Biological Seed Treatments using *Trichoderma harzianum* for Horticultural Crops

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Biological control or biocontrol has the potential to replace or augment conventional plant disease management practices based on the use of synthetic pesticides, Biocontrol provides a nonpolluting means for control of plant pathogens through the use of indigenous or genetically modified organisms. Biological control practices are consistent with the goals of sustainable agriculture and integrated pest management to minimize the use of synthetic pesticides. Biological control agents or bioprotectants may be used alone or in combination with specific chemical pesticides that are compatible with the bioprotectant. Thus, biocontrol has the potential for commercial use; however, a comprehensive system must be in place to ensure adequate plant protection under a wide range of field conditions.

Three components are necessary for effective implementation of biological control: 1) a superior bioprotectant, 2) a process to produce large quantities of effective propagules, and 3) a delivery system that permits full expression of the biological control properties of the strain (Jin et

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al., 1992). The biocontrol agent employed in most of this project was *Trichoderma harzianum* strain 1295-22, abbreviated as strain 22, or 22, This strain was developed by protoplasm fusion, and selection was based on desirable biological control traits, including rhizosphere competence (Jin et al., 1992), *Trichoderma* was produced in petri dishes during the early stages of research and later liquid fermentation was developed to produce large quantities of inoculum, This inoculum (conidia) could be dried without appreciable loss of viability (Jin et al., 1992), This paper concentrates on the application of *Trichoderma* for seed treatments; however, other biocontrol agents have been employed successfully on horticultural crops (Bennett et al., 1992), Other delivery systems also may be employed for application of biocontrol agents, including in-furrow granules, but will not be discussed here,

There are several reasons for pursuing the use of biological seed treatments for use on horticultural as well as other crop seeds. Particular strains of *Trichoderma* have 1) the ability to protect seed and seedlings from organisms that cause damping-off; 2) are rhizosphere-competent and protect the subterranean portions of growing plants from attack by pathogens; and 3) enhance plant growth and development (Harman and Taylor, 1990), Most of the examples provided document the ability of the bioprotectant to protect seed and seedlings from plant pathogens.

Seed treatment technologies

A common goal of all seed treatment technologies is to apply an active material(s) uniformly onto the seed surface and uniformly from seed-to-seed. However, accomplishing this goal alone may be inadequate for effective biological control. Cucumber *(Cucumis sativus* L.) seeds were either nontreated or treated with strain 22 using a slurry and sown in a *Pythium ultimum* Trow-infested soil, There was little disease control, no significant differences between the emergence of the two treatments, and the final stand (after postemergence mortality) was <5% for both treatments (Taylor et al., 1991). Conversely, bean seeds were well-protected by the same slurry treatment (Harman and Taylor, unpublished data). Therefore, specific seed-treatment systems are needed to provide a conducive environment and competitive advantage for the bioprotectant compared to the pathogen. The following section describes several seed treatment methods, summarizing published results and describing the mechanism for enhanced efficacy.

Solid matrix priming (SMP). *Solid* matrix priming is a method for hydrating seeds under controlled environmental conditions by mixing seeds, a solid particulate carrier, and water in known proportions (Kubik et al., 1988; Taylor et al., 1988). The mixture is incubated for a period of time to allow seeds to imbibe water from the solid carrier and for the seeds to become physiologically active. The seed moisture content is elevated, but the water potential of the system is low enough to prevent germination (radicle emergence) (Tay-[or et al., 1992). After priming, the solid particulate is separated physically from the seeds, Different solid particulate materials may be used for this process, and the water-holding capacity of various carriers has been described (Khan, 1992).

