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Biological seed treatment of cereals with fresh and long-term stored formulations of *Clonostachys rosea*: Biocontrol efficacy against *Fusarium culmorum*

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Abstract

In six field experiments, seed treatment with *Clonostachys rosea* (IK726) significantly reduced disease caused by *Fusarium culmorum*. IK726 was active against the pathogen at average soil temperatures at sowing ranging from 6.2 to 12 °C. Both in the field experiments and in growth chamber experiments conducted in sand, dried and stored conidia of IK726 controlled *F. culmorum* as effectively as freshly harvested conidia. A high correlation was found between disease index ratings from field experiments and from corresponding growth chamber sand tests. Amendment with the stickers Pelgel or Sepiret did not influence control activity. The effective dosages of IK726 (cfu/seed) were estimated in bioassays and were very similar for freshly harvested conidia and for dried conidia. With a density of $> 5 \times 10^3$ conidia per seed more than 80% disease control was repeatedly obtained with both types of conidia.

Introduction

Chemical seed treatment effectively controls seedborne diseases of cereals and in many European countries seed lots are routinely treated with chemicals (Rennie and Cockerell, 1994). However, certain limitations and environmental disadvantages have been associated with the use of chemical fungicides. This has increased the demand for alternatives and coating seeds with antagonistic microorganisms may be such an alternative. A Nordic project was initiated in order to screen for microorganisms antagonistic to various important seedborne diseases of cereals and adapted to the North European soil habitats and micro environments (Knudsen et al., 1997). From this project a biological control agent (BCA) based on *Pseudomonas chlororaphis* and the biological product GlioMix (*Gliocladium* spp.) has been developed in Sweden and Finland respectively (Johnsson et al., 1998; Hökeberg et al., 1997; Knudsen et al., 1997). In Denmark, an antagonistic isolate (IK726) of *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Seifert & W. Gams (formerly *Gliocladium roseum* Bain.) (Schroers et al., 1999) was isolated from field soil and tested in field trails. Results showed that seed treatment with freshly harvested conidia of *C. rosea* controlled *Fusarium culmorum* as effectively as seed treatment with a recommended fungicide. In another field experiment, it was demonstrated that IK726 can control disease caused by seedborne *Bipolaris sorokiniana* (Knudsen et al., 1995).

Dry conidia of IK726 germinate more slowly than freshly harvested conidia (Jensen et al., 1996). Such a delay in germination could influence their efficacy against *F. culmorum* especially under unfavourable environmental conditions. The length of storage might also be of significance. For conidia of insect pathogenic fungi such as *Metarrhizium* spp., it has also been shown

that prolonged storage of dried conidia slowed subsequent spore germination which, in some cases, resulted in reduced biocontrol efficacy (Burges, 1998).

BCAs have often proved to be less efficacious and give more variable disease control than the chemical fungicides under field conditions. Such inconsistent biocontrol may be due to initial failure in establishment on the seed or in the rhizosphere related to unfavourable soil conditions such as temperature, moisture content, pH and aeration as well as competition from the indigenous microbiota (Burgess and Keane, 1997; Hubbard et al., 1983; Mathre et al., 1994; Mao et al., 1997). However, for seed bacterization with *P. chlororaphis*, strain MA 342, Johnsson et al. (1998) demonstrated consistent efficacy for several years and under varying climatic zones in Sweden against several seedborne cereal diseases. The inconsistency in biocontrol efficacy under natural growth conditions may also be due to the fact that selection and development of formulations conducted under artifical and controlled conditions in the greenhouse may not correlate well with results obtained in field tests (Duczek, 1994; Knudsen et al., 1997; Teperi et al., 1998). Even so, a reliable greenhouse test would be very valuable in order to facilitate quick and inexpensive evaluation of BCA formulations.

The aim of this study was to investigate the biocontrol efficacy of seed treatment with *C. rosea* (IK726) against seedborne inoculum of *F. culmorum* in relation to sowing date and soil temperature, formulation of IK726 (freshly harvested or stored preparations of IK726) and dosage of IK726 on seeds. The relationship between performance of IK726 in growth chamber sand tests and in field experiments was also investigated.

Materials and methods

Fungal isolates

The fungal antagonist *C. rosea* IK726 was isolated from barley roots infected with *F. culmorum* (Knudsen, 1994). The pathogen isolate of *F. culmorum* (strain 5) used for artificial inoculation of seed was isolated from a barley seed lot (Knudsen, 1994). Stock cultures of the isolates were maintained on potato dextrose agar (PDA) plugs in 10% glycerol at $-80\,^{\circ}$C. For each field experiment and bioassay fresh inoculum of the fungi were prepared from stock cultures recultured on PDA at room temperature for 14 days.

