SESSION 5
BIOLOGICAL SEED TREATMENTS

Chairman and Session Organiser: Professor J M Whipps
HRI, Wellesbourne

Improving bacterial seed treatments - advantages and problems with the use of molecular marker technologies.

C Leifert
TESCO Centre for Organic Agriculture, Department of Agriculture, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK.

ABSTRACT

Control of soil borne damping-off diseases by bacterial antagonists applied to the seed coat or pellet has been frequently reported to be more variable than fungicide seed treatments. However, due to experimental difficulties in monitoring/tracking populations and activities of microbial inocula in the soil environment with classical bacteriological methods, it has proved extremely difficult if not impossible to identify the exact reasons for variable field performance of biological seed treatments. More recently molecular marker technologies have been used as an alternative strategy to identify population dynamics and environmental activity profiles of bacterial antagonists in the soil environment. This paper critically reviews the opportunities and problems associated with the use of molecular marker based assays (in particular the use of *lux* marker technology) for population and activity profiling of bacterial antagonists in soil.

INTRODUCTION

Bacterial antagonists have frequently been described as potential alternatives to fungicide treatments of seed against soil borne diseases (Leifert *et al.*, 1996). In laboratory and controlled glasshouse environments some antagonists have been reported to provide a level of control similar to fungicide controls (Table 1; Jensen *et al.*, 1992; Wenhua *et al.*, 1996). As a result, a wide range of antagonistic bacteria are currently being developed with the aims:

(i) to improve damping-off control in conventional agriculture (where fungicide treatments may not always give the desired level of control) and
(ii) to provide protection for seeds used in organic production systems (where the derogation on the use of fungicide treated seeds is currently being phased out).

However, few bacterial treatments have so far become available as commercial products, mainly because under field conditions, biological seed treatments were frequently shown to have lower activity levels than fungicides (Whipps, 1997).

The underlying reasons for poor field performance are poorly understood for most antagonists. Poor survival of antagonists during seed processing and subsequent storage, is a major problem for non-spore forming antagonists (Harman *et al.*, 1988). After seed have been sown, narrow environmental activity profiles of antagonists and inability to colonise the spermosphere or rhizosphere during early seedling development have frequently been

described as the main reasons for poor performance of antagonists (Taylor & Harman, 1990; Berger *et al.*, 1996; Wenhua *et al.*, 1996). The development of strategies to improve spermosphere and root colonisation and activity by biological control inocula was therefore described as a major aim of biological control research (White *et al.*, 1996).

MONITORING ANTAGONIST SEED INOCULA IN SOIL

Appropriate techniques to monitor antagonist seed inocula in the soil environment are essential to gain an "in depth" understanding of the soil environmental and host plant factors influencing microbial colonisation and activity. For some antagonists with specific metabolic or morphological characteristics, classical microbiological techniques have been successfully used to identify the reasons for poor root colonisation and biocontrol activity.

Table 1. Percentage Pythium damping-off in micropropagated *Daphne* and *Photinia*, and in conventionally raised Dutch White cabbage (*Brassica*) seedlings in peat-based compost after root treatment with *Bacillus subtilis* strain Cot1 or compost treatment with metalaxyl* (Berger *et al.*, 1996)

Plant species	Metalaxyl	*B. subtilis* Cot1	Untreated
Daphne	4a	62b	86c
Brassica	1a	9a	91b
Photinia	4a	7a	99b

* Separate experiments were carried out for the different plant species. For treatments of the same plant species, different letters indicate significant differences ($p < 0.05$) according to Tukey's honestly significant difference test.

For example, the black pigment produced by *Bacillus subtilis* Cot1 was successfully used to demonstrate that poor protection of *Daphne* plants against damping-off was linked to (i) poor colonisation of *Daphne* roots by strain Cot1 and (ii) to the production of exudates by *Daphne* that were inhibitory to strain Cot1 (Tables 1 & 2). However, due to a lack of differentiating characteristics, it has proved to be extremely difficult to monitor accurately the population density and activity of many other microbial antagonists with traditional plating based microbiological methods.

Table 2. Populations of *Bacillus subtilis* Cot1 (log_{10} spores g^{-1} root fresh weight) in the rhizosphere of micropropagated *Daphne* or *Photinia* plants and conventionally raised Dutch White cabbage (*Brassica*) seedlings 28 days after inoculation with 4×10^6 cfu g^{-1} root fresh weight (Berger *et al.*, 1996)

Plant species	Root section (1 cm root sections) from the		
	Shoot base	Centre of roots	Root tip
Daphne	3.5	2.5	2.1
Brassica	5.4	5.2	3.7
Photinia	6.2	4.9	5.2

Analysis of variance and Tukey's honestly significant difference test (HSD) showed significant differences between plant species ($p<0.001$) and root sections ($p<0.001$) and a significant interaction between plant species and root sections ($p< 0.001$). The HSD was 0.7.

MOLECULAR METHODS FOR THE TRACKING OF ANTAGONISTS IN SOIL

Over the last 10-20 years a range of molecular techniques have been developed which allow the density and/or activity of micro-organisms to be monitored in the soil environment. The two most commonly used approaches are:

(i) PCR (polymerase chain reaction) based techniques in which DNA (or RNA) is extracted from the soil, followed by amplification of specific DNA/RNA sequences of the target organism and visualisation of amplified DNA/RNA. This technique is very powerful for detection of specific organisms in soil, but quantification and differentiation between active and non-active/dead propagules of the target organism remains extremely difficult (Stead, 2001). However, one advantage of PCR technology is that it does not require genetic modification of the target organism, thus avoiding potential negative impacts on the environmental fitness or other activities of the biological control agent (see below).

(ii) Molecular marker techniques in which the target microorganism is genetically transformed with genes, which allow the target organism to be differentiated from the soil microbiota by a specific metabolic characteristic (see below).

Antibiotic resistance marker genes

These genes render transformed strains resistant to specific antibiotics; by plating soil extracts onto selective media containing the antibiotic, the target organism can then be semi-selectively recovered. However, colonies of other soil bacteria, which are naturally resistant to the antibiotics may also be recovered and additional characteristics (e.g. colony

morphology, other molecular markers such as as *lux*) have to be used to separate between the target strain and naturally antibiotic resistant soil microorganisms (White *et al.*, 1996; Knox, 2000).

*Lux*AB marker genes.

These *lux*A and *lux*B genes are derived from the *lux* operon of seven genes (*lux* ABCDEI & R) isolated from the marine bacterium *Vibrio fisheri*. Genetic transformation of soil bacteria with the *lux*AB genes enables transformed strains to express the luciferase enzyme and emit light upon addition of an external source of aldehyde (Rattray *et al.*, 1995). No free-living soil bacteria have been found to be capable of producing light (a bacterial symbiont of nematodes has been described), making it an extremely selective marker in the soil environment (White *et al.*, 1996). Population densities of lux marked strains can be determined by traditional plating of soil extracts onto bacteriological media and detection of light emission from colonies of the target organism after addition of aldehyde. Single cells or micro-colonies may also be visualised *in situ* (e.g. in the rhizosphere or bulk soil) by using Charge-Coupled Device-enhanced imagery (Prosser *et al.*, 1994; Rattray *et al.*, 1995; White *et al.*, 1996). Since light emission has been shown to correlate with the metabolic activity *in vitro* (White *et al.*, 1996), light emission allows the accurate measurement bacterial activity and the spatial distribution of bacterial populations within environmental samples (Rattray *et al.*, 1995).

Lux marking of soil bacteria has been used to study the impact of soil environmental variables (matric potential, temperature, competition from other soil microorganisms (White *et al.*, 1996)). Because of its potential for identifying modes of action and environmental activity profiles of biological control agents used to control soil borne pathogens, it was also described as a "strategy to optimise biological control of soil borne bacteria" (White *et al.*, 1996).

