Eur J Plant Pathol (2010) 127:99–112
DOI 10.1007/s10658-009-9575-3

Evaluation of non-chemical seed treatment methods for the control of *Alternaria dauci* and *A. radicina* on carrot seeds

Eckhard Koch · Annegret Schmitt · Dietrich Stephan · Carola Kromphardt · Marga Jahn · Hermann-Josef Krauthausen · Gustaf Forsberg · Sigrid Werner · Tahsein Amein · Sandra A. I. Wright · Federico Tinivella · Maria L. Gullino · Steven J. Roberts · Jan van der Wolf · Steven P. C. Groot

Accepted: 17 December 2009 / Published online: 16 January 2010

Abstract The current study was initiated to evaluate the efficacy of physical methods (hot water, aerated steam, electron treatment) and agents of natural origin (resistance inducers, plant derived products, micro-organisms) as seed treatments of carrots for control of *Alternaria dauci* and *A. radicina*. Control of both *Alternaria* species by seed treatment with the resistance inducers was generally poor. Results were also

E. Koch (✉) · A. Schmitt · D. Stephan
JKI, Institute for Biological Control,
Heinrichstrasse 243,
64287 Darmstadt, Germany
e-mail: eckhard.koch@jki.bund.de

C. Kromphardt · M. Jahn
JKI, Institute for Strategies and Technology Assessment
in Plant Protection,
Stahnsdorfer Damm 81,
14532 Kleinmachnow, Germany

H.-J. Krauthausen
Agricultural Service Centre,
DLR-Rheinpfalz,
67435 Neustadt, Breitenweg 71, Germany

G. Forsberg
Seedgard AB,
Svalövsvägen 3,
75651 Uppsala, Sweden

S. Werner
Hild Samen GmbH,
Kirchenweinbergstr. 115,
71672 Marbach am Neckar, Germany

T. Amein
Department of Cellular and Molecular Biology,
Box 462, 40530 Göteborg, Sweden

S. A. I. Wright
Department of Plant and Environmental Sciences,
Box 461, 40530 Göteborg, Sweden

S. A. I. Wright
Dipartimento di Scienze Animali, Vegetali e dell'Ambiente,
Facoltà di Agraria, University of Molise,
Via Francesco De Sanctis,
86100 Campobasso, Italy

F. Tinivella · M. L. Gullino
Centre of Competence for the Innovation
in the Agro-Environmental Sector (AGROINNOVA),
University of Torino,
Via L. da Vinci 44,
10095 Grugliasco, Italy

S. J. Roberts
Plant Health Solutions,
20 Beauchamp Road,
Warwick CV34 5NU, UK

J. van der Wolf · S. P. C. Groot
Plant Research International,
Droevendaalsesteeg 1,
NL-6708 PB Wageningen, The Netherlands

not satisfactory with most of the formulated commercial micro-organism preparations. Based on the average of five field trials, one of these, BA 2552 (*Pseudomonas chlororaphis*), provided a low but significant increase in plant stand. Among the experimental micro-organisms, the best results were obtained with *Pseudomonas* sp. strain MF 416 and *Clonostachys rosea* strain IK726. A similar level of efficacy was provided by seed treatment with an emulsion (1%) of thyme oil in water. Good and consistent control was generally achieved with the physical methods aerated steam, hot water and electron treatment. Aerated steam treatment was, apart from the thiram-containing chemical standard, the best single treatment, and its performance may at least partially be due to extensive pre-testing, resulting in dosages optimally adapted to the respective seed lot. In some of the experiments the effect of the hot water treatment, which was tested at a fixed, not specifically adapted dosage, was significantly improved when combined with a *Pseudomonas* sp. MF 416 or *C. rosea* IK726 treatment. The results are discussed in relation to the outcome of experiments in which the same seed treatment methods and agents were tested in other seed-borne vegetable pathosystems.

Keywords Organic farming · Biocontrol agents · Physical seed treatment methods · Resistance inducers · Essential oils

Introduction

Leaf blight and black rot caused by *Alternaria dauci* (Kühn) Groves & Skolko and *A. radicina* Meier, Drechsler & Eddy, respectively, are among the most important diseases of carrot (*Daucus carota*). *A. dauci* causes foliar blight on wild and cultivated carrot. *A. radicina* can also cause foliar blight, but is primarily responsible for root and crown rot disease of carrot. The main economic damage by the latter occurs during storage. *A. radicina* has also been reported to cause foliar blight on parsley, stalk/root rot of celery, and to be pathogenic on caraway, dill, fennel and parsnip (Farrar et al. 2004). *A. radicina* is a producer of mycotoxins that adversely affect the germination of carrot seeds (Tylkowska et al. 2003) but apparently do not present a hazard for consumers (Solfrizzo et al. 2005). In most carrot production areas, chemical control of *Alternaria* leaf blight is practised to prevent destruction of the photosynthetic surface area of the plant and to allow for efficient mechanical harvest (Ben-Noon et al. 2003; Bounds et al. 2007). Control of leaf blight is particularly important in carrot seed crops. Both *A. dauci* and *A. radicina* can infect the inflorescences and developing seeds. For *A. radicina* it has been suggested that seed infection develops from infection of umbel parts through the pedicel and ovary wall (Soteros 1979). The fungus enters the pericarp, or occasionally the testa. Similarly, *A. dauci* was reported to be confined to the outer surface and tissues of dried pericarps and did not penetrate the seed coat and endosperm (Strandberg 1983). Both fungi also occur as spores on the seed surface (Pryor and Strandberg 2002, Farrar et al. 2004). Commercial seed lots are frequently contaminated with one or both species (Maude 1966; Soteros 1979). The degree of infestation with *A. radicina* has been shown to reach very high levels, highlighting that contamination of carrot seeds with this pathogen is a continuing problem (Farrar et al. 2004). Seeds that are moderately or heavily infested with *A. radicina* or *A. dauci* fail to germinate, or the hypcotyls of seedlings growing from infested seeds are infected at or just below the soil line, resulting in post emergence damping off within 2–3 weeks of emergence (Pryor and Strandberg 2002, Farrar et al. 2004). Slight seed infections seem to be responsible for non-visible latent infections in the crown part of the carrot root and may become visible as a black rot either at a high temperature (>20°C) at maturation or during cool storage of the carrots (Groot et al. 2004). Under conventional farming conditions, current practise in most EU countries is to use chemical seed treatment with thiram to control seed-borne *Alternaria dauci* and *A. radicina*.

