Session 6
Biological Seed Treatments

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MECHANISMS OF PROTECTION OF SEED AND SEEDLINGS BY BIOLOGICAL SEED TREATMENTS: IMPLICATIONS FOR PRACTICAL DISEASE CONTROL

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ABSTRACT

Seed treatments with biological control agents have been extensively tested, and are beginning to be used commercially. However, there are substantial gaps in our knowledge of mechanisms by which various bacterial and fungal biocontrol agents control plant pathogenic fungi. Good evidence exists for the role of antibiotics or siderophores in seed protection, but other mechanisms probably also occur. Evidence for and against mycoparasitism, competition for iron or key stimulants of microbial propagule germination, and attachment of bacterial cells to hyphae are presented. Criteria for, and progress in, mechanistic studies with various organisms will be considered. The implications for these various mechanisms in the development of biological seed treatment formulations and methods for successful biocontrol will be considered.

INTRODUCTION

Seed treatments with fungal or bacterial biocontrol agents have been proposed, tested, and are likely to be used soon for commercial plant disease control (Harman, 1991). They may be used to control seedborne plant pathogens, control soilborne seed attacking fungi, or as a method of introducing agents that will colonize newly formed roots or other plant parts (Harman et al., 1989; Harman, 1990; Vannacci and Harman, 1987). However, performance of biological seed treatments has been variable. One important component of performance in any biocontrol system is an understanding of the mechanism by which control occurs.

While many, if not most, biocontrol organisms may suppress pathogens through several mechanisms (Ownley *et al.* 1992), principal mechanisms of biocontrol exist and are likely to be different between various organisms. Three mechanisms have been proposed most frequently for biocontrol, as follows (Baker, 1986): 1. Production of antibiotic substances; 2. Competition for space or nutrients; and 3. mycoparasitism. In addition, there are at least two other mechanisms that have been proposed for biocontrol agents applied to seeds, and that are likely also to occur in other application/delivery methods, as follows: 4. Adsorption/catabolism of specific plant metabolites that are necessary for stimulation of propagules of pathogenic fungi (Nelson, 1992); and 5. Attachment of bacteria to fungal hyphae by lectin interactions that inhibit further fungal growth (Nelson *et al.* 1986).

Biocontrol processes are partially determined by the intrinsic ability of biocontrol organisms to grow on and colonize seed surfaces, which is a property we will describe hereafter as spermosphere competence. In addition, the apparent spermosphere competence of any microorganism is strongly affected by the method of application and by the physiological, ecological, and edaphic interactions that occur immediately after treatment and/or planting of the seed. Similarly, the ability to colonize root surfaces (rhizosphere competence) is also determined by both the genetic and physiological characteristics of the organism in question and also by the various environmental factors such as pH, temperature, and water potential. Consequently, if a biocontrol agent has appropriate genetic and physiological attributes to provide effective seed or root disease control, the method of seed treatment may strongly influence the success or failure of the disease control strategy. Logically, seed treatment efficacy can be made more effective if mechanisms of disease control are known, so that the seed treatment composition and method of application can provide maximum levels of the critical metabolites or propagule growth at an appropriate time.

Timing is a fundamentally important factor in any biological seed treatment method. Not only do some pathogens attack seeds very quickly after planting, but competitive microbial populations proliferate rapidly and may successfully compete or otherwise prevent growth of the

biocontrol agent (Hubbard *et al.* 1983). Determination of biological control mechanisms were, of necessity, first determined by in vitro interaction studies, usually on artificial media. Whereas such studies indicated the kinds of mechanisms that may occur between biocontrol agents and target pathogens, such studies cannot prove that similar mechanisms occur in vivo. We consider that the only definitive studies that prove or disprove specific mechanisms of action require a molecular version of Koch's postulates. In such studies, mutant strains are prepared that are deficient in the particular character to be considered. In addition, the gene coding for the specific character is isolated, and added back to the deficient strain. The various strains so constructed are tested for biocontrol ability under realistic conditions, and in this way, the contribution of any gene, and presumably its gene product, can be determined both qualitatively and quantitatively.

This paper will critically examine our knowledge of specific mechanisms of biocontrol by various microbial agents. Further, we will consider the temporal interactions of bioprotectants, seeds, pathogens, and other microorganisms around planted seeds, and how such interactions affect our understanding of mechanisms of seed protection. Finally, we will relate our understanding of mechanisms and temporal relationships to strategies and formulation of biological seed treatments, and indicate how further studies may enhance efficacy and usefulness of this method of seed and seedling protection.

TEMPORAL RELATIONS OF PATHOGEN-SEED-ANTAGONIST INTERACTIONS

For seed-rotting pathogens, early events in seed germination are particularly important in determining the success or failure of seed infections. Generally, the first 12-48 hr of seed germination and seedling development are critical to longer-term plant health. This phenomenon is particularly notable for seed-rotting *Pythium* species. Some critical time periods for particular pathogens and biocontrol agents are as follows (primarily as summarized by Harman and Stasz, 1986).

Time after Sowing Seed	Activity
0-4 h:	Propagules of *Pythium* spp. begin to germinate. Bacterial biocontrol agents metabolically active
4-24 h:	Seed coats are infected by *Pythium* spp. The number of infected seeds increases linearly over this time period (Taylor *et al*, 1991).
5-12 h:	Propagules of fungal biocontrol agents begin to germinate on seed surfaces.
5-60 h:	Chlamydospores of *Fusarium* spp. germinate and infect seeds.
24-40 h:	Embryos of seeds are infected by *Pythium* spp.

These representative data indicate that seeds are at risk very soon after planting, and that if bioprotectants are to protect them, they must react quickly. Clearly there is a window of vulnerability that must be closed if biocontrol of soil-borne seed and seedling pathogens are to be controlled. Further, biocontrol agents must grow if they are to be effective, and pathogens must proliferate if they are to infect their hosts. Therefore, the nature of the nutritive or stimulatory materials released from seeds, which are energy sources for both beneficial and deleterious organisms, must be considered.

Our current knowledge of pathogen stimulants was recently reviewed (Nelson, 1990) and will only be updated here to include more recent findings. Our most complete knowledge of pathogen stimulants from seeds comes from studies of *Pythium* spp. Conclusions drawn from many previous studies of propagule germination among *Pythium* spp. have led to the suggestion that carbohydrates and amino acids are the primary exudate components responsible for initiating *Pythium*-seed interactions in nature (see Nelson, 1990), although sporangia of *Pythium* species clearly respond to other stimuli such as volatiles (Nelson, 1987) and can be manipulated in ways that eliminate their responses to sugars and amino acids while maintaining their responses to seed exudates (Nelson and Craft, 1989). For example, sporangia produced on most synthetic culture media germinate readily in response to sugars and amino acids present in cotton seed exudate, but fail to respond to these same molecules when produced on metabolically active plant tissue such as diseased seeds and radicles or on a lecithin-containing mineral salts medium. Regardless of how

sporangia are reared, they remain fully germinable in response to unfractionated cotton seed exudate (Nelson and Craft, 1989). The inability of plant-produced sporangia to germinate in response to sugars and amino acids under some conditions in vitro raises questions about the types of compounds responsible for stimulating sporangium germination in soil.

The active components of cotton seed exudates stimulatory to *P. ultimum* sporangia consist of unsaturated fatty acids and triglycerides of these fatty acids (Ruttledge and Nelson, submitted). These stimulatory molecules are present in the exudate as soon as seeds begin imbibing water and reach maximum levels around 4 hr after imbibition begins. Interestingly, some degree of unsaturation is required for activity. All of the unsaturated fatty acids tested to date are effective sporangium germination stimulants and active at concentrations of at least 100 ug/ml. On the other hand, none of the saturated fatty acids tested to date have shown stimulatory activity at those concentrations.

The activity of unsaturated fatty acids in stimulating fungal spore germination has been demonstrated before. Papavizas and Adams (1969) showed that endoconidia and chlamydospores of *Thielaviopsis basicola* did not germinate in response to saturated fatty acids such as stearic or palmitic acid, but did germinate in response to linolein and linolenic acid. Additionally, Harman *et al* (1978) showed that unsaturated fatty acids such as oleic, linoleic, and linolenic acid stimulated germination of *Alternaria alternata* conidia while saturated fatty acids such as stearic and palmitic acid, were ineffective. They have proposed (Harman *et al*, 1980; Harman *et al*, 1978) that the volatile peroxidation products of unsaturated fatty acids may be the active germination stimulants in fungi, since as little as 200 µg/l 2,4-hexadienal in aerial solution stimulated germination of *A. alternata* conidia. Although this compound was not stimulatory to *F. oxysporum* f.sp.*pisi* chlamydospores, relatively high concentrations (400nl/petri dish) of trans, trans-2,4-nonadienal were stimulatory (Harman *et al*, 1980). These observations, coupled with the fact that volatiles from seeds and decaying plant tissues are stimulatory to a range of soilborne pathogens (Gilbert and Linderman, 1971; Gilbert and Griebel, 1969; Gorecki *et al*, 1985; Linderman and Gilbert, 1969; Linderman and Gilbert, 1975; Nelson, 1987; Norton and Harman, 1985; Paulitz, 1991) supports the notion of fatty acids and volatile peroxidation products as being important stimulants of propagules of seed-rotting pathogens.

SEED-BIOPROTECTANT INTERACTIONS AND MECHANISMS OF BIOCONTROL

With the background presented above, it is possible to discuss the mechanisms by which biocontrol may operate and to place this discussion in an ecological framework. The biocontrol agents to be discussed will be bacterial strains in the genera *Enterobacter, Pseudomonas*, and *Serratia*, and fungi in the genera *Gliocladium* and *Trichoderma*. These organisms have been more extensively studied than other organisms, particularly as seed treatments. The discussion that follows is not exhaustive, but will provide specific examples of biocontrol mechanisms.

Antibiotics and toxicants

Very compelling evidence indicates that some microbes exert biocontrol ability through production of toxic substances. Howell and his coworkers (Howell, 1987; Howell and Stipanovic, 1983) have demonstrated that biocontrol ability of seed treatments with *Gliocladium virens* is primarily mediated through the production of antibiotics. If these antibiotics were eliminated through the production of antibiotic deficient mutants, the organisms largely lost their biocontrol ability. These studies have been extended and corroborated by other workers; however, these substances may not act alone. Di Pietro *et al* (1993) discovered that the endochitinase produced by this organisms can synergistically enhance activity of the *G. virens* antibiotic gliotoxin, and has the effect of making target fungi more sensitive to the antibiotic.

Bacteria also produce antibiotics effective in biocontrol. Definitive studies in which genes have been identified that code for antibiotics, together with transposon mutagenesis to elucidate their role have been conducted (Farrand *et al*, 1985; Gutterson *et al*, 1986; Jones *et al*, 1986; Keel *et al*, 1992; Laville *et al*, 1992; Slota and Farrand, 1982; Thomashow and Weller, 1988; Vincent *et al*, 1991; Voisard *et al*, 1989). In nearly all of the studies in which bacterial genes involved in fungal suppression have been cloned, they have been involved in antibiotic biosynthesis (e.g. oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol,

hydrogen cyanide) in *Pseudomonas fluorescens*. In these bacteria, antibiotics clearly play an important, and in some cases, definitive role.

Competition: Siderophores

Probably the only conclusive research demonstrating a role for competition for a specific substance comes from the research on siderophore competition for iron. A few studies have also demonstrated that siderophore biosynthesis in *P. fluorescens* plays a role in pathogen suppression (Duijff *et al*, 1991; Loper, 1988), whereas a few studies have found siderophores to play little or no role in these processes, particularly with *Pythium* species (Hamdan *et al*, 1991; Keel *et al*, 1989; Paulitz and Loper, 1991).

Competition: Inactivation of germination stimulants

The first 6-12 h of germination is a key period of vulnerability to *Pythium* infection; after that, developing seedlings become less susceptible to infection (Nelson, *et al.*, 1986; Maloney and Nelson, *unpublished*). The suppressive effects of *E. cloacae* on *Pythium* behavior have been observed as reductions in *Pythium* colonization of bacterized seeds as compared with untreated seeds. These reductions occur as early as 4-6 h after sowing seed (Maloney and Nelson, unpublished). One possible hypothesis to explain the rapid reduction in *Pythium* response and infection of germinating seeds in the presence of *E. cloacae* is that *E. cloacae* can inactivate molecules in seed exudates that *P. ultimum* requires for propagule activation and/or germination. In the absence of these signal molecules, *P. ultimum* cannot establish pathogenic interactions with its host. Growth of various strains of *E. cloacae* on 4-hr cotton seed exudate results in dramatically reduced levels of sporangium germination when cell-free exudate solutions are assayed for stimulatory activity (Nelson, 1992; van Dijk and Nelson, unpublished). Likewise, when seeds are coated with strains of *E. cloacae*, sporangia do not germinate in response to exudate stimulants released into and extracted from sand or unsterilized soils (Nelson, 1990). Further, wild-type strains of *E. cloacae* eliminate the stimulatory activity of linoleic acid in as little as 4 hr (van Dijk and Nelson, unpublished). However, transposon-induced mutants of *E. cloacae* have been identified that fail to inactivate the activity of linoleic acid after 24 h and these same mutant strains no longer protect cucumber seeds from infection by *P. ultimum* (Maloney and Nelson, unpublished). It is likely that in this system, stimulant inactivation is an important mechanism of seed rot suppression.

In other systems, stimulant inactivation appears to be playing a role in biological control activity. Elad and Chet (1987) found a significant correlation between the ability of various bacterial strains to inhibit *Pythium* seed rot of cucumber and their ability to inhibit oospore germination of *P. aphanidermatum*. Effective bacterial biocontrol agents inhibited oospore germination by as much as 57% while ineffective biocontrol strains inhibited oospore germination by only 13-20%. There was no direct interaction between the bacteria and oospores, and the ability of effective strains to inhibit oospore germination was not related to the production of inhibitory metabolites. They speculated that bacterial strains catabolized exudate components responsible for stimulating oospore germination. In other studies, pea, cotton, and soybean seeds evolved significantly lower levels of ethanol and acetaldehyde during germination when treated with *Enterobacter cloacae*, *Trichoderma harzianum*, or *Pseudomonas putida* as compared with untreated seeds (Gorecki *et al*, 1985; Nelson, 1990; Paulitz, 1990). The volatiles released from treated seeds were less stimulatory to sporangium germination of *P. ultimum* than were volatiles from untreated seeds (Gorecki *et al*, 1985; Nelson, 1990). In addition, Ahmad and Baker (1988) observed reductions in sporangium germination of *P. ultimum* in the presence of *Trichoderma*-treated seeds as compared with untreated seeds. Thus, there is a substantial and growing body of evidence indicating that competition for germination stimulants may play an important role in biocontrol.

An explanation of the mechanisms of biocontrol by *E. cloacae* is likely to occur soon. A mutant library has been constructed using a mini-TN5/phoA plasmid (de Lorenzo *et al*, 1990); the phoA reporter gene makes it particularly well-suited to detection of gene production expressed on the outer surface of the plasma membrane. Five mutants possess altered biocontrol phenotypes, and one no longer protects seeds against rots (Maloney *et al*, 1994). A 16 kb clone has been

discovered that completely restores biocontrol activity; this gene likely provides a global function such as nutrient transport of sensing. Elucidation of the role of this gene should indicate the mechanism of action of this biocontrol agent.

