Biopriming of Infected Carrot Seed with an Antagonist, *Clonostachys rosea*, Selected for Control of Seedborne *Alternaria* spp.

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ABSTRACT

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An ecological approach was used to select fungal antagonists effective against the seedborne pathogens *Alternaria dauci* and *A. radicina* on carrot. Twenty-five and 105 isolates originating from cereal and carrot habitats were screened against the pathogens in planta, respectively. Irrespective of isolate origin, fungal isolates belonging to *Clonostachys rosea* controlled pre- and postemergence death caused by *A. dauci* and *A. radicina* as effectively as the fungicide iprodione. Isolate IK726 of *C. rosea* was used in biopriming a seed lot with 29% *A. radicina* and 11% *A. dauci* (highly infected), and a seed lot with 4% *A. radicina* and 7% *A. dauci* (low infection). Seeds were primed with water alone (hydropriming) or with addition of *C. rosea* IK726 (biopriming). The occurrence of *A. radicina* and *A. dauci* increased twofold and fivefold, respectively, during 14 days hydropriming, irrespective of the initial infection level. On highly

infected seed, biopriming reduced the incidence of *A. radicina* to <2.3% and that of *A. dauci* to <4.8% while the level of both pathogens was <0.5% on bioprimed seed with a low initial infection rate. In sand stand establishment tests, hydroprimed seeds had a lower healthy seedling stand than nonprimed seeds, mainly due to a high degree of postemergence seedling death. In contrast, biopriming resulted in a seedling stand that was better than that of both nonprimed and hydroprimed seeds. *C. rosea* IK726 multiplied fivefold to eightfold, and microscopic observations using *C. rosea* IK726 transformed with a green fluorescent protein (GFP) reporter gene showed that seeds were covered with a fine web of sporulating mycelium of *C. rosea*. The positive effect of biopriming on healthy seedling stand remained after 5 months of storage at 4°C and IK726 survived at high numbers on these seed. In this study, we demonstrated that biopriming with the biocontrol strain *C. rosea* IK726 facilitates priming of infected seeds without risking adverse effects on seedling establishment.

Additional keywords: bio-osmopriming, drum priming, *Gliocladium roseum*, shelf life.

Alternaria dauci (Kühn) Groves & Skolko and *A. radicina* Meier, Drechs., & E.D. Eddy (syn. *Stemphylium radicinum* (Meier, Drechs., & E.D. Eddy) Neergaard) are both important seedborne pathogens, causing seed decay and damping-off in carrot (*Daucus carota* L.). In addition, leaf blight caused by *A. dauci* can be a major constraint to carrot production worldwide and black rot caused by *A. radicina* is especially damaging to stored carrots (2,25,28,35). Fungicide seed treatments can control *Alternaria* pathogens on carrot seeds efficiently (34,39,41), but the use of fungicides is increasingly restricted in Europe and not allowed in organic farming.

There are only a few examples of biological control of seedborne *Alternaria* pathogens in carrot. Seed treatment with an isolate of *Bacillus subtilis* (FZB Biotechnik GmbH, Berlin) improved seedling emergence from seeds artificially inoculated with *A. radicina*, but mainly at temperatures above 20°C (3,16). Chen and Wu (7), using seeds artificially inoculated with *A. radicina*, selected nine isolates after in vitro screening of 1,897 isolates originating from soils, carrot seeds, or taproots. The efficacy of the bacterium *Burkholderia capacia*, isolate 229, on plant emergence was as good as seed treatment with iprodione. On the other hand, Jahn and Puls (15) found no effect on seedling emergence after treatment with *Pseudomonas* strain W 24 on carrot seed naturally infected by *Alternaria* spp. However, strain W 24 was originally selected for biocontrol efficacy against soilborne plant

pathogens. Furthermore, Hermansen et al. (12) found no effect on field emergence from seed naturally infested with *A. dauci* using the commercial biocontrol agents (BCAs) Mycostop (*Streptomyces griseovirides*) and T-22 (*Trichoderma harzianum* Rafai strain KRL-AG2). These strains also were selected for biological control of soilborne diseases. This emphasizes that isolates selected for efficacy against soilborne pathogens are not necessarily suited for control of diseases caused by seedborne pathogens.

An ecological approach for selection of effective antagonists has been superior for obtaining new commercial BCAs (13,23). Following this approach, selected organisms should be able to function in the same environmental niches as that of the pathogen they are expected to control. Therefore, the potential antagonists are isolated from the growth habitats of the host plants. However, antagonists selected with the ecological approach for control of a seedborne disease in one crop might also lead to effective control of another seedborne disease, especially if the pathogens have a similar ecology and epidemiology.

Seed priming is used commercially in many horticultural crops, including carrot, as a tool to increase speed and uniformity of germination and to improve final stand under environmental stress conditions (4,8,29,36). However, if seeds are infected or contaminated with pathogenic or saprophytic fungi, fungal growth can be enhanced during priming (31,40), thus resulting in undesirable effects on plant health. Fungicides have been applied for elimination of microbial growth during priming processes (29), but have limited use as stated previously. Therefore, application of antagonistic microorganisms during priming (biopriming) could represent an environmentally friendly strategy for control of seedborne pathogens. Inoculation of seeds with BCAs in combination with priming has, in several cases, been reported to enhance and stabil-

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ize the efficacy of BCAs, but such approaches have been used mainly for control of soilborne diseases (5,6,11,42).