Seed material

The barley variety, 'Alis Abed', was used in the field experiment in 1994 and in the dose–response bioassays. The seed lot had less than 1% infection with *Fusarium* spp. according to the blotter test (Neergaard, 1979). Seed of Alis Abed was surface sterilized for 10 min in 2.5% NaOCl before treatment with pathogen and antagonist. In the field experiments sown in 1995 seed lots naturally infected with *F. culmorum* were used. Seed of barley variety, 'Alexis', and of wheat, 'Sleipner', had 30% and 5% infections of *F. culmorum*, respectively, measured in the blotter test and disease indices (DI) in a growing-on sand test of 0.75 and 0.28 respectively (DI described below).

Inoculation with the pathogen

Both naturally-infected seeds and surface-disinfected seeds were inoculated with spore suspensions of *F. culmorum* (strain 5) in order to obtain a high level of infection. The pathogen was grown on potato dextrose broth (PDB) in shake culture (130 rpm) for 8 days. Spores were harvested by filtration through 2 layers of cheese cloth after mixing the culture in a blender (Waring, model 8011G) for 1 min. The suspension was adjusted to $1.5 \times 10^6 \pm 0.5 \times 10^6$ cfu/ml. Seeds were inoculated by soaking in the spore suspension (1 : 2 w/v) for 1 h followed by 24 h of drying in a sterile laminar flow chamber. Seeds were stored at $4\,^{\circ}$C until use.

Production of IK726 inoculum

Freshly harvested conidia of IK726 were obtained from liquid culture. Erlenmeyer flasks (250 ml) containing 94 ml of sterilized PDB were inoculated with 2 agar plugs (5 mm) of the fungus. Flasks were incubated on a rotary shaker at 130 rpm for 8 days at $21 \pm 3\,^{\circ}$C. After 24 h incubation, 6 ml polyethylene glycol 200 was added to each flask. The biomass was blended for 1 min in a Waring blender (Model 8011G), filtered through 4 layers of cheese cloth and centrifuged for 10 min at 8000 rpm and $4\,^{\circ}$C. The pellet, consisting mainly of conidia, was resuspended in sterile water and conidia concentrations were adjusted within a range from 1×10^4 and 1.5×10^8 conidia/ml.

Stored formulations of IK726 were prepared from growth on a mixture of sphagnum peat, wheat bran and

water (15 : 26 : 59 v/v). Before inoculation with IK726 the media was autoclaved for 20 min on 2 successive days. IK726 was incubated at $22 \pm 2\,^{\circ}$C for 14 days. The inoculum was air-dried for 2 days, milled in a blender (Waring, model 8011G) and stored in glass petri dishes at $4\,^{\circ}$C. For the barley field experiment, preparations were stored for 8 weeks (8×10^8 cfu/g) and 32 weeks (6×10^8 cfu/g) respectively and for the wheat experiment, preparations were stored for 30 weeks (5×10^8 cfu/g). The stored preparations were either used directly by suspending inoculum ground in a mortar in sterile water in order to facilitate even application or by washing conidia from the peat–bran formulation by shaking in water for 1 min followed by filtration through 38 μm nylon mesh. The dry preparations used in dose–response bioassays were stored from 1 to 11 months.

Biological seed treatment

For the 1994 field trial and in the dose–response bioassays, seeds were coated by soaking in conidial suspensions of the antagonist (1 : 2 w/v) for 10 min. For field trials, seeds were dried overnight while seeds for bioassays were dried for 1–2 h. For the field trials in 1995 and the corresponding bioassays, seeds were coated by a laboratory seed treater (Hege II) followed by 2 h of drying at room temperature. When the stickers Pelgel (The Nitragin® CO, USA) and Sepiret® (Sepiret film coating, Seppic, France) were used in the coating procedure they were applied to the antagonist suspension at rates of 2% and 1% w/v respectively. In all experiments, the *Fusarium* controls and the healthy controls (disinfected seed) were treated with sterile water. Coated seeds were not stored before sowing.