Solid matrix priming may be integrated with the application of bioprotectants to increase the efficacy of the biological (Harman and Taylor, 1988). The bioprotectant is applied first in an aqueous suspension, followed by the addition of a finely ground solid particulate, One carbonaceous material that was found to be well-suited as a solid particulate was Agro-Lig, a Leonardite shale (American Colloid Co., Arlington Heights, Ill.). The Agro-Lig has a pH of 4.1 and contains an abundance of inorganic nutrients, In this study, the mixture was incubated for 4 days at 20C and the excess solid particulate was sifted from the seeds prior to sowing,

Laboratory efficacy studies consisted of sowing seeds in an Arkport sandy loam soil infested with *Pythium ultimum* Trow (Harman and Taylor, 1988), The pathogen level was adjusted to result in ≈ 10% stand from nontreated seeds, and there were from 200 to 250 propagules per gram of soil, Seeds were treated either with strain 22 via SMP with Agro-Lig or with a slurry containing strain 22. The percentage of healthy plants was significantly greater for SMP with strain 22 compared to the slurry treatment for cucumber (96% vs. 64%, respectively) and pea *(Pisum sativum* L.) (92% vs. 68%, respectively) (Harman and Taylor, 1990). The priming effect alone was not responsible for the greater percentage of healthy seedlings in these studies (Harman et al., 1989). In a similar study, seeds were treated either with strain 22 via SMP or with thiram. Significant improvements in stand were measured in SMP-treated cucumber (96% vs. 72%, respectively), pea (92% vs. 12%, respectivel y), and snap bean *(Phaseolus vulgaris* L.) (84% vs. 4%, respectively) seeds compared to the chemical check.

Several mechanisms appear to account for the improved efficacy of *Trichoderma* applied via SMP (Taylor and Harman, 1990). The priming process creates a relatively high water potential, and the water potential of the medium has been reported to range from –1.1 to –1.9 MPa(Taylor et al., 1988). The number of *Trichoderma* propagules (colony forming units, cfu) increased 10-fold during priming ($10^3$to 10^4cfu/seed) (Harman and Taylor, 1988). Therefore, more propagules were present on the seed surface. The acidic pH of the Agro-Lig combined with nutrients provided an ideal environment for growth of the *Trichoderma.* Therefore, there were at least two factors responsible for the improved efficacy of the bioprotectant via SMP-colonization of the seed surface prior to sowing, and the development of a favorable pH plus nutrients.

Film-coating (liquid-coating). Film-coating is a process by which a suspension or solution is sprayed onto seeds to develop a uniform layer of material over the entire seed surface (Halmer, 1988). The ingredients consist of a binder or binder plus solid particulate and other materials, including colorants, plasticizers, and active ingredients. These materials may be formulated as a dry powder for handling and storage and then mixed with water prior to use. The result is a high-solids suspension of low viscosity that may be sprayed onto seeds either in a coating pan or fluidized bed (Halmer, 1988).

We developed a variation of the film-coating process, termed liquid-coating (Taylor et al., 1991). This method consists of making a suspension of binder, solid particulate, and bioprotectant.The binder was either Pelgel (LiphaTech, Milwaukee, Wis.), a methyl cellulose-based material, or Polyox N-10 (Union Carbide, Danbury, Corm.), a high-molecular-weight polyethylene oxide. The most-effective solid particulate were Agro-Lig or finely ground muck soil, Strain 22 was added to the suspension and sprayed onto the seeds in a tumbling drum, The proportion of individual components used to make the liquid-coating suspension was described previously (Taylor et al., 1991), Unlike film-coating, the coating material was not dried extensively during the coating process, but remained moist in a manner similar to pelleting.

An additional step conducted on several treatments was to incubate the freshly coated seeds at high relative humidity for 4 days at 25C. Another modification of the basic technique was termed double-coating, The bioprotectant was suspended in Pelgel and sprayed onto seeds without drying, The second outer coating was a suspension of solid particulate in Polyox. In this manner, the bioprotectant was placed in close proximity to the seed surface, followed by an outer protective layer that encapsulated the seed and bioprotectant.