Mycoparisitism and production of cell wall degrading enzymes

Mycoparisitism is a complex process by which biocontrol fungi may attack pathogenic fungi and involves the following steps, as inferred from in vitro studies (primarily from (Chet, 1987): (1) the biocontrol fungi grow tropically toward the target fungi, (2) hyphae of the biocontrol fungi bind to lectins on the surface of the target fungi via attachment of carbohydrate receptors on the surface of the biocontrol fungus to lectins on the target organism, (3) cell wall degrading enzymes are produced that attack the target fungus and destroy its integrity; these enzymes have recently been shown to be complex mixtures of synergistic proteins that act together against pathogenic fungi (Lorito *et al*, 1993a), and (4) appressoria-like structures are produced that apparently initiate penetration of the target fungus by the bioprotectant. While mycoparasitic structures have been observed on *Trichoderma*-treated seeds (Hubbard *et al*, 1983), Lifshitz *et al* (1986) considered this mechanism to be unlikely to protect seeds against *P. ultimum*. They based this hypothesis on the fact that (a) mycoparasitic structures were rarely observed, and (b) infection by this pathogen occurs too rapidly for growth of *Trichoderma* and mycoparasitism to occur on the seed surface. These considerations may well be correct, however, the time required for *P. ultimum* to breach the seed coat may indeed provide the requisite time for mycoparasitism to occur. Definitive results concerning the role of mycoparasitism by *Trichoderma* in control of a range of pathogens should be available soon. The genes for cell wall degrading enzymes are being isolated (Hayes *et al*, 1994), and the first chitinase deficient mutants have been prepared (Harman and Hayes, 1993). Once a series of mutants deficient in specific cell wall degrading enzymes have been prepared, as well as strains to which the genes have been restored, we will be able to definitively assess the role of the enzymes in biocontrol.

Chitinolytic enzymes from *Serratia marcescens* can play a role in biocontrol. A gene (ChiA) from this bacterium was inserted into the nonbiocontrol agent *Escherichia coli*, and the transgenic bacterium achieved biocontrol ability (Shapira *et al*, 1989). Similarly, *T. harzianum* was transformed with plasmids that resulted in integration of ChiA from *S. marcescens* into the *T. harzianum* genome (Haran *et al*, 1993). The gene was under control of the CaMV 35S promoter and so the heterologous enzyme was produced constitutively. The transformed strains were more capable of overgrowing *Sclerotium rolfsii* in vitro than the original strain from which it was derived. Further, it produced wider lytic zones in the area of contact with the pathogen than the wild type. Both the wild type and the transformed strains had similar growth rates and conidiation levels. These results indicate that the biocontrol ability of this strain was improved by transformation.

Adherence of *E. cloacae* to *P. ultimum*

One of the more conspicuous traits of *E. cloacae* in its interaction with *Pythium* spp. is its ability to adhere to hyphae. Empirical relationships between adherence of *E. cloacae* to hyphae of *P. ultimum* and biological control properties in the bacterium have been established (Nelson *et al*, 1986). However, pretreating cell suspensions of *E. cloacae* with various mono-, di-, and trisaccharides, as well as certain amino sugars, or a-linked glucosides, prevents cells from attaching to intact hyphae or agglutinating hyphal fragments. Addition of these same sugars also eliminates the ability of cells to inhibit fungal growth. On the other hand, pretreatment of cells with certain other monosaccharides, methylated sugars, or α-linked glucosides does not interfere with the ability of *E. cloacae* to attach to hyphae and inhibit the growth of *P. ultimum* (Nelson *et al*, 1986). The same carbohydrates that block binding of *E. cloacae* to *P. ultimum* hyphae also block ammonia production by *E. cloacae* (Howell *et al*, 1988) and allow cells to disperse through water films adjacent to *P. ultimum* hyphae (Maloney and Nelson, unpublished). These results suggest that bacterial adherence to hyphal cell walls might involve the binding of a fimbrial adhesin to specific sugar residues, possibly glucosides, that are associated with the fungal cell wall. Analogous interactions between E. coli and animal cells have been described (Duguid and Old, 1980).

Microscopic studies of the interactions between bacteria and live mycelium revealed bacterial cells clustered primarily around hyphal tips which were devoid of organized and streaming cytoplasm, around tips which were sealed off from their hyphae by septa, or along hyphae which contained cytoplasm that was no longer streaming and was withdrawn from the mycelial cell wall (Maloney & Nelson, unpublished). Only rarely were bacteria clustered in large numbers along hyphae which had actively-streaming cytoplasm. These observations have led us to speculate about the nature of the *Pythium* cellular changes occurring as a result of the close interaction of *E. cloacae* with hyphal tips. It is unclear from our preliminary experiments whether *E. cloacae* cells bind preferentially to regions of hyphae devoid of cytoplasm, or whether binding of *E. cloacae* results in the observed loss of cytoplasm and hence viability.

Although relationships between bacterial adherence and biological control activity appear to firm, molecular genetic analysis of these interactions has not supported the contention that adherence properties in *E. cloacae* are in any way related to biological activity. After screening a mutant library of *E. cloacae* transconjugants, several have been identified from three types of adherence assays, that are deficient in adherence properties. However, all of these strains have remained suppressive to *Pythium* seed rot of cucumber (Maloney & Nelson, unpublished).

IMPLICATIONS FOR DEVELOPMENT OF BIOLOGICAL SEED TREATMENTS

In spite of the efforts described here, as well as information from other studies and reviews, it is apparent that biological seed treatments can still be dramatically improved. This improvement can logically come from enhancement of the biocontrol agents themselves, and from developments in seed treatment methodology. These will be discussed separately.

Genetic improvement

Once a full understanding of the genetic basis for mechanisms of biocontrol agents is in hand, we will have a series of "molecular bullets" for improvement of biocontrol agents. Such genes may be used for other purposes such as producing disease resistant plants. Key to making rapid progress are understanding (a) of synergistic interactions between biocontrol organisms, and various biological and chemical components of plant disease control, and (b) use of promoters that change development or regulatory control of metabolites critical for biocontrol. We recently have discovered high levels of synergy between enzymes from *T. harzianum* and various other organisms or materials. Chitinolytic and glucanolytic enzymes from *T. harzianum* are strongly synergistic; each individual protein requires 40 to 150 ug/ml to achieve ED50 levels, while for combinations of enzymes, less than 2 ug/ml of total protein is required to achieve the same effect (Harman *et al*, 1993; Lorito *et al*, 1993a; Lorito *et al*, submitted). Further, these same enzymes are synergistic with *E. cloacae*. If levels of both enzymes and bacteria too low to have an effect are mixed with the test pathogen are added to the pathogen *Botrytis cinerea*, dramatic effects can be seen. First, within a few minutes the bacterium binds to hyphae of the pathogen,; binding ordinarily does not occur in this medium. In the presence of the enzyme, but not in its absence, proliferation of the bacteria occurs on the hyphal surface and within 24 hr the target fungus is nearly completely destroyed (Lorito *et al*, 1993b). Further, these same enzymes are strongly synergistic with chemical fungicides; the presence of the enzyme may increase the sensitivity of target fungi to the fungicide by more than 100-fold (Lorito *et al*, 1993c). Other synergistic combinations will no doubt be determined. Addition of such synergistic combinations of enzymes to appropriate biocontrol organisms (*E. cloacae* is an obvious choice) should result in much more potent biocontrol agents. As noted earlier, the first few such strains have already been produced, and even more effective strains should follow rapidly (Haran *et al*, 1993, Shapira *et al*., 1989).

Changes in regulatory control of specific gene products also are likely to be effective. For example, chitinolytic enzymes are usually only produced by bioprotectants after the two organisms come into contact. If genes coding for these enzymes are placed under the control of constitutive rather than inducible promoters, the temporal relationships of pathogen and protectant are likely to be improved in favor of the biocontrol agent. Promoters active at different developmental or in response to various metabolites are available and a number of strategies can be devised for use of such promoters for improvement of biocontrol agents.

Improved biocontrol seed treatment strategies

A number of strategies to improve temporal, edaphic, or competitive interactions of seed treatment bioprotectants have been developed. Concepts and practices of such treatments recently have been reviewed (Harman, 1991; Jin *et al*, 1992; Taylor and Harman, 1990; Taylor *et al*, submitted). The level of improvement, which can usually be attributed to an apparent enhancement of spermosphere competence, can be dramatic. For example, if *T. harzianum* is used to treat cucumber seeds and is applied as a slurry, biocontrol efficacy is marginal. However, if the organism is applied to the seed in a nutritive base, and then an inert layer placed over this (double coating), biocontrol efficacy is considerably enhanced. Further, if the seeds are then incubated for a few days at 100% relative humidity, the bioprotectant grows and colonizes the seed surface. Such seeds can then be dried and nearly perfect biocontrol can be obtained. Similar excellent results can be obtained with the use of solid matrix priming (SMP) with either fungal or bacterial biocontrol agents. In SMP, seeds are treated and then added to moistened substrate that provides sufficient moisture for microbial growth but not for seed sprouting. After a few days, biocontrol microbes proliferate by an order of magnitude and colonize the seed coat. Additives to seed treatments, such as specific food bases or materials to control pH can also be useful.

However, biological seed treatments can and must be further improved. Even though such treatments as double coating and SMP provide excellent biological control, the processes are too labor-intensive, and hence to expensive, for many crop seeds, including most agronomic crops. Ideally, biological seed treatments should meet the following criteria: (1) provide excellent and reliable control of pathogens on a wide range of crop seeds, (2) be inexpensive and simple to apply, (3) have no deleterious effects upon seed viability during storage, and (4) the biocontrol agent must retain viability and efficacy during storage for over one year at room temperature. This latter property is primarily a function of the production and formulation technique (Jin *et al*, 1992; Harman, unpublished) employed, but no components of the seed treatment formulation can adversely affect viability of the biocontrol agent.

Are such treatments possible? The authors feel that the answer is definitely yes. Seed treatment techniques described above indicate that *T. harzianum* and, to a lesser extent, *E. cloacae*, can be effective on a wide range of crop seeds, even when existing strains are employed. Seed treatments that enhance rapid growth and development, i.e. that increase apparent spermosphere competence, which leads directly to improved rhizosphere competence in strains possessing this property (Harman, 1991) can provide the necessary level of enhancement of biological seed treatments. Microbial nutrition added to the seed treatment is likely the key to development of more economical and successful treatments. Such development is likely to arise from a knowledge of the nutritive requirements of the agent of interest, and from the nature of seed exudates from various species. To illustrate this concept, we can consider the relative efficacy of *T. harzianum* and *E. cloacae* on different seeds. *T. harzianum* is quite effective, even as a slurry treatment on snap bean seeds, but requires a relatively complex double coating or SMP treatment to achieve similar efficacy on cucumber seeds. Conversely, *E. cloacae* is effective on cucumber, but has little or no effect on snap beans (Nelson *et al*, 1986). For *T. harzianum*, the difference lies on its ability to colonize these two seeds. The fungus readily colonizes bean seeds, but rather poorly and slowly colonizes cucumber seeds. Seed exudates from beans are richer and support growth of *T. harzianum* much better than do exudates from cucumbers. *E. cloacae*, on the other hand, is blocked from hyphal attachment by the level of sugars from snap beans, and this apparently prevents its biocontrol activity on this seed type. Clearly, however, a greater knowledge of the qualitative and quantitative differences in seed exudates, can assist in developing inexpensive formulation additives that enhance activity of at least *T. harzianum*. However, an additional requirement also exists. Any such formulation must be capable of stimulating growth of *T. harzianum* without a commensurate enhancement of growth of pathogens and competitive microflora. To meet this criterion, knowledge of stimulants for germination of pathogen propagules is essential; such materials either must be avoided or be formulated in such a way that they are available only, or primarily to, the biocontrol agent. The example of the relative efficacy of simple treatments on snap beans and cucumbers, plus the demonstration that the fungus is indeed effective on these and many more crops if treatments permit sufficient spermosphere competence, demonstrate that solutions exist. The challenge will be to gather the appropriate data and then, through extensive empirical testing, devise economically reasonable solutions.

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BACTERIZATION TO PROTECT SEED AND RHIZOSPHERE AGAINST DISEASE

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ABSTRACT

Microbiological protection against plant diseases is gaining momentum, partly because of the negative attitude towards chemical pesticides. In order to protect the plant against a pathogen, a biocontrol agent should not only produce a protective factor, it should also deliver it by colonization at the right site at the right time in optimal levels. The present knowledge of mechanisms of protection and colonization are discussed in this paper. Major factors limiting further application of microbiological control agents that will be discussed are colonization, survival on seed, microbiological activity during seed germination, and public acceptance.

INTRODUCTION

The interaction with microbes is of utmost importance in a plant's life. Therefore microbe-plant interactions are also economically important. Pathogenic microbes are responsible for the loss of approximately one third of the world's crop. Fortunately, many microbe-plant interactions are beneficial for the plant, e.g. the interaction of leguminous plants with Rhizobium and Bradyrhizobium can result in biological nitrogen fertilization, whereas microbial antagonists can cause a reduction of plant diseases. Finally, some microbe-plant interactions are neither harmful nor beneficial to the plant. Campbell & Greaves (1990) reported on experiments in which roots of wheat seedlings were inoculated with fluorescent Pseudomonas spp. from the rhizosphere of field-grown wheat. Of 150 isolates studied, approximately 40 % inhibited root growth, 40 % stimulated it, whereas the remaining 20 % had no effect.

Benefical effects of naturally-occurring plant-associated bacteria on plant growth can be direct of indirect, i.e. in the absence of pathogens or through limitation of the growth of the pathogen, respectively. Indirect microbial plant growth stimulation is often referred to as biocontrol in order to stress the contrast with chemical control.

Direct microbial plant growth promotion is due to the fact that a large number of soil bacteria produce plant growth promoting hormones, e.g. Azospirillum, Azotobacter , Pseudomonas and Bacillus spp. Other microbes which directly promote plant growth are for example nitrogen-fixing bacteria (e.g. Rhizobium, Bradyrhizobium, Frankia spp.).

Although most studied biocontrol microbes attack microbial pathogens, it is interesting to note that other biocontrol microbes act by attacking insects or weeds. Interest in biocontrol has gained momentum, partly because of the negative attitude towards chemical pesticides. This review will focus on the bacteria that can be used in biological protection against disease.

PLANT GROWTH - PROMOTING RHIZOBACTERIA (PGPR's)

Monoculture of crops can result in decrease of crop yield due to an increase of pathogens in soil. After several years of monoculture plant yield can increase again as a result of changes in the microbial population. It is said that disease-conducive soil changes to disease-suppressive soil. Microbial biocontrol agents can be isolated from plant roots present in the latter soil. They belong to the PGPR's. PGPR's often are fluorescent Pseudomonas spp. (e.g. Weller & Cook, 1983) but strains of Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Enterobacter, Erwinia, Flavobacterium, Serratia, Streptomyces and Xanthomonas have also been reported to promote plant growth.

MECHANISMS OF INDIRECT PLANT GROWTH STIMULATION

In order to protect the plant against a pathogen, a biocontrol agent usually must fullfil two criteria. Firstly, it must produce a protective factor that somehow selectively harms the pathogen, e.g. an antibiotic or cell wall-degrading enzyme. Secondly, it must be present at the right site in the rhizosphere at the right time to deliver optimal levels of the protective factor. Substantial knowledge has been collected, at least at the level of laboratory know-how, of the protective factors and their biosynthesis. However, knowledge of the molecular basis of rhizosphere colonization is virtually lacking as is know-how on spatial and temporal colonization strategies of pathogens and biocontrol agents.

PROTECTIVE FACTORS PRODUCED BY PGPR's

Antibiosis is the best-known strategy of PGPR's (Tomashow, 1991). The P. fluorescens strain Pf-5 produces pyrrolnitrin and protects cotton against a variety of fungi (Howell, 1990). Similarly, P.fluorescens 2-79 which protects wheat against Gaeumannomyces graminis var. tritici, produces phenazine-1-carboxylic acid (Gurusiddaiab *et al.*, 1986). P. fluorescens strain CHAO, which can protect tobacco against Thielaviopsis basicola, produces hydrogen cyanide (HCN), 2,4-diacetyl-phloroglucinol, monoacetyl-

phloroglucinol, pyoluteorin, salicylic acid (Défago *et al.*,1990) as well as indole-3-acetic acid (Oberhänsli *et al.*, 1991). Agrocin 84 is produced by Agrobacterium radiobacter var. radiobacter strain 84 which is applied to protect stone fruit and rose cuttings against A. tumefaciens (Ryder & Jones, 1990).

The ability of biocontrol strains to produce antibiotics does not necessarily mean that the antibiotic is involved in biocontrol. For example, work using mutants deficient in antibiotic production has in one case shown lack of evidence (Kraus & Loper, 1992), and in another case strong evidence (Tomashow & Weller, 1988), for a major role of the antibiotic in biocontrol. Interestingly, Laville *et al.* (1992) reported that a gacA mutant of P. fluorescens strain CHAO is impaired in the production of the two antibiotics 2,4 diacetylphloroglucinol and pyoluteorin as well as in the production of HCN. The authors provide evidence for a role of GacA protein as a global regulator of secondary metabolism whose expression is increased in the stationary phase.