Field trials

All field trials were conducted on sandy loam soils at The Royal Veterinary and Agricultural University's (KVL) field station 'Højbakkegaard' in Taastrup, Denmark. Seeds were sown using an experimental sowing machine. Records of climatic conditions with daily measurements of soil temperature (10 cm depth) and precipitation from 'Højbakkegaard' were provided by the Department of Agricultural Science, KVL. For each sowing date a critical soil temperature defined as the average daily temperature from sowing until 7 days later was calculated.

Barley 1994. Seeds of 'Alis Abed', both pathogen inoculated and surface sterilized, were treated with freshly harvested conidia of IK726 (1×10^7 conidia/ml) with or without Pelgel amendment. Plots consisted of 1 m rows (100 seeds per row) arranged in a completely randomized block design with 4 replicates per treatment. Four experiments with the same experimental design were sown on the 13th, 20th and 27th April and 3rd May.

Barley 1995. Seeds of 'Alexis' were coated using the Hege II seed treater and 20 ml suspension per kg seed. Suspensions of fresh IK726 conidia (1.5×10^8 spores/ml), of conidia (SC) stored 8 weeks (2×10^8 spores/ml) and of preparations (SD) stored for 8 or 32 weeks (0.5 g/ml) were applied. All coating suspensions were amended with Sepiret. Plot size was 1.0 m × 1.25 m and corresponding to approximately 600 seeds per plot. Plots were arranged in a completely randomized block design with 5 replicates per treatment. The experiment was sown on 3rd May.

Wheat 1995/96. Seeds of 'Sleipner' were coated using the Hege II seed treater. Fresh conidia (1×10^8 conidia/ml) were applied at a rate of 16 ml/kg seed. A suspension of an IK726 preparation stored for 30 weeks (0.067 g/ml) was applied at 20 or 27 ml/kg seed and amended with either Sepiret or Pelgel. The fungicide Sibutol (bitertanol (280 g/l) + fuberidazol (18 g/l)) was applied in the recommended dosage of 1 ml/kg seed. The plot size was 1.5 m × 10.0 m. Plots were arranged in a completely randomized block design with 5 replicates. Plots were sown on 3rd October.

Growth chamber sand test

Seeds, 3 per pot (4.2 × 4.2 cm; Vefi, Larvik), were sown in sand moistened with tap water (3 : 1 v/v). A strip of 6 serial pots with a total of 18 seeds represented a replication. Each replicate was placed on a saucer to avoid cross contamination. Ten days after sowing, seedlings were watered with 50 ml fertilizer solution (5 ml/l Hornum, P. Brøste Industri A/S, DK) applied to the saucers. The treatments were randomized within each of 4 replicate blocks and each was covered with a plastic bag to maintain high humidity. In all bioassays there were 4 replications per treatment. Plants were incubated in a growth chamber at $15\,^{\circ}$C with a 12 h light period (110 μmol/m^2/s measured outside the bags). The

light was supplied by fluorescent tubes (Phillips TLD 36W/83).

Before sowing, the viability of IK726 on the seed was evaluated by plating washing water from treated seeds onto PDA. The washing test was performed on surface sterilized seeds given the same IK726 treatments as pathogen-inoculated seeds. Twenty randomly selected seeds were washed by shaking with 20 glass beads and 3 ml water on a whirlimixer for 1 min. Series of 10-fold dilutions were made and 100 µl aliquots were spread on PDA. Colonies of IK726 were counted after incubation at 22 ± 3 °C for 3–4 days.

Coating suspensions of IK726 with concentrations from 10^4 to 10^8 spores/ml were produced for the dose–response experiments. The dose–response investigation with freshly harvested conidia and for conidia stored at 4 °C are based on 8 and 13 independent bioassays respectively.

Assessments and data analysis

Plants from the sand test were assessed after 19 days incubation. Disease severity was evaluated on washed roots and coleoptiles using the DI described by Knudsen et al. (1995) where 0 = healthy plants and 4 = dead plants. The percentage of emerged plants was calculated and shoot dry weight was determined after 2 days of oven drying at 60 °C. In the field experiments plant emergence was assessed 3 weeks after sowing by counting the number of plants in 3 × 1 m rows per plot. Disease index was scored and dry weight measured 4 weeks after sowing on 3 × 20 plants per plot. At harvest grain yield and 1000 grain weight were measured.

Analysis of variance were performed by the SAS procedure Proc glm (Anonymous, 1987). Duncan multiple range test was used to compare means.