The objective of the present work was to screen and compare the biocontrol efficacy of various strains isolated from cereal and carrot habitats against *A. radicina* and *A. dauci*. One isolate, IK726 of *Clonostachys rosea*, with high biocontrol efficacy was subsequently selected, and the effect of priming carrot seed with this antagonist on the incidence of *A. dauci* and *A. radicina* and on pre- and postemergence infection was investigated. Furthermore, the development of the fungal antagonist during and after the priming process was studied using a green fluorescent protein (GFP) transformant of *C. rosea* IK726.

MATERIALS AND METHODS

Seed material and fungal pathogens. Two naturally infected seed lots of carrot cv. Royal Chantenay Rola were used in the experiments. The infection level was determined by deep freeze blotter test (14). On seed lot 142568, infection percentages were 29% *A. radicina*, 11% *A. dauci*, and 30% contamination with *A. alternata*. On seed lot 133087, infection percentages were 4% *A. radicina*, 7% *A. dauci*, and 13% contamination with *A. alternata*. Germination percentages of lots 142568 and 133087 on filter paper were 55 and 86%, respectively. Seed of the same cultivar (lot 142849) with less than 1% *Alternaria* pathogens and a germination percentage of 85% were used for artificial inoculation in screening experiments. Seed were surface-sterilized in 1.5% NaOCl (7) and inoculated with *A. radicina* isolate IK1926, which previously was isolated from a diseased carrot seedling. Spores of *A. radicina* were harvested from 14-day-old potato dextrose agar (PDA, Difco Laboratories, Detroit) cultures after filtration through three layers of cheesecloth. Spore suspensions were adjusted with water to 5×10^4 spores per ml. Seeds were inoculated by shaking 10 g of seed in a 40-ml water suspension for 10 min at 150 rpm. Subsequently, seeds were air-dried overnight on filter paper in a laminar flow hood and stored at 4°C until use.

Isolation and cultivation of fungal antagonists. Microorganisms were isolated from different carrot habitats by washing, plating, and particle filtration techniques. Isolations were made from (i) peels of three independent batches of stored carrots, (ii) freshly harvested carrots obtained from a carrot field trial in a conventional farming system and a carrot field trial in an organic farming system, (iii) seed coats of a seed lot infected with *Alternaria* spp., and (iv) hydroprimed carrot seeds. Three different media were used for isolation of fungi. Two were nutrient rich: PDA amended with 2.2 g of Triton X-100 per liter and 0.5 g of chloramphenicol per liter and potato carrot agar amended with 0.5 g of chloramphenicol per liter. The third medium was special low-nutrient agar (ADSA Micro) (33). Two media were used to isolate yeasts: acidified yeast media (YM), pH 3.7 (24), and osmophilic YM amended with 40% glucose, pH 4.5 (24). In total, 211 isolates of saprophytic fungi and yeast were obtained. Of these, 105 isolates (avoiding duplicates or triplicates in taxa of unknown antagonistic properties) were tested in a plant bioassay with naturally infected seeds. For each screening experiment, the isolates to be tested were grown on PDA at room temperature for 14 days.

A group of isolates originating from cereal habitats that previously had shown antagonistic properties against seedborne cereal seedling diseases caused by *Fusarium culmorum* and *Bipolaris sorokiniana* (22) were tested in plant bioassays with naturally infected seeds and seed artificially inoculated with *A. radicina*, respectively. Twenty-six fungal isolates originating from bulk soil (12 isolates), barley straw (9), roots infected with *F. culmorum* (2), barley seed (2), and barley leaves (1) were recultivated from -80°C agar plug freeze cultures on PDA for 1 week. Subsequently, 5-mm-diameter agar disks were transferred to new PDA plates and incubated at room temperature for 14 days, except isolates IK17 and IK194 which were grown for 21 days before use.

Dried formulations of BCAs. Several commercial biocontrol products were tested also: Promot (*T. harzianum* and *T. koningii*), 5×10^7 CFU/g; Supresivit (*T. harzianum*), 2×10^9 CFU/g; TRI003 (*T. harzianum*), 3×10^8 CFU/g; GlioMix (*Gliocladium catenulatum*), 1×10^8 CFU/g; and Subtilex MB600 (formerly Epic), (*Bacillus subtilis*), 5×10^{10} CFU/g. Dry formulations of the near commercial isolates *C. rosea* IK726 (18) and IK726d11, a *gfp* transformant of IK726 (26) also were included. Both dried peat-bran preparations of IK726 and IK726d11 (4×10^8 CFU/g), and a dried clay preparation of IK726 (2×10^9 CFU/g), were prepared according to Jensen et al. (19). All dried formulations were stored at 4°C until use.