Siderophores, i.e. compounds with an extremely high affinity for Fe^{3+}, are secreted by most bacteria upon sensing Fe^{3+}-deficiency. Fe^{3+}-concentrations in neutral and alkaline soil are so low that under those conditions soil micro-organisms are subject to Fe^{3+}-limitation. They can produce a range of different siderophores. The resulting siderophore-Fe^{3+} complexes are taken up through often specific receptors in the bacterial outer membrane (Neilands, 1982). Certain fluorescent biocontrol pseudomonads are very efficient in the competition for Fe^{3+}-ions, thereby further limiting the amount of Fe^{3+} available for the pathogen (Kloepper *et al.*, 1980; Geels & Schippers, 1983; Weller & Cook, 1983). The plant apparently can cope with this situation since it grows even better in the presence of the biocontrol bacterium.
The production of siderophores appeared to be a prerequisite for the biocontrol activity of these pseudomonads, since siderophore-negative mutants were unable to increase potato yields in contrast to the wildtype strain (Bakker *et al.*, 1986; Kloepper *et al.*, 1980).
Interestingly one of these plant growth-promoting strains, P. putida strain WCS358, possesses multiple siderophore-uptake systems which are induced by the presence of heterologous siderophores (Koster *et al.*, 1993) These multiple uptake systems enable this strain to utilize the siderophores of many other microorganisms which presumably is an advantage in the rhizosphere.

A role of volatile substances like ammonia and cyanide in biocontrol has been implicated. Voisard *et al.* (1989) provide evidence for the role of HCN in controlling black root rot of tomato by P.fluorescens strain CHAO. Circumstancial evidence for quite another role of cyanide, namely in inhibiting plant development by affecting root energy metabolism, has been proposed by Schippers *et al.* (1990).

Chitinase secreted by the soil bacterium Serratia marcescens plays a role inprotecting beans against certain fungi (Jones *et al.*, 1986; Ordentlich *et al.*, 1987).

Competition for niches is best-known from the use of non-pathogenic "ice-minus" mutants of P. syringae to protect plant leaves against the wild type bacterium which can cause frost damage (Lindow, 1990). Similarly, avirulent mutants of P. solanacearum are effective as antagonists for the control of bacterial wilt of tomato (Trigalet & Trigalet-Demery, 1990).

Competition for nutrients is considered as both a directly protective factor as well as a colonization factor. A non-pathogenic Fusarium oxysporum strain was able to reduce the occurence of Fusarium wilt in carnation. When P. putida strain WCS358 was combined with this non-pathogenic F. oxysporum strain, an increased suppression of the disease was observed. For this cooperative plant-disease control the production of siderophores by the P. putida strain was essential, since siderophore-negative mutants did not increase the effects of the F. oxysporum strain (Lemanceau *et al.*, 1992). The non-pathogenic F. oxysporum was shown to be less sensitive for competition for Fe^{3+} than the pathogen and is therefore supposed to more succesfully compete for carton with the weakened pathogen. (Lemanceau *et al.*, 1992)

Co-operation of (serveral) microbial biocontrol agents is supposed to be responsible for the prevention of particular soil-borne diseases in disease-suppressive soils (Schippers, 1992). Preinoculation of carnation roots with Pseudomonas fluorescens WCS 417r protects the plant against a vascular disease caused by F. oxysporum f. sp. dianthi wilt (van Peer *et al.*, 1991). The protection was related to accelerated and increased phytoalexin accumulation at the site of infection, which occurs only after infection with the pathogen. It was hypothesized that signals triggered by the Pseudomonas bacterium in the root system induce sensitization of defence responses against the fungus in the stem, including increased accumulation of phytoalexins. This protection seems to be a form of systemic acquired resistance, which can also be brought about in plants by exogenously applied chemicals like 2,6-dichloroisonicotinic acid and salicylic acid (Uknes *et al.*, 1992).

RHIZOSPHERE COLONIZATION

Colonization is of crucial importance to deliver the protective biocontrol factor at the right site in the rhizosphere at the right time. Colonization will be influenced by the root's surface components, the exudate composition, the local bacterial growth conditions in the various niches of the rhizosphere, the soil type, and various other biotic and abiotic factors. Our poor knowledge of colonization is directly related to our poor knowledge of these factors, both individually as well as in the way they mutually influence each other.

We have approached the question of which bacterial traits are important for colonization by genetic studies. In the first approach we tried to list putative bacterial colonization traits, e.g. mobility. We subsequently isolated mutants deficient in such a trait and compared their colonizing ability to that of the wild type strain. Using this approach we have shown that the presence of flagella, and perhaps mobility or even

chemotaxis (de Weger *et al.*, 1987), as well as the ability to produce O-antigen side chains of lipopolysaccharide (de Weger *et al.*, 1989) are crucial factors for colonization of potato roots by the tested Pseudomonas strains. Similarly, we intend to indentify quantitatively the major components of root exudates in order to test whether their utilization as a carbon source is involved in colonization. The second genetic approach assumes that we cannot predict all colonization traits. After screening random mutants for their colonizing ability, we subsequently plan to isolate colonization (col) genes. Nucleotide sequence analysis of these genes will presumably lead to novel traits involved in colonization.

BACTERIAL BIOCONTROL PRODUCTS AND THEIR FUTURE

Quite a few bacterial products for plant growth stimulation are commercially available (Kloepper, 1991; Lewis, 1991).
For seed companies it is interesting to sell their products with a coating containing one or more biocontrol strains, thereby using the know-how that the microbial population in a plant's rhizosphere can be determined by that present in the spermosphere (Lynch, 1990).
A limiting factor for the efficacy of the bacterial biocontrol products, as well as for the number of products, is our poor insight in the molecular basis of interactions in the spermosphere and in the rhizosphere. Often the bacterial factors which cause biocontrol are unknown. If they are known, knowledge of the effect of environmental factors on the levels of productive factor produced in situ are important. A description of spacial-temporal colonization patterns as given by Bahme and Schroth (1987) should be carried out for both biocontrol organisms as well as for pathogens under a range of relevant conditions. In this respect it is interesting to note that bacteria isolated from different sites along the wheat root differ in their physiological properties (Liljeroth *et al.*, 1991) Finally, mechanisms used for colonization are unknown, despite the fact that in several field trials colonization is the limiting step for biocontrol (Schippers *et al.*, 1987; Weller, 1988).

Whereas survival is hardly a problem with spore-formers, survival during coating on seed and during storage on the shelf is a problem when Gram-negative bacteria are used as biocontrol organisms. This problem may be overcome by increasing our knowledge of survival strategies of Gram-negative bacteria. Similarly, knowledge of bacterial physiology could be important to ensure a fast and correct response of the biocontrol strain upon germination of the seed.

The composition of root exudate is of prime importance for interactions between biocontrol strain, pathogen and resident microflora. Quantitatively the major exudate compounds are neutral sugars, organic acids and amino acids (Vancura, 1964). However, for many crop plants this knowledge, if available, is not public. Moveover, exudates can contain a number of other compounds that can have profound effects on interactions in the rhizosphere. Various flavonoids have been reported to activate the NodD protein as an early step in nodulation of leguminous plants by Rhizobium

bacteria (see chapters of Long *et al.*, Redmond *et al.*, Wijffelman *et al.* and Firmin *et al.* in Lugtenberg, 1989). Flavonoids have also been reported to increase the growth rate of specific soil bacteria (Hartwig *et al.*, 1991), to promote development of spores of the vesicular-arbuscular mycorrhizal fungus Glomus etunicatum (Tsai & Phillips, 1991), and to function as chemoattractants for zoo-spores of the pathogenic fungus Phytophtora sojae (Morris & Ward, 1992).
Microbial genes which have a function in the interaction with a plant are often induced by secreted plant components. Also biotic and antibiotic conditions proposed to be valid at the site of the interaction may stimulate the induction (e.g. Lugtenberg & de Maagd, 1991; Stachel *et al.*, 1985; Mantis & Winans, 1992; Schulte & Bonas, 1992). Minor exudate components which can play major roles in such interactions can be purified by using reporter genes behind the promoter of the induced gene (e.g. Zaat *et al.*, 1987).

Although some information is available about concentrations of various components in soil and in exudate, only local conditions in their particular niche are relevant for micro-organisms in the rhizosphere. In our group we have started to study local rhizosphere conditions with respect to phosphate-availability (de Weger *et al.*, in press) and amino acid availability (Simons *et al.*, in preparation). Another technique, which uses magnetic beads containing specific antibodies, provides the possibility of isolating cells of a specific bacterial strain from a majority of other bacteria (de Weger *et al.*, in preparation). This technique, therefore, seems to offer the possibility of introducing a specific bacterium (e.g. with an inducible promoter in front of a reporter gene), allowing it to grow in the rhizosphere, and subsequently reisolating and analysing specifically the cells of the introduced strain (e.g. after activation of the studied promoter in situ).

Increased know-how about rhizosphere events will allow us to construct genetically improved strains for biocontrol purposes. Indeed, Maurhofer *et al.* (1992) have shown that increased antibiotic production in P. fluorescens strain CHAO resulted in better protection of cucumber against Pythium ultimum. In the absence of a pathogen the increased antibiotic production had a deleterious effect on cress and sweet corn. The authors conclude that, depending on the host-pathogen system, enhanced antibiotic production by P. fluorescens may result in improved disease suppression or, in contrast, in a toxic effect on the plant.

As far as we know, resistance of pathogens towards protective factors has not been reported, but can certainly not be excluded. Therefore strategies for using microbial control agents should take this possibility into account. A combination of at least two protective factors with different mechanism of action can overcome the putative problem of resistance. Co-operation between several microbial biocontrol agents seems to be responsible for the efficiency of suppression of particular soil-borne diseases in natural disease-suppressive soils. Co-inoculation of biocontrol agents therefore may also improve protection against disease by seed bacterization (Schippers, 1992).

We agree that before application genetically modified strains will have to go through a risk assessment procedure. We disagree with certain environmental activists who oppose more or less on principle the application of genetically modified bacteria in the environment. Public acceptance is negatively influenced by their opposition and sometimes also their ignorance as well as by lack of public knowledge of these matters. In this respect it is important to note that Rhizobium an Bradyrhizobium inoculants have been used in massive amounts for almost a century without a single reported accident. Considering the poor sterilisation facilities in the earlier decades, it is likely that the inoculants not only contained the namedbacteria but also a broad range of other organisms. We see a role for scientists to educate the public on what is really going on and for companies to co-operate in an attempt to explain the balance of advantages and disadvantages of their future products.

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BIOLOGICAL SEED TREATMENTS - THE DEVELOPMENT PROCESS

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ABSTRACT

Biological seed treatments for plant pest and disease control have been the focus of significant research effort for the past 15 years, in the hope of replacing chemical seed treatments with biologically based products. Commercial products are beginning to reach the market place, but growth of the field has been slowed by formulation difficulties, limited understanding of rhizosphere ecology, poor reproducibility of field effects, and uncertainties over the size of the commercial opportunity, the strength of patents, and the cost of registration.

INTRODUCTION

Biological treatments may be applied to seed in order to introduce rhizobia or other beneficial symbionts, or to achieve biological control of pests or diseases. While production of legume symbionts is a well-established industry, it is only during the past 15 years that application of biological control agents to seed has received serious attention. The purpose of this paper is to discuss the potential for biocontrol agents and plant growth-promoting rhizobacteria (PGPR) applied to seed, the development process through which such products must pass before reaching the market place, and the effect of development costs on the growth prospects for seed-applied biocontrol agents.

Market for biopesticides

The market for biopesticides has grown considerably in recent years from negligible levels in the 1970s to at least $100 million today, and is expected to grow to $400-500 million by the end of the decade. This growth has been driven by environmental concerns, the high cost of re-registering existing chemical products, resistance, and reduced tolerance for chemical residues on food. In contrast, the crop protection industry as a whole, after several decades of growth, has begun to decline in real terms (Anon., 1993a). The growth prospects for biological products have prompted considerable interest in biopesticide research on the part of industry. The majority of this research is not directed towards seed treatment, however, since the vast majority of biocontrol products are bioinsecticides applied to foliage, water or soil. Products based on *Bacillus thuringiensis* accounted for 92% of biopesticide sales in 1990 (Anon., 1991), although nematode-based products are growing in importance.

Biological seed treatments

Although the research effort devoted to biological seed treatments has increased sharply in recent years, the concept is not new. Seed treatments with bacterial inoculants such as *Azotobacter chroococcum* and *Bacillus megaterium* has been practised for many decades in the former Soviet Union with the objective of enhancing crop growth and yield, while other organisms such

as *Pseudomonas* and *Streptomyces* species have been investigated as potential seed treatment additives (Cooper, 1959; Brown, 1974; Filippov, 1989).

Western interest in biological seed treatments has increased greatly during the past 20 years, beginning with the demonstration by Merriman *et al.* (1974) that applications of bacteria to seed resulted in increased plant growth in the field. Kloepper *et al.* (1980) demonstrated that such growth increases were associated with aggressive colonization of the rhizosphere by the applied bacteria, and it was subsequently demonstrated by Weller & Cook (1983) that a major disease, take-all of wheat, could be controlled by application of fluorescent pseudomonads to seed. Harman et al. (1980) demonstrated that such affects could also be obtained by application of fungi to seed. The past decade has been characterised by attempts to understand the ecology and mode of action of organisms with potential as seed treatments, and also to exploit such organisms industrially.

AVAILABLE PRODUCTS

Chemical products

A number of chemical products are applied to seed in order to control various pests and diseases. This method of application is increasing in popularity since it makes efficient use of active ingredient, is relatively inexpensive, avoids the need for specialised equipment where seed is treated off-farm, and minimises the volume of chemical applied per unit area. Although the range of active ingredients which can be applied by seed treatment is increasing, as is the range of pests and diseases which can be controlled in this way, seed treatment still represents a very small proportion of total agrochemical use. Some of the most important targets for seed treatment products, both registered and in development, are listed in Table 1.

Biological products

The majority of microbial seed treatment products currently available (Table 2) have been developed for control of soilborne damping-off diseases, caused mainly by *Pythium*, *Rhizoctonia*, and *Fusarium* species, or for stimulation of early plant growth. Biological seed treatments have made essentially no impact in the substantial market for cereal seed treatments, which are mostly used for control of seedborne pathogens, or on the market for seed-applied insecticides. A novel seed-applied biological insecticide is, however, being investigated by Crop Genetics International Inc., consisting of a strain of the endophyte *Clavibacter xyli* pv. *cynodontis* which expresses a Bt endotoxin. It is hoped eventually to introduce the bacterium into seed, possibly by vacuum infiltration, for control of European Corn Borer (*Ostrinia nubilalis*). The biological seed treatment products which are currently available, or in late stages of development, are listed in Table 2.

TABLE 1. Major pests and pathogens controlled by seed treatment

Crop	Pest or pathogen
Sugar beet, wheat, maize, vegetables, legumes	Soil pests, aphids
Cereals	*Tilletia caries* *Ustilago* spp. *Pyrenophora* spp. *Fusarium* spp. *Septoria* spp.
Cotton	*Pythium spp.*, *Rhizoctonia solani* *Xanthomonas campestris*
Vegetables	*Pythium spp.*, *Rhizoctonia solani*
Maize	*Pythium*, *Fusarium* spp.
Sugar beet	*Pythium*, *Aphanomyces* spp., *Phoma betae*
Potatoes	*Fusarium* spp., *Rhizoctonia solani*
Peas, beans, soya	*Pythium* spp., *Rhizoctonia solani* *Peronospora* spp.