Results

Effect of sowing date (field experiments 1994)

Treatment with *C. rosea* gave a significant reduction in *F. culmorum* DI at all sowing dates varying from 40% to 73% control compared to the untreated *Fusarium* control (Table 1). Amendment with Pelgel did not influence biocontrol efficacy significantly nor did it influence the level of disease. The average DI in the untreated *Fusarium* controls varied from 0.79 to 1.28 but there was no significant effect of sowing date ($p = 0.11$) although the critical soil temperature increased from 6.2 °C at the first sowing date and up to 10.2 and 9.2 °C when sown respectively 14 and 20 days later. Plant emergence and plant dry weight were assessed 4 weeks after sowing but there were no significant effects of seed treatments (data not shown).

Table 1. The effect of coating seed of barley with freshly harvested conidia of *C. rosea* (IK726) on the control of *F. culmorum* in the field assessed 4 weeks after sowing at different sowing dates in 1994

Treatment	Disease index (DI) 4 weeks after sowing and date of sowing			
	13 April	20 April	27 April	3 May
F. culmorum control (F)[1]	1.11 a[7]	1.08 a	1.28 a	0.79 ab
F + Pelgel[2]	0.91 ab	0.98 a	1.36 a	0.94 a
F + *C. rosea*[3]	0.58 c	—	—	0.50 bc
F + *C. rosea* + Pelgel	0.64 bc	0.62 b	0.39 b	0.48 bc
Healthy control[4]	0.18 d	0.20 c	0.30 b	0.29 c
Healthy control + *C. rosea*	0.09 d	0.21 c	—	0.23 c
Average control with *C. rosea*[5]	40%	40%	73%	43%
Critical soil temperature (°C)[6]	6.1	8.2	10.5	9.2

[1]Seeds were surface sterilized and inoculated with *F. culmorum* (1.5×10^6 spores/ml).
[2]The sticker, Pelgel, was added to the coating suspension (2% w/v).
[3]100 g of seeds were treated with 200 ml suspensions of *C. rosea* (1×10^7 conidia/ml).
[4]Surface sterilized seeds.
[5]The DI values for *F. culmorum* control treatments (± Pelgel) and the DI values for *C. rosea* treatments (± Pelgel) of *F. culmorum* inoculated seeds are averaged.
[6]Critical soil temperature: average daily soil temperature from sowing until 7 days later.
[7]Within columns means with different letters are significantly different ($p = 0.05$) according to the Duncan multiple range test.

Only freshly harvested conidia of IK726 were used in the 1994 experiments.

Effect of storage and formulation on disease control in barley

Compared to the untreated *Fusarium* control, all treatments with IK726 gave a significant reduction in the DI irrespective of formulation (35–54% control). There was no significant difference in the biocontrol efficacy of fresh conidia or stored IK726 conidia nor did storage time affect the biocontrol efficacy (Table 2). Plant emergence and plant dry weight were unaffected by IK726 seed treatments (data not shown). Amendments with the sticker Sepiret did not influence disease development or plant emergence. In Table 3 the results of the corresponding sand test are given. Biological seed treatment significantly reduced the DI with 70–80% compared to the untreated *Fusarium* control. The level of disease was considerably higher in the sand test than in the field experiment.

Effect of storage and formulation on disease control in winter wheat

All seed treatments with IK726 gave a significant reduction in the *F. culmorum* DI ranging from 37% to 68% control dependent on dosage (Table 4). Sibutol,

Table 2. The effect of seed treatment with fresh or stored conidia of *C. rosea* (IK726) on seedborne *F. culmorum*, on field grown spring barley, 1995

Treatments	Disease index[7]	Control (%)	Emergence plants per row	Cfu/seed
F. culmorum control (F.c)[1]	1.24 a[8]	—	72	—
F.c + Sepiret(S)[2]	1.25 b	—	72	—
F.c + Fresh IK726 (1.2×10^8/ml)[3] + S	0.62 b	50	76	9.9×10^3 a
F.c + 8 weeks IK726 (2×10^8/ml)[4] + S	0.69 b	54	80	7.0×10^3 ab
F.c + 32 weeks IK726 (0.5 g/ml)[5] + S	0.78 b	37	79	3.9×10^3 b
F.c + 8 weeks IK726 (0.5 g/ml) + S	0.66 b	47	71	4.8×10^3 b
Healthy control[6]	0.36 c	71	78	—

[1]Seeds naturally infected with *F. culmorum* (30% infection) were inoculated with a suspension of *F. culmorum* (1.5×10^6 spores/ml).
[2]The sticker, Sepiret, was added to the coating suspension (1% w/v).
[3]20 ml per 100 g of seed of a suspension of freshly harvested conidia of IK726.
[4]20 ml per 100 g seed of a suspension of dried conidia of IK726 stored for 8 weeks at 4 °C.
[5]20 ml per 100 g seed of a dried preparation of IK726 stored for 32 weeks at 4 °C.
[6]Seeds were surface sterilized.
[7]4 weeks after sowing.
[8]Means followed by different letters are significantly different ($p = 0.05$) according to the Duncan multiple range test.