For organic farming, carrot is one of the economically most important crops. *Alternaria dauci* and *A. radicina* pose a particular hurdle to the organic production of carrot seeds, because the use of effective chemical fungicides to combat foliar and umbel blight is not allowed. Biological control of foliar *Alternaria* blights has been attempted (Chen and Wu 1999; Köhl et al. 2004) but has not yet been developed sufficiently for practical use. Therefore, organically produced carrot seed lots are often contaminated, and effective non-chemical methods for seed sanitization are needed. The suitability of hot water treatment (45–55°C), with or without the addition of NaOCl, for control of

A. dauci and *A. radicina* as a viable alternative to fungicidal seed treatment has been repeatedly demonstrated (Strandberg and White 1989; Pryor et al. 1994; Hermansen et al. 1999; Nega et al. 2003). In experiments by Jahn and Puls (1998), seed treatment with accelerated electrons reduced the level of seed infestation with *Alternaria* spp. and was particularly efficacious (50–100%) against *A. radicina.*

A number of studies have been conducted to explore the potential of seed treatment with micro-organisms to eradicate *Alternaria* spp. After treatment of seeds infested with *A. radicina* with a strain of *Bacillus subtilis*, Hentschel (1991) observed improved germination and plant health. Using carrot seeds artificially inoculated with conidia of *A. radicina*, similar results were obtained by Chen and Wu (1999) with isolates of *Pseudomonas cepacia* and *B. amyloliquefaciens*. Hermansen et al. (1999), however, found no effect on field emergence from seed naturally infested with *A. dauci* using the commercial biocontrol preparations Mycostop (*Streptomyces griseoviridis*) or T-22 (*Trichoderma harzianum* Rifai strain KRL-AG2). On the other hand, different strains of the fungus *Clonostachys rosea,* isolated from cereal or carrot habitats, controlled pre-and post-emergence death caused by *A. dauci* and *A. radicina* as effectively as the fungicide iprodione, and biopriming of a highly infested carrot seed lot with *C. rosea* strain IK726 reduced the incidence of *A. radicina* from 29% to <2.3%, and that of *A. dauci* from 11% to <4.8% (Jensen et al. 2004). In experiments by Slusarenko et al. (2008) imbibition treatment of *Alternaria*-infested carrot seeds with garlic juice resulted in improvements of the germination rate comparable to treatment with a commercial, thiram-containing seed treatment.

The above compilation of the literature shows that a number of positive approaches for non-chemical control of seed-borne *Alternaria* spp. on carrot exist. However, due to differences in the methodology used and because most of the studies were only dealing with a single alternative treatment method, a detailed appraisal of the suitability of the different technologies is difficult. The current study was initiated to evaluate and compare the efficacy of different physical methods and agents of natural origin as seed treatments for control of *Alternaria* spp. on carrot seeds. Initial tests were performed in seed trays under controlled conditions using an identical seed lot and with inclusion of a chemical standard. Selected treatments, including combinations of physical methods with alternative agents, were evaluated in field experiments and in tests in seed trays with different seed lots. In the season of 2006, field experiments were conducted in four European countries. The work was part of a larger project aimed at identifying non-chemical seed treatment methods for organic vegetable production. The project involved fungal seed-borne pathogens of cabbage, parsley (Amein et al. 2006), beans, peas (Tinivella et al. 2009), lamb`s lettuce (Schmitt et al. 2009), carrots (the present paper) and bacterial seed-borne pathogens on cabbage and carrots (Schmitt et al. 2006).

Materials and methods

Plant material

The work was carried out with carrot seeds (*Daucus carota* L.) observed in pre-tests on blotter paper (not shown) to be naturally infested with *Alternaria dauci* and *Alternaria radicina.* Unless stated otherwise, the experiments were performed with a highly infested seed lot of the variety ‘Laguna’ (seed lot 1). A second seed lot of the same variety, but infested to a lesser degree (seed lot 3) and one seed lot of variety ‘Narome’ (seed lot 2) were each used in one and two experiments, respectively.

Seed treatment with physical methods

The hot water (HW) treatments were applied by immersing the seeds in a water bath of a set temperature followed by re-drying in a ventilated drying room. The treatment parameters selected were 50°C for 30 min (“HW1”) and 53°C for 10 min (“HW2”) (Nega et al. 2003). The aerated steam (AS) treatments were performed using a treatment device described by Forsberg et al (2002). With the different seed lots, germination tests on blotters were performed in order to determine treatment parameters optimised for each seed lot (data not shown). Based on the results of these pre-tests, the treatment parameters selected for seed lot 1 were 754 kJ m^{-3} / 252 g H_2O m^{-3} for 2 min (“AS1”), 637 kJ m^{-3} / 215 g H_2O m^{-3} for 5 min (“AS2”), or 673 kJ m^{-3} / 223 g H_2O m^{-3} for 2 min (“AS3”). For seed lot 2 the treatment parameters were 754 kJ m^{-3} / 252 g H_2O m^{-3} for 2 min (“AS4”), and for seed lot 3

they were 712 kJ m^{-3} / 237 g H_2O m^{-3} for 2 min ("AS5").

The electron (EL) seed treatment was performed with the e-ventus WESENITZ 2 device (Jahn et al. 2005). The treatment parameters applied were 100 kV / 24 kGy ("EL1") and 110 kV / 24 kGy ("EL2").

Seed treatment with non-chemical agents

The agents evaluated and the rates used were largely identical to those employed by Tinivella et al. (2009) on legumes and Schmitt et al. (2009) on lamb`s lettuce. They comprised micro-organisms and agents of natural origin and were assigned to one of three groups, resistance inducers / plant-derived products, commercial microbial products and experimental microbial preparations (see Tinivella et al. 2009 for details). In cases where the same treatments were evaluated in parallel both in seed trays and in the field, the seeds used were treated once and thereafter partitioned for both uses. As chemical standard, Aatiram (Stähler, Stade, Germany; 670 g×kg^{-1} thiram) was included in all experiments except in the field experiments performed in 2006 on organic land. It was applied by shaking an excess amount of the product together with the seeds in a flask.

Resistance inducers and plant derived products

The following concentrations of the resistance inducers and plant derived products were tested: Bion 50 WG, 1 mg l^{-1}; Chitoplant, 0.5% (w/v); salicylic acid 10 mg l^{-1}; jasmonic acid, 1 mg l^{-1}; Comcat 0,5 mg l^{-1}; Milsana flüssig 1% (v/v); Kendal 1% (v/v); thyme oil 1% (v/v) (Tinivella et al. 2009). The seeds to be treated were placed in the different solutions/ emulsions (usually in volumes of 50 ml in 100 ml beakers) which were stirred for 4 h. The controls ("Untreated") were seeds submerged in water under stirring for 4 h. The mustard powder product Tillecur was used at 130 mg per 10g seeds. Thyme oil was used as an emulsion (1%) prepared by sonification in 40°C warm water.

Commercial and experimental micro-organisms

The formulated microbial products (some of them commercialised) and the rates used per 10 g of carrot seeds were: FZB 24 (based on *Bacillus subtilis*), MBI 600 (based on *Bacillus subtilis* strain MBI 600), and Serenade (based on *Bacillus subtilis* strain QST 713), 100 mg; Mycostop Mix (based on *Streptomyces griseoviridis* strain K61), 50 mg; F251/2 (based on the non-pathogenic strain *Fusarium oxysporum* 251/2), 300 mg; BA 2552 (based on *Pseudomonas chlororaphis* strain MA 342), 300 µl; IK726(F) (a clay formulation of *Clonostachys rosea* strain IK726), 100 mg. All dry / powder formulations as well as the liquid microbial product BA 2552 were applied by shaking the seeds with the product for about 60 sec in a flask.

The following experimental micro-organisms were used: strain E183 (*Pseudomonas putida* biotype B), strain G12 (*P. putida* biotype A), strain G53 (*P. putida* biotype B), strain I112 (*Pseudomonas* sp.), strain Z 17 (*Burkholderia* sp.), strain MF 416 (*Pseudomonas* sp.), strain Ki 353 (*Pseudomonas* sp.), strain K 3 (*Bacillus subtilis*), strain L 18 (*P. fluorescens*), strain RG11 (*Pichia guilliermondii*), strain R11 (*Curtobacterium* sp.), strain RG6 (*Serratia plymuthica*), strain RG68 (*S. plymuthica*), strain M8 (*Pichia guilliermondii*), strain MSA35 (*Fusarium oxysporum* apath.), strain TV69039 (*Trichoderma viride*) and strain IK726 (*Clonostachys rosea*). Their cultivation on laboratory media has been described elsewhere (Schmitt et al. 2009). The seeds were immersed for 15 min in the microbial cultures or spore suspensions, respectively, and thereafter used immediately or allowed to dry overnight and sown the following day. Seeds immersed for 15 min in water served as controls. In experiments in which several different treatments were included (e.g. physical methods, resistance inducers, experimental micro-organisms) the seeds of the controls ("Untreated") did not receive any treatment.