Other marker systems

Other genes used to mark soil organisms included gfp (green fluorecent protein from the jellyfish *Aequorea victoria*; transformant colonies are bright green; Inouye & Tsuji, 1994), GUS (*β*-glucuronidase; transformants turn blue; Wilson, 1995), *xyl*E (2,3-dioxygenase; transformants turn yellow; de Leij *et al.*, 1994), TFD monooxygenase (transformant colonies turn red; King *et al.*, 1991) and *lac*ZY (*β*-galactosidase; Ryder, 1994).

LIMITATIONS AND PROBLEMS OF MOLECULAR MARKER STRATEGIES

A wide range of potential bacterial biological control agents has been successfully transformed with the *lux*AB genes (White *et al.*, 1996; Knox, 2000). However, several limitations and problems were identified with the use of *lux*-marked soil bacteria.

Gram negative bacteria

Gram negative bacteria have been relatively easily transformed with the *lux*AB genes, both through plasmid and chromosomal insertion of the genes (Knox, 2000). However, for high

light output (required for single cell and *in situ* monitoring) high copy number plasmids (rather than chromosomal insertion of the genes) were found to be required. Although the production of light is thought to be energetically inexpensive, the expression of the luciferase and/or antibiotic resistance genes (which are frequently introduced together with the *lux* genes) may impair host fitness (de Weger, 1991). Also, when genes are inserted into the chromosome of the target bacterium, the transcription of other genes may be affected. Strains successfully transformed frequently show reduced growth rate characteristics and different colony morphologies *in vitro*. Clearly, such *lux*-marked strains are undesirable for soil ecological studies since their activity profiles are likely to differ significantly from the wild type. It has therefore become routine in most laboratories to

(i) test growth rates and/or metabolic activity (e.g. dehydrogenase activity) *in vitro* or sterile sand/soil assays and
(ii) select *lux*-transformed strains which have similar growth characteristics and metabolic activity (Prosser *et al.*, 1994; Rattray *et al.*, 1995; White *et al.*, 1996).

However, a risk remains that genes and/or physiological capabilities of the bacterium, which do not impact on the growth rate or metabolic activity *in vitro* do have an effect *in vivo*. For Gram negative antagonists (e.g. *Enterobacter cloacae*, *Pseudomonas fluorescens* and *P. corrugata*) the biological control activity has not been reported to be affected by transformation with the *lux*AB genes (Fravel *et al.*, 1990; Knox, 2000).

Gram positive bacteria

Gram positive bacteria were generally found to be more difficult to transform with the *lux*AB genes and light output was frequently reported to be lower than in Gram negative bacteria (Knox, 2000). There was no light output after transformation of two biocontrol strains of *Bacillus subtilis* (MBI600 and MBI205) with different plasmids (pCSS115, pCSS117), even though kanamycin resistance was also transferred indicating that the plasmid was successfully transferred into *Bacillus* cells (Knox, 2000). When strains were transformed with plasmid pSB340 high light output was obtained with both *Bacillus* strains (see Table 3). However, when *lux*-marked strains were tested for biological control activity *in vitro* they showed no biological control activity. From cultures of *lux* transformed strains, several "spontaneously cured" strains could be isolated which did not emit light and in which plasmid pSB340 could no longer be detected. These cured strains showed *in vitro* biocontrol activity in the majority of assays performed (Knox, 2000). This clearly demonstrates that specific metabolic activities may be lost as a result of the genetic manipulation, without an effect on the growth rates or the overall metabolic activity of the transformed bacterium.

Interestingly the *lux* (pSB340) transformed strains showed low levels of bioluminescence even in the absence of externally supplied aldehyde substrate (Table 3). The biological control activity of *B. subtilis* strain MBI600 was shown to be at least partially due to the production of volatiles (Fiddaman & Rossall, 1993; 1994). These volatiles are thought to be chemically similar to the long chain (aliphatic) aldehydes which are known substrates for the luciferase enzyme. It was therefore hypothesised that the low level of light emitted by the *lux*-marked strains in the absence of an external aldehyde source may be "fuelled" by the antifungal volatiles known to be produced by the *Bacillus*, resulting in loss of biological control activity.

Table 3. Light output (relative light units) with and without application of external aldehyde substrate and *in vitro* biological control activity in wild type, *lux* (plasmid pSB340) -transformed strains of *Bacillus subtilis* MBI600 and MBI340 and strains spontaneously cured of plasmid pSB340 (Knox *et al.*, 2000)

	B. subtilis MBI600			*B. subtilis* MBI205		
Characteristic assessed	WT	*Lux*	*Lux*C	WT	*Lux*	*Lux*C
Light (RLU[a]) without aldehyde	0	7*	0	0	7-11*	ND[c]
Light (RLU[a]) with aldehyde	0	> 5000***	0	0	> 5000***	ND
In vitro antagonism[b]	+	-	+	+	-	ND

[a] relative light units (RLU) above background light levels (background light levels were approx. 2.5 RLU) in 18 h bacterial cultures
[b] against *Fusarium oxysporum* (+ = present; - = absent)
[c] Not determined
* significantly higher than wild type ($p<0.05$)
*** highly significantly different ($p<0.001$)
lux = MBI600 and MBI205 strains transformed with plasmid pSB340
*lux*C = MBI600 WP-strains which were transformed with plasmid pSB340, and were then spontaneously "cured" of the plasmid during culture *in vitro*

CONCLUSIONS

Substantial progress has been made in understanding the factors limiting biological control activity of seed inocula under field conditions. However, progress in applying molecular marker technology for the tracking of biological control agents applied as seed inocula has been slow, especially for Gram positive bacteria. Furthermore, there are serious questions concerning the ability to guarantee environmental fitness and physiological integrity of genetically transformed biological control strains.

The development of reverse transcriptase PCR technology may provide an alternative to the use of marker technology, since the technique has the potential to overcome both

(i) the problems of current DNA based PCR detection methods (inability to differentiate between active and non-active/dead propagules of the target organism) and
(ii) the problem of unforeseen side effects of molecular marker technology.

There also has been a realisation that the creative use of classic soil microbiological methods (e.g. plating onto semi-selective media, use of specific physiological characteristics of the wild type organism, selection of antibiotic resistant mutants) and well designed empirical

approaches, can provide reliable results both more quickly and more cost effectively than the molecular techniques currently available.

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Biocontrol activity of *Pythium oligandrum* and *Coniothyrium minitans* in pelleted and film-coated seed

J M Whipps
Plant Pathology & Microbiology Department, HRI, Wellesbourne, Warwick, CV35 9EF, UK

M P McQuilken
Department of Plant Biology, SAC, Auchincruive, Ayr, KA6 5HW, UK

ABSTRACT

Oospores of *Pythium oligandrum*, produced in a cane molasses liquid medium, were coated onto cress and sugar-beet seeds by commercial seed pelleting or film-coating procedures. Oospore germination was unaffected by the coating treatments. In glasshouse pot trials, both types of treatment gave significant control of damping-off in cress and sugar beet caused by *Pythium ultimum* and *Aphanomyces cochlioides*, respectively. In some cases, the control was equivalent to fungicide drenches or standard seed treatments, but little control was achieved with any treatment when the pathogen inoculum potential in soil was high. Oospores survived in pelleted sugar beet seed for up to 6 years at 8 °C and retained their biocontrol efficacy against *Pythium* spp., but only at low pathogen inoculum potentials. To mimic treatment of infested seed lots, conidia of *Coniothyrium minitans*, were film-coated onto *Sclerotinia sclerotiorum*-infected sunflower seeds and sclerotia by polymer film-coating, using a fluidised-bed seed system. *C. minitans* had little effect on seedling disease in tests conducted in a peat-soil mix, but completely suppressed apothecial production of sclerotia when placed in soil. The film-coating process decreased the germination of conidia recovered from sunflower seeds, but after storage for 1 year at 10 °C, *C. minitans* still grew from 97% of seed. The potential for developing *P. oligandrum* and *C. minitans* as commercial seed treatments for biocontrol of seedling diseases is discussed.