TABLE 2. Biological seed treatment products

Trade name	Manufacturer	Active ingredient	Crops treated
Quantum 4000	Gustafson	*Bacillus subtilis*	Various
Kodiak	Gustafson	*Bacillus subtilis*	Cotton, peanuts, beans
Mycostop	Kemira	*Streptomyces griseoviridis*	Vegetables, ornamentals
F-Stop	Under negotiation	*Trichoderma harzianum*	Various
Blue Circle	Stine Microbial Products	*Pseudomonas cepacia*	Various
Nogall	Bio Care Technology	*Agrobacterium radiobacter*	Tree seedlings

STRAIN SELECTION STRATEGIES

Two basic strategies are available for selecting the strain which comprises the active ingredient in a biological product; the strain may be deliberately manipulated in order to confer the required characteristics or may be selected from the natural microflora according to the ability to fulfil various criteria. To date, only one of the commercially-available seed treatment products contains a strain which was genetically modified using recombinant DNA technology, the *Agrobacterium radiobacter* strain sold in Australia as Nogall, in which the transfer region has been deleted from the plasmid coding for agrocin production (Ryder, 1991). There are several reasons for this. Firstly, although considerable progress has been made in recent years in understanding the molecular basis of the interactions between biocontrol agents and plant pathogens, the ability to achieve significant improvements in strain performance through genetic manipulation has yet to be proved in the field. Achieving this is, in turn, hampered by the reluctance of regulatory authorities to permit release of genuinely "improved" microbial strains, although a number of genetically marked strains have been released into the environment for tracking studies. Given this situation, commercial interest in genetically engineered strains for seed treatment is likely to be limited, since the market opportunity is too small to allow the substantial research and development costs to be recouped. However, authorities in both Europe and the USA have sought to remedy this situation by funding basic research on ecology and genetic stability of recombinant microorganisms which should allow rational decisions on field release and registration to be made in future. Despite the present difficulty of developing products based on recombinant strains, other strain improvement strategies are possible, such as the protoplast fusion approach described by Harman (1991).

Selection of strains from nature presents a number of challenges, given the essentially infinite number of strains available in nature, and the multiple factors which must be taken into account during the screening process. These factors are summarised in Table 3. Strain selection is, in general, heavily dependent on biological efficacy data. Efficacy data may have been over-emphasised, since a number of promising strains have not progressed beyond the research phase due to difficulties with formulation, production, or inconsistency of effect across a range of environmental conditions.

In general, as the complexity of the screening process increases, the throughput (i.e. the number of strains which can be tested per unit time) decreases. The challenge in designing a screening cascade is to ensure that the assays reflect, as closely as possible, the situation which is encountered in the field, without compromising throughput unduly. The throughput which is required in order to select an effective strain depends on the strictness of the criteria which are imposed during the screening process (for example, biocontrol activity against more than one pathogen), and the abundance of the necessary traits in nature. It is not possible to calculate the necessary rate of throughput until a screening cascade has been running for some time. Screens may consist of mechanistic assays, in which strains are tested for some pre-defined trait, such as hyperparasitism, antibiotic or siderophore production, which is believed to contribute to the biological effect. An example is the *in vitro* screen used by Geels & Schippers (1983) to select *Pseudomonas* strains which counteract yield depression in potato. Non-mechanistic *in planta* assays, such as those developed by Kloepper (1991) incorporate plant-surface interactions, and do not require the mode of action to be defined at the outset. A rapid throughput can usually be achieved using

assays of this type, although at some cost in terms of the environmental factors which can be incorporated into the assay. The third approach which has been employed for selecting microbial strain is the use of microcosms or mesocosms, ecosystems which incorporate as many features as possible of the field situation. Microcosms are simulated ecosystems, constructed in the laboratory or glasshouse, for example those of Nelson & Craft (1992), while mesocosms consist of field plots, as described by Hagedorn *et al.* (1993). Our experience has been that *in planta* screens are preferable to *in vitro* assays, but that miniaturisation is required in order to screen an adequate number of strains (typically several thousand). This strategy was outlined by Renwick *et al.* (1990).

TABLE 3. Strain selection strategies for microbial seed treatments

Component of the screening cascade	Available strategies
Source of isolates	Seeds Random soil samples Suppressive soils Hyperparasites from pathogen biomass Isolation under selection pressure e.g. for rhizosphere competence
Nature of isolates	Random Genera selected for ease of production or formulation
Biological efficacy	Mechanistic *in vivo* screening Non-mechanistic *in planta* screening Screening in microcosms or mesocosms Spectrum of biocontrol activity
Ecology	Host specificity Colonisation of plant surfaces Effect of temperature, moisture, soil type
Secondary evaluation	Production parameters (growth rate, stability) Evaluation of strains in seed-applied formulation

PATENTING

Microorganisms, unlike chemicals, can easily be isolated and propagated by individuals other than the original producer. It is therefore even more essential to protect microbial products from unlicensed manufacture than is the case with chemicals. In the past, patenting of microbial strains has been a controversial issue, but this has now largely been resolved in the major industrialised nations (Anon, 1993b). The fact that a microbial product consists of a living organism is no longer considered to be a barrier to patentability. Large numbers of patents covering both microorganisms and microbial genes have now been granted in Europe, the USA and Japan. However,

as with any other patent, the criteria of novelty, non-obviousness and utility must be fulfilled. While the number of challenges to granted patents covering wild-type organisms has been limited, and while novelty and non-obviousness have become perhaps more difficult to prove as the prior art has expanded, there is no evidence to suggest that patents of this type are intrinsically weak. Protection of intellectual property has also been greatly strengthened by availability of DNA profiling and other diagnostic tools which allow precise identification of microbial strains.

PRODUCTION AND FORMULATION

Fortunately, the majority of bacterial agents can be produced by liquid (submerged) fermentation, which is the standard method for producing microbial biomass in Europe and North America. Although more technically difficult, it is also possible to produce fungal biomass, for example that of *Trichoderma*, in this way (Harman, 1991). Following production, sufficient numbers of viable propagules, usually in a stabilised form, must be applied to seed to achieve the required biological effect. This has clearly been achieved in the case of *Rhizobium* and related inoculants using peat-based and liquid inocula, but is proving more difficult where numbers of cells per seed is critical as with biocontrol agents. The storage stability requirements for biological agents used to treat seed vary widely. Seed is sown immediately after hopper box treatments, but may also be treated soon after harvest and stored until the following growing season. The technology available for formulation and application of biological products to seed has been discussed in a number of reviews (Connick, 1990, Harman, 1991; Lewis, 1991; Rhodes, 1993) and is summarised in Table 4.

TABLE 4. Formulation technology for application of biological agents to seed

Dry formulations	Aqueous fomulations	Coatings
Gum/talc powders	Sprays	Pellets
Dusts	Dips	Liquid
Dry spores	Fluid drilling gels	coatings
	Solid matrix priming	

REGISTRATION

Microbial products are regulated, not only according to pesticide regulations, but by regulations governing the handling and release of microorganisms. Although the cost of registering microbial products may still be considerably lower, in most countries, than that of chemical products, registration costs and delays are still a major factor in determining the commercial viability of products in development. It is necessary to interact with regulatory authorities at a number of points throughout the development process. Significant differences exist between countries; indeed most countries have not yet formulated specific guidelines for the registration of microbial products, but deal with microbial products on a case-by-case basis according to chemical legislation. Specific legislation covering microbial products is probably best advanced in the USA, under the guidelines produced

by Subdivision M of the Environmental Protection Agency. Specific legislation also applies in Canada and France, and will soon cover all the member states of the European Community as Directive 91/414/EEC Part B comes into force.

It is impossible, therefore, to describe a universal scheme for registration of microbial crop protection products. However, a typical example of the evidence required by regulatory authorities in the course of development of a microbial product is presented in Table 5. Requirements may be waived where the organism concerned is thoroughly documented in the scientific literature, or has a long history of safe use.

TABLE 5. Typical registration requirements for a biological control agent.

Development phase	Evidence required
Field release	Identity with indigenous organism Genetic stability Ecotoxicology
Registration	Identity and biological properties of the microorganism Description of the formulation Manufacturing process and product analysis Residues (frequently not required) Efficacy Toxicology, pathogenicity and infectivity Effects on non-target organisms Environmental fate

CONCLUSIONS

A large number of potential biological seed treatments have been described in the literature, while others are in the process of commercial development. Given the inherently limited commercial opportunity for such products, it is clear that not all will be commercially viable. Success will depend not only on possessing strains which are clearly differentiated from those of competitors, but on strong patents, formulation technology, and the ability to minimise delays during registration.

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THE SEED INDUSTRY'S VIEW ON BIOLOGICAL SEED TREATMENTS

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Abstract

Biological seed treatments are attractive because of the combination of their specific effect and limited environmental impact in comparison with agrochemicals or fertilizers. Inoculation of legume crops with rhizobia already is widely employed. Other plant growth-promoting microorganisms have been tested, as well as bacterial and fungal strains that deter a number of soil-borne diseases. The first products are on the market. Limitations for biological seed treatments do exist in the following fields: their efficacy, in an absolute sense against the disease or pest, and in comparison to resistance in the host or to chemical control measures, cost, and legislative restrictions on use. Biological seed treatments will find their place where an acceptable effectiveness, reliability and competitive pricing can be provided.

INTRODUCTION

Biological seed treatments have many potential uses, but currently only the first applications are emerging, both for growth/yield improvement and for control of soil-borne diseases. The use of microbial inocula has a longer history of practical use, with nitrogen-fixing bacteria employed in legume fields and mycorrhiza in tree nurseries being the prime examples.

Commercialization of nitrogen-fixing rhizobia already had begun last century after a U.S. patent on the use of pure cultures was issued to Nobbe and Hiltner in 1896. Inoculation of legume crops became widespread in the first quarter of this century. Currently inoculation is standard for legume crops such as soybeans and alfalfa grown under relatively low nitrogen levels and, as the application technology improves, new products also emerge (Smith, 1992).

Practical use of mycorrhiza is restricted to environmental extremes, such as nutrient deficiency (especially phosphorus) or prolonged drought. Under such conditions, vesicular-arbuscular mycorrhizae and ectomycorrhizae have been shown to benefit plants (Linderman, 1988).

Although biocontrol of insects employing *Bacillus thuringiensis*, baculoviruses or predators beautifully demonstrates the possibilities that exist for successful biocontrol practices, these successes are almost exclusively with foliar applications, and for that reason are not discussed here (Jutsum, 1988; Reinecke, 1990).

SEED TREATMENTS

Using the seed as a carrier can improve the efficacy of microbial inocula. Examples are biocontrol of *Pythium* and *Rhizoctonia* damping-off by several fungi and bacteria. Less obvious are the significant effects of seed treatments on diseases that affect the plant after its

seedling stage; for instance the biocontrol of *Aphanomyces* root rot of pea caused by *A. euteichis* f.sp. *pisi* (Parke et al., 1991), control of take-all in wheat (Thomashow & Weller, 1990), and also in our work with a fluorescent *Pseudomonas* isolate we repeatedly found that a seed treatment was more effective against *Fusarium* than a soil drench. Apparently the seed treatment positioned the inoculum where it could most effectively colonize the emerging root and therefore deter the pathogen.

Seed treatments have been attempted in various forms: commercial or semi-commercial are only simple dustings such as with a *Streptomyces griseoviridis* strain now marketed as Mycostop (Tahvonen, 1988; White et al., 1990), or with a *Bacillus subtilis* marketed as Kodiak and Quantum 4000. Much work has also been done on biocontrol with *Gliocladium virens* as the active ingredient, some of it on seed coatings (Howell, 1991), more on soil or substrate applications for which one product, Gliogard is now on the U.S. market (Fravel et al., 1985; Lumsden & Locke, 1989; Lynch et al, 1991). Incorporating a biocontrol agent in seed pellets was done for *Pythium oligandrum* (Lutchmeah & Cooke, 1985), but the same was also coated onto seeds (McQuilken, 1990). The most subtle seed treatments tested were based on combinations of priming (also known as osmoconditioning) of seeds in the presence of the biocontrol agent, which is thought to establish itself on the seed during the process (Harman et al., 1989; Callan et al. 1990). The examples of seed treatments outperforming soil or substrate applications are not rare, but apparently in some cases the amount of inoculum needed cannot be applied as a seed treatment. An example of this may be control of *Fusarium* by a non-pathogenic *Fusarium* isolate, which was shown to be effective if the antagonist could be applied at a much higher level than the pathogen (Alabouvette, 1990; Lemanceau & Alabouvette, 1991).

BIOLOGICAL SEED TREATMENTS: LIMITATIONS AND PROSPECTS

If very low inoculum densities in the soil cause serious crop losses already, or if relatively mobile organisms such as nematodes or insect larvae have to be controlled, the inoculum dose feasible with a seed treatment may be insufficient, although seedling protection still can be a valid reason for development of a such a product.

An example is white rot of onion, caused by the fungus *Sclerotium cepivorum*. After failure of an onion crop due to the pathogen hundreds of sclerotia can be present per kg of soil which will remain viable for many years. However, less than one sclerotium per kg soil already precludes growing onions. Any control has to be extremely effective to reduce the inoculum potential in the soil far enough to keep the infection level acceptable (Entwistle, 1990).

The efficacy of biological seed treatments in comparison with alternatives such as resistance in the host or chemical control is an important issue. The outcome of a comparison of the various options will depend on the individual crop/parasite combination.

Resistance to a pest or disease in the host plant is attractive because of its often absolute character. Obviously, sources of resistance have to be available before breeding for resistance becomes an option. The long time frame for a breeding success and therefore the high costs, the specificity of breeding for resistance (only the newly bred varieties carry the desired gene) and the negative correlation with yield can be arguments against resistance.

Novel seed treatments with fungicides and insecticides are being developed that combine improved efficacy with the use of lower amounts of the active ingredients. Large

reductions in the quantities of pesticides can be realized by employing such seed treatments. An example of this is the seed treatment we developed to control the cabbage root fly (*Delia radicum*) in cauliflower and Brussels sprouts. Use of seeds coated with chlorpyrifos reduced the use of insecticide by over 99% and combines efficacy, a very acceptable environmental impact, and safe use for the grower (Kosters et al., 1993).

Biological seed treatments will find their place where alternatives are not cost effective or not available.

The economics of biological seed treatments are most complicated. If a biological seed treatment is extremely crop, parasite and probably even environment specific, that minimizes the overall environmental impact of the 'biological'. However, such a specificity also restricts use so much that for a company it may be impossible to do the research needed to develop a practical application based on an already repeatedly demonstrated effect. Therefore, a certain generality will probably be needed; the few current commercial products such as Mycostop are indeed not extremely crop and pathogen specific.

A final limitation in the application of microbial inocula is in the legislative restrictions on use. Despite the low intrinsic risks in comparison with agrochemicals, government regulations are complex, quickly changing and very different from country to country. Standards on acceptable environmental impacts associated with the introduction of beneficial microorganisms are badly needed.

Outlined above are a number of limitations and opportunities that do exist for microbial seed treatments. Combinations of efficacy, economic constraints and legislative restrictions will determine the products that will be developed. If an acceptable efficacy, reliability and competitive pricing can be provided to the grower, a product will find its place in the market. Environmental benefits will convince both the grower and the consumer.

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RHIZOSPHERE CONSTRAINTS AFFECTING BIOCONTROL ORGANISMS APPLIED TO SEEDS

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ABSTRACT

The ecological constraints affecting success of seed-applied biocontrol agents of soil-borne pathogens are discussed and new approaches for overcoming them are reviewed. Both evidence and theory suggest that progress has been limited by the use of single clonal strains of biocontrol agents, which inevitably are ecologically constrained in competitive soil environments. They achieve transitory, localised dominance of the rhizosphere, and in only some soils and seasons, leading to inconsistency and poor efficacy of applied biocontrol, compared with natural biocontrol which is multifactorial. The more promising new approaches attempt to exploit the diversity of biocontrol agents by identifying ecotypes of individual control agents, for use singly or combined, and by combining different biocontrol agents for synergism or more persistent control.

INTRODUCTION

There is a truism in plant pathology: most plants are resistant to most pathogens; disease is the exception rather than the rule. It is equally true that biological control is omnipresent in natural and agricultural environments, contributing to the suppression of pathogens and moderation of disease. This natural biocontrol is particularly important in soil. However, it is complex and multifactorial. It operates not only by protection of infection courts but also through competition and antagonism in the soil-borne phase of pathogens; and it involves not just one biocontrol agent but several, against a background of other soil properties (physical, chemical) that favour biocontrol agents but limit pathogen activities.