Cucumber seeds were treated with the liquid-coating techniques containing the bioprotectant and sown in the *Pythium ultimum* Trow-infested soil in the laboratory (as previously described), Efficacy of strain 22 was enhanced by liquid coating, and either Agro-Lig and muck soil were effective as solid particulate; seedling emergence from liquid-coated seeds were comparable to SMP-treated seeds (Taylor et al., 1991). Both binders were equally effective, but Pelgel was best-suited for the high-relative-humidity incubation treatments, The high-relative-humidity incubation of liquid-coated seeds was found to increase further the percentage of seedling emergence compared with either liquid-coated alone or SMP treatments. The final seedling emergence from cucumber seeds sown in a *Pythium* -infested soil for SMP, liquid coating, and liquid coating plus high-relative-humidity incubation was 34%, 46%, and 80%, respectively,

Several mechanisms may account for the improved performance from liquid-coated seeds (Taylor and Harman, 1990). The attributes of Agro-

Lig to acidify the medium and provide inorganic nutrients are the same as described for SMP. The uniform film coating, which was <0.1 -mm thick, was shown to act as a barrier for the ingress of *Pythium ultimum* Trow (Taylor et al., 1991). The coating barrier may have allowed time for the bioprotectant to become active prior to attack by the pathogen, The high-humidity incubation allowed further proliferation of the *Trichoderma,* and a 60-fold increase in cfu was measured after incubation. This incubation is similar to the priming condition of SMP, but, unlike SMP, the solid particulate containing much of the *Trichoderma* is not discarded prior to sowing. It was later shown that Pelgel is a carbon source (food base) for *Trichoderma* and other microorganisms, including *Pythium ultimum* Trow, *Rhizoctonia solani* Kuhn, and *Fusarium graminearum* Schwabe, while Polyox is relatively inert (Harman and Taylor, unpublished data). Pelgel was therefore beneficial during the high-relative-humidity incubation because it acted as a food base for the growing bioprotectant. Pelgel was also effective as the first layer in double-coating and was protected from the environment by the outer layer of inert Polyox and solid particulate.

Compatibility with synthetic pesticides

Biological control agents may be used as the sole protestant against seed- and soil-borne pathogens, as previously described. However, the bioprotectant will have broader utility if it can be formulated with other compounds, including synthetic pesticides, to protect seeds and seedlings, Compatibility of strain 22 was tested in combination with a number of commercial fungicides and insecticides (Table 1). Soybean *[Glycine max* (L.) Merr.] seed was treated first with the synthetic pesticides (Meister Publishing, 1991) listed in Table 1, and then strain 22 was applied as a second, or outer, coating in Pelgel. The seeds were sown in Arkport sandy loam soil, removed after 24 or 48 h, then the seed coats were removed and stained with a vital stain, fluorescein diacetate. The rate and amount of *Trichoderma* growth was evaluated, and a rating system was developed to evaluate compatibility.

Most fungicides showed some degree of compatibility with strain 22, except for benomyl (Benlate), imazalil, and flusilazol (Nustar) (Table 1). Imazalil and flusilazol are both sterol demethylation inhibitors, whereas benomyl inhibits the tubulin synthesis required for mitosis (Koller, 1992). Apron(metalaxyl) was fully compatible and was used in 1991 field studies with sweet corn (described later), while broad-spectrum fungitides such as captan and thiram were somewhat compatible, All insecticides tested showed good compatibility with strain 22.

Table 1. Compatibility of synthetic pesticides with T. harzianum 1295-22

Material	Chemical name(s)	Compatibility[z]
	Fungicides	
Apron	Metalaxyl: N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester	+++
Baytan	Triadimenol: Beta-(4-chlorophenoxy)-alpha-(1,1-dimethylethyl)-1H 1,2,4-triazole-1-ethanol	++
Benlate	Benomyl: methyl1-(butylcarbamoyl)-2-benzimi-dazolecarbamate	–
Botran	DCNA: 2,6-dichloro-4-nitroaniline	++
Captan	N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide	+
Demosan	Chlorneb: 1,4-dichloro-2,5-dimethoxybenzene	+++
Imazalil	1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1H-imidazole	–
Maneb	Manganese ethylenebisthiocarbamate	+
Nustar	Flusilazol: 1-[[Bis(4-flurophenyl)methylsily]methyl]-1H1,2,4-triazole	–
PCNB	Pentachloronitrobenezene	++
Thiram	Tetramethylthiuram disulfide	+
Vitavax 34	Thiram + Carboxin (5,6-dihydro-2-methyl-N-phenyl-1,4-oxathiin-3-carboxamide	+
Vitavax 200	Carboxin	++
	Insecticides	
Diazinon	O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl)phosphorothiote	+++
Lindane	gamma isomer of benezene hexachloride	++
Lorsban	Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl)phosphorothioate	+++
Malathion	O,O-dimethyl phophorodithioate of diethyl mercaptosuccinate	+++
Methoxychlor	2,2-bis (p-methoxyphenyl)-1,1,1-trichloroethane	+++
Orthene	Acephate (0,S-Dimethyl acetylphosphoramidothioate	+++
Sevin	Carbaryl (1-napthyl N-methylcarbamate)	+++