INTRODUCTION

Pythium oligandrum and *Coniothyrium minitans* are well documented mycoparasites, which have shown considerable promise as biocontrol agents of a range of damping-off plant pathogens (e.g. *Pythium ultimum*, *Rhizoctonia solani*) and *Sclerotinia sclerotiorum*, respectively (Whipps, 1997). Solid substrate preparations of the mycoparasites have often been incorporated into potting mixes and soil prior to sowing or planting. The development of this delivery method for commercial use has limitations because it is potentially expensive and inconvenient to use on a large-scale. In contrast, the application of biocontrol agents by commercial seed-coating procedures is a more economical method of delivery. Although there have been numerous reports of successful application of biocontrol agents to seeds (see Whipps, 1997; McQuilken *et al.*, 1998a), few potential biocontrol agents have been successfully applied to seeds using commercial seed-coating processes.

This paper reviews studies conducted over the last decade to apply spores of *P. oligandrum* and *C. minitans* to seeds using commercial processes, and to demonstrate the ability of these

treatments to control damping-off and *S. sclerotiorum*, respectively, compared to fungicides. Oospores of *P. oligandrum* were coated onto cress and sugar-beet seeds by pelleting or film-coating, and the biocontrol activity of coated seed tested against *P. ultimum* and *Aphanomyces cochlioides*. The effects of long-term storage on the survival and germinability of *P. oligandrum* oospores in the coating material of pelleted sugar-beet seeds were studied. The biocontrol activity of stored, pelleted seed was also investigated. Conidia of *C. minitans* were applied to sunflower seed and sclerotia using a fluidised-bed film-coating process. The effect of such film-coatings on the control of seed-borne *S. sclerotiorum* and the germination of sclerotia in simple bioassays was also investigated.

MATERIALS AND METHODS

Inoculum production of fungi

Oospore inoculum of *P. oligandrum* (isolate IMI 133857) for seed-coating cress and sugar-beet was produced in cane molasses liquid medium (McQuilken *et al.*, 1990 a, b; 1998). Harvested inoculum was washed in three changes of sterile distilled water and air-dried overnight in a laminar flow cabinet at 18-21 °C. Air-dried inoculum contained 1.4-1.8 x 10^4 oospores/mg.

Spore suspensions of *C. minitans* (isolate IMI 134523) for film-coating sunflower seed and sclerotia, were prepared by flooding 14-day-old potato dextrose agar (PDA) cultures with sterile distilled water and gently scraping the colony surfaces with a spatula. Sclerotia of *S. sclerotiorum* were produced on sterile wheat grain following incubation at 20 °C for 3 weeks (McQuilken *et al.*, 1997). Washed, air-dried sclerotia (2-4 mm diameter) were selected for film-coating.

Seed-coating of cress and sugar-beet

Seed-coating of cress (cv. Curled) and sugar-beet (cv. Amethyst) was undertaken by Germain's (UK) Ltd., King's Lynn, Norfolk, UK, employing two commercial coating processes. Fungicides and *P. oligandrum* oospores were incorporated separately into 'EB3' pellets, which consisted of clay and wood particles, or formulated with liquid based polymeric adhesives and coated onto seed surfaces using a film-coating binder system (McQuilken *et al.*, 1998a and references therein). Prior to coating, sugar-beet seeds were steeped for 12 h at 25 °C, either in water or in 0.2% aqueous thiram solution (Agrichem Flowable Thiram 60% FS). Thiram was also added as a dust formulation to the EB3 pellet and the film-coating at a rate of 4.8 g a.i. per Unit (100000 seed). Thiram-treated seed was then treated with one of two fungicides (g/Unit): hymexazol (Tachigaren 70% WP), 10.5 g a.i., or metalaxyl (Apron 25% WP) 0.29 g a.i.. Hymexazol controls both *Pythium* spp. and *A. cochlioides*, whereas metalaxyl controls only *Pythium* spp. Cress seeds were not treated with fungicides. Cress seed (1 kg) and a Unit of water-steeped sugar-beet seed were treated with 4.0 and 4.5 g of oospore inoculum to achieve 35 x 10^3 or 50 x 10^3 oospores/seed. Cress and water-steeped sugar-beet seeds were pelleted and film-coated without additives, and a treatment in which seed was steeped in water alone was included for sugar-beet. Pelleted seed was dried at 30 °C, whereas film-coated seed was dried at 25 °C. All seed was stored in plastic bags at 8 °C. Survival and germinability of

oospores removed from coated seeds was assessed immediately, and after 2, 4 and 6 years of storage (see McQuilken *et al.*, 1998b).

To mimic treatment of infested seed lots, iprodione (Rovral WP; 50% a.i.), gamma-HCH + thiram + fenpropimorph (Lindex Plus FS; 82.5 % a.i. + 11% a.i. + 6.5 %g a.i.) and conidial suspensions of *C. minitans* were applied to *S. sclerotiorum*-infected sunflower seeds (30% (w/w) infection level) and sclerotia by polymer film-coating, using a fluidized bed seed system (Maude & Suett, 1986). The seeds are suspended in a moving column of warm air and are sprayed with a polymer sticker that dries on to seeds as it coats them (Wurster, 1959). Batches of seed (100 g) were film-coated (per kg of seed) with gamma HCH (25.1 g) + thiram (3.4 g) + fenpropimorph (1.9 g) and iprodione (9.8 g). Batches (100 g) were also film-coated with a conidial suspension of *C. minitans* (94 ml; 1.5-1.7 x 10^7 conidia/ml) to achieve 5.0-5.3 x 10^6 conidia/g. A polymer (polyvinylacetate) concentration of 1% (w/w) was used as sticker for all applications, and was applied for 30 min at 20-25°C. Controls consisted of batches of seed treated with the polymer alone. Film-coated seeds were stored at room temperature as described before. Survival and germinability of conidia removed from coated seeds was assessed after 1 week, 6 months and a year. (McQuilken *et al.*, 1997). Batches of sclerotia (30 g) were also film-coated (per kg of sclerotia) with gamma HCH (25.4 g) + thiram (3 g) + fenpropimorph (2 g) and iprodione (9.6 g), and a conidial suspension of *C. minitans* (McQuilken *et al.*, 1997).

Effect of seed-coating treatments on damping-off of cress and sugar-beet

The ability of *P. oligandrum* seed treatments to reduce damping-off of cress and sugar beet was tested in sand artificially infested with *P. ultimum* (10 sporangia/g sand), and in soil naturally-infested with *A. cochlioides* and low levels of *P. ultimum*. Glasshouse and growth chamber experiments were conducted and assessed for disease according to McQuilken *et al.* (1990). Following 6 years of storage, the ability of *P. oligandrum*-pelleted sugar-beet seed to reduce damping-off was evaluated in soil-sand mixtures infested with *Pythium* spp. and *A. cochlioides* (McQuilken *et al.*, 1998b).

Effect of film-coating treatments on seed-borne *S. sclerotiorum* and germination of sclerotia

To determine the effect of film-coating treatments on the control of seed-borne *S. sclerotiorum*, film-coated sunflower seeds were sown in a peat/loam (1:1 v/v) mix in compartmented seed trays (60 compartments/tray, one seed/compartment). Seed trays were placed on a bench in a glasshouse chamber maintained at 22±2 °C (80% r.h.) with 14 h light/day. Trays were watered daily, and counts of surviving seedlings were made after 14 days. The experiment was repeated four times.

The effect of film-coating treatments on carpogenic germination of sclerotia was assessed using a field pot bioassay based on the method of McQuilken & Whipps (1985). Soil (brickearth, silt-loam, Hamble series) was collected to a depth of 15 cm, sieved (10 mm mesh) prior to filling rectangular plastic pots (11 x 10 cm). Twenty sclerotia (isolate JN1), enclosed in Terylene net bags (5 x 5 cm, mesh< 2 mm) were buried in 10 replicate pots of soil, 1 cm beneath the surface in mid-October. Numbers of apothecia produced from the sclerotia in each pot were counted at fortnightly intervals from late April until early June. Apothecia were not

removed after counting. Recovery and infection of sclerotia by *C. minitans* was assessed 34 weeks after the start of the experiment according to McQuilken *et al.* (1997).