Blotters tests

Blotter tests using seeds placed on moist filter paper were performed as described (Schmitt et al 2009) according to ISTA rules (Anonymous 2000). Two weeks after start of the experiment the percentage of germinated seeds was recorded, and the seeds and seedlings were inspected for the presence of conidiophores and mycelia of *A. dauci* and/or *A. radicina*.

Plant experiments under controlled conditions

The experiments were performed in a growth room (20°C; 16 / 8 h day / night) in household polypropylene

containers (27.5 × 17.5×7 cm; from herewith called "seed trays") filled with horticultural potting substrate as described by Schmitt et al. (2009). Per seed tray, 100 seeds were sown, and three seed trays per treatment were used (unless stated otherwise). The number of healthy seedlings per tray was generally determined 3 weeks after planting.

Field tests

Field tests were performed on conventional agricultural land in the season 2004 at Schifferstadt (Germany) (drilling date: June 17) and Berlin-Dahlem (Germany) (drilling date: July 16), and in the season 2005 at Schifferstadt (drilling date: July 12). In 2006 field tests were carried out on certified organic land at Schifferstadt (Germany), Örebro (Sweden), Moncalieri, Turin Province (Italy) and Wellesbourne (United Kingdom). The drilling dates in 2006 were April 19 (Schifferstadt), June 13 (Örebro), May 26 (Moncalieri) and April 28 (Wellesbourne).

The field experiments were organised in a randomised block design in 6–8 × 1.2–1.6 m plots with three replications. The seeds were sown with a single seed drill in four rows per plot with a row distance of 35–40 cm and 65–76 seeds m^{-1} row. The number of plants in two 1m lengths of row in the central two rows was counted at the 3–4 leaf stage.

Statistical analysis

The Generalised Linear Modelling procedures of Genstat (Payne et al. 2005) were used for all analyses. Data in the form of the number of infested (blotter experiments), healthy (tray experiments) or emerged (field trials) seedlings out of the total number examined/tested were analysed by fitting a series of Generalised Linear Models with a binomial distribution and logit link function. Analysis of deviance tables were used to assess the importance of different terms in the model, and means and approximate standard errors were formed as predictions based on models containing only the important terms and using the residual deviance as a dispersion parameter. Upper and lower 95% confidence intervals were calculated from the standard errors. Treatment means with non-overlapping confidence intervals were considered to be significantly different.

Results

Identification of effective non-chemical agents

Resistance inducers and formulated micro-organisms

In the seed tray tests with seed treatment with the resistance inducers significant increases in the number of healthy plants were only observed after seed treatment with the chemical standard Aatiram (on average from 10.3 healthy plants per tray in the controls to 76.3 after treatment with Aatiram). The number of healthy plants per tray was not significantly different from that of the control for any of the resistance inducers, nor were there any significant differences among the resistance inducers (data not shown).

Likewise, no significant increases in the number of healthy plants compared to the control were observed after treatment of the same seed lot with the formulated micro-organisms. However, based on the average of three tests, the treatments with Mycostop Mix (*S. griseoviridis*) or F251/2 (*F. oxysporum*) were slightly more efficacious than treatment with BA 2552 (*P. chlororaphis*) or FZB 24 (*B. subtilis*) (Fig. 1).

Experimental microrganisms and Tillecur

Of the 15 strains of experimental micro-organisms tested, 10 increased the number of healthy carrot seedlings in at least one of the experiments performed. In the two experiments in which *Pseudomonas* sp. MF 416, *P. fluorescens* L 18 and *C. rosea* IK726 were included, seed treatment with these strains consistently resulted in a comparatively high number of healthy carrot seedlings (Table 1).

Tests with single applications of selected agents and physical seed treatments methods

For further testing, one resistance inducer (Milsana), two formulated microbial products (Mycostop Mix and BA 2552) and two experimental microbial strains (*C. rosea* IK726 and *Pseudomonas* sp. MF 416) were chosen, together with two hot water treatments, two aerated steam treatments and two electron treatments which were selected on the basis of pre-tests using a range of parameter combinations. Because the results generally indicated a similar efficacy for the two

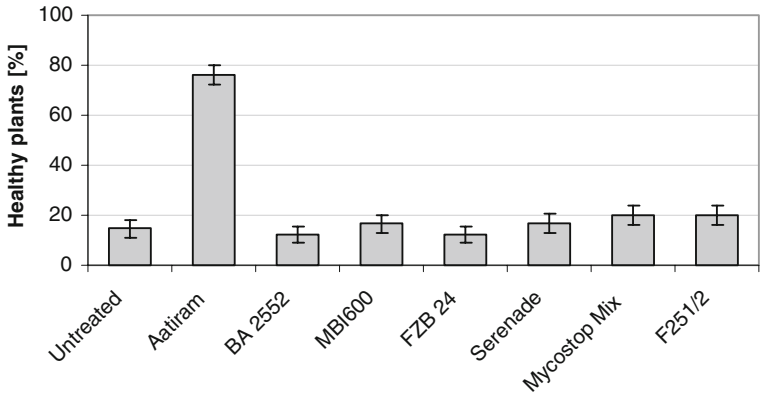

Fig. 1 Effect of treatment with formulated micro-organisms on emergence and early plant establishment in seed tray tests with carrot seeds naturally infected with *Alternaria dauci* and *A. radicina* (seed lot 1). Means of three experiments, each with three seed trays with 100 seeds per tray. Error bars show approximate 95% confidence intervals; means with non-overlapping confidence intervals were considered to be signficantly different

dosages of each physical method, with a tendency for better performance at the higher dosage, in Figs. 2 and 3 only the results for HW2, AS2 and EL2 are presented.

Efficacy on blotter paper

Germination of the untreated seeds of seed lot 1 was much better in the blotter test (Fig. 2a) than in the tests in seed trays (compare Figs. 1, 3a, 4a, 5b). Treatment with Aatiram, aerated steam and electrons caused a significant increase in the germination. An increase in germination, although not statistically significant, was also recorded after seed treatment with hot water and *Pseudomonas* sp. MF 416 (Fig. 2a).