Seed coating with antagonists. Fungal spores were gently scraped from PDA cultures in water and filtered through nylon mesh (38 µm). All spore solutions were adjusted with sterile water to a density concentration of 1×10^7 CFU/ml. Seeds were coated by shaking 1 g of seed per treatment with 4 ml of the adjusted conidial suspension on a shaker (IKA Vibrax; IKA Works, Wilmington, NC) for 10 min at approximately 130 rpm. Subsequently, the seed were air-dried on filter paper for 1 h in a laminar flow hood before planting. For coating of seed with dry formulations of BCA, amounts of the respective dry formulations of BCA resulting in 1×10^7 CFU/ml were suspended in sterile water and shaken for 1 min on a vortexer. Treated seed were subsequently coated and dried as described previously. Effects of antagonist treatments were compared with water treatment and with the chemical fungicide iprodione (20 ml per kg of seed).

Hydropriming and biopriming. Seed of carrot lots 142568 and 133087 were imbibed in aerated water (50 g of seed per 500 ml of water) for 16 h. Seed were surface-dried for about 1 h, and the water content was determined whereupon portions of seeds were dried to predetermined moisture contents (MC) of 38 and 40%. Thereafter, seed were incubated in plastic containers with two 1-mm holes in the lid at 15°C for an additional 13 days. For biopriming with *C. rosea* IK726, the clay inoculum of IK726 was applied during imbibition of seeds (2.5 g of clay inoculum per 500 ml of water) or the IK726 inoculum was dusted onto the seed after drying the seed (0.01 g of inoculum per g of seed at 40% MC). The MC during priming was tested after 7 and 14 days priming by oven-drying 1-g seed samples at 130°C for 1 h (14). After priming, seeds were dried in a laminar hood overnight at $22 \pm 3^\circ\text{C}$ to an MC of $7.8\% \pm 0.2\%$ before use in bioassays and blotter tests. Samples of hydro- and bioprimed seed were stored in airtight containers at 4°C. Hydropriming and biopriming experiments were conducted three times for seed lot 142568 and twice for lot 133087.

Bioassay. Treated seed were sown in washed, coarse sand moistened with tap water (3:1, vol/vol). Plastic boxes (11 × 17 cm) were filled with 300 ml of moist sand, and 50 seed were planted in 1-cm-deep holes and covered with 50 ml of moist sand. Each treatment was replicated four times (in total, 200 seeds per treatment) and arranged in a fully randomized block design. Each block included a single replicate of each treatment and consisted of a tray that was covered with transparent plastic foil. Planted boxes were placed in a growth chamber at $20 \pm 1^\circ\text{C}$, with a 12-h photoperiod at 200 µm/m/s light intensity. Seedlings were watered after 8 and 14 days with tap water using a water atomizer. For screening bioassays, seedling emergence was recorded after 14 and 21 days, whereas in priming experiments, seedling emergence was recorded regularly from 5 to 28 days after sowing. Dead and wilted plants were removed and postemergence infection was confirmed by incubating the wilted plants on three layers of moistened filter paper. After 2 to 5 days incubation under near-UV light (NUV) with a 12-h light period at $20 \pm 1^\circ\text{C}$, which allowed *Alternaria* spp. to sporulate, the plants were examined by dissection microscopy and all species of *Alternaria* were recorded. For the hydropriming treatments, seedling establishment of one set of samples was evaluated immediately after drying seeds to the

predetermined MC (1 day priming), a second set after 7 days incubation (8 days priming), and a third set after 13 days (14 days priming). Furthermore, subsamples of stored seeds were evaluated in bioassays after 1, 2, 3, 4, and 5 months of storage at 4°C.

Examination of *Alternaria* spp. on seed. The occurrence of *Alternaria* spp. on carrot seed during different steps of priming was assessed with a deep freeze blotter test (14). Samples were

taken during the priming process at 1, 8, and 14 days priming. Seed (40 × 10 seeds per treatment) were incubated in petri dishes on moist filter paper for 3 days in darkness at 20°C. Seed were subsequently kept at -20°C for 24 h followed by incubation under NUV at 20°C. The presence of *A. radicina*, *A. dauci*, and *A. alternata* on the seed was recorded under a dissection microscope (Olympus SZH; Olympus, Lake Success, NY) after 7 and 10 days

Fig. 1. Screening of fungal isolates originating from cereal habitats. Spore suspensions were coated on carrot seeds naturally infected with *Alternaria radicina* and *A. dauci*. Emergence of healthy seedlings relative to the disease control (100, solid line) and the fungicide-treated control (162, dashed line) 3 weeks after planting in sand. Treatments marked with a ★ are significantly different ($P = 0.05$) from the water-treated disease control.

Fig. 2. Screening of fungal isolates originating from cereal habitats. Spore suspensions were coated on carrot seeds artificially inoculated with *Alternaria radicina*. Total percentage of emerged seedlings and of healthy seedlings 3 weeks after planting in sand. Treatments marked with a ★ are not significantly different ($P = 0.05$) from the iprodione control.

incubation. The occurrence of *Alternaria* spp. on seed was expressed as the percentage of seed where a fungal species was identified.

