# Effect of dsRNA-containing and dsRNA-free hypovirulent isolates of *Fusarium oxysporum* on severity of Fusarium seedling disease of Essex soybean

by

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**Effect of dsRNA-containing and dsRNA-free hypovirulent isolates of *Fusarium oxysporum* on severity of Fusarium seedling disease of Essex soybean**

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(ABSTRACT)

Sixty-six isolates of *F. oxysporum* and *F. solani* were recovered from healthy and necrotic Essex soybean seedlings grown in naturally infested soil. These were tested for pathogenicity at 20 C and -0.01 MPa water potential in artificially infested, autoclaved field soil. Highly pathogenic, moderately pathogenic, and hypovirulent isolates of both species were identified. Fifty-seven *F. oxysporum* and nine *F. solani* isolates were tested for the presence of dsRNA. The presence of dsRNA was not associated with hypovirulence in *F. oxysporum* since some hypovirulent isolates contained dsRNA while other hypovirulent isolates did not. Furthermore, of six dsRNA-containing *F. oxysporum* isolates, three were hypovirulent, two were moderately pathogenic, and one isolate was highly pathogenic. Four segments of dsRNA, with sizes of 4.0, 3.1, 2.7, and 2.2 kb, were detected in extracts of all six *F. oxysporum* isolates. No morphological differences were found between dsRNA-containing and dsRNA-free *F. oxysporum* isolates. Attempts to cure dsRNA-containing hypovirulent *F. oxysporum* isolates, either by single-sporing of isolates or by using a range of concentrations of cycloheximide, were not successful. No dsRNA was found in any of the *F. solani* isolates tested.

*Pythium ultimum*, an associate in Essex seedling disease, was isolated from water-soaked lesions and interfered with evaluations of disease caused by the *Fusarium* spp. Metalaxyl was used to control *P. ultimum* and had no apparent effect on symptoms associated with *F. oxysporum* and *F. solani* in field soil. Prior inoculation of Essex soybean seeds with conidia of dsRNA-free hypovirulent *F. oxysporum* isolates, plus metalaxyl seed treatment, significantly ($p<0.05$) reduced disease severity on both cotyledons and hypocotyls and increased the rate of seedling emergence in field soil, compared to the control plants treated with metalaxyl alone or not treated with metalaxyl. No significant ($p>0.05$) differences were found between dsRNA-containing and dsRNA-free hypovirulent *F. oxysporum* isolates in their effects on the reduction of disease severity. A mixture of two hypovirulent *F. oxysporum* isolates was significantly ($p<0.05$) more effective than single hypovirulent *F. oxysporum* isolates in increasing the rate of seedling emergence. Symptoms associated with *P. ultimum* were not affected by the prior inoculation of seeds with individual hypovirulent *F. oxysporum* isolates.

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# CHAPTER 1: INTRODUCTION

In the sandy soils of the King and Queen County area of eastern Virginia, *Fusarium oxysporum* (Schlecht.) Emend. Snyd. and Hans. and *F. solani* (Mart.) Appel and Wr. Emend. Snyd. and Hans. were identified as part of a fungal complex that causes damping-off, delayed seedling emergence, root rot and stunting of the soybean [ *Glycine max* (L.) Merr.] cv. Essex. This syndrome is known as "Essex disease" (23, 24).

The Essex disease of soybeans is economically important in Virginia. Yield losses on Essex and other susceptible cultivars can be severe. After breeding for resistance for several years, one cultivar, Chesapeake, was found to produce significantly higher yields than Essex in a field with a history of Essex disease (12). However, during the cool, wet conditions of 1996, a high level of Essex disease on Chesapeake was found in one field by the Department of Plant Pathology, Physiology, and Weed Science Plant Disease Clinic, and *F. oxysporum* and *F. solani* were the only fungi associated with these diseased plants (G. J. Griffin, personal communication).

Previous research (22, 23, 24) showed that *F. oxysporum* and *F. solani* were more frequently associated with Essex disease than the other fungi involved in the fungal complex, *Rhizoctonia solani* Kuhn or *Pythium ultimum* Trow. Both fusaria were equally pathogenic at 20 C and -0.01 MPa water potential. As a pathogen, *F. oxysporum* was more important on cotyledons, and *F. solani* was more important on roots. However, both species were equally important on hypocotyls and hypocotyl-root transition zones. Furthermore, colonization of soybean tissues by hypovirulent (low virulence) and nonpathogenic *F. oxysporum* and *F. solani* isolates was observed (22, 23, 24); this may play an important role in the ecology and control of *Fusarium*-incited diseases. *Rhizoctonia solani* was isolated from symptomatic soybean cotyledons with moderate frequency (5-10 %) and from hypocotyls with higher frequency (13-26 %) at 25 C for both -0.03 and -0.01 MPa water potential. *Rhizoctonia solani* was not pathogenic to roots. Griffin (30) indicated that *P. ultimum* appeared to be the main component of a fungal complex associated with the early phase of the disease (delayed emergence and damping-off) and a contributor to the late phase of the disease (stunting and wilt) producing characteristic water-soaked lesions, seed rot, and pre-emergence damping-off. It was also reported that treatment of seeds of cv. Essex with metalaxyl significantly increased seedling emergence in the growth chamber and field and decreased the disease severity index.

Similar interactions among *Fusarium* spp., *R. solani*, and *Pythium* spp. have been found to influence disease incidence and severity on other hosts (59, 27). Pieczarka and Abawi (59) reported a synergistic effect between *F. solani* f. sp. *phaseoli*, a causal agent of

bean root rot, and *P. ultimum.* They isolated both fungi from bean plants showing symptoms of severe necrosis of roots and hypocotyls and stunting. It was concluded that damage to healthy roots by *P. ultimum* may predispose beans to infection by *F. solani* f. sp. *phaseoli*, resulting in more damage than produced by either pathogen alone. They also found that seed rot and damping-off diseases were caused mainly by *Pythium* spp. and *Rhizoctonia* sp., while plants infected with *F. solani* f. sp. *phaseoli* exhibited stunting and premature defoliation. On peanuts, Frank (27) found a synergistic interaction between *F. solani* and *P. myriotylum* in the pod-rot complex. Frank indicated that *F. solani* predisposes the pods to the activity of *Pythium*, but that *Pythium* was the decisive factor in symptom development.

Although the association of the presence of dsRNA with virulence has been reported for several plant pathogenic fungi (7, 9, 13, 18, 51, 57, 69) (Table 2.1), the genus *Fusarium*, which includes many plant pathogens, has not been investigated extensively in this respect, and also no information exists on the use of hypovirulence, associated with dsRNA, to control *Fusarium*-incited diseases.

The objectives of the present study were: 1. To obtain *F. oxysporum* and *F. solani* isolates from diseased and healthy soybean plants grown in naturally infested soil, and identify highly pathogenic, hypovirulent, and nonpathogenic isolates. 2. To determine if prior inoculation of cv. Essex soybean seeds with nonpathogenic or hypovirulent *F. oxysporum* and *F. solani* isolates reduces the incidence and severity of seedling disease in naturally infested soil. 3. To determine if there is an association between the presence of dsRNA and virulence in *F. oxysporum* and *F. solani* isolates from soybean plants.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Biological control of *Fusarium*-incited diseases with nonpathogenic isolates

Nonpathogenic isolates of *F. oxysporum* and *F. solani* colonize the surface and cortex of plant roots without causing disease symptoms and may play an important role in the ecology and control of *Fusarium*-incited diseases. Several diseases caused by formae speciales of *F. oxysporum* have been controlled, with varying degrees of success, by prior inoculation of the plant with (i) pathogenic or nonpathogenic fungi of genera different from *Fusarium* (16, 29), (ii) nonpathogenic and mildly pathogenic *Fusarium* species such as *F. solani* (47, 50), (iii) nonpathogenic formae speciales of *F. oxysporum* (6, 16, 17, 67), (iv) nonpathogenic races of the same forma speciales (6, 37, 49), and (v) nonpathogenic *F. oxysporum* isolates (37, 41, 46, 48).

McClure (50) found that freshly cut sweet potato sprouts inoculated with mildly pathogenic isolates of *F. solani*, cause of Fusarium black foot rot, were protected from subsequent infection by the sweet potato wilt fungus, *F. oxysporum* f. sp. *batatas.* This protection occurred when as short a time as 1 day or less elapsed between inoculations. It was concluded that tyloses were abundant in the xylem in advance of the foot rot pathogen, thus leaving no unclogged vessel by which the wilt pathogen could by-pass tylosis-clogged vessels.