The presence of colonies of *A. dauci* and *A. radicina* on 86% and 55% of the seeds/seedlings respectively, reflected the high level of infestation of seed lot 1 with these pathogens (Fig. 2b and c). Overall, the effect of the treatments was very similar for both fungi. Treatment with the chemical Aatiram led to a significant, although only partial, decrease in the % infection. All physical treatments were at least as effective as Aatiram, with aerated steam treatment providing the strongest eradicative effect. Seed

Table 1 Performance in seed tray tests of selected non-commercial micro-organisms and the plant-derived product Tillecur as seed treatments for protection against infection by *Alternaria dauci* and *A. radicina* (seed lot1). Pooled results (means) of trials in seed trays with 100 seeds per seed tray and three trays per treatment with carrot seeds infested with the pathogens

Treatment / Strain (*species*)	Number of trials		Healthy plants per seed tray [2]
	Total	With significant positive effect [1]	
Aatiram	10	10	73.1
P. putida E183	4	3	32.8
P. putida G12	2	2	19.7
P. putida G53	2	1	15.2
P. fluorescens L 18	2	2	45.9
Pseudomonas sp.MF 416	2	2	49.7
Pseudomonas sp. I112	3	2	29.3
B. subtilis K 3	1	1	25.3
S. plymuthica RG68	2	1	19.6
C. rosea IK726	2	2	37.0
T. viride TV69039	4	3	22.5
Tillecur	3	2	15.4
Untreated	10	–	8.4

[1] Statistically significant increase in the number of healthy seedlings per seed tray in relation to "Untreated"

[2] Trials with significant positive effect only

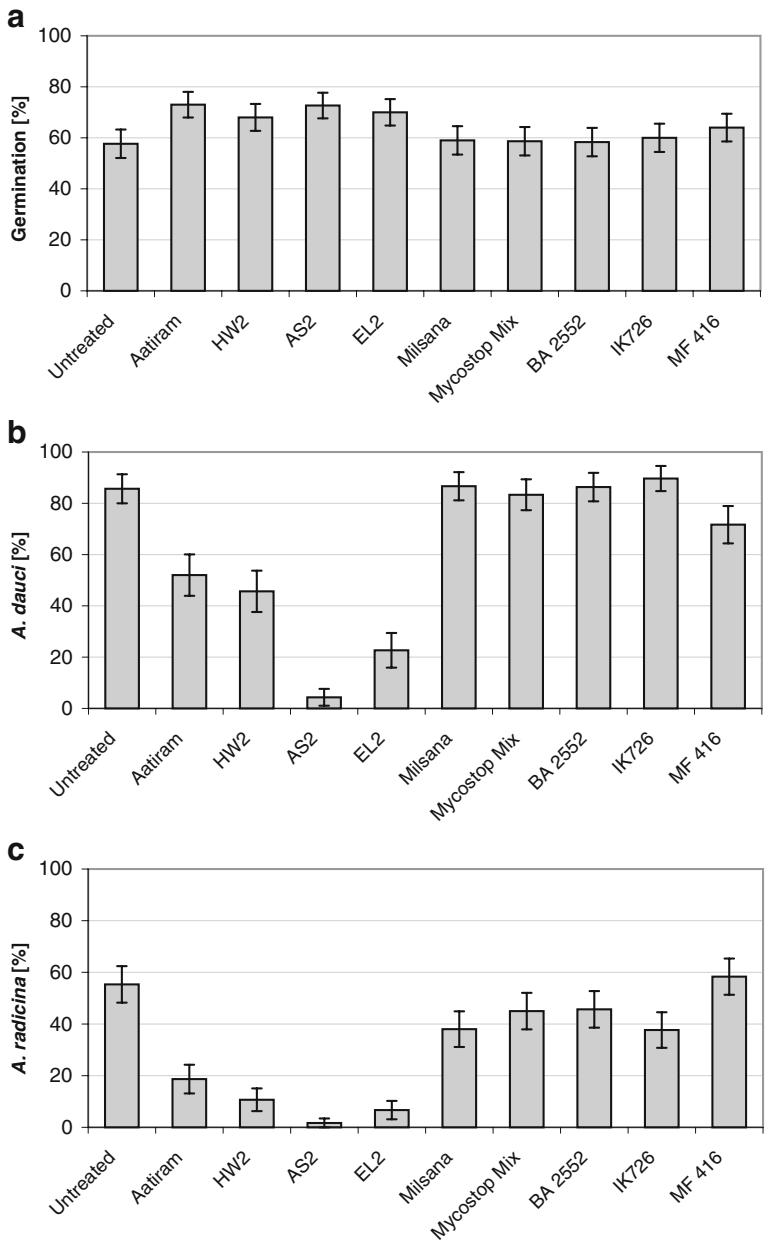

Fig. 2 Effect of selected seed treatments on germination **a** and percentage of carrot seeds infected with *Alternaria dauci* **b** and *A. radicina* **c** on blotter paper (seed lot 1). Means of three separate tests with naturally infested seeds, each with four plates per treatment and 25 seeds per plate. Error bars show approximate 95% confidence intervals; means with non-overlapping confidence intervals were considered to be signficantly different

treatment with Milsana and the formulated and experimental micro-organisms had either no significant effect, or the reduction of the infestation rate was substantially lower than recorded after treatment with the physical methods.

Efficacy in seed tray tests and field trials

Seeds from the same treated batches were employed in the seed tray test under controlled conditions, and in two field trials performed in the season of 2004 at two different sites in Germany. In the seed tray tests all treatments except Milsana and BA 2552 (*P. chlororaphis*) increased the number of healthy plants significantly (Fig. 3a). The most effective treatments were Aatiram, aerated steam and electron seed treatment.

In the two field experiments performed in 2004 at Schifferstadt and Kleinmachnow, the treatments with Aatiram, aerated steam, electrons, hot water and

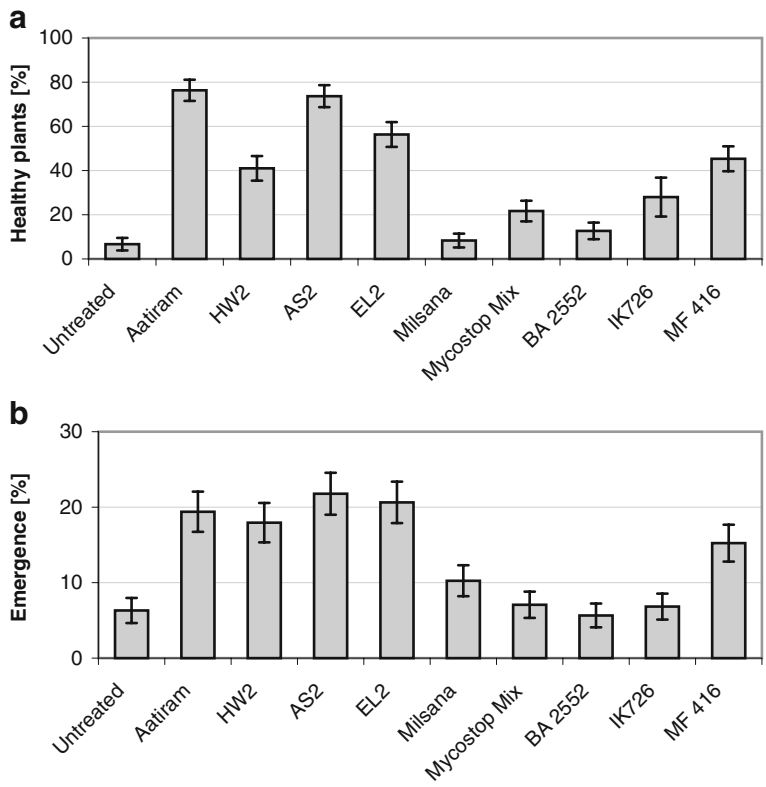

Fig. 3 Effect of selected treatments of carrot seeds naturally infected with *Alternaria dauci* and *A. radicina* (seed lot 1) on emergence and plant establishment in seed tray tests **a** and field trials **b**. **a**: Means of three seed trays with 100 seeds per tray. **b**: Means of two field experiments performed in 2004 at Schifferstadt and Kleinmachnow (Germany), respectively. Stand counts of 2 × 2 metre row per plot (3 plots per treatment). Error bars show approximate 95% confidence intervals; means with non-overlapping confidence intervals were considered to be signficantly different

Pseudomonas sp. MF 416 resulted in the greatest increase in germination percentage. The treatments with Mycostop Mix, BA 2552 or IK726 had no significant effect (Fig. 3b). The effect of Milsana was significant only at Kleinmachnow (not shown).