Monitoring the population of *C. rosea* on seeds. The population density of *C. rosea* on carrot seed was determined at 1- to 3-day intervals during the priming period. Subsamples of 3 ×

20 seeds per treatment were shaken with 3 ml of sterile water and 20 glass beads for 1 min on a vortexer at full speed. Serial dilutions of washing water were plated on PDA amended with chloramphenicol (0.5 g/liter) and Triton X-100 (2.1 ml/liter), and the plates were incubated at 20 ± 2°C. Colonies were counted after 2 to 4 days, and the mean number of CFU per seed was calcu-

Fig. 3. A and B, Screening of fungal isolates originating from carrot habitats. Spore suspensions were coated on carrot seeds naturally infected with *Alternaria radicina* and *A. dauci*. Emergence of healthy seedling 3 weeks after planting in sand. Treatments marked with a ★ are significantly different ($P = 0.05$) from the water-treated disease control.

lated. The same method was used for evaluation of CFU on seed dried to $7.8\% \pm 0.2\%$ MC and stored for 0, 16, 21, 25, and 37 weeks.

The growth and development of *C. rosea* on seed during priming was monitored using the *gfp*-transformed isolate (IK726d11) of biocontrol strain *C. rosea* IK726 (26). Seed were primed with a peat-bran formulation of *C. rosea* IK726d11 by dusting the formulation onto seed at a predetermined MC (0.1 g of IK726d11 per g of seed at 40% MC), as previously described. Seed primed with water only or with wild-type *C. rosea* were used as controls. Seed for microscopy examination, 30 at each time, were sampled immediately after application at day 0 and after 1, 2, 3, 4, 5, 7, 8, 11, and 13 days (14 days of priming). Heavy mycelial growth and sporulation with verticillate and penicillate conidiophores were visualized with a stereo fluorescence microscope (SFM) (Leica MZ FLII; Leica Laser Technik, Heidelberg, Germany) equipped with a GFP 2 filter block with excitation at 480/40 nm and a barrier filter at 510 nm LP. Fine hyphal growth, spore germination, and individual spores were examined with an epifluorescence microscope (EFM) (Olympus BX 60) equipped with an MF filter block (455 to 495 nm excitation wavelength, mirror at 505 nm, and barrier filter at 515 to 555 nm) or a WIB filter block (460 to 490 nm excitation wavelength, mirror at 505 nm, and barrier filter at 515 to 550 nm) and by a confocal laser scanning microscope (TCS4d; Leica Laser Technik).

Statistics. Data were analyzed by analysis of variance and treatment means were compared with a least significant difference test at $P = 0.05$. The bioassay for screening isolates included a maximum of eight antagonist treatments. The comparison of isolates was based on significant differences from the water and fungicide controls within each bioassay. The CFU data were $\log_{10}$ -transformed before analysis of variance. Analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC).

RESULTS

Screening of fungal isolates. Among fungal isolates that previously showed antagonistic activity against *F. culmorum* and *Bipolaris sorokiniana*, 10 of 24 isolates significantly improved

seedling emergence from seeds naturally infected with *A. dauci* and *A. radicina* (Fig. 1). The five *C. rosea* isolates and one isolate each belonging to *Humicola*, *Aureobasidium pullulans*, *F. merismoides*, *Phoma*, and *Acremonium*, respectively, had a biocontrol efficacy not significantly different from the iprodione control. When the same isolates were tested on seeds artificially infected with *A. radicina*, only the *C. rosea* isolates controlled pre- and postemergence infection of the pathogen as well as iprodione (Fig. 2). Several other isolates significantly improved healthy seedling stand, but not to the level of the healthy control and the fungicide control (Fig. 2). Of the commercial products, only TRI003 and Gliomix significantly increased emergence from naturally infected seeds (Fig. 1), but only Gliomix was as effective against artificial *A. radicina* infection as the iprodione treatment (Fig. 2). Of isolates obtained from carrot habitats, 24 of the 105 isolates screened significantly improved seedling emergence from seeds naturally infected with *A. dauci* and *A. radicina* (Fig. 3). Of the effective isolates, 13 belonged to *C. rosea*. The remaining isolates belonged to *C. catenulatum* (isolate 1878), *C. solani* (isolate 1889), *Clonostachys* sp. (isolate 1908), *Acremonium strictum* (isolate 1806), *F. tabacinum* (isolates 1755 and 1786), *F. oxysporum* (isolate 1838), *Cylindrocarpus* sp. (isolate 1741), and unidentified fungi (isolates 1758, 1834, and 1835). The biocontrol efficacy of isolates 1897 and 1907 of *C. rosea* was not significantly different from control provided by iprodione.