Specific examples of this stable, multifactorial biocontrol are seen in suppressive soils. Suppression of the cyst nematode *Heterodera avenae* in cereal monoculture involves at least two fungi and requires suitable moisture conditions for one of them - the zoosporic fungus *Nematophthora gynophila* (Kerry *et al.*, 1980). Natural soil suppressiveness to fusarium wilts probably involves both non-pathogenic *Fusarium* spp. and fluorescent pseudomonads (e.g. Lemanceau & Alabouvette, 1991); also, it is associated with specific soil properties such as the type of clay constituents (Stotzky & Martin, 1963). Take-all decline or equivalent take-all suppression in cereal monoculture is a complex phenomenon (Hornby, 1979) even where fluorescent pseudomonads have been implicated as the primary biological cause (Weller, 1983). One crucial line of

evidence shows the multifactorial nature of biocontrol; we can seldom, if ever, reproduce it by introducing a single biocontrol agent into untreated, natural soil.

Most current research on biocontrol of soil-borne plant pathogens is far removed from these natural situations, being aimed at the development of single biocontrol agents - usually single clonal strains - for use as commercial inocula. It has succeeded in a few cases, notably with *Phlebia gigantea* for protection of pine stumps against *Heterobasidion annosum* and with strain K84 of *Agrobacterium radiobacter* for control of crown gall. But these successes can be attributed to special factors (Deacon, 1991). Usually the result has been inconsistent control across sites and seasons (Table 1) or early-season control that is not carried through to harvest (e.g. Geels & Schippers, 1983) or a degree of control sufficient only for low pathogen levels (e.g. Whipps *et al.*, 1993). So, biocontrol now has the reputation of being inefficient and inconsistent compared with control by chemicals or plant breeding (Becker & Schwinn, 1993).

BIOCONTROL BY INOCULATION: HOW SHOULD WE PROCEED?

It remains to be seen whether biocontrol of soil-borne pathogens with inoculant organisms must remain a restricted activity or will flourish. But it is clear that the approaches used of late have not produced the hoped-for success and so must be reappraised. It is also clear that this reappraisal must involve a return to first principles of microbial ecology, which tell us this: that individual organisms (and especially individual strains) can occupy only a restricted niche when in competition with a multitude of other organisms, and especially when those others are resident in a site and thus adapted to the site factors. The niche that an introduced organism can occupy is likely to be much narrower than the range of conditions that it can exploit in laboratory culture or even in experimental work in a glasshouse.

Should we expect a single clonal strain to protect all of the root system? No, it may flourish for a while by virtue of its high initial inoculum level, but will progressively be restricted to specific sites or microenvironments that suit it more than they suit any resident organism. Should we expect that strain to function in all soils, on all crops or in all seasons? No, for the same types of reason. And should we expect it to confer protection against a range of pathogens as it does in laboratory culture or in glasshouse trials? No, because each pathogen also has specific ecological attributes - a different site of infection, time of infection, or set of conditions in which it is active. The single clonal biocontrol strain cannot be expected to cover all these situations in a competitive environment where its own activities are constrained.

There is nothing inherently complex, or new, in these statements. They are obvious. But it has taken many years of failure for us to recognise them, and still the research literature (and attempted commercialisation) is dominated by single clonal strains. The rest of this paper is devoted to expansion of these points and discussion of how they are being, or could be, overcome. But

first it is necessary to consider (1) a special case in which single strains might prove effective and (2) a case that seems special at first sight but is not so.

Biocontrol in glasshouses

Glasshouses and similar protected cropping environments are highly artificial, so that many of the ecological constraints on biocontrol are removed. Control agents can be selected for activity in the specific environmental conditions of the crop. Moreover, in soil-less rooting media or pasteurised soil the biocontrol agent should experience little competition during its initial establishment. These highly favourable conditions may not persist through the life of the crop, but at least the control of seedling diseases or damage to cuttings should be achievable with relative ease. There are several impending products for these circumstances, including 'Mycostop' (*Streptomyces griseoviridis*; Mohammadi, 1992) and 'Gliogard' (*Gliocladium virens*). It is envisaged that *G. virens* will be added to rooting media and formulated in a prill with nutrients to support its production of antibiotics, and that cuttings of ornamentals will be introduced later to coincide with the high antibiotic levels (R.D. Lumsden, pers. comm.). Even so, there might be an additional benefit in using mixtures of strains because Howell *et al.* (1993) have identified two groups of strains of *G. virens* with different antibiotic profiles. One group produces an antibiotic active against *Pythium* but not *Rhizoctonia solani*; the other group has the opposite attributes.

Control of seedling diseases in field sites

Control of seedling diseases requires only short-term activity of a biocontrol agent and has been demonstrated repeatedly in glasshouse experiments with seed-applied inocula. Seed-derived nutrients often promote the activities of biocontrol agents so that it should be possible to achieve an overwhelmingly high, localized population of the control agent, sufficient to overcome any ecological constraints, even in field conditions. However, this apparently special case has two principal limitations in practice. First, the seedling pathogens - especially *Pythium* spp. - can initiate infection extremely quickly, before a seed-applied biocontrol agent can exert its effects. Second, the seedling pathogens are seldom confined to infection of the seed but also attack the young root and shoot tissues. Protection of these requires that the biocontrol agent spreads rapidly from its site of application, and this is often the stage at which biocontrol breaks down. G.E Harman and E.B. Nelson (this volume) discuss the future options for this form of control.

MICROSITES ON ROOTS

In this section it will be argued that roots and other plant surfaces provide many microsites (or microenvironments or niches) that favour specific microorganisms, that single biocontrol agents thus cannot give generalized protection, and that future work must focus on mixtures of compatible biocontrol agents to achieve effective crop protection.

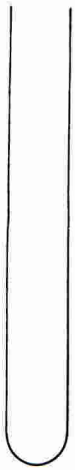
Application to seeds would be the favoured means of delivering biocontrol agents to the root zone for control of root diseases in field sites. Many laboratory, glasshouse and field experiments show that seed-applied microbes can spread down roots, either by carriage in percolating water (e.g. Bahme & Schroth, 1987; Lascaris & Deacon, 1991; U. Krauss, this volume) or in some cases perhaps by physical carriage by the root tip. Gammack *et al.* (1992) reviewed the subject. Some bacterial biocontrol agents, especially pseudomonads, can even maintain a partial or complete dominance of the microflora at the extending root tip. This ability to proliferate on roots in competitive conditions is termed rhizosphere competence (e.g. Ahmad & Baker, 1987). But the term is potentially misleading because it can obscure the fact that different organisms exploit different regions of roots - at least in competitive conditions. There are rhizosphere competences, not just rhizosphere competence.

The consequence is that spread of a seed-applied biocontrol agent down roots does not necessarily confer protection of the whole root system, only where the control agent can proliferate. Such localized proliferation is reflected in the fact that populations of individual microorganisms on plant surfaces typically show a lognormal distribution (Hirano *et al.*, 1982; Loper *et al.*, 1984), i.e. exponential growth where the conditions especially suit them but only poor growth elsewhere.

Weller (1983) reported on a specific case in which the spatial (and temporal) variation in population of a biocontrol agent influenced the efficiency of disease control. In field studies a take-all suppressive fluorescent pseudomonad initially grew well on the seedling roots of autumn-sown wheat, keeping pace with the rate of root tip extension. It also gave effective early-season control of take-all. But its population level decline markedly over winter, and in spring it was found mainly on the younger root regions, with only low levels on the more mature root regions. The roots subsequently developed take-all lesions in their older regions, and the bacterial strain was then found to proliferate on the lesions. The likely explanation is that this strain, like most rhizosphere pseudomonads, is specialized to exploit the relatively large amounts of simple soluble nutrients released from root tips or disease lesions but competes poorly for other types of nutrient elsewhere on the roots.

In terms of the distribution of microsites (niches) for microbial growth on roots, we know perhaps more for cereals and grasses (Deacon, 1987) than for any other type of plant. Fig. 1 depicts a cereal root in a simplified way, considering only the major sources of root-derived nutrients and not other features such as differences in root surface properties or physico-chemical factors that can influence microbial activities.

Figure 1. Simplified succession of zones for microbial growth along cereal roots, relating to the supply of root - derived nutrients.



4. Zone of secondary invaders, including mycoparasites, that exploit tissues occupied by primary invaders.

3. Zone of progressive, natural root cortical senescence, favouring weak parasites that exploit incipiently senescing cells.

2. Progressive limitation of simple, soluble nutrients as bacterial population reaches carrying capacity. Deleterious microorganisms are now favoured.

1. Root tip; abundant supply of soluble nutrients, including amino acids, favouring pseudomonads.

Even from such a simplified picture we see that there is a progression of zones that selectively favour particular types of microbe along the root. The root tip and youngest root region favour at least some types of pseudomonad, which multiply rapidly in a nutrient pool. Behind this zone the microbial population level stabilizes because it uses all the continuing supply of soluble nutrients just to maintain itself (Newman & Watson, 1977). Deleterious microorganisms will be favoured in this zone because only they can gain access to further nutrients, by damaging the plant cells in their vicinity. These organisms will include those that invade the tissues, but also those that grow on the root surface and produce phytotoxic metabolites such as hydrogen cyanide (e.g. Bakker & Schippers, 1987). Further back, the cortex senesces progressively - a natural, programmed phenomenon. This favours weakly parasitic fungi that exploit incipiently senescing cells and that are known to control take-all in glasshouse conditions. They include *Phialophora graminicola* (principally in grass turf) and *Idriella bolleyi* which is common on cereals in agricultural field conditions. Still further back, the conditions become favourable for secondary invaders, including mycoparasites. The mycoparasites are of different types, with different modes of action (Deacon & Berry, 1992), but have one feature in common and it may be more important than the parasitism of other fungi *per se*: the ability to invade substrata that have already been colonized by other fungi. This seems to be a rare ability among fungi in general, and it forms the basis of a selective isolation technique for presumptive mycoparasites: soil or root pieces (preferably air-dried) are placed directly on agar precolonized by other fungi. There is even evidence of fungus-specific effects - at least *in vitro* - because the type of mycoparasite that grows on the agar plates depends on the fungus used as the primary colonizer (Mulligan & Deacon, 1992). The implication is that zone 4 (Fig. 1) might select for different secondary colonizers, depending on the microbes that became

established in the earlier zones - a further dimension to the microsites along roots.

Microsite-based biocontrol

Understanding of the ecology of the rhizosphere - even at the rather gross level represented by zones of activity in Fig. 1 - should enable us to improve the prospects for biocontrol. It is notable that different potential biocontrol agents are favoured by the different zones on cereal roots: pseudomonads near the tip (or on take-all lesions), *Phialophora* and *Idriella* further back, and mycoparasites still further back. Even the deleterious agents of zone 2 (Fig. 1) might function as biocontrol agents if they induce host resistance to more aggressive pathogens (reviewed by Kuc & Strobel ,1992, and an example in agricultural practice was described by Komada , 1990). This zonation of biocontrol agents might explain why natural take-all suppression is relatively stable and long-lasting. An obvious future approach would be to explore the use of mixtures of inoculant organisms, selected for occupation of the different niches. For example, provided that they are compatible as seed-applied inocula, we could expect a combination of a pseudomonad and *Idriella bolleyi* to give better and more durable protection against take-all than would either alone. They would distribute themselves naturally on the developing roots according to their niches.

This kind of approach has already been reported for control of fusarium wilt diseases, where combinations of fluorescent pseudomonads and non-pathogenic fusaria were found to be highly successful in experimental conditions, even when the pseudomonads did not show biccontrol activity alone (e.g. Park *et al.*, 1988; Lemanceau & Alabouvette, 1991).

SOIL AND SEASONAL FACTORS IN BIOCONTROL

Becker & Schwinn (1993) pointed to the 'actual or perceived' inconsistency of biocontrol as a major factor that limits the acceptance of this technology. To overcome it they advocated technical solutions such as better quality control for inoculum preparations and attention to the genetic stability of biocontrol strains. These are important considerations, but are not the solution. Inconsistency is inevitable if single clonal biocontrol strains are expected to compete with the panoply of indigenous microbes in different sites and seasons. This is illustrated in Table 1, with data from the field trials of Suslow & Schroth (1982) on plant growth-promoting bacteria, which act at least partly by controlling soil-borne pathogens. The strains in these trials were the best of numerous bacteria screened in glasshouse and laboratory tests and they were carefully maintained. Yet none gave a consistently significant growth response across a range of sites and (or) growing seasons. Cook (1992) described similar variations in control of take-all by selected fluorescent pseudomonads in north-western U.S.A. Soil factors seemed to be the important variables in this case.

The solution that Cook and his colleagues are exploring is a 'customised' approach, in which fluorescent pseudomonads for take-all control are isolated from soils/regions where they are intended for use as inoculants. As an extension of this approach in general, it could be useful to identify strains that perform well in seasons where others have failed (e.g. from studies such as those in Table 1). Then it might be possible to develop mixed-strain inocula that give consistent control across sites and seasons. Schroth & Becker (1991) identified the use of strain mixtures as an important area for future research. It will require the answers to many questions. Would a mixture of seed-applied strains be mutually cancelling so that none proliferates enough to give control? How rapidly would one strain be selected from the mixture according to environmental conditions? And how many strains would be necessary in order to cover most eventualities?

TABLE 1. Per cent increase in yield of sucrose from sugar beet crops when seeds were treated with individual plant growth-promoting *Pseudomonas* strains and grown in different field sites in California (CA) or Idaho; data abstracted from Suslow & Schroth (1982)

Bacterial strain+	Field location and year of trial					
	Westside, CA, 1977	Davis, CA 1977	Idaho 1977	Davis, CA 1978	Imperial,CA 1979	Tracy, CA 1979
B4	-5	5	12*	10*	10	nd
SH5	15*	nd	0	13*	19	8
RV3	nd	16*	12*	10*	8	-13
A1	nd	nd	nd	12*	nd	5

+ All strains were isolated from sugar beet roots. * Significant yield increase compared with untreated control. nd, no data.

HOW MUCH DIVERSITY HAS GONE UNNOTICED IN BIOCONTROL AGENTS?

The questions in the section above take us into largely uncharted territories of biocontrol: we have little knowledge of the ecological variation that exists between strains of biocontrol agents. Meanwhile, population geneticists have made quite rapid progress in charting the variation within plant pathogens, with evidence that new, host-adapted species of *Phytophthora*, for example, have arisen within the relatively recent period of developed agriculture in North America (Hansen, 1989). For successful biocontrol we need as much information about our friends as about our enemies.

There is preliminary evidence that rhizosphere bacteria (though not necessarily biocontrol strains) can be 'host-adapted' and that this is partly

associated with their ability to be agglutinated by root-surface polysaccharide (e.g. Chao *et al.*, 1988; but see Glandorf, 1992). This could be important in the selection of effective biocontrol strains (or mixtures) of either broad or narrow host range. For leaf surface (phylloplane) bacteria there is evidence of inter-strain differences in microsite selection (resource utilisation patterns). Lindow (1992) reviewed this in relation to biocontrol of ice-nucleation active bacteria. These bacteria, such as *Pseudomonas syringae*, have a surface protein that acts as a nucleus for ice crystal formation, leading to frost damage of the leaves. They can be controlled by mutants (Ice^-) that lack the protein, and the basis of control is simple competitive exclusion: if the Ice^- mutants are applied early then they can use the leaf surface resources and exclude the natural Ice^+ population. But Lindow and his colleagues found that, in experimental conditions, the best control was obtained by Ice^- mutants derived from individual Ice^+ strains: the progeny were better at controlling their parents than at controlling non-parental strains, even though there was no apparent difference between the wild-type strains. Thus it was suggested, first that wild-type populations of *P. syringae* consist of a mixture of strains with different resource utilisation patterns and, second, that the Ice^- mutants retain these patterns so that they can better compete with their parents. The question (Lindow, 1992) is: how many such patterns are there in a wild-type population, and how many Ice^- strains might be needed in an inoculum mixture to give the best biocontrol? Identical questions face us in the rhizosphere.