Komada (41) demonstrated that sweet potato plants were protected against Fusarium wilt, caused by *F. oxysporum* f. sp. *batatas*, in naturally infested soil by prior inoculation with nonpathogenic *F. oxysporum* isolates, which were often found in the vessels of healthy sweet potato plants and natural soils. It was concluded that the control effect is due to the cross protection associated with the host response, because no antagonistic interaction was observed between nonpathogenic and pathogenic isolates *in vitro.* This biological control method gave nearly the same effect as chemical control with benomyl.

Biological control of Fusarium wilt diseases has been accomplished by introducing nonpathogenic *Fusarium* spp. into soil or infection courts. Three hypotheses were proposed to explain mechanisms involved in the suppression of the pathogenic *Fusarium* that result in a decrease in disease incidence and severity (48). These include competition for nutrients, competition for infection sites, and induced host resistance.

Competition for nutrients affects the activity of the pathogen in soil and consequently plays an important role in suppressiveness of the soils to Fusarium wilts. Alabouvette (1) compared root colonization, chlamydospore germination, and population dynamics of *Fusarium* in conducive and suppressive soils, and concluded that disease

suppression was due to nutrient competition between pathogenic and nonpathogenic *Fusarium* spp. in the rhizosphere.

Schneider (61), on the other hand, proposed that the nonpathogenic *Fusarium* spp. compete with the pathogen for infection sites at the root surface. He isolated nonpathogenic strains of *F. oxysporum* from suppressive soils in California, and demonstrated that their addition to soil infested with *F. oxysporum* f. sp. *apii* limited the severity of Fusarium wilt of celery.

Enhanced resistance induced by nonpathogenic *Fusarium* spp. also was demonstrated in protection against pathogenic *Fusarium* spp. Induced resistance is accomplished by the inoculation of a plant with an inducer agent (nonpathogenic isolate) prior to, or concomitant with, a second ("challenge") inoculation with a pathogen. Working with Fusarium wilts of tomato, cabbage, flax, carnation and watermelon, Davis (16) observed that different formae speciales of *F. oxysporum* were more effective in inducing resistance to a given host's pathogenic forma specialis than were other root pathogens (*Verticillium albo-atrum* and *Rhizoctonia solani)* or nonpathogens (*Penicillium notatum* and *Neurospora crassa).* He indicated that effective inducers of resistance were often closely related physiologically and taxonomically to the challenger pathogenic isolate. Biles and Martyn (6) and Martyn et al. (49) found that nonpathogenic races of *F. oxysporum* f.sp. *niveum* (races 0 and 1) were better inducers of resistance on watermelon than the related forma specialis *F. oxysporum* f. sp. *cucumerinum.* Although significant protection was obtained with a time interval of 24 h between induction and challenge, resistance was enhanced when 3 days elapsed between inoculations.

Gessler and Kuc (29) reported that several formae speciales of *F. oxysporum* induced resistance in cucumber to *F. oxysporum* f. sp. *cucumerinum* in flask culture, and that a 3-day interval between induction and challenge was necessary for adequate protection. Resistance was also induced by foliar infection with *Colletotrichum lagenarium* or tobacco necrosis virus (TNV), but not with *F. oxysporum* f. sp. *melonis* when the interval between inducer and challenge inoculations was increased to 7 days.

Hervas et al. (37) found that prior inoculation of germinated chickpea seeds with nonpathogenic races of *F. oxysporum* f. sp. *ciceris* or nonpathogenic isolates of *F. oxysporum* significantly reduced disease incidence and severity after challenge inoculation with the highly pathogenic *F. oxysporum* f. sp. *ciceris* race 5. However, the extent of protection decreased when the inoculum concentration of the challenger was close to that of the inducing agent. Results suggested that in cultivar ICCV 4, resistance against *F. oxysporum* f. sp. *ciceris* race 5 was best induced by the nonpathogenic isolate of *F. oxysporum*, Fo 90105. However, this isolate did not protect cultivar JG 62 against the

same race. Thus, the induced resistance varied with the nature of the inducing agent and the genotype of the host.

Kroon et al. (43) used the experimental design of split root system to observe the induced resistance in tomato plants against *F. oxysporum* f. sp. *lycopersici.* Because *F. oxysporum* f. sp. *dianthi* reduced disease symptoms caused by *F. oxysporum* f. sp. *lycopersici* without any direct interactions with this pathogen, it was concluded that *F. oxysporum* f. sp. *dianthi* was able to induce resistance against *F. oxysporum* f. sp. *lycopersici* in tomato plants.

Larkin et al. (46) indicated that nonpathogenic isolates of *F. oxysporum,* indigenous to "Crimson Sweet" suppressive, monoculture soil, were the dominant organisms responsible for suppression of Fusarium wilt of watermelon. In split-root experiments, in which the antagonist and the pathogen were physically separated from each other, root colonization by selected nonpathogenic isolates of *F. oxysporum* reduced disease incidence, verifying the mechanism of action as induced systemic resistance. Several nonpathogenic isolates of *F. oxysporum* from this suppressive soil had potential for development as biocontrol agents.

## 2.2 Biological control of plant disease with hypovirulent isolates associated with dsRNA

The presence of dsRNA was found to be associated with the ability of hypovirulent *sensu stricto* (=low virulence) (32) strains of *Cryphonectria parasitica* (Murr.) Barr [=*Endothia parasitica* (Murr.) P. J. and H. W. Anderson] to bring about biological control of chestnut blight. Biocontrol is associated with non-lethal, superficial, slow-growing cankers produced by hypovirulent strains (2, 18, 32).

Hypovirulence has been reported for numerous plant pathogenic fungi (Table 2.1) and, in many cases, associated with the presence of mycoviruses or unencapsidated dsRNA (7, 9, 13, 14, 18, 34, 51, 57, 69); some of the latter have been classified within the unique virus family Hypoviridae (38). The dsRNA was found to be associated with the phenomenon of transmissible hypovirulence in *C. parasitica* (2,18). The hypovirulent phenotype was transmitted during hyphal anastomosis between *C. parasitica* isolates. It was also noted that anastomosis between a hypovirulent and virulent isolate is controlled to a large extent by vegetative compatibility of the isolates. Five to seven nuclear genes are thought to involved in vegetative compatibility in *C. parasitica* (2). Considerable diversity was found to exist in the amount, size, and number of segments of dsRNA recovered from individual isolates of *C. parasitica* (58). Frequently, isolates that contain hypovirulence-associated dsRNA have, alone or in combination, reduced growth rates, and changes in

pigmentation (57). However, not all dsRNA has been associated with detectable alterations in fungal phenotypes. Evidence for the existence of dsRNA-containing, virulent *C. parasitica* isolates in nature had been presented (3, 20, 33). Griffin et al. (33) isolated *C. parasitica* from apparent superficial cankers naturally developed on American chestnut trees in managed forest clearcuts and a plantation. Cluster analysis of pathogenicity of the fungal isolates to American chestnut trees revealed four virulence groups designated virulent, intermediate virulent, intermediate hypovirulent, and hypovirulent. Isolates that were positive for dsRNA were found in all four virulence groups. Dunn et al. (20) characterized thirty-five isolates of *C. parasitica* for culture morphology, growth rate, virulence on apple and live chestnut sprouts, and the presence of dsRNA. Five isolates were identified as hypovirulent but dsRNA was consistently isolated from only three. In several cases, virulent isolates were found to contain detectable concentrations of dsRNA. Anagnostakis (3) found that three of the eleven *C. parasitica* iaolates isolated from sunken cankers contained large dsRNA molecules. When two of these were tested for virulence, they appeared to be as virulent as the virulent, dsRNA-free control isolate #38755. In *C. parasitica*, a 12-kb segment of dsRNA, present in 25 % of the isolates recovered from lethal or sunken cankers in the central Appalachians, was not associated with hypovirulence or altered morphology (21). However, lethal cankers are probably not a good place to find hypovirulent isolates of *C. parasitica*. Superficial, non-killing cankers commonly contain hypovirulent *C. parasitica* with dsRNA (33). Hypovirulent, abnormal morphology isolates of *C. parasitica* were cured of detectable levels of dsRNA by treatment with cycloheximide (28). Curing was accompanied by a dramatic increase in virulence, and by recovery of a normal colony morphology. All cured isolates could be converted to the hypovirulent form by pairing with a hypovirulent isolate.