Effect of biopriming on occurrence of *Alternaria* spp. Hydropriming and biopriming of carrot seeds naturally infected with *A. radicina*, *A. dauci*, and *A. alternata* significantly affected occurrence of these pathogens (Table 1). After 8 days of hydropriming, the occurrence of *A. radicina*, *A. dauci*, and *A. alternata* on seed lot 142568 had each increased to more than 60%. However, for both biopriming treatments, the occurrences of all *Alternaria* species were <6% and furthermore were significantly lower ($P < 0.0001$) than the initial incidence of each species before biopriming. The occurrence of *A. dauci* increased fivefold after 14 days of hydropriming ($P < 0.0001$). In contrast, biopriming strongly suppressed *Alternaria* spp., as occurrences of all three species were below 4.8%, which corresponded to a reduction in incidence of $\geq 94\%$ of each *Alternaria* species compared with

TABLE 1. Results of blotter tests where the occurrences of *Alternaria radicina*, *A. dauci*, and *A. alternata* were recorded on two carrot seed lots hydroprimed and bioprimed with *Clonostachys rosea* IK726, at 15°C

Seed lot	Seed treatment	Fungal species identified in blotter tests ^y	Occurrence of fungi on seeds (%) ^w		
			Priming period		
			0 days	8 days	14 days
142568	Hydroprimed ^x	<i>A. radicina</i>	27.5 ± 7.2	67.0 ± 7.0	53.3 ± 3.0
		<i>A. dauci</i>	13.8 ± 1.5	64.3 ± 4.2	75.3 ± 3.4
		<i>A. alternata</i>	32.8 ± 6.5	82.5 ± 5.4	87.8 ± 4.0
	Bioprimed IK726 (applied at imbibition) ^y	<i>A. radicina</i>	27.5 ± 7.2	4.8 ± 2.5	1.0 ± 0.8
		<i>A. dauci</i>	13.8 ± 1.5	2.0 ± 0.8	2.3 ± 1.0
		<i>A. alternata</i>	32.8 ± 6.5	4.8 ± 2.5	2.0 ± 0.8
	Bioprimed IK726 (applied after imbibition) ^z	<i>A. radicina</i>	27.5 ± 7.2	6.0 ± 0.8	2.3 ± 1.7
		<i>A. dauci</i>	13.8 ± 1.5	6.0 ± 2.8	4.8 ± 1.3
		<i>A. alternata</i>	32.8 ± 6.5	6.3 ± 2.2	4.0 ± 1.4
133087	Hydroprimed	<i>A. radicina</i>	7.6 ± 3.7	12.3 ± 3.0	14.5 ± 2.2
		<i>A. dauci</i>	5.1 ± 2.8	31.8 ± 8.5	39.3 ± 4.3
		<i>A. alternata</i>	13.0 ± 5.9	44.3 ± 3.2	67.8 ± 4.4
	Bioprimed IK726 (applied at imbibition)	<i>A. radicina</i>	7.6 ± 3.7	0.8 ± 0.4	0
		<i>A. dauci</i>	5.1 ± 2.8	2.5 ± 2.1	0.5 ± 0.9
		<i>A. alternata</i>	13.0 ± 5.9	0.3 ± 0.4	0

^w A total of 400 seeds per treatment were incubated on moist filter paper at 20°C in darkness for 3 days, placed at -20°C for 24 h, and subsequently incubated at 20°C under near-UV light for 7 days.

^x Mean percentage of seeds where *A. radicina*, *A. dauci*, or *A. alternata* occurred. Mean values are from four replicates ± standard deviation.

^y Seeds were imbibed in aerated water (50 g of seed per 500 ml of water) for 16 h, dried to a predetermined moisture content (MC) of 40%, and subsequently incubated at 15°C. The procedure was also used for biopriming seeds.

^z Clay inoculum of IK726 was applied at imbibition of seeds (2.5 g of clay inoculum per 500 ml of water) before drying seeds to 40% MC.

^z Clay inoculum of *C. rosea* IK726 was dusted onto seeds (0.01 g per g of seed at 40% MC) after seeds were imbibed and dried to 40% MC.

hydroprimed seeds. For seed lot 133087, which had a substantially lower infestation rate, a similar result was obtained. The occurrence of *A. radicina* increased twofold during hydropriming, whereas that of *A. dauci* increased about eightfold. In contrast, biopriming almost eradicated the three *Alternaria* spp.

Effect of biopriming on seedling emergence. Seedling emergence was affected significantly after 1, 8, and 14 days of hydropriming or biopriming (Table 2). When seeds were primed for 1 day, the initial emergence of primed seeds was as high as for nonprimed seeds. However, in the standard priming treatment, about one-third of the emerged seedlings subsequently died due to postemergence infection, resulting in a final seedling stand of 32.5%. Priming of carrot seeds with water for 8 and 14 days affected the establishment of carrot seedlings even more (Table 2). Thus, although the total emergence was about 30%, postemergence death reduced the final seedling stand to 7%. In contrast, biopriming resulted in more than 46% of emerged seedlings and more than 43% of healthy seedlings, irrespective of the method for application of *C. rosea* IK726. Priming treatments affected seedling emergence of seed lot 133087 in a similar manner (data not shown), although the negative effect of hydropriming was less dramatic due to the lower *Alternaria* infestation level. Based on examination of wilted and dead seedlings from 14-day primed seed, infection by *A. radicina* and *A. dauci* corresponded to 24.3 and 43.3% of the total number of emerged seedlings, respectively (Fig. 4). For bioprimed seed, 1.8 to 6.5% of emerged seedlings were infected by *A. radicina*, whereas 2.3 to 6.5% were infected by *A. dauci* (Fig. 4). All dead seedlings from nonprimed seeds (9.8% of emerged seedlings) were infected by *A. radicina*.