Recent work in this laboratory suggests that there is substantial inter-strain variation within *Idriella bolleyi*. This fungus is common on both roots and stem bases of cereals, and is implicated in control of several cereal pathogens including take-all (Kirk & Deacon, 1987a), eyespot (Reinecke & Fokkema, 1981), *Fusarium* spp. (Reinecke *et al.*, 1979) and *Pythium graminicola* (Waller, 1979). Such a broad range of activities might be expected to be associated with ecologically different strains, especially as the basis of control seems to be competitive exclusion (Kirk & Deacon, 1987b). With this in mind, L. I. Douglas (unpubl.) has compared the tolerance of different strains to water stress *in vitro*, because this correlates with the conditions in which different cereal root-rot and foot-rot pathogens occur in field sites (Cook & Baker, 1983). Of three strains of *I. bolleyi* taken at random from a culture collection, one showed low stress-tolerance, equivalent to that of the take-all fungus; one showed extreme stress-tolerance like that of *Fusarium culmorum*, and one was intermediate in behaviour. These differences were evident on osmotically (KCl) adjusted agar and in liquid media supplemented with polyethylene glycol, which exerts a matric water stress. They were also evident when assessed by different criteria - spore germination, mycelial growth or ability to sporulate.

These *in vitro* findings must be extended to soil conditions, and to comparisons of strains isolated from cereal roots as opposed to stem bases in field sites. But the variation seen to date is astonishing because it spans almost the whole range of *in vitro* water-stress responses of the pathogens that *I. bolleyi* might be used to control. Equivalent work is needed on the potential ecotypic variation in other biocontrol agents.

CONCLUSION

As we look back over the history of biocontrol of plant disease (Garrett, 1965) and especially the more recent history (Deacon, 1991) there is a clear dichotomy. On the one hand, real progress has been made in understanding the processes and mechanisms that limit pathogen activities in nature. And the focused application of molecular tools and mathematical modelling (e.g. Lindow, 1992) will yield even more rapid progress in this area. On the other hand, the achievements in applying this understanding to practical cropping systems have been modest. In fact, we have moved backwards because the few notable successes in the use of microbial inocula for biocontrol do not compensate for the abandonment of traditional cropping practices that fostered natural biocontrol.

The approaches advocated here could provide the bridge between natural and inoculant-based biocontrol. It is the responsibility of the research community to build this bridge and to ask the relevant questions. For example, how many types of control agent would be needed for effective control of a pathogen, and how many strains of each would ensure that this control is consistent across sites and seasons. This information could then provide the basis for rational decisions by companies that are interested in producing biocontrol inocula. Of course, single control agents, and single strains, would be preferred, especially under the current regulatory conditions. But there should be no inherent barrier to the development of mixed inocula, because these are already available in commercial silage additives. The overriding criterion must be efficacy in practice.

Truman Capote is said to have recommended taking an instant dislike to Simone de Beauvoir because 'it saves time'. For the same reason perhaps we should take an instant dislike to single clonal biocontrol agents.

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INCORPORATION OF MICROENCAPSULATED BENEFICIAL ORGANISMS INTO ENVIRONMENTALLY ACCEPTABLE SEED COATINGS TO ENHANCE CROP PERFORMANCE IN SOYBEANS

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ABSTRACT

The effectiveness of microencapsulation technology to deliver beneficial organisms on seeds was evaluated using *Rhizobia/Glycine* as a model system. The survival of microencapsulated *Rhizobia* applied to the seed was evaluated by standard bacteriological techniques and by nodulation tests. The effects of coating material on seed quality and *Rhizobia* survival was also determined. A total of 8 seed lots with various coating material combinations were evaluated. A multi-coating delivery system was developed for microencapsulation which included both fluidized-bed and pan coating processes. Both warm and cold germination test results indicate no significant effects on germination due to the microencapsulation. Microencapsulated seed lots showed a significant amount of live *Rhizobia* on the seed at 7 and 30 days after microencapsulation. This result was further verified by the nodulation test in the greenhouse. These results indicate that the 'sandwich' seed microencapsulation technology provides a suitable environment for survival of *Rhizobia* on seeds which can then be stored and handled safely.

INTRODUCTION

Microencapsulation technology had its origin in the initial work of Barry Green of the National Cash Register Company who developed the first process of microencapsulation by coacervation, Green 1956. This process provided the world with the first carbon-less copying paper, and, more importantly, described a technology which would have far reaching applications beyond the initial objectives in office systems. Green's successful use of microencapsulation stimulated researchers to adapt microencapsulation techniques to other areas such as agriculture, advertising, pharmaceuticals and to other industries. Seed is a suitable and effective delivery system for many biotechnically altered organisms designed to enhance crop performance or replace pesticides. However, survival of these organisms on the seed is often very low. Microencapsulation of these organisms in a suitable buffered substrate may increase their survival. In 1978, Lim patented a process by which viable cells, tissues, and other labile biological substances could be microencapsulated within a semipermeable membrane (Lim 1984). To date, however, this technology has not been applied to living organisms

which are routinely or appropriately applied to seed. These microcapsules could then be incorporated into biodegradable polymeric seed coatings which are being developed, to provide an environmentally safe alternative to fungicide seed treatment of agricultural crops. To assure successful microencapsulation of living cells and/or

tissues, several conditions are necessary including the selection of coating materials and conditions which are totally nontoxic to living organisms (Lim 1984). For seed microencapsulation, an additional concern relates to the effect of the coating on seed performance. The current research attempts to develop the technology to incorporate beneficial organisms into environmentally acceptable seed coatings to enhance crop performance. *Rhizobial* bacteria are an important component of soybean production and this technique was used as a model system.

MATERIALS AND METHODS

Soybean seed (*Glycine max* L.) variety Wells provided by the Committee for Agricultural Development, Iowa State University, and *Rhizobia japonicium* carried in peat (manufactured by Titre Inc., Ryegate, MT) was used for seed microencapsulation. *Rhizobia* was applied by Pharmaceutical Services (UI) in a fluidized bed and by the Seed Science Center (ISU) using both fluidized bed and pan coating equipment. Three biodegradable polymeric coating materials, Sepiret (Seppic, Paris, France), Polyvinylpyrrolidone (PVP) and Aquacoat (FMC Pharmaceuticals, Philadelphia,PA), were used. The formulations are listed on Table 1.

TABLE 1. Coating materials evaluated as delivery systems for application of *Rhizobium j.* to Soybean *Glycine max.* L

10% Sepiret + *Rhizobia*/Peat + 5% PVP, applied by ISU
15% Sepiret + *Rhizobia*/Peat + 5% PVP, applied by ISU
20% Sepiret + *Rhizobia*, applied by UI
5% Polyvinylpyrrolidone + Talc + *Rhizobia*,applied by UI
7% Polyvinylpyrrolidone + Talc + *Rhizobia*, applied by UI
10% Polyvinylpyrrolidone + Talc + *Rhizobia*, applied by UI
5% Aquacoat + *Rhizobia*, Cured 48hrs at 40C, applied by UI
5% Aquacoat + *Rhizobia*, Not cured, applied by UI

Coatings were applied using both conventional pan and fluidized bed coating equipment designed and constructed by ISU for laboratory research and having a coating capacity of upto 500 gm of seed. Seeds were initially coated with 5% PVP (% seed weight) as a base coat, and then followed with a peat-based *Rhizobia* coat evenly over the PVP base coat. The *Rhizobia* coat was then coated with a 10 to 15% (% seed weight) coat of Sepiret to form a sandwich coating(Figure 1). The effectiveness of *Rhizobia* on microencapsulated seed was evaluated either 3 days after coating or stored at room

temperature for later evaluation. Sixty seeds were soaked in 50 ml of autoclaved water for 24 hours with shaking. A yeast mannitol agar medium was used to count the viable colonies of *Rhizobia* (Vincent, 1970). *Rhizobium* colonies were characterized by morphological, staining and cultural tests, and finally verified by the invasiveness test, i.e. nodulation of soybean under bacteriologically controlled conditions. All steps and equipment were under bacteriological control. Soybean were grown in a lighted and temperature controlled greenhouse. Seeds were planted in autoclaved sand. Root nodules were then counted at 21, 28, and 35 days after planting to evaluate the effectiveness of the microencapsulation. The standard warm germination and a sand/soil (4:1) cold test were conducted. Warm germination was evaluated after 7 days at 25°C, and the cold

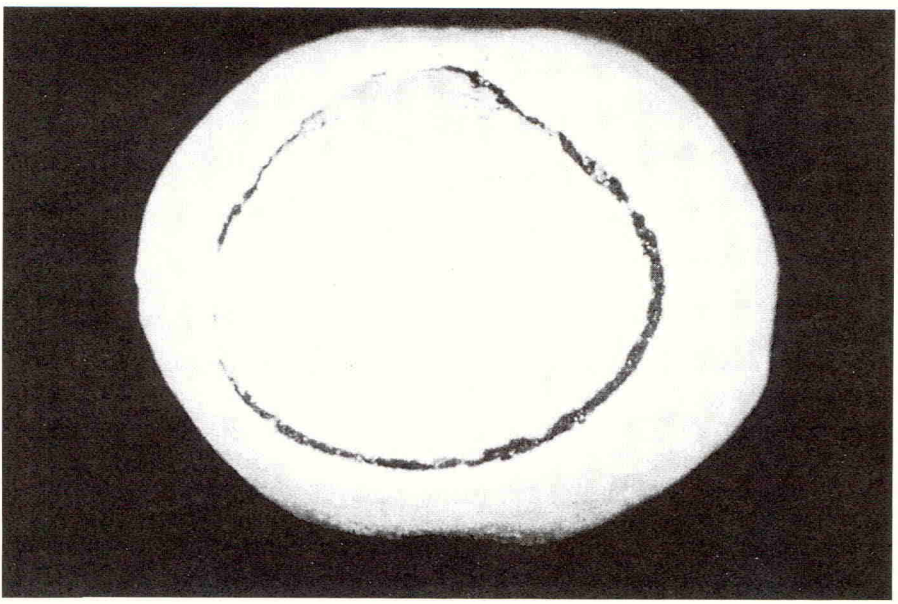

Figure 1. Soybean seed microencapsulated by the ISU technique showing the "sandwich" structure.

germination test was evaluated after 14 days, the first 7 days at 10°C, followed by 7 days at 25°C. The AOSA (Association of Official Seed Analysts) seed testing rules were used as a guide for seedling interpretation (Anon. 1986).

RESULTS AND DISCUSSION

The effectiveness of seed microencapsulated *Rhizobia* was evaluated by a bacterial plate count test after 7 and 30 days of storage at room temperature (Table 2).

TABLE 2. Viable number of *Rhizobium* cells on microencapsulated soybean seed

Coating Materials	Rhizobia Carrier	Method	Days After Microencapsulation	
			7	30
			MPN (Rhizobia/seed)	
10% Sepiret	Peat	ISU	9.96×10^6	6.97×10^6
15% Sepiret	Peat	ISU	9.25×10^6	8.95×10^6
20% Sepiret	Polymer	UI	0	0
5% PVP	Polymer	UI	0	0
7% PVP	Polymer	UI	0	0
10% PVP	Polymer	UI	0	0
Aquacoat-C	Polymer	UI	0	0
Aquacoat-NC	Polymer	UI	0	0
Untreated		UI	0	0

Two seed lots microencapsulated by ISU showed 9.96×10^6 and 6.97×10^6 live *Rhizobia* per seed for the 10% Sepiret coating, and 9.25×10^6 and 8.95×10^6 for 15% Sepiret coating after 7 and 30 days storage, respectively. No live *Rhizobia* were found on the 6 lots of microencapsulated by UI or on untreated seeds. The bacteriologically controlled nodulation test (Table 3) further confirmed the plate count results. Peat-base *Rhizobia* microencapsulated with 10 and 15% Sepiret began to form nodules at about 3 weeks after planting and the nodule number per plant was similar to peat-base *Rhizobia* inoculated on the seed immediately before planting. Storage of microencapsulated seeds at room temperature for 30 days result in slight decreases in nodule number (Table 3). The live *Rhizobia* number by plate count tests also exhibited a slight decline (Table 2). However, both live *Rhizobia* number on seed and nodule number per plant of 7 day-old peat-base *Rhizobia* inoculated seed were much less than that of 30 day-old microencapsulated seed. These results indicate that the 'sandwich' microencapsulation technology employed by ISU provided a suitable microenvironment for the survival of *Rhizobia* on seed. In this technology, *Rhizobia* were buffered against the stress of the coating conditions by the peat carrier. Preliminary storage studies showed that the 'sandwich' microencapsulated seeds can be stored and handled safely (Table 2, 3). When the coating polymer was directly used as the *Rhizobia* carrier, the bacteria did not survive the coating conditions. In addition, the coating materials also may directly effect the survival of the *Rhizobia*.

TABLE 3. Nodulation of microencapsulated soybean with *Rhizobium japonicum*

Storage Days	Coating Materials	*Rhizobia* Carrier	Method	Days After Planting 21	28	35
				Nodules / Plant		
3	10% Sepiret	Peat	ISU	9	14	13
3	15% Sepiret	Peat	ISU	0	21	24
7	20% Sepiret	Polymer	UI	0	0	0
7	5% PVP	Polymer	UI	0	0	0
7	7% PVP	Polymer	UI	0	0	0
7	10% PVP	Polymer	UI	0	0	0
7	Aquacoat-C	Polymer	UI	0	0	0
7	Aquacoat-NC	Polymer	UI	0	0	0
0		Peat	Inoculate	10	17	19
0	Untreated			0	0	0
30	10% Sepiret	Peat	ISU	4	9	8
30	15% Sepiret	Peat	ISU	5	15	20
30	20% Sepiret	Polymer	UI	0	0	0
30	5% PVP	Polymer	UI	0	0	0
30	7% PVP	Polymer	UI	0	0	0
30	10% PVP	Polymer	UI	0	0	0
30	Aquacoat-C	Polymer	UI	0	0	0
30	Aquacoat-NC	Polymer	UI	0	0	0
30	Untreated			0	0	0
7		Peat	Inoculate	2	5	7

The interaction of two living organisms, seed and microorganisms, make seed microencapsulation complicated. Therefore, the effects of coating materials on seed and seedling performance were determined (Table 4). Both warm and cold germination results indicate no significant effect on germination by microencapsulation as compared to control seed, except for the cold germination of the 10% PVP coated seed. In fact, microencapsulated seed exhibited an increased germination in most cases. Aquacoat coated seed exhibited significantly higher incidence of abnormal seedling in the warm germination test, however, it also exhibited higher seed vigor in the cold test. Aquacoat coated seed also appear to be free of bacteria in the *Rhizobium* plate count test. This coating material may have some toxic effect on both seed and microorganisms.

CONCLUSIONS

These results indicate the need for caution when coating materials are used directly as microorganism carriers. The use of a safe carrier as a buffered medium for microorganism could be a key factor in the success of microencapsulation to incorporate the beneficial microorganisms into seed coatings. The application of the microencapsulation technology to *Rhizobia/Glycine* system will greatly improve the soybean seed inoculation with superior *Rhizobia* and the microencapsulated seed can be handled safely. Once this technology is developed it should be applicable to other microorganisms and other seed types.

TABLE 4. The effect of microencapsulation materials on seed and seedling performance.

Coating Material	*Rhizobia* Carrier	Method	Warm Nor.	Warm Abnor	Cold Nor.	Cold Abnor.
				Germination %		
10% Sepiret	Peat	ISU	82.3	12.0	41.5	13.3
15% Sepiret	Peat	ISU	83.3	13.0	45.3	12.0
20% Sepiret	Polymer	UI	84.3	9.8	50.5	6.3
5% PVP	Polymer	UI	87.0	9.8	59.8	7.0
7% PVP	Polymer	UI	86.7	8.8	50.5	6.3
10% PVP	Polymer	UI	83.8	9.5	13.8	3.8
Aquacoat-C	Polymer	UI	8.8	90.3	84.3	2.5
Aquacoat-NC	Polymer	UI	13.8	84.3	83.0	2.8
Untreated			75.3	15.5	36.3	14.5

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TESTS FOR BIOLOGICAL CONTROL OF SEED AND SEEDLING DAMPING-OFF DISEASES OF PEAS AND BEANS USING *BACILLUS* SPECIES.