[z]*–= noncompatible, +=somewhat compatible, ++=moderately compatible, and +++= fully compatible,*

Field studies

Field studies were conducted during the past several years to evaluate the efficacy of different biological seed treatments This work was conducted on several vegetable and agronomic seed types; however, the most consistent response was measured with sweet corn. Two genotypes of sweet corn were studied—the sugary *(su)* genotype, cultivar Jubilee, and the shrunken-2 *(sh2)* genotype, cultivar Super Sweet Jubilee, Sweet corn seed, especially the *sh2* genotype, may have poor stand establishment potential that can be attributed to physiological and pathological causes. Several seed-borne pathogens have been detected from *sh2* seeds, including *Fusarium* spp., *Rhizopus* sp., *Aspergillus* sp., and *Pythium* spp. (Parera and Cantliffe, 1991). A condition known in the industry as five-leaf dieback may occur, in which seeds germinate but a portion of the seedlings exhibit reduced plant growth and frequently die (Harman et al., 1989).

1988 trials. Two separate studies were conducted, one for each cultivar, and each consisted of four treatments: nontreated, captan-treated at the commercially recommended rate, the parental strain T12, and the progeny strain 22. The treatments were applied with and without SMP in a factorial arrangement (4 x 2). Treatments applied without SMP were performed with a slurry of Pelgel; SMP was conducted as previously described. The percentage of healthy seedlings was recorded, and the weak seedlings were determined to assess the five-leaf dieback problem. Yield of marketable ears was recorded at crop maturity,

With 'Jubilee', interactions were measured in both emergence parameters, and mean separation was performed on individual treatments, The percentage of healthy seedlings overall was high, except for a reduced stand in the nontreated seed that received SMP (Table 2). This poor stand may be attributed to the proliferation of deleterious organisms rather than beneficial organisms on seeds during priming, Differences in weak seedlings were recorded; however, variation within the plot revealed little statistical differences, and no yield differences were found. With 'Super Sweet Jubilee', all biological treatments had a greater number of healthy seedlings than the nontreated controls, and no differences were measured between the captan slurry, T12, or strain 22 applied via SMP. Only the main treatment effect was significant for weak plants, and both bioprotectants had fewer weak seedlings than either nontreated or captan-treated seeds. Yield of marketable ears was numerically greatest for the strain 22 applied via SMP, but not statistically different from the captan slurry.

Collectively from these studies, it was shown that all seed treatments had little influence on 'Jubilee'. In 'Super Sweet Jubilee', improvements in seedling emergence and yield and a reduction in weak plants was observed from bioprotectants

Table 2. ***The influence of seed treatment and solid matrix priming on emergence and yield of `Jubilee' and 'Super Sweet Jubilee' in field trials conducted in 1988.***

Cultivar	Treatment	SMP	Seedlings (%) Healthy	Seedlings (%) Weak	Marketahle ears (kg/plot)
Jubilee	Nontreated	—	85a*	5 abc	1.4a
		+	67 b	9 a	1.5a
	Captan	—	84a	5 abc	1.4a
		+	81 a	8 ab	1.4a
	T 12	—	80a	7 ab	1.7a
		+	86a	4 bc	1.4a
	22	—	86a	4 bc	1.4a
		+	87a	1 c	1.3a
Super Sweet Jubilee	Nontreated	—	42 cd	13 a**	1.1 c
		+	34 d		1.3 bc
	Captan	—	68a	11 a	.6 abc
		+	43 c		1.3 abc
	T 12	—	61 ab	6 b	1.3 bc
		+	63 ab		1.7 ab
	22	—	59 b	5 b	1.2 c
		+	66 ab		1.9 a

**Mean separation within columns within cultivars by LSD (5%)*
***Only the main effect for treatment was significant for weak plants in 'Super Sweet Jubilee'.*

applied via SMP compared with the nontreated (control) seeds.