Table 3. Effect of seed treatment with fresh or stored conidia of *C. rosea* (IK726) against seedborne *F. culmorum* on spring barley in growth chamber sand test. Results from sand test corresponding to the 1995 barley field experiment (Table 2)

Seed treatments	Disease index[7]	Control (%)	Emergence (%)	Cfu/seed
F. culmorum control (F.c)[1]	2.21 a[8]	—	97	—
F.c + Sepiret (S)[2]	2.10 a	—	97	—
F.c + Fresh IK726 (1.2×10^8/ml)[3] + S	0.70 bc	69	96	9.9×10^3 a
F.c + 8 weeks IK726 (2×10^8/ml)[4] + S	0.38 c	83	94	7.0×10^3 ab
F.c + 32 weeks IK726 (0.5 g/ml)[5] + S	0.78 bc	65	94	3.9×10^3 b
F.c + 8 weeks IK726 (0.5 g/ml) + S	0.60 c	73	96	4.8×10^3 b
Healthy control[6]	1.04 b	53	99	—

[1–6]Conditions as mentioned in Table 2.
[7]After 19 days incubation.
[8]As in Table 2.

Table 4. The effect of seed treatment with fresh or stored conidia of *C. rosea* (IK726) on seedborne *F. culmorum*, on field grown winter wheat, 1995

Seed treatments	Emergence (plants per row)	Relative emergence	Disease index[7]	Disease control (%)	Dryweight (60 plants, mg)	Grain yield (hkg ha^{-1})	1000-kernel weight, (g)	Cfu/seed
F. culmorum control (F.c)[1]	28 a[6]	100	1.01 a	—	29 a	70.8 D	20.0 c	—
F.c + fresh IK726[2] + Sepiret	44 cd	157	0.46 bc	54	33 bc	75.9 BC	20.2 bc	2×10^3
F.c + low IK726[3] + Sepiret	41 c	146	0.51 bc	50	31 ab	75.6 BC	20.2 bc	2×10^3
F.c + high IK726[4] + Sepiret	42 c	150	0.43 c	57	34 c	76.1 BC	20.2 bc	4×10^3
F.c + low IK726 + Pelgel	33 ab	118	0.64 b	37	32 b	74.4 C	20.1 c	2×10^3
F.c + high IK726 + Pelgel	40 bc	143	0.32 c	68	35 c	77.2 ABC	20.2 bc	2×10^3
F.c + Sibutol	50 de	179	0.05 d	95	34 c	79.6 A	20.8 a	—
Healthy control (H.c.)[5]	54 d	193	0.11 d	89	34 c	78.4 AB	20.5 abc	—
H.c. + low IK726 + Sepiret	53 d	189	0.06 d	94	35 c	79.2 A	20.7 ab	2×10^3

[1]Seeds naturally infected with *F. culmorum* (5% infection) were inoculated with a suspension of *F. culmorum* (1.5×10^6 spores/ml).
[2]2 ml per 100 g seed of a suspension of freshly harvested conidia (1×10^8 conidia/ml).
[3]2 ml per 100 g seed of a dried IK726 preparation (0.067 g/ml) stored for 30 weeks at 4 °C.
[4]2.7 ml per 100 g seed of a dried IK726 preparation (0.067 g/ml) stored for 30 weeks at 4 °C.
[5]Seeds were surface sterilized.
[6]Means followed by different letters are significantly different ($p = 0.05$) according to the Duncan multiple range test.
[7]4 weeks after sowing.