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ABSTRACT

Potential bacterial antagonists to the damping-off fungi *Pythium mamillatum* and *Botrytis cinerea* were isolated from the testae and cotyledons of peas and dwarf French beans. The isolates were screened for antagonism by *in vitro* dual culture analysis of pathogen against potential antagonist. Five *Bacillus* isolates showed distinct antagonism to the two pathogens *in vitro* and were selected for further screening as seed treatments together with *Bacillus brevis* strain Nagano and a *Bacillus licheniformis* isolate previously shown to be antagonistic to *B. cinerea*. When applied as seed treatments to peas and beans sown on agar inoculated with the pathogens, six of these antagonists reduced *Pythium* and *Botrytis* infection levels compared to controls. When pea seeds were sown in infested compost a reduction of *Pythium* infection levels was again observed with these seed treatments.

INTRODUCTION

Damping-off diseases cause considerable yield losses in many important agricultural crops worldwide. These losses may occur before seed germination or seedling emergence (pre-emergence damping-off) or the juvenile tissues of older seedlings may be attacked producing brown, watery lesions and eventual tissue collapse (post-emergence damping-off). A number of soilborne pathogenic fungi have been implicated in damping-off diseases including species of *Botrytis* and *Pythium* (Lucas et al, 1985).

Elimination of soilborne fungi by control measures including chemicals is unrealistic and only limited success has been achieved with the use of single control measures to reduce crop disease caused by such pathogens (Martin et al). Therefore control of these pathogens by the utilisation of integrated strategies including biological control should be considered in order to reduce the general overuse of fungicides in the environment (Rishbeth, 1988; Nelson, 1989). The use of microbial antagonists already present in the spermosphere as biocontrol agents applied as seed treatments against these fungi is one possible option. Antagonists must remain viable and withstand desiccation, heat treatment, formulation and field application (possibly in association with a reduced level of fungicide). Bacterial spores are considered to be ideal candidates for such applications (Roberts & Hitchins, 1969; Rhodes, 1990).

Screening for antagonism *in vitro*

Five *Bacillus* isolates showed distinct antagonism to the two pathogens *in vitro* on all three types of media tested. *B. brevis* strain Nagano and *B. licheniformis* showed antagonism to *B. cinerea* but limited antagonism to *P. mamillatum*. The results are presented in Table 1.

TABLE 1 Antagonism of isolates to *Botrytis cinerea* and *Pythium mamillatum in vitro*

Isolate	*Botrytis cinerea*			*Pythium mamillatum*		
	NA	MEA	PDA	NA	MEA	PDA
B3	Z1	7	11	*	*	3
C1	Z1	9	9	5	*	*
D4	Z1	6	*	10	4	5
J7	Z1	4	*	3	*	*
M10	Z1	5	1	10	3	6
Bacillus brevis (strain Nagano)	Z1	-	*	Z1	-	-
Bacillus licheniformis	Z1	*	*	Z1	*	-

Figures represent zone of inhibition in mm.
Z1, zone of inhibition present (unmeasured);
*, some antagonism cf control; -, no antagonism.

Screening for antagonism *in vivo*

Pythium infection levels were reduced most effectively on white dwarf French beans and pea lot 1 where six out of the seven potential BCA's were effective on both seed types. Only *B. brevis* and *B. licheniformis* were effective on black dwarf French beans and only *B. brevis* and treatments C1 and D4 were effective on pea lot 2 (Table 2). *Botrytis* infection levels were reduced by all treatments on all seed types except on black dwarf French beans which appeared to be very susceptible to *B. cinerea* (Table 2).

TABLE 2. Effect of *Bacillus* seed treatments on infection levels of damping-off fungi in peas and beans sown on agar

Pathogen	Seed Type	*Bacillus* seed treatments B3	C1	D4	J7	M10	*Bacillus brevis*	*Bacillus licheni-formis*
Botrytis cinerea	DFB	0	0	0	0	0	0	0
	DFW	3	3	3	3	2	3	3
	P1	3	2	3	3	2	2	2
	P2	3	3	2	3	1	3	3
Pythium mamillatum	DFB	0	0	0	0	0	1	1
	DFW	3	3	3	0	3	2	3
	P1	1	2	2	1	1	0	2
	P2	0	2	2	0	0	2	0

Figures represent number of seeds protected from infection cf controls.
0, No reduction of infection compared to controls; DFB, black dwarf French bean; DFW, white dwarf French bean; P1, pea lot 1; P2, pea lot 2.

Six of the isolates tested consistently reduced pre-emergence infection by *Pythium* in infested compost (Figure 1). However this work needs to be repeated on a greater number of replicate seeds to produce statistical comparisons.

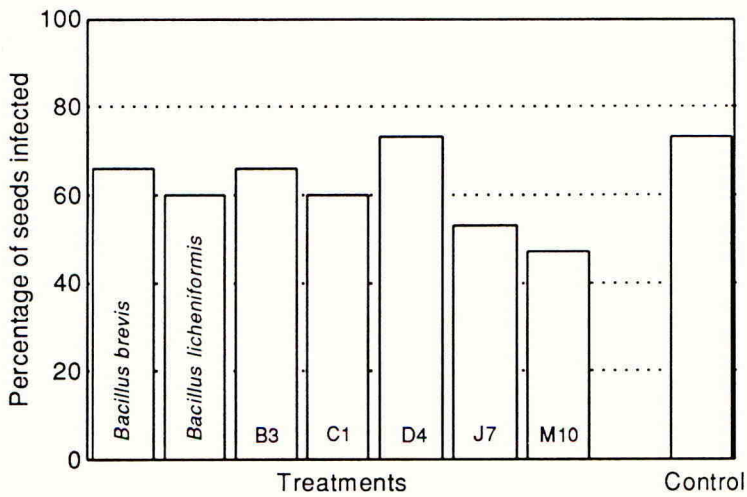

Figure 1. Effect of *Bacillus* seed treatments on *Pythium* infection of peas sown in infested compost.

DISCUSSION

Ninety two potential antagonists were isolated from the testae of peas and dwarf French beans. Five isolates of *Bacillus* showed distinct antagonism to both *B. cinerea* and *P. mamillatum in vitro* on three types of media. These five isolates together with *Bacillus licheniformis* and *Bacillus brevis* strain Nagano reduced *Botrytis* infection levels on peas and white French beans sown on agar. Six of the seven isolates appear to consistently reduce *Pythium* infection levels in pea seeds sown in infested compost. Further work is underway to investigate the mechanism of antagonism and to determine if these treatments can yield significant reductions in disease incidence.

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SPORE MOVEMENT OF *MUCOR HIEMALIS* IN THE RHIZOSPHERE OF GROUNDNUT IN NATURAL FIELD CONDITIONS

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ABSTRACT

A pimaricin-resistant strain of *Mucor hiemalis* was applied as spores to the hypocotyl of groundnut seedlings and to the soil surface of unplanted soil. Its movement in the soil profile was studied in natural field conditions in Malawi. The procedures for monitoring spread of the inoculant organism in field sites are described and their general applicability is discussed.

Fungal spread was greatest along the groundnut tap root, exceeding 40 cm after 51 days. Soil below inoculated plants was colonized to a depth of 22 cm, but non-planted soil only down to 7 cm. Although transport along the tap roots was high, their colonization was patchy, as the fungus was diluted out with increasing depth in the profile. Fungal establishment on lateral roots was poor.

It is concluded that soil application of BCAs is unlikely to yield success. In contrast, inocula applied to seed or stem bases have the potential to keep up with the rapidly expanding root system. The development of biological seed inocula is particularly promising for tropical climatic conditions. A novel method for the selective isolation of rhizosphere-competent fungi is suggested.

INTRODUCTION

The use of biocontrol agents (BCAs) against soil-borne diseases requires an efficient delivery system of the inoculum into the root zone of the crop. Population dynamics of seed-applied bacteria in the rhizosphere have received appreciable attention (recently reviewed by Elsas & Heijnen, 1990; Gammack *et al.*, 1992). Knowledge of fungal spore movement in natural soil is scarce.

In a podsol profile in Cheshire, U. K., spores of *Mucor ramannianus* freely passed through the sandy A horizon, but were unable to penetrate the more compacted B_1 horizon (Hepple, 1960). Tropical soils lack the structural horizons typical for soils in temperate areas (Lowole & Banda, 1986). Additionally, the rainfalls during the cropping season typically exceed precipitations in the UK.

Little information is available on the influence of a root system on fungal spore movement. Spores in percolating water, rather than relatively slow-growing hyphae, are believed to be responsible for colonization of root apices which can grow several centimetres per day (Huisman, 1982). Secondary sporulation on seeds and roots of wheat has been shown for seed-applied spores of the fungus *Idriella bolleyi* in artificial conditions (Lascaris & Deacon, 1991). Bahme & Schroth (1987) demonstrated the importance of irrigation water in movement of *Pseudomonas fluorescens* down potato roots; they also reported a log-normal distribution of the inoculant bacterium on roots, indicating that population levels fall off rapidly with distance from the inoculum source.

The present study investigates the movement of *Mucor hiemalis* spores in a ferric luvisol in Malawi and the influence of the root-soil interface on the transport process. The work was conducted in a natural, agricultural soil profile, because sieving and repacking disrupts the continuity of natural channels and increases the surface area available to entrap organisms (White, 1985).

MATERIALS AND METHODS

Groundnut plants (cv. JL 24) were planted on one half of either side of a trench. Twelve days after sowing, hypocotyls and non-planted soil were inoculated with 3 ml of a spore suspension (total 0.5×10^7 spores) of a pimaricin resistance-marked *M. hiemalis* strain. The wild type originated from healthy groundnut roots in the same field.

During sampling the soil from the trench wall was carefully removed. When the root system was approached, soil cores (diameter 8 mm) were taken at 2 cm intervals down the profile. Afterwards the plants were excavated and the tap roots were cut into 2 cm segments. Laterals were severed 2 cm away from the tap root and the following 2 cm pieces were collected (Fig. 1).

Air-dried samples (0.25 g soil or 2 cm root) were enriched for *M. hiemalis* in submerged liquid culture in potato-sucrose broth containing 40 μg ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. After three days incubation, the cultures were streaked onto potato-dextrose agar containing 40 μg ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 20 μg ml^{-1} pimaricin.

Rainfall was measured in a gauge adjacent to the trench, and evaporation by Chitedze Meteorological Station. The minimum depth D of the wetting front in non-saturated soil was calculated according to the equation $D = R_e/\theta_{FC}$, assuming dry soil, where R_e is the excess rainfall (rainfall minus evaporation) and θ_{FC} the volumetric water content at field capacity, which was 20.1 %. A detailed description of procedures will be published elsewhere.

RESULTS

Pimaricin-resistant *M. hiemalis* was never recovered from non-inoculated soil, nor from the trench floor. In all enriched rhizosphere samples examined by microscopy, *Mucor*-type spores were observed, but only occasionally in bulk soil. Sensitive detection of the inoculant strain was possible by simultaneous use of (1) enrichment by employing the fungus's ability to proliferate rapidly in liquid culture and (2) the genetic marker by exploiting the fungicide resistance.

Fig. 2 shows the average depth in the profile from which the marked strain was recovered. In bare soil movement increased over time but did not exceed 7 cm. In soil below inoculated plants, *M. hiemalis* was gradually transported to a depth of 22 cm. Apart from day 4, when soil and root colonisation coincided, the fungus moved approximately twice as far along the root than it did in adjacent soil, exceeding 40 cm at 51 d. Downward transport was significantly correlated with cumulative rainfall in bare soil ($r = 0.874$, $P < 0.05$), planted soil ($r = 0.880$, $P < 0.01$) and along the tap root ($r = 0.957$, $P < 0.001$). The calculated wetting front soon exceeded the length of the tap root and surpassed spore movement in bulk soil.

With increasing depth, tap root colonization became patchy as the fungus was diluted out (Fig. 1). Colonisation of lateral roots was poor in comparison to the tap root. The greatest recorded depth of occurrence on laterals was 9 cm at 18 d. At later

Fig. 1: A. Summary representation of occurrence (solid symbol) of inoculant strain at different sampling depths (open symbol = no detection) (a) non-planted soil, (b) planted soil, (c) tap root and (d) lateral roots. B. Exposed root system in trench; cork-borer (for sampling) arrowed.

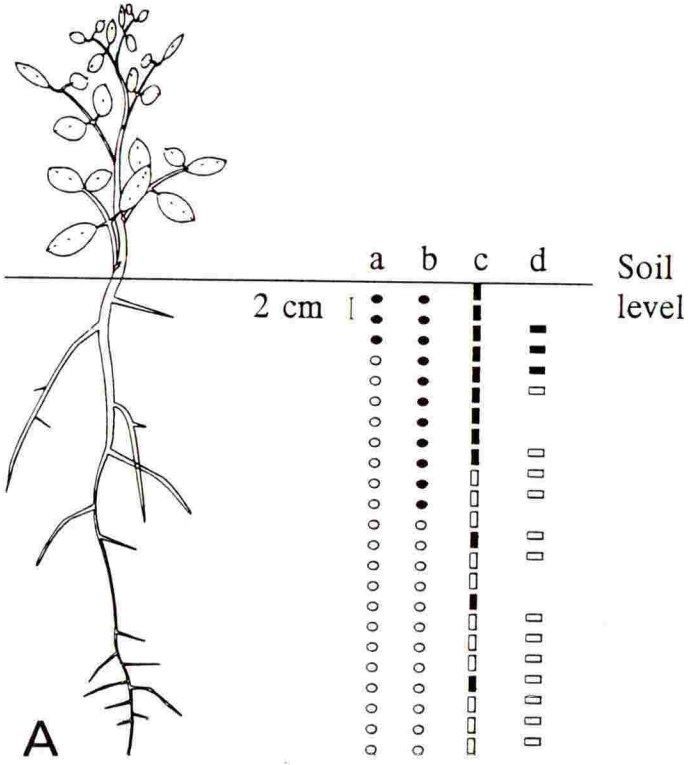
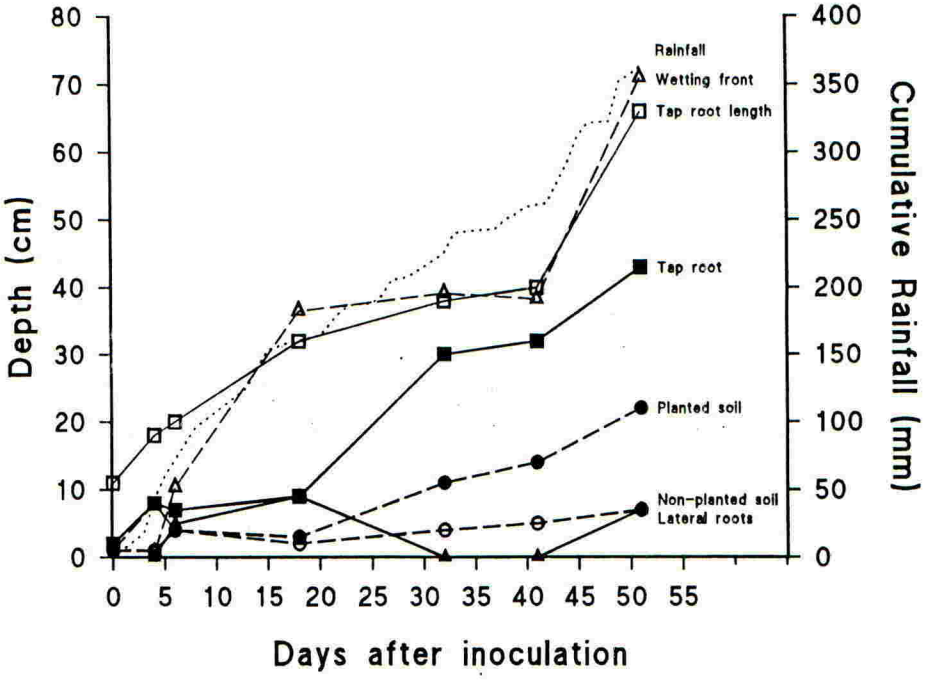


Fig. 2: Tap root length and maximum distance of pimaricin-resistant *Mucor hiemalis* down the tap root, root laterals or in soil, in planted or non-planted inoculation sites. Data points are means of two replicates for roots and root-free soil. Cumulative rainfall and calculated wetting front are also indicated.



sampling dates, the first-order laterals in the upper regions of the tap root had largely senesced and decomposed. Younger laterals were formed further down the profile, but *M. hiemalis* was not found on them at 32 and 41 d, and by 51 d, the fungus was only detected down to 7 cm depth on laterals, the same depth it had reached in non-planted soil.