Tests with combinations of treatments

In order to select suitable treatment pairs, pre-tests in seed trays were run in which the three physical treatment methods and the best non-chemical treatment agents were tested in different combinations (data not shown). As a result, the following combinations were chosen: Hot water+*C. rosea* IK726 or *Pseudomonas* sp. MF 416, Aerated steam+BA 2552 and Electron seed treatment+thyme oil (1%).

Performance in seed tray tests with different seed lots

Figure 4a shows the performance of the selected combinations together with the single treatment partners with the highly infested seed lot 1. All treatments except BA 2552 increased the number of healthy plants per tray significantly. Apart from the thiram-containing chemical standard, the most efficacious single treatments were aerated steam, electron treatment, *C. rosea* IK726 and hot water. The efficacy of the hot water treatment increased significantly when it was combined with a treatment of *Pseudomonas* sp. MF 416. An increase, although not statistically significant, was also noted when hot water was combined with *C. rosea* IK726. No such increase was obtained by combining aerated steam with BA 2552 and electron treatment with thyme oil.

The untreated seed lots 2 (Fig. 4b) and 3 (Fig. 4c) were much less infested than seed lot 1. The results obtained in the seed tray tests with these seed lots were nevertheless in good agreement with those obtained with seed lot 1: The number of healthy plants per tray was again significantly increased by all treatments except when using BA 2552. Also, aerated steam was, apart from the chemical standard Aatiram, the most effective single treatment, and combinations of hot water with *C. rosea* IK726 or *Pseudomonas* sp. MF 416 (the latter was only employed in the test with seed lot 2) were more effective than the hot water treatment alone, while the efficacy of aerated steam and electron seed treatment was not greater in the

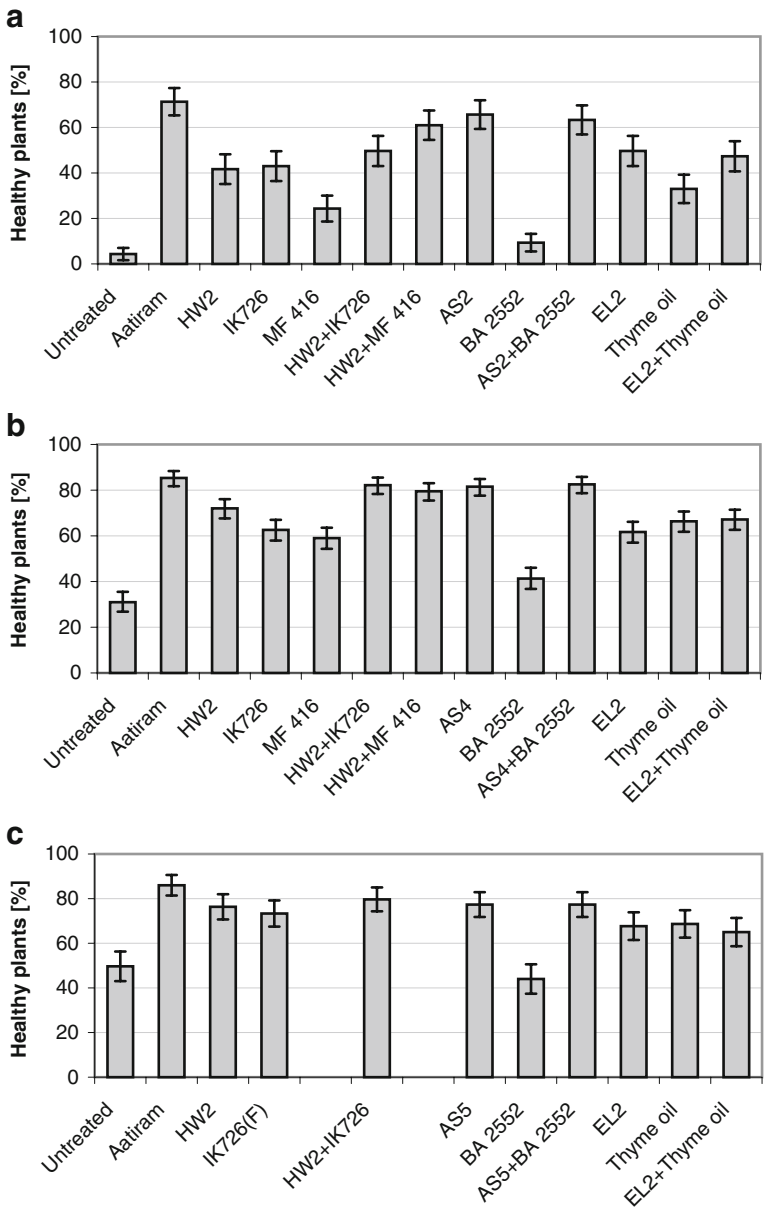

Fig. 4 Effect of selected seed treatments and treatment combinations on emergence and early plant establishment of carrots developing from seeds naturally infected with *Alternaria dauci* and *A. radicina* in seed tray tests. **a**: Seed lot 1. The seeds were taken from the treated batches used for the field experiment at Schifferstadt 2005. **b**: Seed lot 2. **c**: Seed lot 3 (the treatments MF 416 and HW+MF 416 were not tested on this seed lot). Means of one (seed lots 1 and 3) or two (seed lot 2) experiments per seed lot, each with three seed trays with 100 seeds per tray. Error bars show approximate 95% confidence intervals; means with non-overlapping confidence intervals were considered to be signficantly different

combined treatments. Despite these similarities in the results obtained with the three seed lots, it was obvious that in case of the less infested seed lots 2 and 3 the differences among the treatments were smaller and less often statistically significant than with seed lot 1.

Performance in field experiments

When seeds of seed lot 1 treated with the selected combinations and the corresponding single treatment partners (compare Fig. 3a) were sown in the field in the season of 2005, all treatments provided some increase in plant stand. However, due to high variability, the increase was statistically significant only for the aerated steam treatment (from 32 plants$\times$m^{-1} row in the "Untreated" to 58 in the aerated steam treatment, corresponding to an increase of 81%). The least effective treatments were *Pseudomonas* sp. MF 416 and BA 2552 (12 and 14% increase, respectively). All other treatments, including the chemical Aatiram, performed very similarly, with increases in the range of 42–53% (not shown).