RESULTS

Survival and germination of *P. oligandrum* oospores on coated seeds

Oospores obtained from treated seeds had a similar germination to that of those in the inoculum used to coat seeds. Mycelium of *P. oligandrum* grew within 48 h from all *P. oligandrum*-coated seed when seeds were plated on corn meal agar following 0, 2 and 4 years storage at 8±2 °C, and from 93% of seeds after 6 years.

Effect of seed-coatings on damping-off in cress and sugar-beet

In sand artificially infested with *P. ultimum*, *P. oligandrum* pelleted cress seed gave increases in seedling stand equivalent to the fungicide drench of propamocarb HCl (Table 1). Film-coating cress with *P. oligandrum* increased seedling stand, but was not as effective as the fungicide treatment. *P. oligandrum* pelleted sugar-beet seed gave significantly greater seedling stands than the pelleted control, and were equivalent or just less than the two pelleted fungicide treatements. Film-coating sugar-beet with *P. oligandrum* gave significantly greater seedling stand compared with the film-coated control and was equivalent to the film-coated fungicide treatments. Both pelleted and film-coated controls gave significant improvements in seedling stand compared with uncoated controls.

Table 1. Effect of seed treatments on seedling stand of cress and sugar-beet in sand artificially infested with *Pythium ultimum*

Treatment	Seedling stand (%) of crop (days after sowing)	
	Cress (16 days)	Sugar-beet (21 days)
Control (uncoated)	-	33 a
Pelleted	47 a[b]	57 b
+ *P. oligandrum* (30 °C)[a]	96 b	86 c
+ *P. oligandrum* (45 °C)[a]	94 b	89 c
+ propamocarb HCl drench	94 b	-
+ metalaxyl seed coating	-	93 c
+ hymexazol seed coating	-	97 d
Film-coated (25 °C)	38 a	61 b
+ *P. oligandrum*	51 b	92 c
+ Propamocarb HCl drench	93 b	-
+ metalaxyl seed coating	-	91 c
+ hymexazol seed coating	-	93 cd

[a] Temperature at which the pelleted or film-coated seeds were dried after treatment
[b] Figures in columns followed by a different letter differ at the 5% level calculated from the least significant difference (LSD) derived from analysis of variance (ANOVA)

In soil naturally infested with *A. cochlioides* and low levels of *P. ultimum*, control of damping-off in sugar-beet with both fungicide and *P. oligandrum* seed treatments was obtained only

when infested soil was diluted 1:10 or more with soil of low inoculum potential (Table 2). Both these treatments consistently reduced the number of seedlings killed compared with the pelleted control. When the low inoculum potential soil was used (0% infested soil) the number of seedlings killed was equivalent in all treatments.

Table 2. Effect of seed treatments on post-emergence damping-off of sugar-beet caused by *Aphanomyces cochlioides* 28 days after sowing in naturally-infested soil

	Seedlings killed (%)					
	Aphanomyces cochlioides infested soil (v/v)[a]					
Treatment	100	50	10	5	1	0
Control (uncoated)	55 a[b]	51 a	63 a	58 a	54 a	4 c
Pelleted[c]	67 a	65 a	86 a	66 a	67 a	15 bc
+ hymexazol	67 a	75 a	10 bc	28 b	10 bc	6 c
+ *P. oligandrum*	67 a	68 a	42 a	28 b	22 b	3 c

[a] % by volume; soil infested with a high inoculum potential of *A. cochlioides* was diluted with soil of low inoculum potential. Both soils contained a low inoculum potential of *Pythium* spp.
[b] Figures followed by a different letter differ at the 5% level calculated from LSD derived from ANOVA
[c] Seeds were dried at 30 °C after treatment

Effect of storage on control of damping-off by *P. oligandrum* pelleted sugar-beet seeds

The germination of untreated pelleted sugar-beet seed in uninfested sand was 48-64% after 6 years storage. Storage of *P. oligandrum* pelleted seed for 6 years affected subsequent biocontrol activity (Table 3). *P. oligandrum* pelleted seed had no effect in reducing total damping-off (damping-off due to *Pythium* spp. and *A. cochlioides*) in either 5 or 1% (v/v) soil-sand mixtures containing a natural infestation of *Pythium* spp. and *A. cochlioides*. In the 1% (v/v) soil-sand mixture, *P. oligandrum* pelleted seed significantly reduced *Pythium*-induced damping-off from 33 to 26%. However, in the 5% (v/v) soil-sand mixture with higher pathogen inoculum potential, *P. oligandrum* failed to control *Pythium*-induced damping-off when 54-55% of seedlings were killed.

Table 3. Effect of storage on control of *Pythium* and *Aphanomyces cochlioides* damping-off by *Pythium oligandrum* pelleted sugar-beet seeds

	Total damping-off (%)		*A. cochlioides* damping off (%)		*Pythium* spp. damping off (%)	
Treatment[a]	5% (v/v)	1% (v/v)	5% (v/v)	1% (v/v)	5% (v/v)	1% (v/v)
EB3 pellet (control)	77 a[b]	48 a	23 a	15 a	54 a	33 a
+ *P. oligandrum*	81 a	47 a	26 a	21 a	55 a	26 b

[a] Pelleted seeds were stored in plastic bags at 8±2 °C for 6 years, and then sown in 5 and 1% (v/v) soil-sand mixtures naturally-infested with *Pythium* and *A. cochlioides*
[b] Figures followed by a different letter differ at the 5% level calculated from LSD derived from ANOVA

Effect of film-coating treatments on seed-borne *S. sclerotiorum*

Seedling survival was reduced to 83% in the *S. sclerotiorum*-infected sunflower seed, compared to 98% in the uninfected control (Table 4) The film-coating sticker alone did not affect either uninfected or infected seedling survival rate. Only the fungicide seed treatments significantly increased the percentage of seedling survival in comparison with the infected control, achieving survival rates similar to that of the uninfected control.

Table 4. Effect of film-coating sunflower seed with *Coniothyrium minitans* and fungicides on control of seed-borne *Sclerotinia sclerotiorum*

Seed treatment	Seedling survival (%)
Uninfected	98 a[a]
Uninfected + sticker	98 a
Infected	83 b
Infected + sticker	82 b
Infected + sticker + *C. minitans*	86 b
Infected + sticker + iprodione	93 c
Infected + sticker +thiram + fenpropimorph	94 c

[a] Figures followed by a different letter differ at the 5% level calculated from LSD derived from ANOVA

Survival and germination of *C. minitans* conidia film-coated on seeds

Conidia obtained from film-coated seeds 1 week after coating gave a lower germination rate (59-76% after 36 h at 18 °C) compared with those in the inoculum used for film-coating (73-87% after 36 h at 18 °C). Germinability of conidia declined from 77 to 54% over the 1-year period of storage. Mycelium of *C. minitans* grew within 72 h from all *C. minitans* film-coated seeds, when seeds were plated on PDA following 24 weeks storage at 10 °C, and from 97.5% of seeds after 1 year.

Effect of film-coating treatments on germination of sclerotia

In the field pot bioassay, *C. minitans* and fungicide treatments completely inhibited production of apothecia (Table 5). Thirty-four weeks after burying sclerotia, no sclerotia were recovered from *C. minitans* treatment pots and only 9 and 1% were found in fungicide treatment pots, compared with over 70% recovered in control pots. Amongst the sclerotia that were recovered, *C. minitans* had spread to infect 3% of untreated sclerotia, over 19% of sticker-only treatment sclerotia and 27% of the few remaining iprodione film-coated sclerotia.

