcat	Brassinosteroides	Berger et al. (2004)	G.L. Polus, Lindenfels, Germany	0.5 mg l^{-1}
Milsana	Extract of *R. sachalinensis*	Daayf et al. (1995)	Compo, Münster, Germany	1%
Kendal	Plant extracts, oligosaccarines, glutathione, K_2O	Scannavini et al. (2004)	Gerlach, Hannover, Germany	0.1%
Tillecur	Mustard powder	Spiess (2003)	Dr. Schaette AG, Bad Waldsee, Germany	20% (*w*/*v*)
–	Thyme oil	Wolf et al. (2008)	Chi International B.V, Breda, The Netherlands	0.1%

[a] Not applicable

Table 2 Commercial microbial products employed and quantities applied

Product	Microbial active ingredient	Reference	Formulation	Producer/supplier	Quantity applied/ 10 g seed
BA2552	*Pseudomonas chlororaphis* strain MA 342	–	Water-based formulation of bacterial cells 10^{10} cfu ml^{-1}	Bioagri AB, Uppsala, Sweden (www.bioagri.se)	300 µl
MBI600	*Bacillus subtilis*	Bennet et al. (2003)	Powder-based 5×10^{10} cfu g^{-1}	Becker Underwood Inc., Iowa, USA (www.beckerunderwood.com)	100 mg
FZB24	*Bacillus subtilis*	Grosch et al. (1999)	Powder-based 5×10^{10} cfu g^{-1}	Abitep GmbH, Berlin, Germany (www.abitep.de)	100 mg
Serenade	*Bacillus subtilis* strain QST 713	Olanya and Larkin (2006)	Wettable powder 5×10^{9} cfu g^{-1}	Intrachem Bio Italia, Cesena, Italy (www.intrachem.it)	100 mg
Mycostop Mix	*Streptomyces griseoviridis*	Kortemaa et al. (1994)	Dried spores and mycelium $>10^{8}$ cfu g^{-1}	Verdera, Espoo, Finland (www.verdera.fi)	50 mg
F251/2	*Fusarium oxysporum* strain 251/2	Minuto et al. (1997)	Powder-based (currently not marketed)	ISAGRO, Novara, Italy (www.isagro.it)	300 mg

sary to get the desired concentration. In case of Comcat the solution was stirred for 2 h, after which the beaker was placed in a sonication bath for 5 min. Jasmonic acid was made up in SDW after predissolving 100 mg of the compound in 2 ml of ethanol (96%). For each preparation 200 ml were poured into a beaker and approximately 50 seeds were added. Each beaker was then covered with a lid

Table 3 Experimental antagonistic microorganisms used

Isolate	Identity	Method of identification	Accession number (GenBank)	Reference
E183	*Pseudomonas putida* biotype B	FAME (0.87)		Koch (1997)
G12	*Pseudomonas putida* biotype A	FAME (0.63)		This work
G53	*Pseudomonas putida* biotype B	FAME (0.62)		This work
I112	*Pseudomonas* sp.	16S rRNA gene (partial sequencing)		Koch et al. (1998)
Z17	*Burkholderia* sp.	FAME		This work
Ki353	*Pseudomonas* sp.	16S rRNA gene (partial sequencing)	AY366185	Johansson (2003)
K3	*Bacillus subtilis*	16S rRNA gene (partial sequencing)		This work
L18	*P. fluorescens*	FAME		Amein and Weber (2002)
MF416	*Pseudomonas* sp. RNA group I	16S rRNA gene (partial sequencing)	EU266580	Johansson and Wright (2003)
RG11	*Pichia guilliermondii* (anamorph *Candida fukuyamaensis*)	Sequencing of the ITS 1–5.8S–ITS2 ribosomal region	EU266584	This work
R11	*Curtobacterium* sp.	16S rRNA gene (partial sequencing)	EU266585	This work
RG6	*Serratia plymuthica*	16S rRNA gene (partial sequencing)	EU266583	This work
RG68	*Serratia plymuthica*	16S rRNA gene (partial sequencing)	EU266582	This work
M8	*Pichia guilliermondii*	Sequencing of the ITS 1–5.8S–ITS2 ribosomal region		Spadaro et al. (2005)
MSA35	*Fusarium oxysporum* (apath.)	Morphology		Minuto et al. (1997)
TV69039	*Trichoderma viride*	Morphology		Koch et al. (2006)
IK726(F)	*Clonostachys rosea*	Morphology		Jensen et al. (2000)