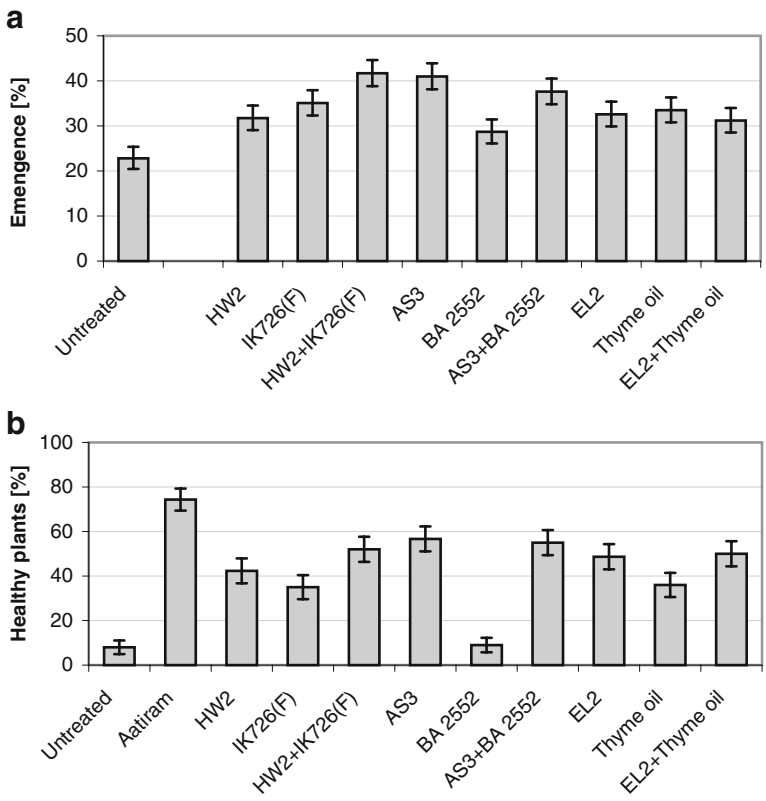

Fig. 5 Effect of selected seed treatments or treatment combinations on emergence and establishment of carrot plants developing from seeds naturally infected with *Alternaria dauci* and *A. radicina* (seed lot 1) in field trials **a** and seed tray tests **b**. **a**: Means of the five field experiments performed in 2006 (compare Table 2). The treatment Aatiram was not included. **b**: The seeds were taken from the treated batches used for the field experiments in 2006. Means of three seed trays with 100 seeds per tray. Error bars show approximate 95% confidence intervals; means with non-overlapping confidence intervals were considered to be signfi-cantly different

In order to substantiate the results, seeds of seed lot 1 were newly treated and employed in the season of 2006 in five field experiments in different countries and in one seed tray test. The field experiments were arranged in an identical design at all sites. The treatments were the same as employed in the previous seed tray tests (Fig. 4) and in the field experiment in 2005, with the following modifications: *Pseudomonas* sp. MF 416 was not included, the conidial suspension of *C. rosea* IK726 used in the previous experiments was replaced by a clay formulation, and the chemical standard had to be omitted because the trials were performed on organic land. The results are presented for individual sites in Table 2 and summarized in Fig. 5a. The emergence rate in "Untreated" differed among the different sites. At Örebro, all treatments caused a significant increase in plant stand. At Wellesbourne, this was true for all treatments except for the electron treatment and BA 2552. There were differences between the results of the two experiments performed at Schifferstadt. Only aerated steam gave a significant increase in emergence in both experiments. At all sites,

Table 2 Effect of seed treatment on germination and crop establishment (3–4 leaf stage) in small plot field trials in four European countries (seed lot 1). Figures within columns marked with an asterisk are significantly different from "Untreated"

Treatment	Trial site[1] / emergence [%]									
	1		2		3		4		5	
Untreated	2.2		12.6		28.5		33.0		36.5	
HW2	13.3	*	25.3	*	30.5		43.8		44.5	
HW2+IK726	35.2	*	38.1	*	38.5		50.7	*	45.5	
AS3	27.9	*	35.2	*	41.2	*	48.4	*	51.5	*
AS3+BA 2552	28.5	*	28.2	*	39.9	*	43.0		48.1	*
EL2	18.7	*	19.1		33.6		40.6		50.5	*
EL2+Thyme oil	19.4	*	27.6	*	28.3		39.4		40.3	
IK726(F)	20.7	*	21.0	*	37.9		42.3		53.1	*
BA 2552	12.5	*	17.7		27.6		39.9		45.0	
Thyme oil	19.4	*	28.7	*	36.4		38.2		43.7	

[1] 1: Örebro, Sweden; 2: Wellesbourne, United Kingdom; 3: Moncalieri (Turin Province), Italy; 4 and 5: Schifferstadt, Germany. Overall means and their confidence intervals are shown in Fig. 5a

the combination of hot water treatment with IK726(F) performed better than hot water treatment alone, in particular at Örebro and Wellesbourne (Table 2) where the difference was statistically significant (not shown).

Computation of the average plant stand from all five sites revealed that all treatments resulted in statistically significant increases. Aerated steam treatment was the most effective of the physical seed treatments. The alternative seed treatment agents *C. rosea* IK726(F) and thyme oil were as effective as hot water and electron seed treatment and significantly better than BA 2552. Among the combinations, only hot water treatment+*C. rosea* IK726(F) provided a denser plant stand than application of the physical method alone (Fig.5a).

When seeds from the same treated batches were used in the seed tray test, the results were overall in good agreement with those obtained in the field experiments: all treatments, except for BA 2552, provided a significant increase in plant stand. Aerated steam was the best single physical treatment, and the combination of hot water treatment with *C. rosea* IK726(F) performed significantly better than the hot water or *C. rosea* IK726(F) treatments alone (Fig. 5b).

Discussion

In tests performed in seed trays, treatment of carrot seeds with the putative resistance inducing agents failed to control seed-borne *A. dauci* and *A. radicina* and the only resistance inducer (Milsana) included in the two field experiments in 2004 provided significant control only at one site. The same compounds also failed to protect peas against seed-borne *Ascochyta* spp. (Tinivella et al. 2009) and lamb's lettuce against *Phoma valerianellae* (Schmitt et al. 2009). The reasons for this failure are not known. It can certainly not be expected that the mechanisms of induced plant resistance work in all plant-pathogen combinations, especially under high infection pressure. Many of the positive reports of resistance induction deal with biotrophic fungi, but none of the above fungi are biotrophs. The failure of the inducers to protect against seed-borne pathogens may also be due to insufficient uptake of the inducing agent. Resistance inducers are commonly applied to green foliage. Correspondingly, after the treatment of seeds an effect can only be expected if sufficient amounts of the agent penetrate into the seed and reach the physiologically active tissues of the developing seedling. With carrot seeds this may not be the case. A certain level of control by the above-mentioned resistance-inducing agents was reported in the case of seed-borne *Colletotrichum lindemuthianum* on bean, but for some of the substances this was associated with a reduction in emergence (Tinivella et al. 2009). Therefore, control of seed-borne fungi by treatment of the seeds with resistance inducers remains problematic.

In the first round of testing in seed trays the commercial/formulated micro-organism products failed to provide significant control of *A. dauci* and *A. radicina* on carrots. Seed treatment with these preparations was also ineffective for pea seeds infested with *Ascochyta* spp. (Tinivella et al. 2009) and for lamb's lettuce seeds infested with *P. valerianellae* (Schmitt et al. 2009).

In further tests in seed trays, Mycostop Mix showed some efficacy, but testing was discontinued after it failed to provide significant control in the two field experiments performed in 2004. Despite relatively low efficacy in the initial tests, BA 2552 was included in the further tests, both alone and in combination with aerated steam treatment. Its performance in these tests remained unsatisfactory. In the end, the 2006 field experiments showed that on average BA 2552 provided a low but significant increase in germination. In greenhouse experiments by Amein et al. (2006) with cabbage seeds infested with *Alternaria* spp., all of the above commercial/formulated micro-organism preparations reduced the incidence of *Alternaria* infections on the seedlings, and Mycostop Mix and BA 2552 were the most efficacious products. On the other hand, the number of healthy bean seedlings emerging from seeds infested with *C. lindemuthianum* did not increase after treatment with Mycostop Mix, whereas it did after treatments with BA 2552, FZB 24, MBI 600 and Serenade (Tinivella et al. 2009). This illustrates that general statements regarding the usefulness of these products as seed treatments for vegetables cannot be made, and that individual testing in the different pathosystems is required. In the carrot / *Alternaria* pathosystem none of the tested commercial/formulated microbial preparations was particularly effective.