Presence of dsRNA was found to be associated with virulence in several other plant pathogenic fungi (7, 9, 13, 18, 34, 51, 57, 69) (Table 2.1). Castanho et al. (13, 14) reported that the decline of root disease caused by *R. solani* had been associated with hypovirulence. They found that diseased isolate 189a of *R. solani* contained three segments of dsRNA with molecular weights approximately equal to 2.2, 1.5, and $1.1 \times 10^6$. Healthy isolate 189HT5 usually contained no detectable dsRNA, but occasionally preparations contained traces of the same-sized segments of dsRNA as in 189a. The association of dsRNA with disease was shown by converting healthy 189HT5 to the diseased-type by anastomosis with diseased 189a. It was observed that the resulting converted 189HT5 mycelium contained the same three segments of dsRNA as in 189a. However, no general correlation between the presence of dsRNA and virulence were reported for *R. solani* by Bharathan et al. (4, 5) and

by Kousik et al. (42), although associations between specific segments of dsRNA and hypovirulence in *R. solani* were proposed (4).

There is increasing evidence that hypovirulence or the d-factor (diseased factor) in *Ophiostoma ulmi*, the causative agent of the Dutch elm disease, is associated with certain multiple segments of dsRNA (10, 57). Multiple unencapsidated dsRNA segments have been found in both healthy and diseased strains of *O. ulmi*, and were efficiently transmitted between strains and to conidia of an infected strain. Transmission of the diseased state by extrachromosomal elements (d-factors) coincided with the transmission of a specific set of 10 dsRNA segments that ranged in size from 0.34 to 3.5 kb (10).

Tooley et al. (64) reported the existence of dsRNA in 14 isolates of *Phytophthora infestans* (Mont.) de Bary, the causal fungus of potato late blight, from Mexico. Three banding patterns and four dsRNA segments were identified. The dsRNA occurred in both A1 and A2 mating-type isolates. Other isolates examined, from the United States and Europe, were dsRNA-free. Virulence assays performed with the 14 Mexican isolates originally found to contain dsRNA had shown high overall levels of virulence in all but one isolate. Thus, it was concluded that there was no apparent connection between presence of dsRNA and lowered virulence. Newhouse et al. (54) surveyed additional isolates of *P. infestans* for the presence of dsRNA. It was found that the dsRNA was most abundant in Mexican isolates (83.3 %), followed by isolates from the Netherlands (20 %) and Peru (2.9 %). In addition to the dsRNA segments and patterns previously reported in Mexican isolates (64), three new segments and six new patterns were discovered. They observed that Dutch and Peruvian isolates contained dsRNA segments that were identical in size with dsRNA segments recovered from Mexican isolates.

Zhou and Boland (69) evaluated 132 isolates of *Sclerotinia homoeocarpa* F. T. Bennett, the causal agent of dollar spot of turfgrass, for virulence and for the presence of dsRNA. It was observed that in at least four isolates, the hypovirulent phenotype was associated with the presence of specific segments of dsRNA. In addition, these hypovirulent isolates often grew slowly on potato-dextrose agar (PDA), formed thin colonies with atypical colony margins, and failed to produce typical black stroma. They found that the hypovirulent phenotype and dsRNA were transmitted from hypovirulent isolate Sh12B to virulent isolate Sh48B, and the converted isolate was hypovirulent and contained dsRNA. Subcultures of hypovirulent isolate Sh12B, that did not contain dsRNA, were obtained through curative treatment using cycloheximide-containing medium and heat. They indicated that cured subcultures grew faster on PDA, had more typical colony morphologies, were more pathogenic on bentgrass leaves, and did not contain dsRNA.

Although hypovirulence and the presence of dsRNA have been reported for a number of plant pathogenic fungi, no information exists on the use of hypovirulence, associated with dsRNA, to control *Fusarium*-incited diseases. Only few studies presenting a precise description and detection of virus-like particles (VLPs) and dsRNA elements in some *Fusarium* spp. were performed (15, 44, 45, 55, 56, 25, 66).

Chosson et al. (15) reported the presence of virus-like particles in some *Fusarium* species. They indicated that a strain of *F. oxysporum* f. sp. *lini*, a wilt pathogen of flax, and *F. roseum* cv. *Culmorum* harbored some virus-like particles. The frequency of virus-infected strains was found to appear around 1 in 30 for *F. oxysporum* f. sp. *lini*. For *F. roseum* cv. *Culmorum*, particle diameter was estimated to be 25-40 nm. They stated that any modifications of the pathogenic power of these *Fusarium* species by the presence of the viral complex would result in agronomic consequences.

Lapierre et al. (44) isolated some virus-like particles (VLPs) from *F. oxysporum* (2 out of 50 isolates) and *F. roseum* cv. *Culmorum* (10 out of 18 isolates). They observed some thermostability properties of viral particles of *F. roseum* cv. *Culmorum*, and found that *in vitro* the optimum temperature for multiplication of particles was at 20 C. At 14 C, multiplication was less. At 31.5 C, VLPs were reduced 40 % as compared to the optimum temperature (14 C). It was observed that in the sucrose gradient, the heaviest molecule became less and less concentrated, compared to the light molecule, when the temperature arises. *In vitro*, with low ionic force, the heavy particles were denatured between 50 C and 80 C. The light particles only began to be denatured at 70 C. Their complete denaturation was accomplished at 85 C. The elevated ionic forces did not protect the VLPs of *F. roseum* cv. *Culmorum* from thermal denaturation. They indicated that a thermo-therapy of the virus-infected strains might be envisaged at a temperature around 32 C. In 1975, Lapierre et al. (45) reported a systematic study of the viruses of the genus *Fusarium*. This study contained 45 *F. oxysporum*, 30 *F. roseum*, and 6 *F. solani* strains with a various number of formae speciales. They found two (out of two) *F. oxysporum* f. sp. *lini*, seven (out of 17) *F. roseum* cv. *Culmorum* , and no *F. solani* virus-infected strains. They studied pathogenicity of *F. roseum* cv. *Culmorum* strains *in vitro* on roots of wheat and found a slightly greater frequency of virus-infected strains that were not very pathogenic than virus-free strains.

Fekete et al. (25) assayed 55 geographically different strains of *F. poae*, a secondary pathogen of small grain cereals, for the presence of extrachromosomal nucleic acid elements. All strains were found to harbor dsRNA elements and encapsidated virus-like particles. The numbers of dsRNAs ranged from 1 to 12 among strains with sizes between 0.55 and 12 kb. They found that there were great individual differences in dsRNA patterns of the various strains, but numbers and sizes characteristic for a given isolate remained

unchanged after repeated subculturing of the fungi. Sporulation was normal, and no morphological alterations or signs of degeneration were observed in dsRNA-containing isolates.

Nogawa et al. (56) detected two kinds of dsRNA, estimated to be 1.9 (M1) and 1.7 (M2) kb in size, in extracts from one strain of *F. solani* f. sp. *robiniae*, a pathogen of pseudorobiniae (*Robinia pseudoacacia* L.). They classified this virus in group C of the mycoviruses (11) based on the nonenveloped isometric particle size (30 nm), dsRNA species (1.9 and 1.7 kb), and major capsid polypeptide (38 kDa). They proposed the designation of FusoV for this virus according to the terminology suggested by Pryor and Boelen (60). They indicated that the dsRNA molecules extracted from virus-like particle preparations were identical in electrophoretic mobility to the dsRNAs obtained directly from cells. To investigate the transmission of the dsRNAs through the asexual cycle, 42 independent sublines were obtained by isolating single microconidia from the dsRNA-containing strain. They found that dsRNA were transmitted at a frequency of 85 % through microconidia. They also investigated that the sublines cured of dsRNAs showed no apparent difference in colony morphology (growth rate, pigment production, and spore formation) as compared to the original dsRNA-containing strain. In 1996, Nogawa et al. (55) described a replicase and a transcriptase activity associated with FusoV (54) particles and proposed a replication mechanism for the FusoV dsRNA genome. It was indicated that a nucleotide sequence analysis of cDNAs synthesized from FusoV genomic dsRNAs revealed that M1 (1.9 kb) and M2 (1.7 kb) encoded RNA-dependent RNA polymerase and capsid protein. They reported that an *in vitro* RNA polymerase reaction using purified FusoV particles, that was supplemented with nucleotidetriphosphates, caused the synthesis of single-stranded RNA species and a subsequent formation of dsRNAs having the same size as M1 and M2. Since the ssRNA species synthesized in the first stage were proved to be of positive polarity (coding strand) for both M1 and M2 by dot blot hybridization analysis, they suggested that FusoV genomic dsRNA replicates in a conservative manner.