Identities in parenthesis are tentative (probability according to analysis of fatty acids (FAME) <0.6)

and placed for 1 h on a rotary shaker at room temperature. After the treatment seeds were dried on filter paper under a laminar flow.

Tillecur was applied by dipping the seeds in a 20% suspension (*w*/*v*) of the powder in water. The treated seeds were dried with the use of talcum powder. Thyme oil was applied to the seeds at the concentration of 0.1% according to the protocol described by Wolf et al. (2008) with few modifications. Seeds were weighed and transferred into a glass bottle. A 0.1% emulsion of thyme oil was prepared in 40°C warm, demineralised water by sonication (sonicator Vibra Cell, Sonics & Materials Inc., Danbury CT, USA) in a measurement cylinder in which the tip of the sonication probe was sufficiently in contact with the emulsion until a light milky emulsion was formed (Output 85, Tune 30). The emulsion was kept at 40°C and the seeds were placed in a closed bottle (volume in ml about five times the seed weight) for 15 min before adding the thyme oil emulsion. Emulsion was added until the seeds were fully submerged. The bottle was closed with a cap and put on a roller bank for 30 min at 40°C. Finally, seeds were rinsed under tap water at 25°C and dried on filter paper under a laminar flow.

Commercial microbial products

The products, their producers/suppliers and rates used are listed in Table 2. They were formulated as dry (MBI600, FZB24, Serenade, F251/2, Mycostop Mix) or liquid (BA2552) preparations and applied by shaking the seeds together with the formulation in a plastic bag for 60 s. If a dry dressing gave an uneven distribution on the seeds, they were slightly moistened with a light spray of water prior to the treatment.

Experimental microorganisms and growth conditions

The microorganisms used and their identities are listed in Table 3. The filamentous fungi and yeasts were identified based on morphology or sequencing of the internal transcribed spacer (ITS) region, respectively. For identification of the bacteria, partial or complete sequencing of the 16S DNA or gas chromatography of cellular fatty acids (MIS, Microbial ID Inc., Newark, Delaware, USA) (Miller and Berger 1985) were employed.

The bacterial strains MF 416, RG68, RG6, R11 and K3 were identified by amplifying and sequencing the 16S rRNA. Bacterial cultures were grown overnight at 23°C in Tryptic soy broth (Difco, Becton Dickingson, Le Pont de Claix, France). Genomic DNA from 2 ml of the culture was extracted using the GenElute Bacterial Genomic DNA Kit, Mini (Sigma, Stockholm, Sweden). The final volume of eluted genomic DNA was 400 µl per sample. Two microliters of the DNA of each strain was used as template in the PCR reaction. The following two primers for amplification of the 16S region used were 27f (AGAGTTTGATCCTGGCTCAG) and 1525r (AAG GAGGTGATCCAGCC) (Lane 1991). Amplification was carried out in a final volume of 100 µl, containing template DNA, 10×PCR buffer, 2 µl DMSO, 1 µg ml^{-1} BSA, 5 mM $MgCl_2$, 50 µM KCl, 200 µM of each dNTP, 0.5 µM of each primer, 0.5 U *Taq* DNA polymerase (Perkin Elmer, Upplands Väsby, Sweden) under the following conditions: DNA denaturing at 94°C for 5 min, annealing at 55°C for 90 s, and elongation at 72°C for 2 min for 30 cycles. The amplified DNA was run on a 1% agarose gel, and the PCR products were purified using the E.Z.N.A Gel Extraction Kit, Omega bio-tek (Stockholm, Sweden). For sequencing of the amplified products, primers 27f and1525r (Lane 1991), and 704f (GTAGCG GTGAA ATGCGTAGA) and 765r (CTGTTTGCTCCCCAC GCTTTC) were used.