Table 5. Effect of film-coating sclerotia of *Sclerotinia sclerotiorum* with *Coniothyrium minitans* and fungicides on apothecial production and subsequent sclerotia recovered and infected by *C. minitans*

Treatment	Sum of apothecia	Sclerotia recovered (%)	Sclerotia infected by *C. minitans* (%)
Control	16 ± 3.8[a]	74 ± 8.3	3 ± 1.9
Sticker only	25 ± 6.9	58 ± 7.3	19 ± 5.9
Sticker + *C. minitans*	0	0	-
Sticker + iprodione	0	9 ± 5.3	27 ± 13.3
Sticker + thiram + fenpropimorph	0	1 ± 1.0	0

[a] Values are means ± SE, based on observations of 10 replicate pots

DISCUSSION

The ability to survive a commercial seed coating process is an important attribute for any potential biological control agent that is to be applied to seed. Both the oospore preparation of *P. oligandrum* and the conidial inoculum of *C. minitans* clearly have this property with both fungi demonstrating excellent survival on seeds for at least one year. Indeed, *P. oligandrum* could still be recovered from pelleted seed after six years. Nevertheless, although reproducible control of damping-off was achieved by seed coating with *P. oligandrum*, the control was dependent on the level of the pathogen present in the soil or sand, with control failing at higher pathogen levels or with seed storage age, which was associated with declining levels of viable *P. oligandrum*. Biocontrol in the presence of a high pathogen inoculum potential is a common problem. Similarly, *C. minitans* seed coating of sunflower seeds infected with *S. sclerotiorum* failed to provide any control of disease also reflecting the high level of pathogen present in the seed due to the artificial infection system used. However, the film coating of *S. sclerotiorum* sclerotia with *C. minitans* successfully prevented all apothecial production and killed the sclerotia demonstrating considerable potential for clean–up of seed lots containing contaminating sclerotia of *S. sclerotiorum*.

Improved inoculum quality or activity could be a way to increase biocontrol efficacy of these biocontrol organisms. For *P. oligandrum*, pre-treatment of oospores with cellulase (Holmes *et al.*, 1998), or use of more germinable propagules such as encysted zoospores (Madsen *et al.*, 1995), may have potential. Growth of *C. minitans* to provide conidia with improved surface characteristics to aid binding of recognition of host hyphae or sclerotia (Smith *et al.*, 1999) is another option.

Another simple approach may be to increase the amount of inoculum of the biocontrol agent applied. This may be possible if the inoculum can be produced in a cost-effective manner and the coating process can be adapted to apply higher amounts without adversely affecting survival of the biocontrol agent. However, a simpler procedure may be to allow the biocontrol agent to grow naturally on the seed prior to any coating process. Application during seed priming may be a method by which this can be achieved (Callan *et al.*, 1997), and a Horticulture LINK project has begun to examine this concept for seeds important in UK horticulture and agriculture.

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Effect of seed treatment with acetic acid for control of seed borne diseases

A Borgen
Holmemosevej 7, DK-4780 Stege, Denmark

B J Nielsen
Danish Institute of Agricultural Sciences, Research Centre Flakkebjerg, DK-4200 Slagelse, Denmark

ABSTRACT

In field trials, seed treatment with acetic acid reduced common bunt (*Tilletia tritici*) by 92-96% in winter wheat, and by 83% in spring wheat, without negative effects on germination vigour of the seeds. Leaf stripe (*Pyrenophora graminea*) in spring barley was reduced by 93%. Acetic acid is a cheap and environmental friendly fungicide with a potentially wide scope of application especially in organic agriculture, where conventional pesticides are prohibited.

INTRODUCTION

Lime has been used as a seed treatment against common bunt, *Tilletia tritici* (syn. *T. caries*) since the 18th century (Olsen, 1791), probably acting through a pH effect since lime is a strong base. However, the control of common bunt using lime is not complete, and has found only minor use since the development of more effective seed treatments like copper (Kühn, 1866) and hot water treatment (Jensen 1888a, 1888b). With the development of organic mercury seed treatment (Riehm, 1913) common bunt has been controlled almost exclusively by synthetic pesticides in the industrialised world.

Nevertheless, recently, increasing focus has been placed on the environmental side-effects of synthetic pesticides and there is now a requirement in public opinion and in legislation in some countries to reduce the amount of pesticides used in general. One way to do this is to replace the conventional pesticides with naturally occurring substances (Nielsen *et al.*, 1998). In organic agriculture normal pesticides are not used, and here, seed borne diseases have become a severe problem. Consequently, the use of pH extremes to control seed borne diseases deserves to be reassessed. The treatment of seed with acid to create a very low pH has never been studied for pathogen control, but Hahne (1925) showed that acetic acid has a strong inhibitory effect on spore germination of common bunt *in vitro*. Consequently, the aim of this study is to investigate whether seed borne diseases can be controlled by seed treatment with acetic acid without adverse side effects on seed germination and vigour.

MATERIALS AND METHODS

Field trials were conducted at three sites on Zealand, Denmark: Common bunt at Højbakkegård in 1997 and 1998, leaf stripe (*Pyrenophora graminea* syn. *Dreschlera graminea*) of barley at Flakkebjerg in 2000 and both leaf stripe and common bunt at Mørdrupgård in 2000.

The effect of dose rates of acetic acid was tested by applying increasing amounts of increasingly concentrated acid to seeds of wheat and barley. For experiments with common bunt the winter wheat variety Pepital was used at Højbakkegård in 1997 and 1998 and the spring wheat variety Dragon was used at Mørdrupgård in 2000. The spring wheat seed used had a very low germination ability in order to increase the possibility of achieving a high infection in the field and to detect possible side effects on germination from the seed treatment. The seeds were contaminated with 5 g spores per kg seeds, which resulted in a contamination between 1.7 - 2.0 x 10^6 spores per g seed when tested by the ISDA haemocytometer method (Kietreiber, 1984).

The tests for the effect on leaf stripe were carried out in both years using the variety Alexis which by blotter test was shown to be heavily infected.

In 1997 and 1998, normal fermented 5% vinegar for household use was used (*FDB Lagereddike*). In 2000, different concentrations were made by adding increasing volumes of inert water into concentrated acetic acid (99.9%). After treatment the seeds were stored at 5 °C. Samples were removed for field tests 2-6 days after seed treatment. Germination tests were conducted 1-3 months later at Højbakkegård and Mørdrupgård. At Flakkebjerg, effects on germination were tested by counting the number of emerging plants in the field.

Germination tests in lab were done in the form of a cold sand test in plastic plates containing 1.5 kg sand with water (65ml H_2O/kg quartz sand). Sowing depth was 1.5 cm and temperature was 10 °C. The emergent number of seedlings was counted every day for 5 consecutive days after first emergence. There were 3-4 replicates.

In the field trials at Højbakkegård and Mørdrupgård, treatments were sown in 1.25 m rows with 8 or 10 replicates. The total number of plants assessed in these trials was 1-2000 on average in each treatment. The seeds in the trial at Flakkebjerg were sown in 9 m rows with 200 seeds with 4 replicates. After heading, the number of infected ears (common bunt) or plants (leaf stripe) were counted based on visible macro-symptoms.

Data of diseased plants and germination rate was analysed by a generalised linear model (GENMOD in SAS ver. 6.12).

RESULTS

Common bunt

In winter wheat, common bunt was controlled by 96% and 92%, respectively, in the years 1997 and 1998 at the dose of 20 ml of 5% acetic acid per kg seed (Figure 1). No negative effect on seed vigour was recorded at this dose. However, at the higher dose of 30 ml/kg in 1997 and 40 ml/kg in 1998, germination vigour was significantly reduced in terms of germination speed.

Even when low vitality seeds of the very susceptible spring wheat variety Dragon were used at Mørdrupgård in year 2000, the bunt frequency was still very low in all plots. The use of low vigour seeds resulted in low field germination in both treated and untreated plots with an average of only 37 ears per plot. Because of a low number of plants, the effect of many treatments were not statistically significant, especially infected plants (Table 1). However, the optimal dose was still found to be about 20 ml/kg in a concentration between 5% and 20%.