**Table 2.1.** Association of dsRNA with virulence in plant pathogenic fungi

| Fungus | Effect on virulence | Reference |
|---|---|---|
| *Cryphonectria parasitica* | decreased or no effect | 18, 57 |
| *Rhizoctonia solani* | decreased or no effect | 13, 14 |
| *Ophiostoma ulmi* | decreased | 57 |
| *Phytophthora infestans* | increased | 54, 57, 64 |
| *Gaumannomyces graminis* var. *tritici* | decreased or increased | 57 |
| *Puccinia graminis* f. sp. *tritici* | no effect | 68 |
| *Pyricularia oryzae* | no effect | 39 |
| *Diaporthe phaseolorum* var. *caulivora* | no effect | 57 |
| *Helminthosporium victoriae* | decreased | 57 |
| *Chalara elegans* | decreased or increased | 9 |
| *Leucostoma persoonii* | decreased | 34 |
| *Sclerotinia sclerotiorum* | decreased | 7 |
| *Sclerotinia minor* | decreased | 51 |
| *Sclerotinia homoeocarpa* | decreased | 69 |

## CHAPTER 3: MATERIALS AND METHODS

### 3.1 Obtaining *F. oxysporum* and *F. solani* Isolates

Seeds of Essex soybean were used for all experiments. Naturally infested soil from the P. Minor field in King and Queen County, Va., which has a history of poor Essex soybean emergence, seedling disease, and plant stunting was used for all experiments. Soil was collected from the area which had a high level of disease. The soil was sieved (4 mm mesh), thoroughly mixed using a paint mixer for at least 20 min, and stored moist in cold room at 4-5 C. To obtain *F. oxysporum* and *F. solani* isolates, necrotic and healthy cotyledon, hypocotyl, and root tissues of laboratory-grown plants were washed and cut into small pieces (2 mm), and placed on Komada's *Fusarium*-selective medium (40) and acidified potato-dextrose agar (APDA). Plants were grown in naturally infested soil in a growth chamber using the rolled, plastic-doll method (22), as described below, at 20 C and -0.01 MPa water potential. Farias and Griffin (23, 24) reported that 20 C appeared to be a favorable temperature for disease development as it occurs very frequently in the field at planting time and during disease development according to soil temperature data collected at Warsaw, Virginia (G. J. Griffin, unpublished). In addition, in greenhouse soil-temperature tanks, disease severity was greatest at 20 C and a soil water potential of -0.01 MPa (22). Therefore, these environmental conditions were used in our studies. Sections of necrotic tissues were surface disinfested in 1 % NaClO for 2 min, rinsed in distilled water, and damp-dried on absorbent paper towels before being plated on the media. Plates were incubated at room temperature (25-28 C). Fungi developing from the plated seedling segments were transferred to APDA and APDA-WA (water agar) to be identified. Fungal colonies were identified by microscopic observation of conidium and microconidiophore morphology, along with the cultural characteristics on APDA, such as pigmentation and colony morphology (8, 22, 53). The APDA-WA technique (8) was used for conidium, conidiophore morphology, and chlamydospore production by placing a small APDA plug (about 1 $cm^2$) with the fungus on a 2 % WA plate. A glass cover slip was placed on the WA to facilitate microscopic observations. Fungal colonies were subcultured on PDA slants. Maintenance of cultures was done by transferring active cultures on PDA slants twice a year. After suitable growth and sporulation, the slants were kept at 4-5 C.

### 3.2 Pathogenicity Trials

Monoconidial isolates of *F. oxysporum* and *F. solani* were prepared by the dilution-plate technique. A turbid conidial suspension of each isolate was prepared in a dilution blank containing 9 ml sterile water. An approximately ten-fold serial dilution was prepared by pouring a small portion (~1 ml) from the tube with the turbid suspension to the next blank and pouring was continued until six serial tubes had been prepared. The conidial

suspension in each dilution tube was poured onto a WA plate, and the excess suspension was drained off the plate. The plates were incubated at room temperature for 16 hr. The surface of each plate for conidial germination was examined with a dissecting microscope. A sterile needle was used to cut small squares of agar containing single germinated conidia, and the transferred conidium was placed on a slant of PDA.

A total of 66 monoconidial isolates of *F. oxysporum* and *F. solani* (Table 4.1) from necrotic and healthy tissues of cotyledons, hypocotyls, and roots were tested for pathogenicity on Essex soybean seeds using the rolled, plastic-doll method in an incubator at 20 C and -0.01 MPa water potential (22, 23, 24). In this method, spore suspensions ($10^5$ conidia/ g soil) were prepared by washing 7- to 14-day PDA slants with sterile distilled water and filtering the conidial suspension through cheese-cloth. Conidial densities in the suspension were determined by the use of a hemacytometer under a compound microscope. Soil from the P. Minor field was autoclaved for 1 hr, air-dried, covered, and stored at room temperature. Autoclaved soil (300 g) was artificially infested with $10^5$ conidia/g soil, moistened to -0.01 MPa (field capacity), and spread on a 30X40 cm plastic (polyethylene) sheet. Ten seeds were distributed evenly in the soil. Another plastic sheet was laid over the soil, and seeds and the whole was rolled into a plastic doll. The plastic-dolls were incubated at 20 C in a growth chamber, and seeds were allowed to germinate and grow for 6 days. After this period, seedlings were removed from plastic-dolls, washed, and disease severity on cotyledons and hypocotyls was determined using the following disease scale: 0= healthy plant; 0.5= trace disease (< 1 % of the plant part with lesions); 1= slight disease (2-10 % of the plant part with lesions); 2= moderate disease (11-25 % of the plant part with lesions); 3= extensive disease (26-50 % of the plant part with lesions); 4= severe disease (more than 50 % of the plant part with lesions); 5= dead plant. According to this disease scale, nonpathogenic isolates give no disease, hypovirulent isolates give trace to slight disease, moderately pathogenic isolates give more than slight disease to moderate disease, and highly pathogenic isolates give extensive or more than extensive disease. Reisolations of the test fungi were made from lesions of all the diseased seedlings, as previously described. All experiments were performed three times. The means of each treatment for each experiment were subjected to analysis of variance and Mann-Whitney U nonparametric rank sum test (63) was applied for the separation of means. Statistical analysis was performed by JMP$^R$, version 3.0 (SAS Institute, Cary, NC).

### 3.3 Assay of dsRNA in *F. oxysporum* and *F. solani*

Hypovirulent, moderately pathogenic, and highly pathogenic isolates of *F. oxysporum* and *F. solani* from soybean seedlings were analyzed for the presence of dsRNA. Isolates were cultured on APDA for 7 to 14 days at room temperature, and fungal mycelia were grown in the liquid glucose-yeast extract medium (GYEM) (glucose, 10g/l + yeast extract, 2g/l + $K_2HPO_4$, 1g/l + $MgSO_4.7H_2O$, 0.5g/l + thiamin, 1ml/l + biotin, 1ml/l). Using a cork borer, four to six plugs of mycelium were transferred into Erlenmeyer flasks, each containing 75-100 ml of GYEM and 0.9 ml of antibiotic (streptomycin, 0.5mg/ml + chlortetracycline, 43mg/ml) solution. After 14 days growth at 25 C, mycelium was harvested through filtration with a Buchner funnel by a vacuum pump, dried and stored at -20 C. Fungal mycelium was frozen in liquid nitrogen and ground with glass beads in a chilled mortar and pestle. The dsRNA was extracted and analyzed using the method described by Morris and Dodds (52). Ground samples (>1 g) were extracted with 11 ml phenol (containing 0.1 % 8-hydroxyquinoline), and 5ml of chloroform-isoamylalcohol (24:1) to isolate cellular nucleic acids. The cellular nucleic acids were collected from the aqueous phase by centrifugation at 8,000 r.p.m. for 30 min at 0 to -5 C. The dsRNA was selectively purified from other nucleic acids by binding to CF-11 cellulose powder (Whatman CF-11 cellulose) using STE buffer (Tris + NaCl + EDTA (disodium), pH=6.8) in 17 % ethanol. At certain ethanol concentrations (15-17 %), dsRNA was found to be the only major class of nucleic acid bound to cellulose (52). The collected aqueous phase was poured onto small columns prepared with 2.5 g CF-11 cellulose powder saturated with STE-17 % ethanol. The sample was washed free of ssRNA (single-stranded RNA) with 40-50 ml of STE-17 % ethanol, and the dsRNA was eluted from the cellulose by the addition of 9 ml of STE buffer without ethanol. Traces of DNA were removed from the dsRNA by adding 0.5 M $MgCl_2$ and 20 µl DNase (DNase I from Promega, Madison, WI) , and incubating the mixture at room temperature for 60 min. The dsRNAs were analyzed by agarose gel (0.7 %) electrophoresis in TBE buffer (Tris + boric acid + EDTA (disodium), pH=6.8) for 45 min at 100 V. Gels were stained in ethidium bromide (0.2 µl/ml)-TBE buffer for 10 min and destained in distilled water for 40 min, and then photographed with Polaroid film using a red filter and ultraviolet light source (302 nm). Isolates in which dsRNA was detected were designated as dsRNA-containing isolates. As markers, two or three concentrations of 12.7 kb dsRNA from *C. parasitica* strain EP-713, and a dsDNA ladder (Kb DNA Ladder from Stratagene, La Jolla, CA) containing 15 fragments ranging in size from 250 bp to 12 kb were used. Size of dsRNA bands in *F. oxysporum* isolates was estimated by interpolation on a plot of dsDNA size standards versus band position in the gel. Confirmation of dsRNA

was accomplished by RNase (RNase-A from Sigma, St. Louis, MO) treatment in 0.3 M NaCl for 30 min.