The yeast strain RG11 was identified by amplifying and sequencing the ITS region. Total genomic DNA was extracted, and the rDNA internal transcribed spacers and 5.8S DNA were amplified by using primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). The following PCR conditions were used: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplification was run over 30 cycles. The amplified product was purified using the E.Z.N.A Gel Extraction Kit, and sequenced using the same two primers, ITS5 and ITS4. Sequences of bacterial and yeast strains were compared to the GenBank database records by using Basic Local Alignment Search Tool.

The bacteria and the yeasts were routinely cultured on slants on nutrient yeast dextrose agar (NYDA; 1 l water; 4 g yeast extract; 8 g nutrient broth (MERCK); 1.5 g dextrose; 16 g agar). For preparation of inoculum for seed treatment, a loop-full of the respective culture grown overnight on NYDA was

suspended in 250 ml sterilised nutrient yeast dextrose broth-medium (composition as NYDA above, but without agar) and the flask was incubated for 48 h on a rotary shaker (120 rpm) in darkness at room temperature in order to reach a final cell concentration of, at least, 1×10^9 cells ml^{-1}. The seeds were placed in the suspension for 15 min and left in an open Petri dish to dry overnight.

MSA 35, an antagonistic strain of *Fusarium oxysporum*, was maintained on PDA and cultured for 10 days in casein hydrolysate in the darkness at room temperature on a rotary shaker. The concentration of the culture was adjusted with water to 1×10^6 conidia ml^{-1}, and the seeds were placed for 15 min in the culture and dried overnight as described above.

A clay formulation of *Clonostachys rosea* IK726 (F) was kindly supplied by I. Knudsen (Department of Plant Pathology, University of Copenhagen, Denmark). An aliquot of 1.5 g of clay preparation (1×10^8 cfu g^{-1}) was suspended in 10 ml SDW on a Vortex test tube shaker and then diluted in 200 ml water. The seeds were placed for 10 min in the suspension (10 g seeds per 40 ml suspension), dried and either sown immediately or stored at 4°C until use.

Glasshouse trials

Trials were carried out between April and August 2005 at the Centre of Competence for innovation in the agro-environmental sector, University of Torino, Italy and repeated three times. Treated seeds were sown in 0.5 l plastic pots with a surface of 100 cm^2 at one seed per pot. A potting mix composed of one part commercial potting mix (50% white peat, 25% clay, 25% perlite added with N–P–K 1 kg mc^{-1}) and one part sandy soil was used. The pots were placed on benches in a randomised block design (50 seeds in total were used per treatment: five replicates for each treatment, ten pots (seeds) per replicate) in two glasshouses (one for bean plants and one for pea plants) with temperatures ranging from 25°C to 30°C, and watered daily. The relative humidity varied from 50% to 80%. The pots sown with bean seeds were kept under a translucent plastic film, which was moistened daily on the underside in order to maintain a high humidity. Emergence was determined 2 weeks after sowing. On bean, disease incidence was determined 1 month after sowing and expressed as percentage of plants with anthracnose symptoms. On pea, disease was assessed 2 weeks after sowing and a disease index calculated using the Townsend–Heuberger formula:

$$\%\ \text{infection} = \left(\sum (n*v)/(i*N)\right)*100$$

- v class of infection defined as degree of symptom appearance on seedling according to Fig. 2
- i highest class of infection (5 in this case)
- n number of plants in each class
- N total amount of plants

Visible symptoms were scored, and the presence of *C. lindemuthianum* was determined by microscopic examination of typical acervuli and setae emerging from the fruiting bodies. The presence of *Ascochyta* spp. was confirmed by plating the seeds on PDA medium and observing the colonies under a stereo microscope.

Statistical analysis software

Statistical significance was tested with untransformed (emergence) or arcsin square root-transformed data (% healthy plants, % diseased plants, Townsend–Heuberger-Index) using the glm procedure (SAS Inc. 1989) and Student–Newman Keuls test ($P<0.05$).