DISCUSSION

Despite the lack of structural horizons and the relatively high precipitation of 363 mm during the 51 days, movement in non-planted soil was low. The wetting front calculated from excess rainfall in non-saturated soils reached far beyond the detection limit of the spores. A similar lag behind the water front has been reported for zoospores and cysts of *Phytophthora megasperma* and for the bacterium *Serratia marcescens* (Wilkinson *et al.*, 1981). Although spores of the Mucorales are water-dispersed (Dobbs, 1942), water movement was not efficient in carrying the spores through the soil matrix. Establishment of a biocontrol agent applied to bulk soil thus seems unpromising.

In contrast to bulk soil, fungal movement rates in the rhizosphere exceeded those previously reported by an order of magnitude. Water films surrounding the root cortex are believed to be of primary importance for the mobility of microbes (Gammack, 1992; Parke *et al.*, 1986). Chao *et al.* (1986) observed *Trichoderma harzianum* spores on pea roots 5 cm below the inoculated seed if percolating water was present, but only at 2 cm

below the seed in a humid chamber in the absence of water flow. It has been suggested that, under field conditions, the rate of root growth often exceeds the movement of water films in unsaturated soils (Huck & Hoogenboom, 1990). The groundnut tap root can grow at rates greater than 3 cm day^{-1} . Under conditions of tropical rainfalls in this experiment, however, the calculated minimum water front soon caught up with the advancing apex. Even under drier soil moisture regimes, the formation of water films on roots at night time has been reported (Huck *et al.*, 1970). High evapotranspiration rates during hot and dry spells may cause the roots to contract. Changes in root diameter during high transpirational demands have been demonstrated for sunflower (Faiz & Weatherley, 1982) and cotton (Huck *et al.*, 1970), two crops of the semi-arid tropics. Root contraction and thus gap formation at the root-soil interface is quite conceivable, especially since groundnuts are virtually free from root hairs (Chandler, 1978; Yarbrough, 1949) which could bridge gaps (Tinker, 1976). Such voids can readily be flushed with the next rain. It seems likely that water flow within these root channels mediated transport along the tap root and in rhizosphere soil. Thus, under tropical conditions, water-dispersed spores can effectively be transported along root channels.

M. hiemalis was only detected on a proportion of tap root segments and the colonisation of laterals was poor. Competition has been held responsible for the lack of root (Chao *et al.*, 1986) and soil (Wilkinson *et al.*, 1981) colonisation by fungi and bacteria in soil columns packed with non-sterile soil. Transport rates in autoclaved soil were consistently higher. However, this might have reflected a failure to establish measurable population levels, rather than a lack of transport through soil. The same might have been true for tap root segments and their laterals on which *M. hiemalis* was not detected, although it must have passed them. However, *M. hiemalis* is an ubiquitous and highly competitive fungus. In an agricultural soil, even at inoculum ratios as unfavourable as 1:60 it could pre-empt colonization of soil and substrates by, and replace, *T. harzianum*, a fungus often cited for its biocontrol potential (Wardle *et al.*, 1993). The ability of *M. hiemalis* to become established in soil when supplied with a food base merits further study. Spores of *M. hiemalis* dried onto seeds remained viable for over three weeks at room temperature (unpublished). Coating of spores onto seeds could provide a nutrient boost to the fungus during seed germination. Furrow application of the inoculant into the planting hole, however, has the advantage that the agent is not removed from soil when the cotyledons emerge (Windels *et al.*, 1983) or the testa is shed.

Using surface-sterilized seeds, Lascaris & Deacon (1991) provided evidence that seed-derived nutrients and early establishment in root regions close to the seed facilitate root colonisation by the biocontrol fungus *Idriella bolleyi*. In contrast to their investigation, the inoculant in this study was exposed to the natural soil flora and, by inoculating the hypocotyl at twelve days after sowing, was deprived of seed exudates which are highest after 48 h (Subrahmanyam *et al.*, 1983). Nevertheless, *M. hiemalis* was able to colonize proximal regions of the root system and spread downwards along the tap root. This is likely to be at least partly due to secondary sporulation (Windels *et al.*, 1983; Lascaris & Deacon, 1991). Thus, rhizosphere competence might be linked to the ability to sporulate rapidly in water films surrounding roots. The chosen isolation technique - enrichment in liquid culture - mimics this and could be a promising tool for the selective isolation of biocontrol candidates. It marries ecological significance with the advantages of fermentation technology for the mass production of biological crop inoculants.

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BIOCONTROL OF SEED-BORNE BOTRYTIS ALLII USING AN ANTAGONISTIC BACTERIUM

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ABSTRACT

A bacterium, Enterobacter agglomerans, isolated from neck rot infected onions, was antagonistic to the causal fungus (Botrytis allii) in vitro. Film coat applications of the bacterium and also of benomyl, applied at a standard rate, to naturally infected seeds gave similar levels of control of the fungus in laboratory tests and of the disease (neck rot) in stored bulbs produced from field-grown crops of onions.

INTRODUCTION

In 1987 and 1988 while isolating fungal pathogens from commercial consignments of imported onions, some of which were infected with neck rot (Botrytis allii), an unknown bacterium was observed which appeared to be inhibiting the growth of that fungus on some bulbs.

The bacterium was isolated into pure culture and identified as a member of the Enterobacteriaceae using the methods of Lelliot & Stead (1987). It was identified to specific level as Enterobacter agglomerans using an API 20E Bacterial Identification Strip supplied by API System, La Balme Les Grotte, 38390 Montalieu Vercieu, France.

The bacterium was tested for its inhibitory effect on the growth of B allii in culture and for control of onion neck rot when applied to naturally infected seeds.

IN VITRO TESTS

The bacterium produced flat and domed colonies when grown on King's medium B (KB). The flat form was stable when sub-cultured but sub-cultures from domed colonies yielded both types. Tests for antibiosis were made on KB agar and on prune lactose yeast agar (PLYSE).

Bacterial streak test

Materials and methods

Domed and flat colony forms of the bacterium were tested independently. Bacterial cells from agar cultures were spread using a sterile loop across the upper third of a 90mm plate of KB agar. The plates were incubated for 24h at 25°C to establish the bacteria. Each of 4 replicate plates per bacterial colony form was then inoculated with a 6mm disk of mycelium taken from the edge of a colony of B. allii (isolate B4037 which had been obtained from an onion bulb which bore the bacterium). The disk was placed 50mm from the bacterial streak (centre to centre). Plates were incubated for 8 days at 20°C when the radial growth across two diameters of each fungal disk was measured in mm.

The test was repeated on PLYSE agar with five isolates of B. allii (isolate B4037, two isolates from different seed samples, B4072, B3922, one Australian isolate, B4017 and one isolate from shallots, B4089).

Results

By comparison with the control (25.4mm mean radial growth inclusive of disk) bacteria of the domed colony form reduced growth of B. allii on KB agar to a mean of 9.5mm (a 63% reduction in growth). Bacteria of the flat colony form reduced growth to a mean of 13.4mm (a 47% reduction).

Both forms of the bacterium reduced the growth of all five isolates of the fungus from different sources (Table 1).

TABLE 1 Antagonistic effects of Enterobacter agglomerans to isolates of Botrytis allii grown on PLYSE agar

	Growth of B. allii from disks in mm				
		Bacterium (1975D)		% reduction in growth	
Source of isolate	No bacterium	flat	domed	flat	domed
Onion seed (B4072)	18.6	10.8	8.3	42	55
Shallots (B4089)	19.8	8.6	8.6	57	57
Onion (Australia)(B4017)	14.5	13.8	11.0	5	24
Onion seed (B3922)	20.0	7.9	11.8	61	41
Onion (B4037)	19.5	16.6	14.3	15	27

( ) = isolate numbers

Reduction in growth of B. allii isolate B4037 by both forms of the bacterium was less on PLYSE than KB. Reduction in mycelial growth of isolates of different origin ranged from 24 to 57% for the domed colony form of the bacterium and from 5 to 61% for the flat colony form. The predominantly domed colony form was, in general, more antagonistic to the growth of different isolates of the fungus and it was used in all subsequent tests.

SEED TREATMENT TESTS AND FIELD STUDIES

To ensure maximum inhibition of the fungus the bacterium was cultured in nutrient broth and was applied to seed at the highest concentration achievable.

Seed treatment test 1

Method and Materials

Using fluidised bed film coating equipment (Maude & Suett, 1986) E. agglomerans bacteria were applied to seeds in a polyvinyl acetate film. Thirty ml of a mixture of 5ml of cells from a 2-day old culture + 25ml nutrient broth + sterile distilled water (1:1) + 0.25% of seed weight of polyvinyl acetate (PVA), as Vinamul R18160 (Vinamul Ltd., Carshalton, London) was sprayed per 50g infected onion seeds. Colony counts based on serial dilution plates indicated that 2-day old cultures contained 3.4×10^8 viable cells per ml.

Two hundred seeds per treatment (8 replicates of 25 seeds each) of a single stock of naturally infected onion seeds obtained from a commercial source were placed on moist filter paper and on PLYSE agar plates and assessed for colonies typical of the fungus and of the bacterium after 7 days.

Result

There was a significant reduction ($P<0.05$) in recovery of the fungus on filter paper compared with PLYSE (Table 2). The application of PVA caused a further significant reduction ($P<0.05$)in the recovery of seed-borne B. allii on PLYSE. The fungus was not recovered on either substrate from seeds to which bacteria had been applied by film coating.

TABLE 2 Laboratory test of the effect of film coating naturally infected onion seeds with Enterobacter agglomerans cells on the control of seed-borne Botrytis allii

Treatment	Test substrate	% seeds from which B. allii was recovered	% seeds from which bacteria were recovered
Untreated seeds	Filter paper	6	0
" "	PLYSE agar	28	0
PVA sprayed	Filter paper	3	0
" "	PLYSE agar	14	0
Bacteria + PVA	Filter paper	0	100
" "	PLYSE	0	100
	LSD (<0.05)	3.5	-

All seeds treated with the bacterium were enveloped in pools of the organism on both test substrates.

Seed treatment test 2 and field trial

Method and materials

Benomyl and two rates (x and 2x) of the bacterium were applied in a film

coat or in suspension to onion seeds. The x rate of the bacterium was prepared as described above; in this case 6.6ml of the bacterial cell concentrate in nutrient broth was applied to 66g seeds and 13.2ml were used to obtain the 2x rate. Bacteria in suspension were applied to seeds in a rotating bowl and talcum powder was added to dry the bacteria onto the seeds. Benomyl was applied as Benlate (50% wp - Du Pont Ltd.) either as a dust (1g a.i./kg seed) or sprayed in water plus sticker at the same rate of active ingredient to form a film coat. PVA sticker alone was applied to seeds at the rate specified in the previous test.

Two hundred seeds per treatment were plated out on PLYSE as described above. The remaining seeds were drilled in the field on 12 April 1989 in individual plots of 5 rows each 15.25m long and 1.3m apart. There were 4 replicate plots per treatment in a randomised block layout from which plots grown from untreated seeds were excluded. Paired plots of untreated seeds and of the treatments were also sown isolated from each other on the farm at Wellesbourne. Records of emergence of onions per m row were made and the mature bulbs were harvested on 5 October 1989. Onions were stored in nets (about 270 bulbs per net) in an onion store until 9 January 1990 when samples of bulbs were split vertically and recorded for the presence or absence of neck rot (Maude & Presly, 1977). Three nets from each of the three isolation plots per treatment were removed and 50 bulbs taken at random from each net and assessed. This provided a total of 450 bulbs per treatment. Six hundred

TABLE 3 Effect of bacterial seed treatments on the control of *B. alli* on the seeds in the laboratory and on stored bulb onions

	% *B. allii* on agar		% neck rot in bulbs (stored for 3 months) from Isolation plots		from Randomised plots	
Biocide and rate	D	FC	D	FC	D	FC
Benomyl 1g a.i./kg	0	0	1.1	1.1	0.2[a]	1.4[f]
Bacteria x	7.5	0.5	8.1	2.5	3.5[bd]	2.3[f]
Bacteria 2x	6.5	2.0	5.4	1.8	1.7[ce]	1.3[f]
Nil + FC	14.0		8.0		-	-
Nil	27.0		12.9		-	-
LSD^1 (<0.05)	2.09		-			
LSD^2 (<0.05)	4.18		-			

D = dusted or dried on to seeds; FC = film coated onto seeds; LSD^1 = significant differences between application methods and LSD^2 between treatments; different letters indicate significant differences between treatments based on χ^2 analysis

bulbs per treatment comprising 150 bulbs per replicate in 3 sub-samples of 50 bulbs per net were taken for assessment from the randomised block experiment.

Results

In the laboratory and field test, film coat applications of bacteria and benomyl and dust application of benomyl were more effective than suspensions of bacteria dried on to the surface of seeds with talcum powder (Table 3).

Film coating with bacteria (either rate) reduced but did not completely eradicate the fungus in the agar test as it had done in Seed Test 1. The reduction obtained by application of a PVA film only was similar to that obtained in the previous test. Benomyl either dusted or film coated onto seeds eliminated the fungus in the agar test.

None of the seed treatments affected emergence which ranged from 11.7 to 13.7 plants per m row in the randomised part of the experiment. Very similar numbers of onions per m row were obtained from untreated seeds in the isolation plots.

The bacteria applied in a film coat to seeds gave similar levels of control as benomyl applications in bulbs assessed for neck rot after 3 months in store. The bacteria applied as a suspension in nutrient broth and dried on to the seeds with talcum powder were not as effective in reducing neck rot.

In comparison with the laboratory test there was a reduction in transmission of fungus from the seed in field sowings of untreated seeds and seeds treated with PVA alone and this resulted in a lower incidence in neck rot in store.

DISCUSSION

Biological control of plant pathogens by seed treatment with micro-organisms has been directed mainly against soil-borne organisms (Harman, 1991). Fungi and bacteria have been applied to seeds for this purpose (Taylor and Harman, 1990). Generally treatments shown to be effective under artificial conditions in inoculated sterile soil are less effective when exposed to the variability of the soil rhizosphere. However, there are examples where micro-organisms applied as seed treatments have been as effective as manufactured pesticides in achieving control of soil-borne diseases (Parke, 1990; McQuilken *et al.*, 1990).

Micro-organisms have also been applied successfully to control the seed-borne phases of certain fungal and bacterial diseases, for example, black leg of sugar beet (*Phoma betae*) (Walther and Gindrat, 1987; Gordon-Lennox *et al.*, 1987) and black arm of cotton (*Xanthomonas campestris* pv. *malvacearum*) (Randhawa *et al.*, 1987).

In this study a bacterium *Enterobacter agglomerans*, isolated from imported onion bulbs, was shown to be antagonistic *in vitro* to the growth of *Botrytis allii* (onion neck rot) the main source of which in the UK is imported infected onion seeds. Eradication of the seed-borne phase virtually eliminates the disease from the stored bulb crop (Maude, 1983) so the possibility of achieving this by means of a bacterial seed treatment was tested. The film coating method has been used to achieve accurate dosing of agrochemicals onto seeds (Maude & Suett, 1986; Maude,1990) and in research on biocontrol methodology to apply oospores of *Pythium oligandrum* to seeds

(McQuilken et al., 1990).

The method of application of the bacteria to seeds affected the efficacy of seed treatment. Bacteria applied to seeds in nutrient broth which was then dried on to the seeds by the addition of talcum powder were only partly effective. The reason for this is not known. However, bacteria applied by film coating were as effective as benomyl in controlling the seed-borne phase of the fungus on agar. This reduced its transmission in the crop and ultimately resulted in control of neck rot in the stored bulbs. In this respect the microbial treatment appears to have been as effective as the standard rate of benomyl previously used to control the disease in commerce.

The use of sticker alone also reduced the incidence of the fungus and the disease, a phenomenon also reported for seed-borne Alternaria brassicicola when the effect was considered to have been due to toxicity to the more superficial seed-borne inoculum (Maude and Suett, 1986).

The bacterial seed treatments did not increase or reduce crop emergence.

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