### 3.4 Inoculation of Seeds with Hypovirulent Isolates of *F. oxysporum*

Nine selected monoconidial isolates of hypovirulent *F. oxysporum*, identified in pathogenicity trials, were used in seed treatment studies. Monoconidial isolates were prepared by the single-spore isolation procedure described previously. Among these nine hypovirulent isolates, six isolates were dsRNA-free and three were dsRNA-containing isolates. Spore suspensions ($10^6$ conidia/ ml) were prepared by washing the 7-to 14-day old PDA slants with sterile distilled water and filtering the suspension through cheesecloth. Conidial densities in the suspension were determined by use of a hemacytometer under a compound microscope. Essex soybean seed surfaces were covered with conidial suspensions of hypovirulent isolates ($10^6$ conidia/ ml) in water containing 2 % (w/v) Methocel A4C Premium$^R$ (Dow Chemical Co. Midland, MI) as a spreader and sticker. In previous studies with pea and radish, Methocel did not influence seedling disease of either host (34, 35). Preliminary results indicated that *P. ultimum*, the main component of a fungal complex associated with the early phase of the Essex seedling disease, and a contributor to the late phase of the disease, was not controlled by these hypovirulent isolates. This *P. ultimum* incited disease (water-soaked lesions and preemergence-damping off) interfered with assessment of *Fusarium*-incited disease. Therefore, in addition to the prior inoculation of seeds with individual hypovirulent isolates alone, the combination of a *Pythium*-specific fungicide, Apron$^R$ 25 W (metalaxyl), and hypovirulent isolates was used. In this approach, seeds were treated with a combination of a conidial suspension of hypovirulent isolates ($10^6$ conidia/ ml) in water containing 2 % Methocel and Apron$^R$ with the commercially recommended rate (1.25 g a.i. per kg seed). For controls, seeds were treated with the combination of Methocel and Apron$^R$, with Methocel alone, and not treated with Methocel. For each treatment, 30 Essex soybean seeds were used and each treatment was performed three times for each isolate. The soil was thoroughly mixed in a paint mixer for at least 20 min before each experiment. Seeds were dipped into the treatment suspensions for 10 min. Treated seeds were planted in naturally infested soil using the plastic-doll method, as described previously, and incubated in a growth chamber at 20 C and -0.01 MPa water potential for 6 days. After 6 days, seedlings were removed from plastic dolls, washed in running tap water, and disease incidence and severity on cotyledons and hypocotyls were determined using the same disease scale as indicated in methods for pathogenicity trials. Diseased plant tissues were plated out on Komada's selective medium and APDA to recover *F. oxysporum* and *F. solani,* and on WA to recover *P. ultimum,* and *R. solani.* All

experiments were performed three times. The means of each treatment for each experiment were subjected to analysis of variance, and Fisher's LSD test (63) was applied for the separation of means. Statistical analysis was performed by JMP$^R$, version 3.0 (SAS Institute, Cary, NC).

Growth chamber tests on seedling emergence and disease severity were conducted with the best hypovirulent isolates according to the results of the plastic-doll, seed-treatment studies. These seedling tests were conducted in Cone-Tainers containing naturally infested soil at -0.01 MPa water potential. Cone-Tainers were placed in a growth chamber at 20 C and a photo period of 14 hr. Seeds were treated with different treatment suspensions, as indicated above, before planting in Cone-Tainers. Twenty seeds were used for each treatment. Two seeds per Cone-Tainer were planted at a depth of 3.8 cm, and covered with a 1 L glass beaker that retarded moisture loss, but permitted gas exchange through the pouring spout. The soil was adjusted to -0.01 MPa water potential before use. Soil moisture was maintained by weighing Cone-Tainers each day and adding the small amount of water required with an atomizer. Emergence of seedlings was evaluated daily. After 8 days, all seedlings or seeds were removed from the Cone-Tainers, washed in running tap water, and seedling emergence, disease incidence and severity were determined. Diseased plant tissues were plated out on Komada's selective medium and APDA to recover *F. oxysporum,* and *F. solani,* and on WA to recover *P. ultimum,* and *R. solani,* as described previously. All experiments were performed two times. The means of each treatment for each experiment were subjected to analysis of variance, and Fisher's LSD test (63) was applied for the separation of means. Statistical analysis was performed by JMP$^R$, version 3.0 (SAS Institute, Cary, NC).

### 3.5 Curing of dsRNA-Containing Hypovirulent Isolates

To determine the association between the presence of dsRNA and virulence of *F. oxysporum* isolates, curing (elimination) of dsRNA from selected hypovirulent isolates was attempted using cycloheximide (Sigma Chemical Co., St. Louis, MO). Hypovirulent isolates were first cultured on PDA for 7 days, and then plugs from the actively growing margin were transferred onto PDA amended with cycloheximide at 0, 15, 20, 40, 60, 140, and 280 μg/ml. Plates were incubated at room temperature. There were three plates for each cycloheximide concentration. After 14-20 days, hyphal tips were taken from growing margins of individual colonies under the dissecting microscope, and subcultured on PDA plates at room temperature for 14 days. Resulting colonies were evaluated for colony morphology, the presence of dsRNA, and virulence, as described previously.

In addition to attempts to cure mycelial cultures of dsRNA by using cycloheximide, single-sporing of dsRNA-containing hypovirulent *F. oxysporum* isolates was also attempted to obtain dsRNA-free isolates. This approach has been used previously for several fungi (19, 56). Single conidia from each dsRNA-containing hypovirulent isolate were cultured on APDA by using the single-spore isolation procedure described previously. Ten single spore subcultures for each dsRNA-containing hypovirulent *F. oxysporum* isolate were evaluated for colony morphology, and the presence of dsRNA, as described previously.

# CHAPTER 4: RESULTS

## 4.1 Identification of *F. oxysporum* and *F. solani*

Fungal colonies were identified by microscopic observation of conidium and microconidiophore morphology on APDA-WA plates, along with the cultural characteristics on APDA, such as pigmentation and colony morphology. All isolates of *F. oxysporum* produced abundant, single-celled , oval or oblong microconidia. Short and unbranched microconidiophores, and long, four- to five-septate, slightly falcate macroconidia were the most important morphological characteristics to identify *F. oxysporum*. Chlamydospores were usually terminal and one- or two-celled. Isolates of *F. solani* produced mostly sparse, single-celled, oval or oblong microconidia. Macroconidia were abundant, medium to long, stout, and three- to four-septate. Microconidia were characteristically produced on long microconidiophores. Chlamydospores were intercalary and terminal. The isolates had different colony characteristics and pigmentation on APDA. The aerial mycelium of *F. oxysporum* isolates ranged from white to purple and the undersurface of colonies ranged from white to dark purple. The aerial mycelium and the undersurface of colonies for *F. solani* isolates ranged from cream to tan.

## 4.2 Isolates of *F. oxysporum* and *F. solani* recovered from diseased and healthy Essex soybean plant tissues

A summary of the numbers of *F. oxysporum* and *F. solani* isolates obtained from necrotic and healthy cotyledon, hypocotyl, and root tissues of soybean plants is presented in Table 4.1. Plants were grown in naturally infested soil using the rolled plastic-doll method in an incubator at 20 C and -0.01 MPa water potential. Of the 66 isolates of *F. oxysporum* and *F. solani* obtained from necrotic and healthy tissues of cotyledons, hypocotyls, and roots, 57 were *F. oxysporum* (86.4 %), and nine were *F. solani* (13.6 %). From cotyledon lesions, 30 *F. oxysporum* and 5 *F. solani* isolates were obtained. Nine *F. oxysporum* and one *F. solani* isolates were recovered from lesions on the hypocotyl. From root lesions, one of *F. oxysporum* and three *F. solani* isolates were recovered. Of the 17 *F. oxysporum* isolates obtained from healthy tissues, nine were isolated from cotyledons and eight were from hypocotyls. No *F. solani* was isolated from healthy soybean seedling tissues (Table 4.1). *F. oxysporum* was the predominant fungus isolated from lesions on cotyledons (85.7 %) and hypocotyls (90 %), while *F. solani* was the predominant fungus isolated from lesions on roots (75 %), respectively (Table 4.1).