Results

Assessment of seed infection

Rate of infestation of the bean seed lot with *C. lindemuthianum* was 9%. The infestation of the pea seed lot was as follows: 33.8% of pea seeds were infected with *A. pisi* and 6.2% with *A. pinodes*, while 2.9% were infected with both fungi. Peas infected with *A. pinodes* were surrounded by dark colonies that were larger in diameter than those of *A. pisi*. The colonies of *A. pisi* were white and more or less restricted to the vicinity of the seeds from which they originated. Whereas in *A. pisi* the pycnidia were randomly distributed in the colony, they appeared arranged in threads in *A. pinodes* (Fig. 3).

Identification of strains

The Gram stain revealed that the bacterial strains RG68, RG6 and MF 416 were Gram-negative whilst

R11 and K3 were Gram-positive. Almost complete *rrs* (the16S rRNA gene) sequences were obtained for the five bacterial strains examined. The sequences were compared to those in GenBank and were found to be similar to *Serratia plymuthica* (strains RG6 and RG68), *Pseudomonas* spp. (strain MF416), *Curtobacterium* sp. (strain R11) and *Bacillus subtilis* (strain K3). The sequences obtained started at coordinate number 3 for RG6 (and 2 for RG68) and they ended with the coordinate number 1458 for RG6 (and 1468 for RG68) of the *S. plymuthica* sequences (GenBank accession no. AJ233433.1 and DQ365570.1, respectively). The sequence obtained for strain MF416 was most closely related to the 16S rRNA gene of *Pseudomonas* spp, strain BSB9 (GenBank accession no. EU184086), which is a phosphate-solubilising strain isolated from soil, but it was similar also to many other *Pseudomonas* spp. strains in the database. Strain R11 was identified as *Curtobacterium* sp. The closest match was a strain of *Curtobacterium citreum*, strain Z10zhy (accession no AM411064). The 16S rRNA sequence of strain K3 was similar to those of several strains of *Bacillus subtilis* The sequences were aligned with published *rrs* sequences from *S. plymuthica*, *Pseudomonas* spp., *Curtobacterium* sp. and *B. subtilis*, respectively. The sequence of the ITS region of the yeast strain RG11 was most similar to those of *Pichia guilliermondii.* For all the microorganisms investigated there was 99% identity to each closest match. Sequences for strains MF416, RG68, RG6, R11and RG11 were submitted to GenBank, and accession numbers were obtained (Table 3).

Effect of seed treatments on seed-borne *C. lindemuthianum* on bean

Immersion of bean seeds in water for 1 h (untreated control relevant to resistance inducer treatment) reduced the germination rate (Table 4) compared to seeds that did not receive this treatment (untreated controls in Tables 5 and 6). The germination rates were even lower after immersion in the solutions of the resistance inducers. This effect was significant after treatment with Kendal, Comcat, salicylic acid and Chitoplant. Germination was highest after seed treatment with the thiram-containing product Pomarsol. All agents significantly reduced the percentage of diseased plants. However, due to the lower germination rates recorded after treatment with the resistance-inducing agents, an increase of the resulting number of healthy plants compared to the untreated control was only observed after seed treatment with thiram.

In the trials with commercial microbial products, emergence rates were not affected by any of the treatments (Table 5). Treatment with all three *B. subtilis* products (MBI600, FZB24 and Serenade) as well as with the *P. chlororaphis* product (BA2552) had a significant disease-reducing effect similar to

Table 4 Effect of seed treatment with resistance inducers and plant-based products on emergence of bean and pea plants and on disease incidence and disease severity caused by seed-borne *Colletotrichum lindemuthianum* and *Ascochyta* spp. respectively