Table 5. The effect of seed treatment with fresh or stored conidia of *C. rosea* (IK726) against seedborne *F. culmorum* on winter wheat in growth chamber sand test. Results from sand test corresponding to the 1995 wheat field experiment (Table 4)

Seed treatments	Emergence (%)	Disease index[6]	Control (%)	Plant dry weight (mg)	Cfu/seed
F. culmorum (F.c)[1] control	96	1.61 a[7]	—	19	—
F.c + IK726 fresh[2] + Sepiret	100	0.34 bc	79	21	2×10^3
F.c + low IK726[3] + Sepiret	99	0.40 b	75	20	2×10^3
F.c + high IK726[4] + Sepiret	100	0.10 c	94	19	4×10^3
F.c + low IK726 + Pelgel	100	0.43 b	73	19	2×10^3
F.c + high IK726 + Pelgel	100	0.27 bc	83	20	2×10^3
F.c + Sibutol	99	0.12 c	93	19	—
Healthy control (H.c.)[5]	100	0.25 bc	84	19	—
H.c. + low IK726 + Sepiret	95	0.09 c	94	19	2×10^3

[1–5]Conditions as mentioned in Table 4.
[6]After 19 days incubation.
[7]Means followed by different letters are significantly different ($p = 0.05$) according to the Duncan multiple range test.

however gave the significantly highest control (95%). Plant emergence was significantly increased by all biological seed treatments and emergence tended to increase with increasing dosage of IK726, while treatments with Sibutol and fresh IK726 had the highest plant emergence. Plant dry weight was significantly increased by seed treatment and dry weight was significantly increased for the high doses of IK726 to a level equal to the Sibutol treatment (Table 4). At harvest grain yield and thousand grain weight (TGW) were measured. Compared to the untreated *Fusarium* control all seed treatments increased yield significantly from 3.6 hkg/ha at low dosage of IK726 up to 8.8 hkg/ha for the Sibutol treatment. However, for the treatment with high dosage of IK726 + Pelgel the yield was 77.2 hkg/ha which was not significantly different from the chemical treatment. While the effect of seed treatment on TGW followed the same pattern as that of yield, only the Sibutol treatment had a TGW significantly higher than the diseased control (Table 4).

Results from the sand test corresponding to the wheat field experiment are shown in Table 5. The DI was significantly reduced by IK726 treatment and control efficacy varied between 73% and 94% dependent on dosage. For the highest dosages of IK726 the control efficacy was as high as for the chemical treatment. Plant emergence and plant dry weight were, however, unaffected by the various seed treatment. Both for *F. culmorum* inoculated seeds and for the healthy control the level of disease was higher in the sand test than in the corresponding field experiment. Furthermore, in both the field experiment and in the sand test treatment of healthy seed with IK726 had no significant effects on DI, plant emergence, dry weight, yield or TGW compared to the healthy control treated with water (Tables 4 and 5).

Relationship between disease control in the field and in sand tests

The relationship between DI measured in the field and in corresponding sand tests for the IK726 treatments and the water treated *Fusarium* controls is shown in Figure 1. An overall correlation analysis on average disease values for both wheat and barley showed a significantly ($p < 0.0001$) high and positive correlation ($r = 0.94$) between results measured in the sand test and biocontrol effects obtained in the field experiments.

Dose–response for biocontrol effect of fresh conidia and of dried and stored conidia

The relationship between density of cfu of *C. rosea* per barley seed and the control of *F. culmorum* (percent control relative to water treatment) is shown in Figure 2. For the freshly harvested conidia there was a significantly ($p < 0.001$) high correlation ($r = 0.96$) between viable cfu of *C. rosea* per seed and the control efficacy. From linear regression of log cfu/seed and percentages of disease control it was estimated that 80% control of *F. culmorum* could be achieved with approximately 3×10^3 viable cfu of *C. rosea* per seed at sowing

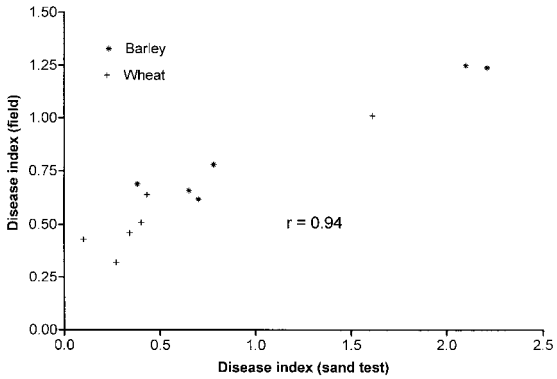

Figure 1. The relationship between severity of attack by *F. culmorum* on barley and wheat seedlings measured in the sand pot test and in the field for seed treated with different formulations of *C. rosea*. Seeds were treated with conidial suspensions of *F. culmorum* and *C. rosea* as indicated in Tables 2 and 4. Disease index: 0 = healthy plants, 1 = slightly brown coleoptile/roots, 2 = moderately brown coleoptile and roots, 3 = severe browning of coleoptile and roots and 4 = dead plants.