Among the experimental, non-commercialized micro-organisms, the best results were obtained with *P. fluorescens* L18, *Pseudomonas* sp. MF 416 and *C. rosea* IK726. *Pseudomonas* sp. MF 416 caused a significant increase in stand, not only in the seed tray tests on two different seed lots, but also in the field experiments performed in 2004 and 2005. Its efficacy

was, however, generally lower than that of the chemical standard and the best physical treatments. In a separate set of experiments in seed trays, seed treatment with lyophilized cells of *Pseudomonas* sp. MF 416 performed similarly well (D. Stephan, unpublished results). To what extent metabolites produced by MF 416 contributed to the observed activity as a seed treatment is not known. *In vitro*, *Pseudomonas* sp. MF 416 suppresses the growth of a range of fungi and bacteria and produces the antimicrobial 2,3-deepoxy-2,3-didehydroxy-rhizoxin (DDR) (Johansson and Wright 2003). Mutants impaired in production of DDR loose a substantial part of their suppressive ability (Wright et al. 2003). On the other hand, *P. chlororaphis* MA 342, the active bacterial ingredient of BA 2552, is also a producer of DDR and has a similar *in vitro* antimicrobial spectrum (Johansson and Wright 2003), but in our tests the activity of BA 2552 against *A. dauci* and *A. radicina* was clearly lower than that of MF 416.

The other non-commercialized micro-organism in this study with good activity against seed-borne *Alternaria* pathogens of carrots was *C. rosea* IK726. Control of *A. dauci* and *A. radicina* by seed treatment with *C. rosea* IK726 has been reported before (Jensen et al. 2004). In our study *C. rosea* IK726 provided a significant increase in plant stand in all experiments performed in seed trays and in three of the five field experiments performed with the clay formulation. As in the case of *Pseudomonas* sp. MF 416, the activity of *C. rosea* IK726 was generally lower than that of the best treatments, although it was the best single treatment in one of the field experiments performed in 2006.

A level of control of *A. dauci* and *A. radicina* comparable to that obtained by treatment with *C. rosea* IK726 was recorded for seed treatment with thyme oil. With thyme oil preparations, good results have also been obtained against *P. valerianellae* on lamb's lettuce (Schmitt et al. 2009) and *Septoria petroselini* on parsley (Amein et al. 2006). A certain activity was also observed against *Ascochyta* spp. on peas (Tinivella et al. 2009). It appears that preparations containing thyme oil are suitable not only for eradicating *Alternaria* spp. from carrot seeds but also from other vegetable pathosystems. Thyme oil contains thymol and various other antifungal compounds (Šegvić Klarić et al. 2006) and has a general antimicrobial activity against seed borne bacteria and fungi (Van der Wolf et al. 2008). However, due to its inherent phytotoxicity, choice of the optimal concentration is critical, and pre-testing is recommended.

In this study, good sanitation effects were generally observed after seed treatment with the physical methods. Good effects of the same physical treatments were also seen in the pathosystems: parsley—*S. petroselini* and lamb's lettuce—*Phoma valerianellae* (Amein et al. 2006; Schmitt et al. 2009). The positive results obtained with the carrot—*Alternaria* pathosystem in the present study are in line with the literature. Successful control by hot water treatment of important seed-borne pathogens (*Alternaria* spp., *Phoma* spp., *Septoria* spp., *Peronospora valerianellae*, *Xanthomonas* spp.) of carrot, celery, parsley and lamb's lettuce has been reported before (Nega et al. 2003). Hot water treatment at 54–55°C for 20 min was shown to eradicate *A. dauci* from carrot seeds in some but not all seed lots (Strandberg and White 1989; Hermansen et al. 1999). Similarly, in the blotter tests performed in the present study, we observed that treatment with hot water at 53°C for 10 min did not completely eradicate *A. dauci* and *A. radicina* from the highly infested seed lot 1.

Aerated steam treatment had the best eradicating effect against both pathogens. This treatment was, apart from the chemical thiram, also the best single treatment in the seed tray tests and in the field experiments. Due to the good sanitation effect of aerated steam alone, its combined application with BA 2552 (*P. chlororaphis*) gave no improvement. Probably, the better performance of the aerated steam treatment as compared to hot water and electron treatments can be attributed to a more extensive pre-testing, which resulted in treatment parameters that were optimally adapted to the respective seed lots. Only limited pre-testing was done with electron seed treatment, and in the case of hot water treatment fixed, not specifically adapted treatments were used for all seed lots. As a result of these apparently sub-optimal treatments, the sanitizing effect was lower. In the case of electron treatment, its combination with thyme oil did not lead to improved efficacy, while the combination of hot water treatment with *Pseudomonas* sp. MF 416 or *C. rosea* IK726 often resulted in an increase in stand when compared to hot water treatment alone. This suggests that combinations of physical methods with suitable treatment agents could also be used commercially. Because all physical methods may adversely affect germinability (Groot et al. 2006, Groot et. al. 2008), application at a

lower dose may reduce the risk of seed damage. In combination with suitable non-chemical agents, like *Pseudomonas* sp. MF416 or *C. rosea* IK726, the overall disease controlling efficacy should be maintained. In addition, there may also be potential for control of soil-borne pathogens.

Most of the experiments of the current study were performed with a single carrot seed lot, which was so heavily infested with *A. dauci* and *A. radicina* that in commercial situations it would most probably be discarded. Therefore, use of this seed lot represented a very stringent test of the treatments. The good overall performance with this and the other two seed lots indicates that the aerated steam, hot water and electron treatments can be recommended for commercial use, both for organic and conventional farming conditions. Furthermore, it appears likely that with "normal" seed lots satisfactory sanitisation effects will also be achieved with thyme oil or micro-organisms like *Pseudomonas* sp. MF416 and *C. rosea* IK726. However, more experiments are needed to substantiate this suggestion. Further development work should also consider the combined use of these agents with the physical seed treatment methods.

Acknowledgement Thanks are due to Petra Zink for excellent technical assistance and to Dr Inge Knudsen (Copenhagen University) for supplying *C. rosea* strain IK726. The research reported in this article was funded by the European Commission in the frame of project 'Seed Treatments for Organic Vegetable Production—STOVE' (QLK5-CT-2002-02239).

References

Amein, T., Wikström, M., Schmitt, A., Koch, E., van der Wolf, J., Groot, S. P. C., Forsberg, G., Werner, S., Krauthausen, H. J., Kromphardt, C., Jahn, M. & Wright, S. A. I. (2006). Non-chemical methods of seed treatments for control of seed-borne pathogens on vegetables. In C. B. Andersen, L. Elsgaard, S. Sondergaard & G. Hansen (Eds.), *Proceedings of the European Joint Organic Congress—Organic Farming and European Rural Development* (pp. 412–413).

Anonymous (2000). Internationale Vorschriften für die Prüfung von Saatgut 1999. *Seed Science and Technology 27, Supplement*, 357 p.

Ben-Noon, E., Shtienberg, D., Shlevin, E., & Dinoor, A. (2003). Joint action of disease control measures: a case study of Alternaria leaf blight of carrot. *Phytopathology, 93*, 1320–1328.

Bounds, R. S., Podolsky, R. H., & Hausbeck, M. K. (2007). Integrating disease thresholds with TOM-CAST for carrot foliar blight management. *Plant Disease, 91*, 798–804.

Chen, T. W., & Wu, W. S. (1999). Biological control of carrot black rot. *Journal of Phytopathology, 147*, 99–104.