Treatment	Bean			Pea		
	Emergence (%)[a]	Diseased plants (% of emerged)[a]	Healthy plants (% of seeds sown)[a]	Emergence (%)[a]	Townsend–Heuberger Index (%)[a]	Healthy plants (% of seeds sown)[a]
Chitoplant	40.7 d	0 b	40.7 c	98.0 a	16.3 a	53.3 a
Salicylic acid	47.3 c,d	0 b	47.3 b,c	97.3 a	17.4 a	48.7 a
Jasmonic acid	50.0 b,c,d	0.9 b	49.3 b,c	96.0 a	16.2 a	50.0 a
Comcat	46.0 c,d	5.2 b	44.0 b,c	97.3 a	19.2 a	46.7 a
Bion	52.0 b,c,d	0 b	52.0 b,c	94.0 a	19.2 a	40.0 a
Milsana	62.7 b,c	0 b	62.7 b	98.0 a	14.5 a	55.3 a
Kendal	41.3 d	6.0 b	38.7 c	97.3 a	14.1 a	48.0 a
Pomarsol (thiram)	84.0 a	0 b	84.0 a	94.7 a	12.8 a	51.3 a
Control (water)	65.3 b	16.4 a	55.3 b,c	98.7 a	18.0 a	42.0 a

Means of three trials (each trial: five replicates/treatment, ten plants/replicate)

[a] Different letters within columns indicate statistically significant differences. Significance was tested with untransformed (emergence) or arcsin square root-transformed data (% diseased plants, % healthy plants, Townsend–Heuberger-Index) according to Student–Newman–Keuls test ($P<0.05$).

Table 5 Effect of seed treatment with commercial microbial products on emergence of bean and pea plants and on disease incidence and disease severity caused by seed-borne *Colletotrichum lindemuthianum* and *Ascochyta* spp. respectively

Treatment	Bean			Pea		
	Emergence (%)[a]	Diseased plants (% of emerged)[a]	Healthy plants (% of seeds sown)[a]	Emergence (%)[a]	Townsend–Heuberger Index (%)[a]	Healthy plants (% of seeds sown)[a]
BA2552 (*P. chlororaphis*)	88.7 a	15.5 c,d	74.7 a,b	95.3 a	16.8 c	38.7 b
MBI600 (*B. subtilis*)	90.7 a	21.9 b,c,d	72.0 a,b	98.0 a	21.6 a,b,c	30.7 b
FZB24 (*B. subtilis*)	94.0 a	15.2 c,d	80.0 a	96.7 a	23.7 a,b	20.0 c
Serenade (*B. subtilis*)	89.3 a	10.9 c,d	79.3 a	97.3 a	26.8 a	6.7 d
Mycostop Mix (*S. griseoviridis*)	90.7 a	38.6 a,b	55.3 b,c	98.7 a	16.7 c	44.0 b
F251/2 (*F. oxysporum* apath)	92.6 a	26.7 a,b,c	68.0 a,b,c	97.3 a	20.9 b,c	18.0 c
Pomarsol (thiram)	93.3 a	6.1 d	87.3 a	98.0 a	9.2 d	64.0 a
Control	92.0 a	43.4 a	53.3 c	97.3 a	19.1 b,c	39.3 b

Means of three trials (each trial: five replicates/treatment, ten plants/replicate)

[a] Different letters within columns indicate statistically significant differences. Significance was tested with untransformed (emergence) or arcsin square root-transformed data (% diseased plants, % healthy plants, Townsend–Heuberger-Index) according to Student–Newman–Keuls test ($P<0.05$).

that of thiram, as measured by the number of healthy plants in relation to the number of seeds sown and/or the percentage of diseased plants (Table 5).

Emergence rates were quite variable after treatment with the experimental microorganisms and the plant-based products thyme oil and Tillecur (Table 6). Compared to the untreated control, all treatments tended to reduce the germination rate. A statistically significant decrease was observed after seed treatment with strains Z17 (*Burkholderia* sp.), Ki353 (*Pseudomonas* sp.), K3 (*Bacillus subtilis*), L18 (*P. fluorescens*), MF416 (*Pseudomonas* sp.) and RG68 (*Serratia plymuthica*). Most treatments caused a significant reduction of the percentage of emerged and diseased plants, which in most cases was similar to that of thiram. However, a significant increase of the number of healthy plants in relation to the seeds sown was only recorded for the treatments with Tillecur and with the *F. oxysporum* strain MSA35. Due to the negative effect on seed emergence, treatment with the bacterial strains Ki353, K3 and L18 reduced the number of healthy plants compared to the untreated control.