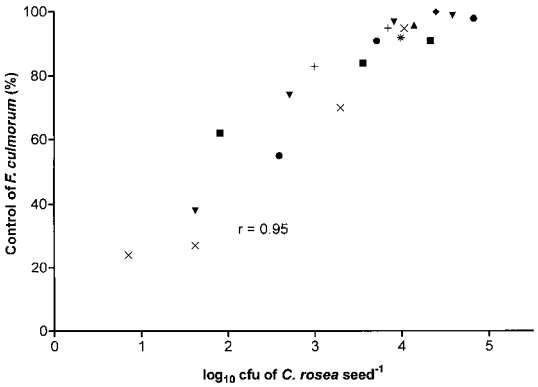

Figure 2. Biocontrol efficacy of different dosages of freshly harvested conidia of *C. rosea* against *F. culmorum* on barley in sand pot tests. Surface sterilized seeds were inoculated with *F. culmorum* (1.5×10^6 spores/ml) and coated with conidial suspensions of *C. rosea* (from 10^4 to 10^8 conidia/ml). Signatures represents different experiments.

(Figure 2). Furthermore one way analysis of variance of data from selected bioassays showed that significant control was achieved with conidial densities as low as 10^2 cfu/seed (data not shown). For the dried conidia stored at 4 °C there was also a highly significant ($p < 0.001$) correlation ($r = 0.73$) between dosage and disease control (Figure 3). To achieve 80% disease control the stored conidia had to be applied at a rate of approximately 5×10^3 cfu/seed.

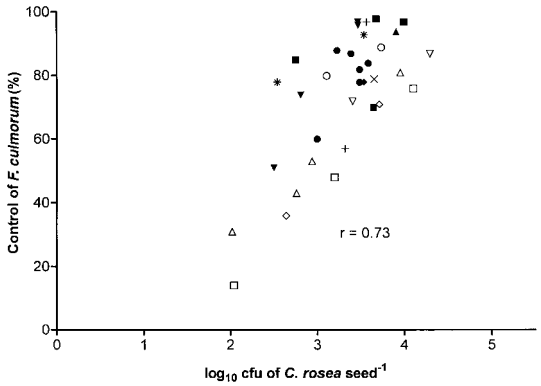

Figure 3. Biocontrol efficacy of different dosages of dried and stored conidia of *C. rosea* against *F. culmorum* on barley in sand pot tests. Surface sterilized seeds were inoculated with *F. culmorum* (1.5×10^6 spores/ml) and coated with conidia washed out of peat/bran formulations of *C. rosea* (from 10^4 to 10^8 conidia/ml). Signatures represents different experiments.

Discussion

The results from field experiments conducted in 1994 and 1995 showed that seed treatment with *C. rosea* (IK726) significantly reduced the *F. culmorum* DI. The minimum temperature for growth of *F. culmorum* is about 0 °C (Domsch et al., 1980), but infection in the field is favoured by relatively dry and warm soil conditions (Parry et al., 1994). According to Malalasekera et al. (1973), seed infection by *F. culmorum* takes place within approximately 7 days after sowing. The average soil temperature in this period (critical soil temperature) will therefore be of great importance for the level of infection as well as for the activity of an antagonist applied with the seed. The critical soil temperature in the present field experiments varied from 6.2 to 12 °C and IK726 was antagonistic to *F. culmorum* at these relatively low soil temperatures, which are typical for cereal production in Denmark. The results indicate that biocontrol of *F. culmorum* by IK726 will be stable under natural growth conditions at least on sandy loam soil types. In a former field experiment with winter wheat in 1992/93, effective biocontrol with IK726 was found at a critical soil temperature of 13.1 °C (Knudsen et al., 1995). Growth chamber experiments have further revealed that IK726 also controls *F. culmorum* effectively at temperatures of 10, 15, 20 and 25 °C (B. Jensen, unpubl.). IK726 is therefore a potential antagonist over a broad temperature range. The disease severity of *Fusarium* infected controls was considerably higher in the growth chamber experiments than in

the field. To some extent, this can probably be explained by the higher temperature in the sand test (15 °C) as the pathogenicity of the used isolate has been shown to increase strongly at temperatures from 10 to 15 °C (Knudsen, 1994).