Effects of biopriming on seed storage. Speed of emergence and seedling establishment of stored bioprimed seed were evaluated during a 5-month period and compared to that of nonprimed seed (Fig. 5). Both primed and bioprimed seeds started to emerge after 5 days in the sand bioassays. At this time, no emergence was observed from nonprimed seeds (Fig. 5B). In addition, maximum stands of healthy seedlings were achieved approximately 7 days after sowing in the priming treatments. In contrast, less than 70% of the seedlings had emerged from nonprimed seeds at this time. Healthy seedling stand after biopriming treatments stabilized between 50 to 60%, irrespective of the storage period (Fig. 5A to D), whereas postemergence death resulted in less than 20% of healthy seedlings during 5 months of storage of seed primed without *C. rosea* IK726.

Multiplication of *C. rosea* IK726 during priming. After application of IK726 to seeds either through imbibition of seeds or after drying seeds to 38 or 40% MC, the initial density of *C. rosea* varied between 0.7×10^4 to 1.2×10^4 CFU per seed (Fig. 6). During the first 2 to 3 days of priming, the population

density decreased significantly ($P < 0.0001$) for all biopriming treatments. However, the densities increased significantly 5 days after seed inoculation, with the best result for biopriming at 40% MC. At the end of biopriming at day 14, the antagonist had further multiplied on the seeds as both of the 40% MC treatments had about 5.5×10^4 CFU per seed while the 38% MC treatment had 4.5×10^4 CFU per seed (Fig. 6).

Survival of *C. rosea* IK726 after storage of primed seeds. After drying seeds to 8% MC, the population density was significantly reduced by 40% ($P < 0.049$) and 61% ($P < 0.007$) for the two treatments where *C. rosea* IK726 was applied during imbibition followed by incubation at 40 and 38% MC, respectively. When *C. rosea* IK726 was applied after the imbibition, seed drying did not result in a significant decrease in population density (Figs. 6 and 7). During storage at 4°C, the CFU per seed gradually decreased (Fig. 7). After 37 weeks, the population density was significantly reduced to 29% of the density at the beginning of storage of seed in which *C. rosea* IK726 was applied to seed at 40% MC ($P < 0.009$), and by 26% ($P < 0.002$) and 40% ($P < 0.045$) for the two treatments in which *C. rosea* IK726 was applied during imbibition followed by incubation at 40 and 38% MC, respectively.

Fig. 4. Effect of priming and biopriming with *Clonostachys rosea* IK726 on postemergence death of carrot seedlings caused by *Alternaria radicina* or *A. dauci*. Isolate IK726 was either applied at imbibition, before drying seeds to 38 or 40% moisture content (MC), or after imbibition but postdrying seeds to 40% MC. Before storage, seeds were dried to 8% MC. Error bars indicate standard errors of the means of four replicates.

TABLE 2. Effect of various priming treatments on total seedling emergence and final stand of healthy carrot seedlings after several days of priming

Treatment	Seedling emergence (%)							
	Nonprimed		Priming 1 day		Priming 8 days		Priming 14 days	
	Total [†]	Healthy [‡]	Total	Healthy	Total	Healthy	Total	Healthy
Hydroprimed, 40% MC ^v	43.0	37.7	44.5 a ^w	32.5 a	22.0 a	0.5 a	30.0 a	6.7 a
Bioprimed, <i>Clonostachys rosea</i> IK726 pre-40% MC ^x	43.0	37.7	62.6 a	58.7 b	62.0 b	59.0 b	54.0 bc	52.0 bc
Bioprimed, <i>C. rosea</i> IK726 pre-38% MC ^y	43.0	37.7	58.5 a	53.5 b	62.0 b	59.0 b	59.0 c	56.0 c
Bioprimed, <i>C. rosea</i> IK726 post-40% MC ^z	43.0	37.7	59.5 a	56.5 b	56.0 b	52.0 b	46.0 b	43.5 b
P value			0.0847	0.0029	0.0001	0.0002	0.0002	0.0001

[†] Total seedling emergence.

[‡] Final stand of healthy seedlings. Wilted and dead seedlings infected by *Alternaria radicina* or *A. dauci* were excluded.

^v Seeds were imbibed in aerated water (50 g of seed per 500 ml of water) for 16 h, dried to a predetermined moisture content (MC) of 40%, and subsequently incubated at 15°C. For biopriming, the method was modified as specified.

^w In each column, means of four replicates followed by different letters differ significantly according to an *F*-protected least significant difference test at $P = 0.05$. The actual *P* value is given below each column.

^x Clay inoculum of *C. rosea* IK726 was applied at imbibition of seeds (2.5 g of clay inoculum per 500 ml of water) before drying seeds to 40% MC.