The overall activity of the chemical standard thiram in the three sets of experiments was variable (Tables 4, 5 and 6). The percentage of emerged and diseased plants was significantly reduced in all experiments, but due to an adverse effect on germinability the increase in the number of healthy plants was significant only in two of the three experiments.

Activity of seed treatments on seed-borne *Ascochyta* spp. on pea

In the experiments with peas infected with *Ascochyta* spp., significant effects of treatment with the resistance-inducing agents on emergence, disease severity and percentage of healthy plants were not observed (Table 4). The thiram product provided the strongest reduction of the Townsend–Heuberger disease index, but the difference compared to the untreated control was not statistically significant. An effect on emergence was also not observed in the experiments with the commercial microbial products. In these tests, a significant disease reduction and increase in the percentage of healthy plants was only provided by the thiram treatment, while the Bacillus-based products Serenade and FZB 24 increased the disease incidence (Table 5).

Among the experimental microorganisms, the strains *P. fluorescens* L18 and *S. plymuthica* RG6 had a significant adverse effect on germination (Table 6). Disease by *Ascochyta* spp. as measured by the Townsend–Heuberger index was only significantly reduced after seed treatment with thiram and thyme oil. A comparatively good disease suppressiveness was also achieved by seed treatment with *C. rosea* IK726(F). Strains *S. plymuthica* RG6 and *Trichoderma viride* 69039 caused a significant increase of the Townsend–Heuberger Index. Thiram, thyme oil and *C. rosea* IK726(F) provided a signif-

Table 6 Effect of seed treatment with experimental microorganisms, thyme oil and Tillecur on emergence of bean and pea plants and on disease incidence and disease severity caused by seed-borne *Colletotrichum lindemuthianum* and *Ascochyta* spp. respectively

Strain (species)	Bean			Pea		
	Emergence (%)[a]	Diseased plants (% of emerged)[a]	Healthy plants (% of seeds sown)[a]	Emergence (%)[a]	Townsend–Heuberger Index (%)[a]	Healthy plants (% of seeds sown)[a]
E183 (*P. putida*)	91.3 a,b,c[a]	6.1 d,e,f	85.3 a,b,c	98.0 a	20.9 b,c,d	42.0 b,c
G12 (*P. putida*)	91.0 a,b,c	3.0 e,f	88.0 a,b,c	N.T.	N.T.	N.T.
G53 (*P. putida*)	93.3 a,b	10.1 d,e,f	84.0 a,b,c	N.T.	N.T.	N.T.
Ki353 (*P. putida*)	72.0 d,e	32.6 a,b	46.0 g	93.8 a	23.3 a,b,c	33.8 b,c
MF416 (*Pseudomonas* sp.)	70.7 d,e	20.1 a,b,c,d	58.0 e,f,g	88.7 a,b	18.3 b,c,d	45.3 b,c
I112 (*Pseudomonas* sp.)	92.7 a,b	13.0 d,e,f	82.7 a,b,c	92.7 a	18.3 b,c,d	44.0 b,c
L18 (*P. fluorescens*)	64.7 e	15.7 c,d,e,f	53.3 f,g	80.0 b	17.8 c,d	35.3 b,c
Z17 (*Burkholderia* sp.)	80.7 b,c,d	20.3 a,b,c,d	63.3 d,e,f,g	93.8 a	23.9 a,b,c	29.1 c,d
K3 (*B. subtilis*)	77.3 c,d	35.1 a	50.7 g	96.9 a	17.9 b,c,d	44.9 b,c
RG11 (*P. guilliermondii*)	95.3 a,b	12.8 d,e,f	84.7 a,b,c	96.0 a	16.4 c,d	42.0 b,c
R11 (*Curtobacterium* sp.)	88.0 a,b,c	0 f	88.0 a,b,c	98.0 a	20.3 a,b,c,d	36.0 b,c
RG6 (*S. plymuthica*)	90.0 a,b,c	1.0 f	89.3 a,b	80.1 b	26.0 a,b	16.8 d,e
RG68 (*S. plymuthica*)	82.0 b,c,d	2.3 e,f	80.0 a,b,c,d	94.0 a	17.1 b,c,d	38.7 b,c
M8 (*P. guilliermondii*)	86.7 a,b,c	0.7 f	86.0 a,b,c	96.5 a	16.6 b,c,d	43.8 b,c
MSA35 (*F. oxysporum* apath.)	92.7 a,b	1.6 f	91.3 a	86.0 a,b	17.2 b,c,d	33.3 b,c
TV69039 (*Trichoderma viride*)	86.7 a,b,c	17.5 b,c,d,e	71.3 c,d,e,f	88.7 a,b	27.9 a	13.3 e
IK726(F) (*C. rosea*)	92.7 a,b	2.7 e,f	90.0 a,b,c	94.7 a	13.4 d	53.3 b
Thyme oil	88.0 a,b,c	0 f	88.0 a,b,c	96.0 a	7.7 e	66.7 a
Tillecur	94.0 a,b	2.1 e,f	92.0 a	90.7 a,b	18.5 b,c,d	32.0 c
Pomarsol (thiram)	88.0 a,b,c	2.2 e,f	86.0 a,b,c	94.0 a	9.6 e	67.3 a
Control	99.3 a	26.0 a,b,c	73.3 b,c,d,e	96.0 a	17.2 b,c,d	24.0 c,d