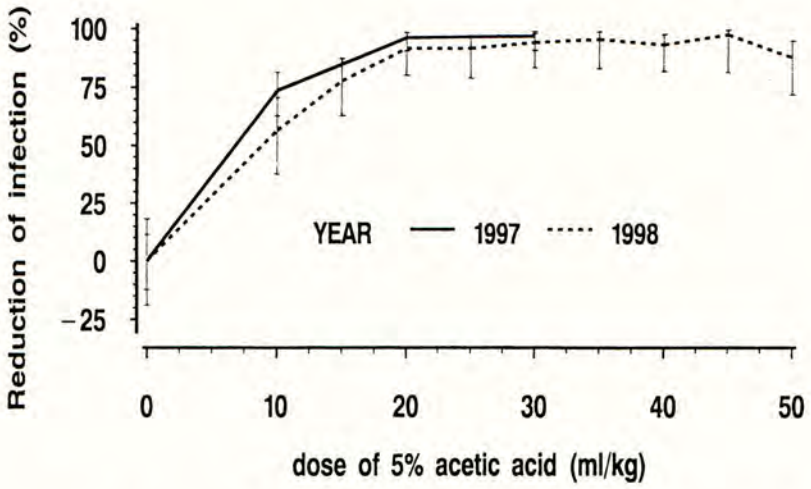

Figure 1. Control of common bunt in winter wheat in two years of field trials. Effect of increasing seed application rate of 5% acetic acid on germination vigour. Højbakkegård 1998. Bars indicate 95% confidence interval.

Table 1. Effect of different combinations of dose and concentration of acetic acid seed treatments on percent reduction in frequency of common bunt in spring wheat. Grey cells indicate treatments with a significant reduction in germination vigour. Experimental year 2000 at Mørdrupgård. Average infection in control plots was 8.0%. (n.s. = not significant).

		Dose of acetic acid (ml/kg)					
		5	10	20	30	40	50
Concentration	5 %	20 n.s.	49	79	62 n.s.	54 n.s.	63
	10 %	25	59 n.s.	83	70	63	70
	20 %	4	39	75	54	45	55 n.s.
	30 %	30	55	30 n.s.	67	60 n.s.	67 n.s.
	99.9%	46	66	86 n.s.	74	69 n.s.	75 n.s.

Barley leaf stripe

In spring barley at Mørdrupgård the infection of leaf stripe was reduced by 93% at the dose of 20 ml/kg of concentrated acetic acid (99.9%) with no significant effect on field emergence (Table 2).

Table 2. Effect of different combinations of dose and concentration of acetic acid as seed treatments on percent reduction in disease frequency of barley leaf stripe (*Pyrenophora graminea*). Grey cells indicate treatments with a significant reduction in germination vigour. Experimental year 2000 at Mørdrupgård. Average infection in control plots was 17.4%. (n.s. = not significant)

		Dose of acetic acid, ml/kg					
		5	10	20	30	40	50
Concentration	0 %	28,6 n.s.	4,2 n.s.	6,4 n.s.	8,3 n.s.	-1,6 n.s.	7,2 n.s.
	5 %	-7,8 n.s.	10,4 n.s.	26,9 n.s.	32,9 n.s.	54,5	84,8
	10 %	-6,2 n.s.	12,7 n.s.	67,8	82,4	95,8	84,5
	20 %	8,1 n.s.	26,7 n.s.	84,3	96,1	93,3	50,6 n.s.
	30 %	29,2 n.s.	36.0 n.s.	90,3	91,0	99,5 n.s.	48,8 n.s.
	99.9%	12,7 n.s.	68,5	93,4	99,7 n.s.	99,7 n.s.	94,4

At Flakkebjerg 2000, fewer combinations of concentration and doses was tested, and an optimal dose was not found and none of the treatments reduced germination vigour significantly (Table 3). The results from Flakkebjerg are consistent with the results from Mørdrupgård, and also with previous published results with increasing doses of 5% acetic acid (Nielsen *et al.*, 2000).

DISCUSSION AND CONCLUSION

Acetic acid is a naturally occurring substance with a high biodegradability and a very low oral toxicity to humans, game birds and others that may come into contact with seeds treated with fungicides. However, acetic acid is a corrosive substance that will evaporate from the seeds during seed treatment and so precautions should be taken to ensure human health and safety at work. We believe that substituting conventional fungicides with acetic acid will reduce the general environmental impact of seed treatments. Seed treatment with acetic acid would be cost effective, since it is a cheap substance and treated seeds remaining unsold could be used for animal feed, while seeds treated with more ecologically-toxic fungicides must be incinerated under controlled conditions.

In the winter wheat experiments, the infection of common bunt was high but a reduction of infection by 92-96% was still achieved when treated with 20 ml/kg of 5% acetic acid, without

Table 3. Effect of different combinations of dose and concentration of acetic acid on the percent reduction of frequency of barley leaf stripe (*Pyrenophora graminea*). Field trial at Flakkebjerg 2000.

Dose ml/kg	Concen-tration	% infected plants (95% confidence intervals)	% reduction
Control		14,0 (10,9 - 17,9)	-
Imazalil		0,2 (0,0 - 2,0)	98,3
5	30 %	6,5 (4,4 - 9,7)	53,4
5	40 %	5,8 (3,8 - 8,7)	58,9
5	50 %	2,9 (1,4 - 5,8)	79,3
10	15 %	13,0 (9,6 - 17,4)	7,5
10	20 %	9,3 (6,6 - 13,0)	34,0
10	25 %	6,4 (6,1 - 6,6)	54,6
20	7,5%	11,2 (10,6 - 11,9)	19,9
20	10 %	10,4 (10,2 - 10,6)	26,0
20	12,5%	5,7 (5,4 - 6,1)	59,4

affecting the germination vigour of the seeds. In spring wheat the infection was lower and a reduction of only 75-83% was recorded. The reasons for the differences in effect between the experiments in winter wheat and spring wheat are unclear. It may be caused by the differences in crop, in differences in infection level or the fact that the spring wheat was grown from very low vitality seed that may have made the seeds more sensitive to the acetic acid treatment.

The experiments with spring barley showed that barley leaf stripe can be effectively controlled by a high concentration of acetic acid (99.9%) at a dose rate of 20 ml/kg. In previous experiments only a low concentration of 5% have been used (Nielsen *et al.*, 2000), but the general pattern of control was similar to these experiments. The ratio surface area to volume is higher in barley than in wheat and *Pyrenophora graminea* is present within the seed coat while *Tilletia tritici* occurs as loosely attached spores on the seed surface. These facts may be the reason that the concentration of acid needed to control the pathogen is higher in barley than in wheat and why barley seeds are less sensitive to acetic acid, exhibiting a relatively lower reduction in seed vigour.

Common bunt is a very devastating plant disease since presence of only a few infected plants can give the whole crop an odour of rotten fish, and consequent crop loss. The disease control treatments must therefore be very effective against this disease (Borgen, 2000a). Even the control level of 92-96% found in these experiments is not adequate for seed lots of susceptible varieties with a high spore load. Therefore, seed treatments with acetic acid cannot stand alone, but must be combined with other measures in an integrated strategy. This could involve physical removal of spores or use only according to specific contamination thresholds dependent on the susceptibility of the varieties to be sown (Borgen, 2000a).

Although barley leaf stripe is an important seed borne disease, reducing yield when disease levels are high, the need for complete control is less critical than for common bunt in wheat. A control

effect of 93% as achieved in these experiments is therefore believed to be acceptable in some cases e.g. the last generation of organic seed production.

In organic agriculture, conventional fungicides are prohibited and the current practice of discarding all infected seed lots is a major constraint on organic cereal propagation. Consequently, acetic acid could be an interesting new weapon to use against seed borne pathogens in organic agriculture particularly if combined with other treatments approved by organic growers (Spieβ, 2000).

In recent years, soil borne infection of common bunt has been of increasing importance in wheat production (Borgen, 2000b). Some systemic pesticides effective against common bunt are also effective against soil borne pathogens, and provide near 100% control of all seed borne infections (Nielsen, 2001). However, organic products or biological products have only a low effect on the soil borne infection of common bunt, which could give be a problem if common bunt was introduced in an organic field

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Use of mustard flour and milk powder to control common bunt (*Tilletia tritici*) in wheat and stem smut (*Urocystis occulta*) in rye in organic agriculture

A Borgen
Holmemosevej 7, 4780 Stege, Denmark

L Kristensen
Agroecology, Royal Veterinary and Agricultural University, Agrovej 10, 2630 Tåstrup, Denmark

ABSTRACT

Common bunt of wheat (*Tilletia tritici*) and stem smut of rye (*Urocystis occulta*) can cause severe problems in organic production. Field trials have been carried out to study the effects of mustard flour and milk powder on these pathogens. Results showed that seed treatment with mustard flour controlled seed borne infection by *T. tritici* in wheat without decreasing the germination vigour of the seeds. Full control of *T. tritici* with milk powder treatments could only be achieved at doses that reduced germination vigour of the seeds. Mustard flour has a potential as a seed treatment in organic agriculture. Both milk powder and mustard flour might be used to control *U. occulta* in rye.