Farrar, J. J., Pryor, B. M., & Davis, R. M. (2004). Alternaria diseases of carrot. *Plant Disease, 88*, 776–784.

Forsberg, G., Andersson, S., & Johnsson, L. (2002). Evaluation of hot, humid air seed treatment in thin layers and fluidized beds for pathogen sanitation. *Journal of Plant Diseases and Protection, 109*, 357–370.

Groot, S. P. C., Birnbaum, Y., Rop, N., Jalink, H., Forsberg, G., Kromphardt, C., et al. (2006). Effect of seed maturity on sensitivity of seeds towards physical sanitation methods. *Seed Science and Technology, 34*, 403–413.

Groot, S. P. C., Birnbaum, Y., Kromphardt, C., Forsberg, G., Rop, N., & Werner, S. (2008). Effect of the activation of germination processes on the sensitivity of seeds towards physical sanitation treatments. *Seed Science and Technology, 36*, 609–620.

Groot, S. P. C., van der Wolf, J. M., Jalink, H., Langerak, C. J., & van den Bulk, R. W. (2004). Challenges for the production of high quality organic seeds. *Seed Testing International, 127*, 12–15.

Hentschel, K. D. (1991). Biocontrol of seed-borne *Alternaria radicina* on carrots by antagonistic *Bacillus subtilis*. *Bulletin SROP, 14*(8), 73–76.

Hermansen, A., Brodal, G., & Balvoll, G. (1999). Hot water treatments of carrot seeds: effects on seed-borne fungi, germination, emergence and yield. *Seed Science and Technology, 27*, 599–613.

Jahn, M., & Puls, A. (1998). Investigations for development of a combined biological-physical method to control soil-borne and seed-borne pathogens in carrot seed. *Journal of Plant Diseases and Protection, 105*, 181–197.

Jahn, M., Röder, O. & Tigges, J. (2005). Die Elektronenbehandlung von Getreidesaatgut—Zusammenfassende Wertung der Freilandergebnisse—(S. 7–61); Electron Treatment of Cereal Crop Seeds—Overview and Appraisal of Field Trials—(p. 66–126). *Mitteilungen aus der Biologischen Bundesanstalt für Land—und Forstwirtschaft Berlin-Dahlem 399,* 126 p.

Jensen, B., Knudsen, I. M. B., Madsen, M., & Jensen, D. F. (2004). Biopriming of infected carrot seed with an antagonist, *Clonostachys rosea*, selected for control of seedborne *Alternaria* spp. *Phytopathology, 94*, 551–560.

Johansson, P. M., & Wright, S. A. I. (2003). Low-temperature isolation of disease-suppressive bacteria and characterization of a distinctive group of pseudomonads. *Applied and Environmental Microbiology, 69*, 6464–6474.

Köhl, J., Langerak, C. J., Meekes, E. T. M., & Molhoek, W. M. L. (2004). Biological control of *Alternaria radicina* in seed production of carrots with *Ulocladium atrum*. *Seed Science and Technology, 32*, 857–861.

Maude, R. B. (1966). Studies on the etiology of black rot, *Stemphylium radicinum* (Meier, Drechsl. & Eddy) Neerg., and leaf blight, *Alternaria dauci* (Kühn) Groves & Skolko, on carrot crops; and on fungicide control of their seed-borne infection phases. *Annals of Applied Biology, 57*, 83–93.

Nega, E., Ulrich, R., Werner, S., & Jahn, M. (2003). Hot water treatment of vegetable seed—an alternative seed treatment method to control seed-borne pathogens in organic farming. *Journal of Plant Diseases and Protection, 110*, 220–234.

Payne, R. W., Harding, S. A., Murray, D. A., Soutar, D. M., Baird, D. B., Welham, S. J., et al. (2005). *The Guide to Genstat Release 8. Part 2: Statistics*. Oxford: VSN International.

Pryor, B. M., Davis, R. M., & Gilbertson, R. L. (1994). Detection and eradication of *Alternaria radicina* on carrot seed. *Plant Disease, 78*, 452–456.

Pryor, B. M., & Strandberg, J. O. (2002). Alternaria leaf blight of carrot. In R. M. Favis & N. Raid (Eds.), *Compendium of Umbelliferous Crop Diseases* (pp. 15–16). St. Paul: Amercian Phytopathological Society.

Schmitt, A., Jahn, M., Kromphardt, C., Krauthausen, H. J., Roberts, S. J, Wright, S. A. I., et al. (2006). STOVE: Seed treatments for organic vegetable production. In C. B. Andersen, L. Elsgaard, S. Sondergaard & G. Hansen (Eds.), *Proceedings of the European Joint Organic Congress—Organic Farming and European Rural Development* (pp. 414-415).

Schmitt, A., Koch, E., Stephan, D., Kromphardt, C., Jahn, M., Krauthausen, H.-J., et al. (2009). Evaluation of non-chemical seed treatment methods for the control of seed-borne infections by *Phoma valerianellae* on lamb`s lettuce. *Journal of Plant Diseases and Protection, 116*, 200–207.

Šegvić Klarić, M., Kosalec, I., Mastelić, J., Piecková, E., & Pepeljnak, S. (2006). Antifungal activity of thyme (*Thymus vulgaris* L.) essential oil and thymol against moulds from damp dwellings. *Letters in Applied Microbiology, 44*, 36–42.

Slusarenko, A. J., Patel, A., & Portz, D. (2008). Control of plant diseases by natural products: allicin from garlic as a case study. *European Journal of Plant Pathology, 121*, 313–322.

Soteros, J. J. (1979). Detection of *Alternaria radicina* and *A. dauci* from imported carrot seed in New Zealand. *New Zealand Journal of Agricultural Research, 22*, 185–190.

Solfrizzo, M., De Girolamo, A., Vitti, C., Tylkowska, K., Grabarkiewicz-Szczęsna, J., Szopińska, D., et al. (2005). Toxigenic profile of *Alternaria alternata* and *Alternaria radicina* occurring on umbelliferous plants. *Food Additives and Contaminants, 22*, 302–308.

Strandberg, J. O. (1983). Infection and colonization of inflorescences and mericarps of carrot by *Alternaria dauci*. *Phytopathology, 67*, 1351–1353.

Strandberg, J. O., & White, J. M. (1989). Response of carrot seeds to heat treatments. *Journal of the American Society of Horticultural Science, 114*, 766–769.

Tinivella, F., Hirata, L. M., Celan, A., Wright, S. A. I., Amein, T., Schmitt, A., et al. (2009). Control of seed-borne pathogens on legumes by microbial and other alternative seed treatments. *European Journal of Plant Pathology, 123*, 139–151.

Tylkowska, K., Grabarkiewicz-Szczesna, J., & Iwanowska, H. (2003). Production of toxins by *Alternaria alternata* and *A. radicina* and their effect on germination of carrot seeds. *Seed Science and Technology, 31*, 309–316.

Van der Wolf, J. M., Birnbaum, Y., Van der Zouwen, P. S., & Groot, S. P. C. (2008). Disinfection of vegetable seed by treatments with essential oils and organic acids. *Seed Science & Technology, 36*, 76–88.

Wright, S. A. I., Bondesson, P., Thorsson, L., Azarang, M. & Gerhardson, B. (2003). Analysis of biocontrol deficient mutants in *Pseudomonas chlororaphis* reveals genes involved in regulation of biocontrol. Pages 241–244. In: Y. Elad, J. Köhl & D. Shtienberg (eds). Influence of A-Biotic and Biotic Factors on Biocontrol Agents, IOBC/wprs Bulletin Vol. *22*(10).