Means of three trials (each trial: five replicates/treatment, ten plants/replicate)

N.T. Not tested

[a] Different letters within columns indicate statistically significant differences. Significance was tested with untransformed (emergence) or arcsin square root-transformed data (% diseased plants, % healthy plants, Townsend–Heuberger-Index) according to Student–Newman–Keuls test ($P<0.05$).

icant increase in the number of healthy plants in relation to the number of seeds sown.

Discussion

Seed quality is a key factor for the full exploitation of a crop in terms of yield and value (McGee 1995). Even when propagated under favourable environmental conditions, seeds may still be contaminated with pathogens. Therefore, the availability of effective seed treatment methods is crucial for cost-effective crop production. In the present study, we tested the efficacy of several microorganisms and putative resistance inducing compounds for control of seed-borne *C. lindemuthianum* and *Ascochyta* spp. on bean and pea, respectively.

Seed treatment with the three commercial formulations of *B. subtilis* (MBI600, FZB24 and Serenade) and one of *P. chlororaphis* (BA2552) had a significant disease-suppressive effect resulting from seed-borne infections by *C. lindemuthianum*. A significant reduction of the percentage of emerged plants showing symptoms of anthracnose was also achieved by most of the tested experimental microorganisms. Interestingly, this reduction was also noted for a non-pathogenic strain of *F. oxysporum* and two yeasts, one of which (*Pichia guilliermondii* M8) is active in post-harvest protection of pome fruit. Yeasts act through nutrient competition with pathogens in plant wounds (Castoria

et al. 2008). Among the most efficacious microorganisms against *C. lindemuthianum* was also a strain of *Curtobacterium* sp. belonging to the group of plant growth-promoting rhizobacteria which can induce resistance in the host (Raupach and Kloepper 2000). The mechanisms by which the seed treatment with microorganisms affected transmission of the pathogen from the seeds to the seedling in the present study are not known. Apart from microbial production of antifungal metabolites (as recorded for MBI600, Serenade, FZB24 and BA2552), plant-mediated effects cannot be ruled out, since in *Phaseolus* beans induction of systemic resistance against leaf pathogens by seed treatment has been reported before. Elbadry et al. (2006) reported induction of systemic resistance against *Bean yellow mosaic potyvirus* and Bigirimana and Höfte (2002) obtained systemic protection against *C. lindemuthianum* by a combined soil and seed treatment with rhizobacteria.