1989 trials. Two separate studies, one for each cultivar, were performed and consisted of five treatments: nontreated, captan, slurry, double-coating, and SMP. Polyox N-10 was the binder and the slurry, liquid coating, and SMP all contained strain 22,

With 'Jubilee', the percentage of healthy seedlings from all biological seed treatments was equal to or better than the chemical check (Table 3). The double-coating treatment had the greatest number of healthy seedlings with the fewest weak seedlings, and results were comparable to SMP. No differences were measured in yield, In 'Super Sweet Jubilee', all biological treatments performed well, and double-coating had a greater percentage of healthy seedlings than captan- or nontreated seeds. There were also very few weak seedlings from seeds treated with strain 22, Yield was greater from double-coated or SMP-treated seeds than nontreated seeds.

The 1989 trials revealed that biological seed treatments had equal to or better seedling emergence than the chemical check. In the case of 'Super Sweet Jubilee', yields were increased by strain 22 and was attributed to rhizosphere competence and/or other growth-promoting factors not directly related to seedling mortality,

1991 trial. The purpose of this study was to compare two chemicals [captan or Apron, (metalaxyl)] and a biological, strain 22, applied separately or in combination as seed treatments, All treatments, except for the nontreated control, were applied as a slurry of Polyox N-10. 'Super Sweet Jubilee' was used, and only total emergence was recorded due to the poor growing season, Chemicals applied alone or in combination performed no better than the nontreated control (Table 4); however, strain 22 in any treatment performed well, and strain 22 applied without chemical pesticides had the highest emergence,

Though improvements were not measured by combining chemicals or chemicals with the bioprotectant, the study does illustrate that the bioprotectant may be used in combination with specific synthetic pesticides, This approach may prove useful for other crops that require several materials to control a pathogen complex.

Table 3. ***Influence of seed treatments on emergence and yield of 'Jubilee' and 'Super Sweet Jubilee' in field trials conducted in 1989.***

Cultivar	Treatment	Seedlings (%) Healthy	Seedlings (%) Weak	Marketable ears (kg/plot)
Jubilee	Nontreated	71 c*	13 a	4.2 a
	Captan	78 b	12 ab	3.7 a
	Slurry +22	81 ab	11 ab	4.8 a
	Double-coating +22	86 a	7 c	4.6 a
	SMP + 22	82 ab	9 bc	4.8 a
Super Sweet Jubilee				
	Nontreated	41 c	17 a	2.1 b
	Captan	62 b	10 b	3.0 ab
	Slurry +22	70 ab	7 b	3.6 ab
	Double-coating +22	73 a	7 b	4.1 a
	SMP + 22	66 ab	7 b	3.9 a

** Mean separation within columns within cultivars by LSD (5%),*

Table 4. ***Combinations of synthetic and biological seed treatments applied as slurries on emergence of 'Super Sweet Jubilee' in field trials conducted in 1991.***

Treatment	Emergence (%)
Nontreated	48 cde*
Binder only	42 de
Captan	39 e
Apron	43 de
22	62a
Captan + Apron	50 bcd
Captan + 22	54 abc
Apron +22	60 ab
Captan + Apron +22	56 abc

** Mean separation by LSD (5%).*

Conclusions

Commercial scale usage of biological seed treatments is feasible for several reasons First, strain 22 is an effective biocontrol strain that has been registered with the EPA for use as a seed treatment, Strain 22 is compatible with many synthetic pesticides used in seed treatments. Second, fermentation technology has been developed to produce high-quality desiccation-tolerant propagules. These spores (conidia) can be formulated for application in seed treatments and other delivery systems. Finally, specific biological seed treatments, SMP, and liquid-coating have been shown to be effective in laboratory and field studies. Other slurry, film-coating, pelleting, and planter box formulations may redeveloped for use with horticultural and agronomic seeds. Further developments and refinements of these seed treatments have the potential to protect different seed types from several pathogens,

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