**Table 4.1.** Number of Fusarium oxysporum and F. solani isolates obtained from necrotic and healthy cotyledon, hypocotyl, and root tissues of plants grown in naturally infested P.Minor field soil, using the rolled plastic-doll method, at 20 C and -0.01 MPa water potential[1]

| | Cotyledons | | Hypocotyls | | Roots | |
|---|---|---|---|---|---|---|
| **Fungus** | **Necrotic** | **Healthy** | **Necrotic** | **Healthy** | **Necrotic** | **Healthy** |
| *F. oxysporum* | 30 | 9 | 9 | 8 | 1 | 0 |
| *F. solani* | 5 | 0 | 1 | 0 | 3 | 0 |

[1] Isolations from necrotic and healthy tissues plated on Komada's medium and acidified, potato-dextrose agar (APDA).

### 4.3 Virulence of *F. oxysporum* and *F. solani* isolates

Of the 66 isolates of *F. oxysporum* and *F. solani* tested in artificially infested, autoclaved P. Minor field soil, using the plastic-doll method, in an incubator at 20 C and -0.01 MPa water potential, 23 isolates (34.9 %) were rated as hypovirulent, 42 isolates (63.6 %) were rated as moderately pathogenic, and only 1 isolate (1.5 %) was rated as highly pathogenic. No nonpathogenic isolates were identified from these trials. A summary of the source, virulence, species, identity and disease severity ratings of 66 isolates tested is presented in Table 4.2. All *F. solani* isolates were found to be moderately pathogenic. Of the 23 hypovirulent *F. oxysporum* isolates, seven were isolated from lesions on cotyledons, six were isolated from healthy tissues of cotyledons, five were isolated from lesions on hypocotyls, and five were isolated from healthy hypocotyl tissues. Isolate *F. oxysporum* 4, obtained from a severe cotyledon lesion, was identified as the only highly pathogenic isolate (Table 4.2). The difference between the mean disease severity of this isolate and the mean disease severity of any hypovirulent isolates was important. Since the data of disease severity ratings of 66 isolates showed the bimodal distribution, none of the multiple comparison procedures, dependent on a normal distribution, could be applied for the analysis of variance and for the separation of means. To be able to demonstrate the differences between the mean of hypovirulent isolates disease severity ratings and the mean of moderately pathogenic isolates disease severity ratings, Mann-Whitney U nonparametric rank sum test (63) was applied. This test ranks all of the observations together, analyzes the ranks and then compares the sum of the ranks for one group to the sum of the ranks of another group and determines a p-value (63). As a result of this nonparametric test, the mean for hypovirulent isolates, 0.72, was found to be significantly ($p<0.0001$) less than the mean of moderately pathogenic isolates, 2.23.

At the termination of each experiment, *F. oxysporum* and *F. solani* isolates added to soil were always recovered from necrotic cotyledon, hypocotyl or root tissues of soybean plants.

**Table 4.2.** Source, virulence, identity and disease severity rating of *Fusarium oxysporum* and *F. solani* isolates on Essex soybean in artificially infested soil at 20 C and -0.01 MPa water potential in plastic-doll assays

| Isolate Number | Species | Source | Disease severity[1,2,3] | Virulence rating[4] |
|---|---|---|---|---|
| 4 | *F. oxysporum* | necrotic cotyledon | 3.05 | Highly pathogenic |
| 42 | *F. oxysporum* | necrotic hypocotyl | 2.96 | Moderately pathogenic |
| 53 | *F. oxysporum* | necrotic cotyledon | 2.94 | Moderately pathogenic |
| 48 | *F .oxysporum* | necrotic root | 2.65 | Moderately pathogenic |
| 19 | *F. solani* | necrotic cotyledon | 2.61 | Moderately pathogenic |
| 50 | *F. solani* | necrotic root | 2.46 | Moderately pathogenic |
| 28 | *F. oxysporum* | necrotic cotyledon | 2.46 | Moderately pathogenic |
| 52 | *F. oxysporum* | necrotic cotyledon | 2.43 | Moderately pathogenic |
| 60 | *F. oxysporum* | healthy hypocotyl | 2.37 | Moderately pathogenic |
| 13 | *F. oxysporum* | healthy hypocotyl | 2.35 | Moderately pathogenic |
| 49 | *F. solani* | necrotic root | 2.35 | Moderately pathogenic |
| 66 | *F. oxysporum* | necrotic hypocotyl | 2.31 | Moderately pathogenic |
| 18 | *F. solani* | necrotic cotyledon | 2.30 | Moderately pathogenic |
| 55 | *F. oxysporum* | healthy cotyledon | 2.30 | Moderately pathogenic |
| 51 | *F. solani* | necrotic root | 2.28 | Moderately pathogenic |
| 64 | *F. solani* | necrotic cotyledon | 2.27 | Moderately pathogenic |
| 44 | *F. oxysporum* | necrotic cotyledon | 2.26 | Moderately pathogenic |
| 24 | *F. oxysporum* | necrotic cotyledon | 2.26 | Moderately pathogenic |
| 65 | *F. solani* | necrotic hypocotyl | 2.25 | Moderately pathogenic |
| 33 | *F. oxysporum* | necrotic cotyledon | 2.22 | Moderately pathogenic |
| 21 | *F. solani* | necrotic cotyledon | 2.21 | Moderately pathogenic |
| 57 | *F. oxysporum* | healthy cotyledon | 2.19 | Moderately pathogenic |
| 37 | *F. oxysporum* | necrotic cotyledon | 2.19 | Moderately pathogenic |
| 9 | *F. oxysporum* | necrotic cotyledon | 2.19 | Moderately pathogenic |
| 38 | *F. oxysporum* | necrotic cotyledon | 2.13 | Moderately pathogenic |
| 35 | *F. oxysporum* | necrotic cotyledon | 2.13 | Moderately pathogenic |
| 2 | *F. oxysporum* | necrotic cotyledon | 2.12 | Moderately pathogenic |
| 27 | *F. oxysporum* | necrotic cotyledon | 2.11 | Moderately pathogenic |
| 5 | *F. oxysporum* | necrotic cotyledon | 2.09 | Moderately pathogenic |
| 20 | *F. solani* | necrotic cotyledon | 2.09 | Moderately pathogenic |
| 45 | *F. oxysporum* | necrotic cotyledon | 2.09 | Moderately pathogenic |
| 11 | *F. oxysporum* | necrotic cotyledon | 2.08 | Moderately pathogenic |
| 63 | *F. oxysporum* | healthy hypocotyl | 2.06 | Moderately pathogenic |
| 39 | *F. oxysporum* | necrotic cotyledon | 2.05 | Moderately pathogenic |
| 12 | *F. oxysporum* | healthy cotyledon | 2.05 | Moderately pathogenic |
| 36 | *F. oxysporum* | necrotic cotyledon | 2.04 | Moderately pathogenic |
| 34 | *F. oxysporum* | necrotic cotyledon | 2.04 | Moderately pathogenic |