^y Clay inoculum of *C. rosea* IK726 was applied at imbibition of seeds (2.5 g of clay inoculum per 500 ml of water) before drying seeds to 38% MC.

^z Clay inoculum of *C. rosea* IK726 was dusted onto seeds (0.01 g per g of seed at 40% MC) after seeds were imbibed and dried to 40% MC.

Microscopic evaluation of seed during priming. The development and establishment of *C. rosea* IK726d11 during bio-priming of carrot seeds was investigated by fluorescence microscopy. The increase in mycelial growth and sporulation on the

seed surface from day 1 to day 14 is visualized in Figure 8A to I. Discrete fluorescent aggregates, mainly associated with peat-bran particles, were visible on seed treated with *C. rosea* IK726d11 on day 0 and day 1 (Fig. 8A). Hyphal growth from some of the

Fig. 5. A to D, The effect of bio-priming with *Clonostachys rosea* IK726 on healthy carrot seedling stand in sand tests for A, unstored seeds; B, seeds stored 3 months; C, seeds stored 4 months; and D, seeds stored 5 months at 4°C. ■ = primed seeds (P), ▲ = bioprimed *C. rosea* IK726 added at imbibition followed by drying seeds to 40% moisture content (MC), ▼ = bioprimed *C. rosea* IK726 added at imbibition followed by drying seeds to 38% MC, ● = bioprimed *C. rosea* IK726 added after imbibition and drying seeds to 40% MC, and □ = nonprimed seeds. Error bars indicate standard errors of the means of four replicates.

Fig. 6. Multiplication of *Clonostachys rosea* IK726 on carrot seed during bio-priming at 15°C. ▲ = bioprimed *C. rosea* IK726 added at imbibition before drying seeds to 40% moisture content (MC), ▼ = bioprimed *C. rosea* IK726 added at imbibition followed by drying seeds to 38% MC, and ● = bioprimed *C. rosea* IK726 added after imbibition and drying seeds to 40% MC. Error bars indicate standard errors of the means of three replicates.

Fig. 7. Survival of *Clonostachys rosea* IK726 during storage of bio-primed seeds at 4°C. Isolate IK726 was applied either at imbibition before (pre) drying seeds to 38 and 40% moisture content (MC) or after imbibition and drying seeds (post) to 40% MC. Primed seeds were dried to 8% MC before storage. Error bars represent standard errors of the means of three replicates.

Fig. 8. Development and establishment of the *gfp* transformant *Clonostachys rosea* IK726d11 on carrot seed during biopriming at 15°C studied with stereo fluorescence microscopy (SFM) and confocal laser scanning microscopy (CLSM). Growth of isolate IK726d11 was visualized with SFM on seeds after a biopriming period of **A**, 1 day; **B**, 2 days; **C**, 3 days; **D**, 5 days; **F**, 7 days; and **H**, 14 days. **E**, Sporulation with verticillate conidiophores was observed after 4 to 5 days. **I**, Penicillate conidiophores were observed after 6 days. **G**, With CLSM, a fine web of hyphae was seen on the pericarp at day 7. **J**, The development in density of *C. rosea* IK726d11 on seeds (CFU/seed) was observed during biopriming. Error bars represent standard errors of the mean of three replicates. **A**, **B**, **C**, **D**, **F**, and **H**, Bars represent 250 µm; **I**, 100 µm; **E**, 50 µm; and **G**, 20 µm.

aggregates was observed with an SFM after 2 days. Conidial germination and the presence of fine hyphae scattered on the pericarp were verified with an EFM. Sporulation, with verticillate conidiophores, was observed from day 4 to 5 and onward (Fig. 8E). The intensity of sporulation was increased on the following days, which corresponded with a significant ($P < 0.001$) increase in CFU per seed (Fig. 8J). From day 7, a fine web of mycelium could be seen (Fig. 8F and G) and at the end of priming penicillate, and verticillate conidiophores covered the whole seed surface (Fig. 8H to I). Fungal growth was also present at the micropylar end of the seed where the radicle and shoot protrude from the pericarp (Fig. 8I). Background fluorescence from the wild-type *C. rosea* or from the untreated primed seeds was generally not confounded with the fluorescence emitted from *C. rosea* IK726d11.

DISCUSSION

Several antagonistic isolates effective against seedborne *A. radicina* and *A. dauci* were identified after in planta screening of fungi isolated from cereal and carrot habitats. Isolates from the cereal group were selected from a group of isolates representing different genera that previously showed a biocontrol efficacy of >50% against seedborne *F. culmorum* in sand tests (21). Isolates from the carrot group were obtained from different carrot-related material. For both groups, isolates belonging to *C. rosea*, in general, had a very high biocontrol efficacy. Moreover, in both isolate groups (not shown for carrot isolates), isolates having a level of efficacy equal to the fungicide iprodione on *A. radicina*-infested seeds belonged to *C. rosea*, *Gliocladium* spp., or *Clonostachys* spp. *sensu stricto*. The overlap in effective antagonistic species between isolates from cereal and from carrot habitats might be related to similarities in infection biology of the pathogens that the isolates are aimed to control. Seedborne *A. radicina* and *A. dauci* are generally superficially located in or on the pericarp (37,38) and infect the seedling through the hypocotyls, as does the cereal seedborne pathogen *F. culmorum* (27,32). The biocontrol potential of *C. rosea* has been shown in several systems. In the field, *C. rosea* isolate IK726 had high efficacy against three seedborne cereal diseases caused by *F. culmorum*, *Bipolaris sorokiniana*, and *Tilletia tritici*, respectively (17,18,22). The isolate also has exhibited effective control against *A. radicina* in growth chamber experiments (26).