Against *Ascochyta* spp., the activity of the tested commercial and experimental microorganism preparations was much lower than against *C. lindemuthianum*. A significant reduction in disease severity of the emerged pea plants by seed treatment with the microorganisms was not observed. Some treatments even tended to reduce the germination rate and to increase the disease severity. The only microbial treatment that resulted in a significant increase in the percentage of healthy plants was that with *C. rosea* IK726(F). Both thyme oil and Tillecur had a clear reducing effect on the symptoms caused by *C. lindemuthianum* on beans, with Tillecur being among the best performing agents in this pathosystem. Thyme oil was, apart from thiram, the most potent agent against *A. pisi* on pea. Since the seeds were treated in thyme oil at 40°C, the temperature may have contributed to the disease controlling effect by facilitating the penetration of the active components into the seed coat and/or by the elevated temperature alone. Very clear differences in performance in the two pathosystems were also observed after seed treatment with the resistance inducers. On pea, treatment with these agents had no statistically significant effect on disease severity. On the other hand, all resistance inducers clearly reduced the percentage of bean plants affected by anthracnose, and most of them caused some reduction in bean germination. The reasons for these differences are not clear, because induced resistance against plant pathogens has also been reported for peas. Morphological and biochemical changes in pea indicating induced resistance after application of various agents (Benhamou et al. 1994; Benhamou and Garand 2001; Singh et al. 2003; Frey and Carver 1998; Katoch et al. 2005) have been described. A decrease in susceptibility of pea to *M. pinodes* after spraying the first leaf with acibenzolar-S-methyl (BTH) has been reported. Effective treatments also enhanced the activities of β-1,3-glucanase and chitinases in untreated upper leaves (Dann and Deverall 2000). There are also a number of reports describing induction of resistance by biotic and abiotic elicitors in the bean-anthracnose pathosystem (Dann and Deverall 1995; Siegrist et al. 1997; Bigirimana and Höfte 2002). Two of these studies (Siegrist et al. 1997; Bigirimana and Höfte 2002) included treatment of the seeds or the soil with the inducing agents. However, in all studies the resistance induction was tested with artificial inoculations of leaves and not with seed-borne infections.

The difficulty of controlling seed-borne *Ascochyta* spp. with any of the alternative treatments in the present study is in agreement with other reports in the literature and may at least partly be explained by the position of these pathogens in the seed (cited by Decker 1957). The author reported that in 80% of the seeds infected with *A. pisi* the fungus was situated beneath the testa, and in about 40% of the seeds the embryo also was attacked. According to Maude and Kyle (1970) hot water treatment, hot carbon tetrachloride and steam/air mixtures all failed to give control of *Ascochyta* infection of pea, whereas seed treatment with the systemic fungicide benomyl was effective. Also in our experiments, seed treatment with the systemic fungicide Octave (Basf Italia; prochloraz 46.1%) gave better control than the thiram product (data not shown). These findings show that *Ascochyta* spp. are particularly difficult to control and may explain the general poor performance of most of the agents tested.

To summarise, the results of our study indicate that seed-borne infections of bean by *C. lindemuthianum* can be reduced by different alternative seed treatments. These include some of the tested commercial and experimental biocontrol agents, thyme oil, the plant-derived product Tillecur and most of the tested resistance inducers. However, many of these treatments, especially among the resistance inducers, adversely affected the germination rate. The resistance inducers were applied as a seed soak treatment, a

method that by itself appears to adversely affect the germination of beans and would probably not be practicable in commercial situations. Further experiments are needed in order to confirm the observed activities against *C. lindemuthianum* also on different been seed lots, to minimise the negative effects on germinability and to develop application methods acceptable for practical use. The observation that almost all seed treatments turned out to be ineffective in controlling the seed-borne *Ascochyta* spp. infections is not surprising in view of the literature describing the difficulty to control these pathogens. Therefore, the only agents that appeared to be efficacious in the present study, thyme oil and a strain of *C. rosea*, should be critically re-tested, and these tests should include seed lots less heavily infected than the one used here. Furthermore, experiments in the field would be desirable to assess the potential of efficacious agents under farming conditions.

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