INTRODUCTION

Common bunt (*Tilletia tritici* syn. *T.caries*) in wheat is routinely controlled in conventional seed lots with synthetic fungicides. However, the disease is a major threat to organic wheat production where these seed treatments are not permitted (Borgen, 2000a; Nielsen *et al.*, 1998). In recent years, new seed treatments have been developed which may be acceptable to organic farming including milk powder (Becker, 1992; Becker & Weltzien, 1993; Borgen & Davanlou, 2000; Borgen & Kristensen, 2000; Borgen *et al.*, 1995; ICARDA, 1996, 1997; Nielsen, 1998; Nordin, 1982; Plakholm, 1993; Plakholm & Söllinger, 2000; Tränkner, 1993, 1996; Winter *et al.*, 1997), mustard flour (Spieß & Dutschke, 1991) and acetic acid (Nielsen *et al.* 2000; Borgen & Nielsen, 2001). Spores of T. tritici can survive for many years in the soil, and from there infect wheat (Borgen, 2000b). For full control of the disease with these treatments it is therefore necessary to study the effects against resting spores in the soil as well.

Stem smut (*Urocystis occulta*) in rye has achieved little attention as a seed borne disease during the past 50 years because the pathogen is easily controlled by synthetic pesticides. However, the disease can gradually build up when untreated seed lots are used for several years, even if it rarely causes severe losses in yield. Nevertheless, periodically the disease does cause some yield loss, and any program for organic seed production should therefore include methods to control this disease.

High doses of a range of different seed treatments can reduce germination vigour of the seeds resulting in a poor or delayed germination in the field. In organic agriculture the phase of establishment of the crop is very important for the yield, since successful competition with weeds will depend on a quick and uniform crop stand. In organic agriculture special attention should be given to negative effects on germination properties. The aim of the present study is to investigate the potential of milk powder and mustard flour as seed treatments against common bunt in wheat and stem smut in rye with special attention being paid to the side effects of the treatment on the seed germination and vigour.

MATERIALS AND METHODS

Field trials were conducted at Højbakkegård on Zealand, Denmark in the period 1995-1997. The effect of different doses of milk powder and mustard flour were tested by applying increasing doses to seeds of wheat and rye. The seeds were contaminated with 5 g spores of either *Tilletia tritici* or *Urocystis occulta* per kg seeds, which resulted in a contamination between 1.7 to 2.0 x 10^6 spores per gram seeds when tested by the ISTA haemocytometer method (Kietreiber, 1984). After treatment, the seeds were stored at 5 °C. Samples were removed for field tests 2-6 days after seed treatment. Germination tests were conducted 1-3 month later.

Germination tests in lab were carried out in the form of a cold sand test in plastic plates containing 1.5 kg sand with water (65ml H_2O/kg quartz sand). 100 seeds were sown 1.5 cm deep in each plate and the temperature was maintained at 10 °C. The number of emergent seedlings were counted every day for 5 days after first emergence. There were 3-4 replicates and the seed vigour was expressed as the time for 50% emergence.

In the field trial with seed borne infection with common bunt in 1995, and with stem smut in 1997, seeds of each treatment were sown in 4 replicate 6 m^2 plots. In the field trials with common bunt 1996-97, treatments were sown in 10 replicate 1.25 m rows. After heading, the number of infected ears of wheat and infected plants of rye were counted based on visible macro-symptoms. Between 1-2000 plants were assessed for common bunt in each treatment and approximately 550 plants assessed for stem smut.

In addition, a field trial with soil borne infection was carried out in 1998 in a field where a highly infected wheat crop was grown in 1996 followed by a barley crop 1997. The experiment was established using winter wheat seeds without any addition of spores of common bunt in plots 8 x 1½ m with 4 replicates per treatment. All plants in all plots were assessed for infection.

Data of diseased plants and germination speed was modelled and analysed by a generalised linear model with logit data-transformation (PROC GENMOD in SAS ver. 6.12). Significant differences were tested by a contrast statement.

RESULTS AND DISCUSSION

Increasing seed treatment doses of mustard flour up to 10 g/kg increased disease control of common bunt in wheat (Figure 1). Rates of application above this gave no further disease reduction. In 1996 and 1997, the highest doses of 33 and 43 g/kg, respectively, significantly reduced germination vigour. This is consistent with previous studies by Spieß & Dutscke (1991). This indicates that mustard flour can be used as an effective fungicide against seed borne infection by common bunt.

In similar trials, flours from other *Brassica* species, brown mustard (*Brassica juncea*), oriental mustard (*Brassica juncea* var. *orientalis*) and oil seed rape (*Brassica napus*) were found to be significantly less effective than the standard mustard flour made from yellow mustard (*Brassica hirta* syn. *Sinapis alba*) (data not presented). A range of compounds derived from *Brassica* glucosinolates were also tested as seed treatments in the form of pure chemicals and although some control was obtained with compounds derived from *Sinapis alba*, no or less effect were seen from compounds from the other species (data not presented).

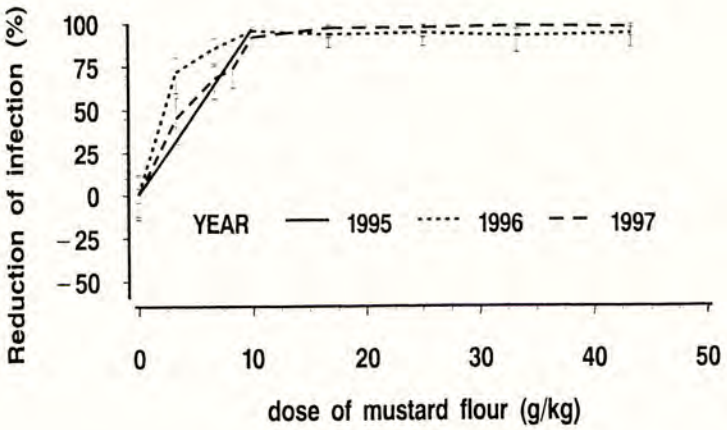

Figure1: Effect of seed treatment with increasing rates of mustard flour on common bunt of wheat in field trials. Bars indicate 95% confidence intervals.

The effect of seed treatment with increasing doses of milk powder on infection of wheat infected by common bunt is shown in Figure 2. In 1996, maximum control was achieved at a rate of 43 g/kg and 1997 this was achieved at 80 g/kg. In both years a significant reduction in germination vigour was recorded at or above these dose rates. This shows that seed treatment with milk powder is not a complete answer to control of seed borne infection by common bunt as proposed by previous studies (Becker & Weltzien, 1993; ICARDA, 1996, 1997; Plakholm, 1993; Plakholm & Söllinger, 2000; Tränkner, 1993, 1996; Winter *et al.*, 1997).