The feasibility of the commercial use of biological seed treatment will be greatly enhanced if stored formulation of the BCAs are effective in small amounts. In two field experiments, seeds were coated with dried formulations of IK726 which had been stored up to 6 months at 4 °C. The stored formulations were as effective as freshly harvested conidia of IK726 in controlling *F. culmorum* since similar doses of the two types of conidia gave the same level of disease control. Dose–response curves for the relationship between density of viable IK726 conidia per seed and the percentage control of *F. culmorum* were established in sand tests in growth chamber. For conidia stored at 4 °C a strong linear relationship ($r = 0.73$) was found between dosage and percentage disease control and it was estimated that about 5×10^3 cfu/seed were sufficient for effective control (> 80%). For freshly harvested conidia an even stronger relationship existed ($r = 0.95$) and the effective dose was of the same magnitude. The growth chamber experiments also showed that control efficacy from batch to batch was reproducible for both types of IK726 conidia. By coating cereal seed with dried peat–bran formulations disease reduction in the field was obtained with as little as 1.3–10 g IK726 preparation per kg seed which is comparable to the dose range for application of chemical fungicides.

The chemical fungicide Sibutol, which was included in the wheat field experiments in 1995, gave 95% disease control. Coating with IK726 had a lower efficacy (69%), which in part could be explained by the relatively low dosage ($2–4 \times 10^3$ cfu/seed) which was below the dosage estimated to give 80% control in the sand tests. Furthermore, the distribution of conidia on the seed after application with the Hege II seed treater was not examined and could have been uneven. It is possible that control efficacy in the field could be improved by increasing the dosage, since the level of disease severity in *Fusarium* controls as well as biocontrol efficacy obtained with the IK726 seed treatment was considerably higher in the growth chamber than in the field when equal dosages were applied. Although disease reduction was lower with IK726, the yield of the best IK726 treatment (dried conidia + Pelgel) was not significantly lower than for the chemical seed treatment.

Formulation with stickers were investigated in all field experiments. Amendment of the water suspensions of IK726 conidia with the sticker Pelgel did not improve biocontrol efficacy in the field experiments (Table 1) when the antagonist was applied by soaking. Furthermore both Pelgel and Sepiret had no side-effects on disease development or plant establishment in the conducted experiments. However the compatibility of IK726 with stickers such as Pelgel and Sepiret can be an important character if more sophisticated coating techniques such as film-coating or double coating should be applied (Cliquet and Scheffer, 1996; Harman, 1991).

It has been reported that treatment of barley seeds with *C. rosea* reduces plant emergence, especially when oxygen availability is limited (Lynch and Pryn, 1977). Possible direct effects of IK726 on plant establishment were examined in the field by including healthy controls treated with IK726. In the absence of seedborne *F. culmorum* inoculum neither negative nor positive effects of IK726 seed coating were observed on plant emergence or on plant dry weight. Growth chamber experiments have shown that the density of IK726 conidia on healthy seed can be increased up to 4×10^4 cfu/seed without any negative effects on plant establishment (Jensen et al., 1996).

The efficacy and reliability of BCAs under natural growth conditions have often proved to be inadequate. This is probably partly due to the fact that much screening and selection of antagonists as well as formulation tests are often conducted under artificial and controlled conditions (Duczek, 1994; Knudsen et al., 1997; Harman, 1991). With regard to disease severity scored on roots and coleoptiles, the results obtained in the sand test were highly correlated with the effects obtained in two field trials. In the wheat experiment, which included two antagonist dosages, the effect of dosage was evident both in the field and in the growth chamber sand test.

In contrast, the biocontrol effects on plant emergence and plant dry weight seen in the wheat field trial were not expressed in the growth chamber test. However, the sand test appears to be a reliable method for a quick and inexpensive evaluation of the efficacy of different formulations of IK726 against *F. culmorum*. In agreement with our results, Teperi et al. (1998) found that *Gliocladium* isolates effective against seedborne *F. culmorum* on wheat could be selected in greenhouse tests in sand. However, the greenhouse tests did not efficiently predict which of the *Gliocladium* isolates

would give the best protection of wheat seedling under field conditions.

In conclusion, consistent efficacy data from field experiments clearly demonstrate that seed treatment with *C. rosea* (IK726) could be a realistic alternative to chemical fungicides for the control of seedborne infections caused by *F. culmorum*.

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