Priming strongly increased the incidence of *Alternaria* spp. In agreement with our results, hydropriming increased the incidence of *A. dauci*, *A. radicina*, and *A. alternata* on carrot seed (40). Baniek and Tylkowska (1) showed that osmopriming increased the general microflora, although the level of *A. radicina* was unaffected or even decreased. On the other hand, Nascimento and West (31) reported that osmopriming increased *Alternaria* spp. on melon seeds from 2 to 50%. Maude et al. (29), however, concluded that osmopriming did not affect the rate of transmission of *A. dauci*. The different effects of priming on the occurrence of *Alternaria* spp. is probably related to both differences in priming procedures as well as location and incidence of the pathogens of the seed lots. In the present study, the enhanced growth of *A. dauci* and *A. radicina* during priming could primarily explain the large reduction in final stand of healthy seedlings compared with that of nonprimed seeds. In contrast, biopriming almost eradicated the two pathogens, which lead to a highly improved seedling stand that was significantly higher than that of non-primed seeds.

Based on dilution plating of bioprimed seed, the population density of *C. rosea* IK726 increased significantly from about 1×10^4 CFU per seed to 3 to 5×10^4 CFU per seed, irrespective of the application method. In replicated experiments with bioprimed seeds at 38 to 40% MC, the final density never exceeded 8×10^4 CFU per seed, which seemed to be the limit for multiplication

of *C. rosea* IK726 on carrot seeds. Data on multiplication of fungi on seed during biopriming are limited. However, Harman and Taylor (10) found that *T. harzianum* strain T-22 increased 10-fold to a level of 10^4 CFU per seed during matrix priming of tomato and cucumber seed. In this study, the temporary decrease in population density observed after 2 to 3 days of priming coincides with the time of conidial germination and hyphal growth of *C. rosea* IK726 as verified with fluorescent microscopy. A similar relationship was found for *T. harzianum* when conidia of the antagonist were introduced to growth medium (9). Based on microscopic evaluation of the growth and distribution of the antagonist during priming, *C. rosea* IK726d11 colonized the whole surface of the pericarp, including the apex of the seed where the primary root emerges. This is of course important for protecting the seedling against inoculum of pathogenic *Alternaria* spp. as well as other seedborne pathogens located on or in the pericarp. Furthermore, it provides an opportunity to protect the seedling from soil-borne pathogens attacking the emerging seedling. In preliminary experiments with carrot, *C. rosea* IK726 biopriming reduced seedling damping-off caused by *Pythium ultimum* mixed in the growing media (B. Jensen, unpublished data), and in field experiments, spraying with *C. rosea* IK726 conidia reduced leaf and head rot of Chinese cabbage caused by *Pythium tracheiphilum* (30).

In the present study, we used a water priming technique to imbibe seeds and subsequently dried them to a predetermined MC. The application of antagonists was made either during imbibition or after the seeds were dried to the predetermined MC. By the latter method, considerably less material of the BCA (<1/3) was applied. At the same time, the efficacy against the seedborne pathogens was approximately equal for the two priming methods. Furthermore, the waste water from the priming process was not contaminated with the antagonist. Application of *C. rosea* IK726 after drying seeds, therefore, seems to be a more economically and environmentally feasible biopriming procedure. Except for the method of adjusting seeds to a predetermined MC, the priming method used in the present experiments very much resembles the drum-priming technique used commercially (36). *C. rosea* IK726 may therefore be a potential candidate for biopriming in practice.

The results of priming two seed lots with a high and a low level of natural infection with *Alternaria* pathogens indicated that *A. dauci* occurred at higher levels than *A. radicina* after application of the hydropriming technique used in the present study. Based on examination of diseased seedlings after emergence, about two-thirds of these seedlings were infected with *A. dauci*. Because the contamination level for *A. dauci* was lower than that for *A. radicina* at the beginning, this further indicates that *A. dauci* develops more efficiently on primed seed. Data on the occurrence of *Alternaria* spp. immediately after the imbibition previously showed that this process increased spread of conidia within the seed lot (20). Tylkowska and Van den Bulk (40) hydroprimed two carrot seed lots using a laboratory technique imitating drum priming (36), but did not find that *A. dauci* was favored in particular. This could be related to the different imbibition procedures for the two hydropriming methods.

In this study, we demonstrate that biopriming with the biocontrol strain *C. rosea* isolate IK726 makes it possible to prime seeds infected with pathogenic *Alternaria* spp. without risking adverse effects on seedling establishment. This is particularly interesting for organic farmers because it is difficult to produce high quality disease-free seed organically.

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