| | | | | |
|---|---|---|---|---|
| 32 | *F. oxysporum* | necrotic cotyledon | 2.03 | Moderately pathogenic |
| 30 | *F. oxysporum* | necrotic cotyledon | 2.02 | Moderately pathogenic |
| 29 | *F. oxysporum* | necrotic cotyledon | 2.02 | Moderately pathogenic |
| 54 | *F. oxysporum* | necrotic cotyledon | 2.00 | Moderately pathogenic |
| 47 | *F. oxysporum* | necrotic cotyledon | 1.97 | Moderately pathogenic |
| 40 | *F. oxysporum* | necrotic cotyledon | 1.89 | Moderately pathogenic |
| 31 | *F. oxysporum* | healthy cotyledon | 0.90 | Hypovirulent |
| 17 | *F. oxysporum* | healthy hypocotyl | 0.89 | Hypovirulent |
| 22 | *F. oxysporum* | necrotic cotyledon | 0.88 | Hypovirulent |
| 26 | *F. oxysporum* | necrotic cotyledon | 0.88 | Hypovirulent |
| 41 | *F. oxysporum* | necrotic hypocotyl | 0.87 | Hypovirulent |
| 25 | *F. oxysporum* | necrotic cotyledon | 0.85 | Hypovirulent |
| 10 | *F. oxysporum* | necrotic cotyledon | 0.85 | Hypovirulent |
| 16 | *F. oxysporum* | necrotic hypocotyl | 0.83 | Hypovirulent |
| 14 | *F. oxysporum* | healthy hypocotyl | 0.82 | Hypovirulent |
| 46 | *F. oxysporum* | healthy cotyledon | 0.80 | Hypovirulent |
| 15 | *F. oxysporum* | healthy hypocotyl | 0.76 | Hypovirulent |
| 8 | *F. oxysporum* | necrotic cotyledon | 0.75 | Hypovirulent |
| 7 | *F. oxysporum* | necrotic hypocotyl | 0.74 | Hypovirulent |
| 43 | *F. oxysporum* | necrotic hypocotyl | 0.70 | Hypovirulent |
| 1 | *F. oxysporum* | necrotic hypocotyl | 0.70 | Hypovirulent |
| 3 | *F. oxysporum* | necrotic cotyledon | 0.64 | Hypovirulent |
| 58 | *F. oxysporum* | healthy cotyledon | 0.60 | Hypovirulent |
| 62 | *F. oxysporum* | healthy hypocotyl | 0.59 | Hypovirulent |
| 6 | *F. oxysporum* | necrotic cotyledon | 0.55 | Hypovirulent |
| 56 | *F. oxysporum* | healthy cotyledon | 0.54 | Hypovirulent |
| 61 | *F. oxysporum* | healthy hypocotyl | 0.52 | Hypovirulent |
| 23 | *F. oxysporum* | healthy cotyledon | 0.52 | Hypovirulent |
| 59 | *F. oxysporum* | healthy cotyledon | 0.49 | Hypovirulent |

[1] Disease severity was rated, after 6 days, using a disease scale from 0=healthy plant to 5= dead plant.

[2] Values are average of three experiments with 10 plants per experiment.

[3] The mean for hypovirulent isolates is significantly less than the mean for moderately pathogenic isolates ($p<0.0001$) according to the Mann-Whitney U nonparametric rank sum test.

[4] The degree of pathogenicity of each isolate was determined according to the disease severity ratings. Hypovirulent isolates give trace, 0.5, or slight disease, 1, moderately pathogenic isolates give more than slight disease, >1, or moderate disease, ≥2, and highly pathogenic isolate gives extensive or more than extensive disease, ≥3.

### 4.4 Characteristics of the symptoms associated with *F. oxysporum* and *F. solani* on Essex soybean seedlings

A summary of the characteristics of the lesions from which *F. oxysporum* and *F. solani* were isolated in all experiments is presented in Table 4.3 and illustrated in Figures 4.1 to 4.4. The appearance and the type of lesions on Essex soybean plants in P. Minor field soil were found to be similar to those in pathogenicity trials with autoclaved, artificially infested P. Minor field soil, and also to lesions in seed inoculation studies with naturally infested P. Minor field soil. *Fusarium oxysporum* was generally isolated from dark brown, deep, sunken cotyledon lesions. Both *F. oxysporum* and *F. solani* were isolated from reddish brown, small, discrete lesions and light brown small dots on cotyledons, from light to dark brown dots or elongated lesions on hypocotyls, and from brown girdling and elongated sunken lesions located on the hypocotyl-root transition zone. Lesions on roots were mostly small, light brown and superficial.

**Table 4.3.** Types of lesions on cotyledons, hypocotyls and roots that were associated with *Fusarium oxysporum* and *F. solani* infection of Essex soybean plants grown in artificially infested and naturally infested P.Minor field soil at 20 C and -0.01 MPa water potential

| | **Lesion type** | | |
|---|---|---|---|
| **Fungus** | **Cotyledons** | **Hypocotyls** | **Roots** |
| *F. oxysporum* | - dark brown, sunken<br>- discrete, small dots<br>- reddish brown, elongated | - reddish-brown, elongated, superficial<br>- small dots<br>- sunken, girdling, elongated on transition zone | - small, light brown, superficial |
| *F. solani* | - reddish-brown small, discrete,<br>- superficial light brown dots | - reddish-brown, sunken<br>- superficial, elongated<br>- light brown dots | - small, light brown, superficial |

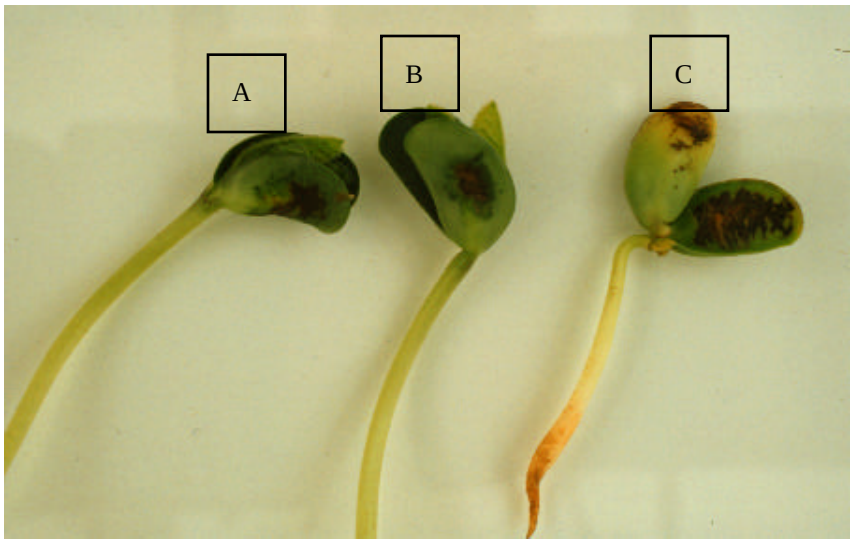


**Figure 4.1.** Lesions on cotyledons and hypocotyls of 8-day old Essex soybean seedlings grown in P. Minor naturally infested soil, using Cone-Tainers, in growth chamber at 20 C, -0.01 MPa water potential and a photo period of 14 hr. A) and B) Large, brown lesions on cotyledons from which *F. oxysporum* was isolated. C) Large, brown, sunken lesions on cotyledons from which *F. oxysporum* was isolated, and brown, sunken lesions on hypocotyl-root transition zone and radicle from which *F. solani* was isolated.


**Figure 4.2.** Lesions on cotyledons and hypocotyls of 8-day old Essex soybean seedlings grown in P. Minor naturally infested soil, using Cone-Tainers, in growth chamber at 20 C, -0.01 MPa water potential, and a photo period of 14 hr. A) Seedling with no lesions on cotyledon and hypocotyl. Some discoloration of root occurred following exposure to air. B) Large, brown, sunken lesions on cotyledons from which *F. oxysporum* was isolated, and brown, sunken lesions on hypocotyl-root transition zone and radicle from which *F. solani* was isolated.


**Figure 4.3.** Lesions on cotyledons and hypocotyls of 6-day old Essex soybean seedlings grown in P. Minor naturally infested soil, using plastic-dolls, in growth chamber at 20 C and -0.01 MPa water potential. A) Brown, sunken lesions on cotyledon and hypocotyl from which *F. oxysporum* was isolated. B) Brown, discrete lesion on cotyledon from which *F. oxysporum* was isolated, and brown, sunken, elongated lesions on hypocotyl and severe lesion on hypocotyl-root transition zone from which *F. oxysporum* was isolated.