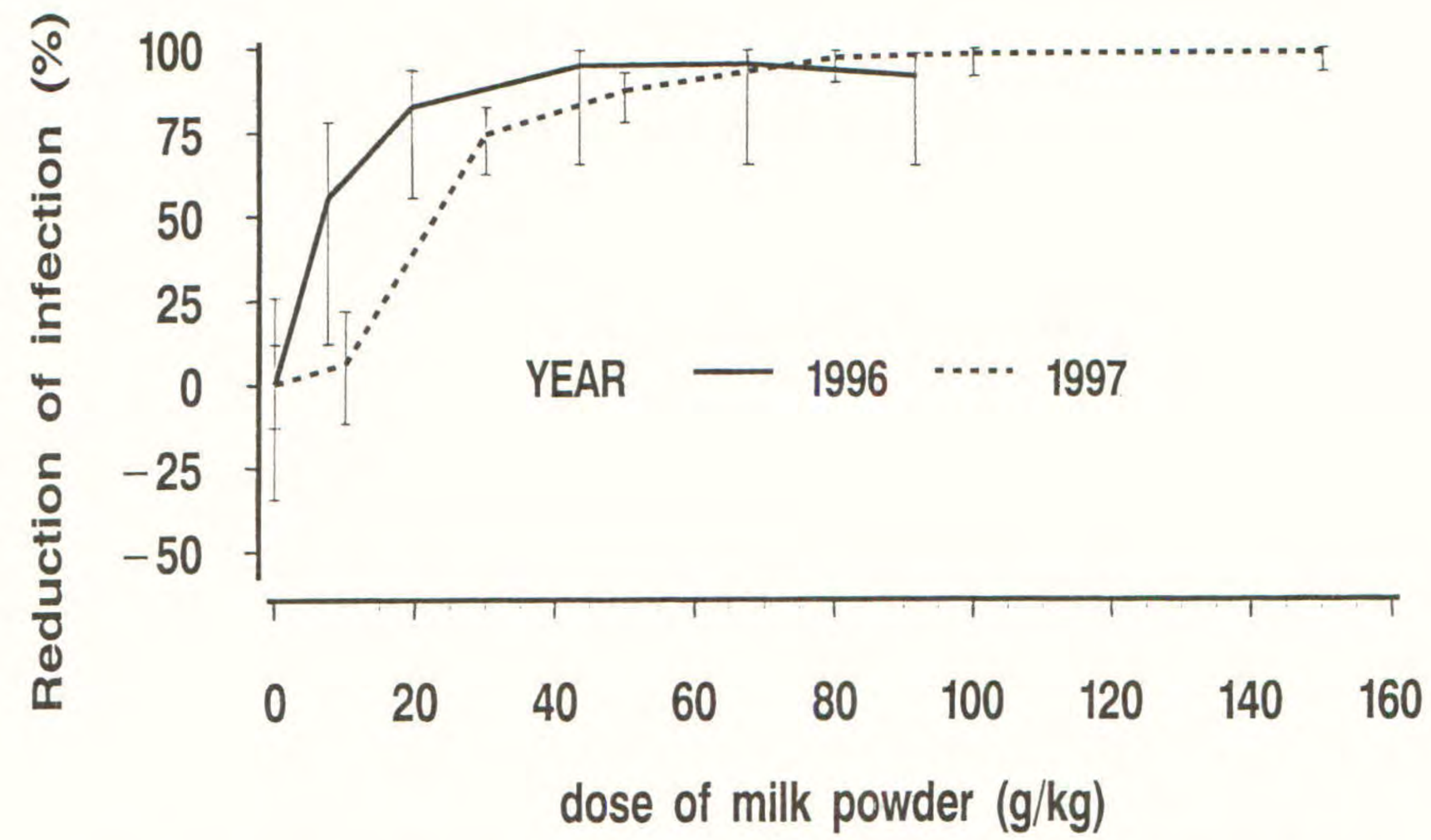

Figure 2: Effect of seed treatment with increasing rates of milk powder on common bunt of wheat in field trials. Bars indicate 95% confidence intervals.

The control of common bunt and the effect on seed germination by seed treatment with milk powder was compared with that achieved with other organic substances (Table 1). As the coating of the compounds on the seeds was visually not uniform in some cases, the differences in effect between the compounds are likely to reflect a combination of both chemical and physical factors.

Becker & Weltzien (1993) have shown that the mechanism of action of the milk powder is probably due to saprotrophic micro-organisms using the milk powder as nutrient source. *T. tritici* is very sensitive the availability of oxygen in competition with other micro-organisms using sugar as a nutrient source (Rabien 1928) and it is therefore likely that this is the basic explanation for the effect of milk powder. The side effect on seed germination at the high doses of milk powder also may be a

result of the decreased availability of oxygen and maybe other factors needed for germination.

Milk powder used alone, stimulating naturally occurring saprophytes in the soil or seed surface, is not a fully effective control measure and has a negative side effect on germination. Recent studies of biological control of common bunt has shown that limited doses of milk powder (20 g/kg) in combination with biological control agents have a synergistic effect and can give an effective control without reducing the germination vigour (Borgen & Davanlou, 2000). This combination is believed to be the future for milk powder and equivalent agents in the control of common bunt.

Table 1: Effect of different organic seed treatments on common bunt in field trials and germination rate in the laboratory (30g/kg applied).

Treatment	% infected plants (95% confidence interval)	Days for 50% germination at 10 °C
Control	27.1 (26-28.2)	8.99
Not contaminated	0.7 (0.2-2.3)	8.74 *
Milk powder	7.0 (4.9-10.0)	9.00 ns
Wheat flour (*Triticum aestivum*)	15.0 (11.9-18.6)	8.91 ns
Maize flour (*Zea mays*)	27.9 (25.2-30.9)	8.79 ns
Mustard flour (*Sinapis alba*)	0.4 (0.1-2.2)	9.51***
Tryptic Soy Broth	2.5 (1.2-5.1)	9.82***
Corn cockle flour (*Agrostemma githago*)	9.0 (6.2-12.9)	9.21 ns
Rye flour (*Secale cereale*)	12.0 (8.6-16.6)	9.26 *
Quinoa flour (*Chenopodium quinoa*)	5.8 (3.6-9.3)	9.43***

* Significantly different from the control at $P<0.05$ or *** $P<0.001$

Milk powder and mustard flour have been tested against natural soil borne infection by common bunt, and milk powder reduced the frequency of diseased plants by 91% ($p<0.001$) (Borgen & Kristensen, 2000) while mustard flour had no significant effect in this study. However, this is based on only one year of field experiments and contradicts conventional conclusions from experiments with artificially contaminated soils that soil borne infection can only be controlled by systemic pesticides (Nielsen, 2001). Further studies should clarify the potential of milk powder in control of soil borne infection by common bunt under practical farming conditions.

Milk powder and mustard flour were tested against stem smut in rye (*Urocystis occulta*) resulting in a reduction of infection by >90% (Table 2). The treatments were

tested in only one dose, and this dose did not affect the germination vigour of the seeds. Whether a higher dose could increase the effect without side effects on germination was not tested. The experiment indicates that both agents may be used as a seed treatment against this pathogen.

Table 2: Effect of milk powder and mustard flour on the infection of stem smut (*Urocystis occulta*) of rye

	percent diseased plants	reduction
Control	53.2 %	0
Milk powder (50g/kg)	4.5 % (P<0.001)	91.5%
Mustard flour (10g/kg)	4.8 % (P<0.001)	91.0%

These studies indicate that the effect of mustard flour seed treatment against stem smut and common bunt is adequate to be relevant for use in organic wheat production, whereas milk powder had too large a negative effect on seed vigour to be acceptable for control of common bunt. However the question arises whether use of these compounds is consistent with the legislation of organic agriculture.

The legislation on organic agriculture is based on the EU-regulation (EEC, 1991). To control plant diseases in this regulation, preventive measures are preferred like protection of natural enemies. However, in cases of an acute threat to the crop, materials from Annex II can be used. In Annex II protective oils are listed, but mustard and milk products are not.

In Germany mustard is allowed in organic production, since the interpretation of the EU-regulation is that the effect of mustard is from the glucosinolates or mustard oils, and therefore included in Annex II as a plant protective oil. The use of milk powder could be interpreted as protecting natural enemies in form of naturally occurring saprophytic micro-organisms. In Germany milk powder is considered as a plant strengthener and is therefore not evaluated as a plant protective agent in Annex II but as a fertiliser listed in Annex I as an animal derived product. Tille-Kur is a product containing mustard flour produced in Germany, sold in many other EU countries and is approved by a number of organic certifying bodies. The product is effective against common bunt (Spieß, 2000). However, both milk powder and mustard flour are prohibited in Denmark as they are not listed in Annex II.

The aim of the EU regulation of organic agriculture is to harmonise production standards for organic agriculture in Europe. This goal has not been met in the regulation of the use of mustard and milk powder in plant protection and needs to be addressed.

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