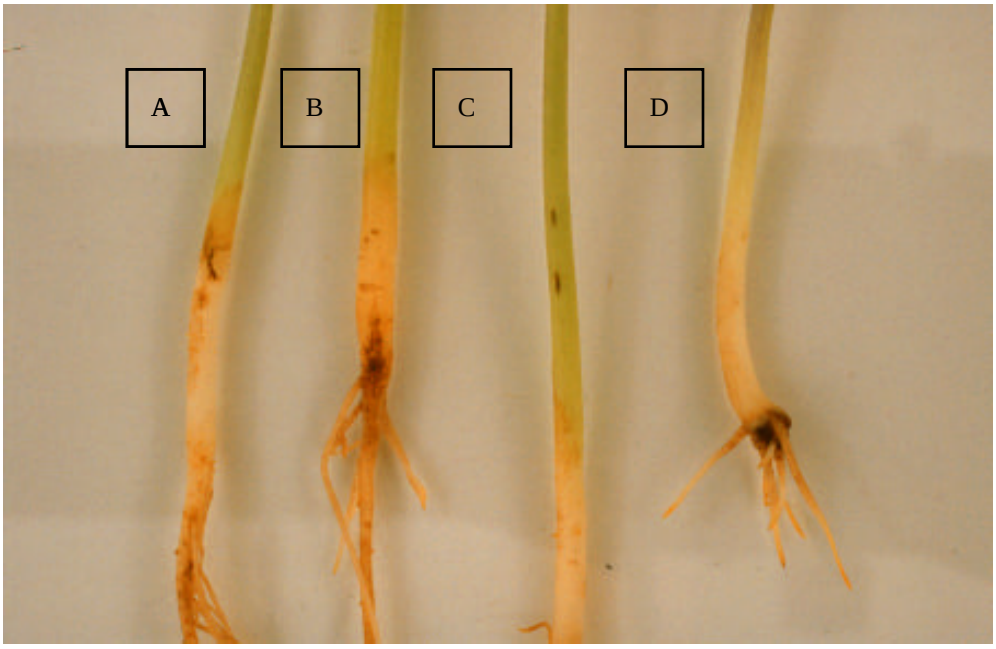


**Figure 4.4.** Lesions on hypocotyls and hypocotyl-root transition zones of 8-day old Essex soybean seedlings grown in P.minor naturally infested soil, using Cone-Trainers, in growth chamber at 20 C, -0.01 MPa water potential, and a photo period of 14 hr. A) Brown, superficial lesion on hypocotyl from which *F. solani* was isolated. B) Large, brown, sunken lesion on hypocotyl-root transition zone from which *F. oxysporum* was isolated. C) Small, reddish-brown, elongated lesion on hypocotyl from which *F. solani* was isolated. D) Brown, sunken lesion on hypocotyl-root transition zone from which *F. solani* was isolated.

### 4.5 Assay of dsRNA in *F. oxysporum* and *F. solani*

One highly pathogenic *F. oxysporum*, thirty-three moderately pathogenic *F. oxysporum*, nine moderately pathogenic *F. solani*, and twenty-three hypovirulent *F. oxysporum* isolates from soybean seedlings were analyzed for the presence of dsRNA. Isolates in which dsRNA was detected were designated as dsRNA-containing isolates. DsRNA was detected in six of the 66 isolates (9.1 %) that were assayed. No dsRNA was detected in *F. solani* isolates. Of the six dsRNA-containing *F. oxysporum* isolates, three were hypovirulent (*F. oxysporum* 1, *F. oxysporum* 3, *F. oxysporum* 6), two were moderately pathogenic (*F. oxysporum* 2 and *F. oxysporum* 5), and one isolate (*F. oxysporum* 4) was highly pathogenic. A summary of the virulence and colony characteristics of dsRNA-containing isolates is presented in Table 4.4. Some hypovirulent isolates contained dsRNA while other hypovirulent isolates did not. After agarose gel electrophoresis, four segments (bands) were visible for all six dsRNA-containing isolates. Based on the dsDNA plot, the sizes of dsRNA segments were estimated to be 4.0 kb, 3.1 kb, 2.7 kb, and 2.2 kb, and were the same for all six dsRNA-containing *F. oxysporum* isolates. These segments were resistant to digestion by DNase and resistant to RNase at high ionic strength (0.3 M NaCl) (Figures 4.5 and 4.6) and, therefore, were confirmed to be dsRNA. As size markers, the kb dsDNA ladder, containing 15 fragments, ranging in size from 250 bp to 12 kb, and two or three concentrations of 12.7 kb dsRNA from *C. parasitica* strain EP-713 were used (Figures 4.6 and 4.7). Two trials on each isolate were done on dsRNA and results were the same for each trial.

**Table 4.4.** Virulence and cultural characteristics of dsRNA-containing *Fusarium oxysporum* isolates

| **Isolate** | **Virulence**[1] | **Color On PDA** | |
|---|---|---|---|
| | | **mycelium**[2] | **reverse**[3] |
| *F. oxysporum* 1 | Hypovirulent | purple | purple |
| *F. oxysporum* 3 | Hypovirulent | white and light purple | light purple |
| *F. oxysporum* 6 | Hypovirulent | white | purple |
| *F. oxysporum* 2 | Moderately pathogenic | purple | purple |
| *F. oxysporum* 5 | Moderately pathogenic | white | purple |
| *F. oxysporum* 4 | Highly pathogenic | white | purple |

[1] The degree of pathogenicity of isolates was determined according to the disease severity ratings as a result of pathogenicity trials.

[2] Color of mycelium from top of colony.

[3] Color of colony from below.

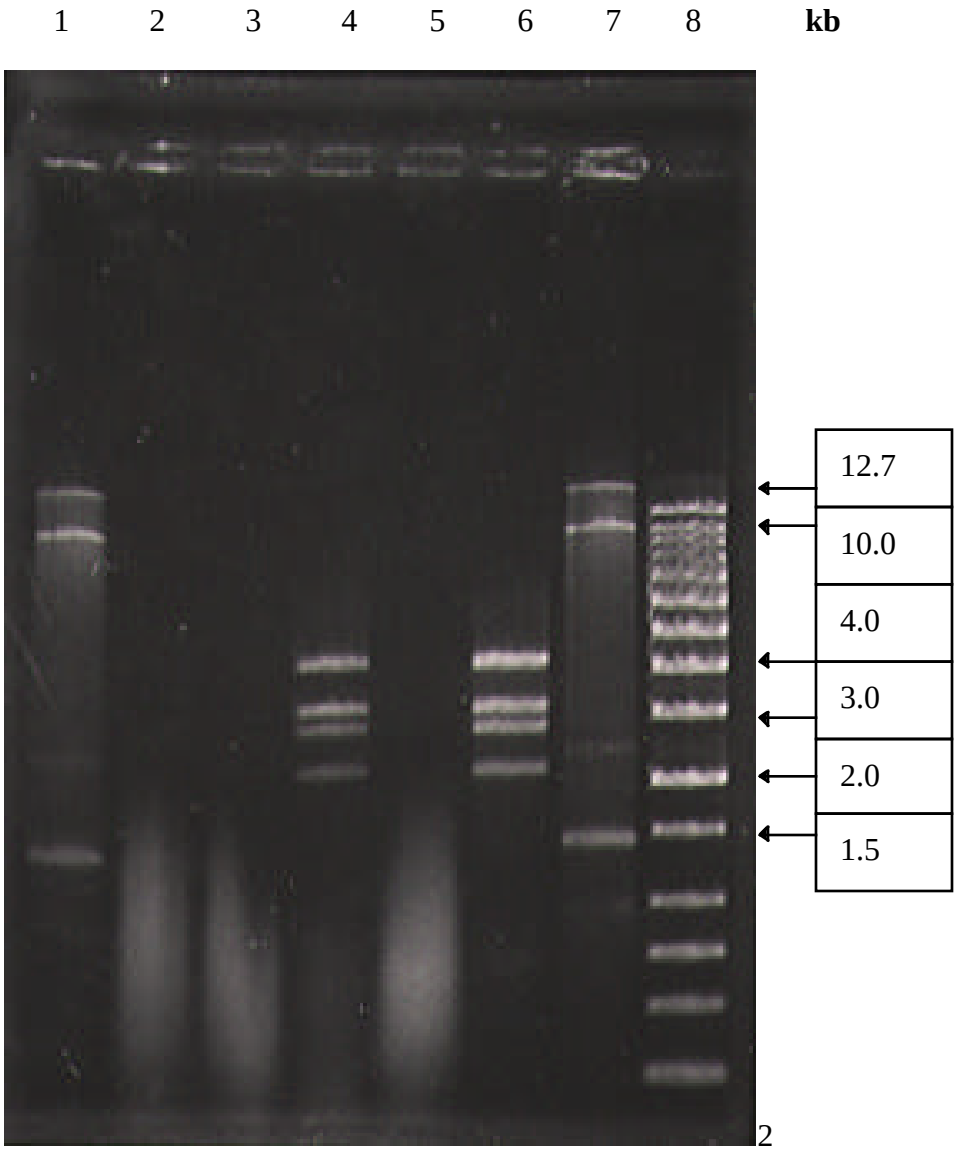


**Figure 4.5.** Double-stranded (ds) RNA segment patterns of two hypovirulent *F. oxysporum* isolates on agarose gel (0.7 %), stained with ethidium bromide, before RNase treatment. Lanes 1 and 7, 1/10 concentration of dsRNA from *Cryphonectria parasitica* strain EP-713 and undiluted dsRNA from *C. parasitica* strain EP-713 (1.3, 10.0, and 12.7 kb) used as size standards. Lanes 2 and 3, dsRNA-free hypovirulent isolates *F. oxysporum* 10 and 31. Lane 5, dsRNA-free moderately pathogenic isolate *F. oxysporum* 11. Lanes 4 and 6, dsRNA from hypovirulent *F. oxysporum* 6 and *F. oxysporum* 1. Lane 8, kb DNA ladder (0.25 to 12.0 kb) used as a size standard. Kb sizes shown are for EP-713 dsRNA and kb DNA ladder.

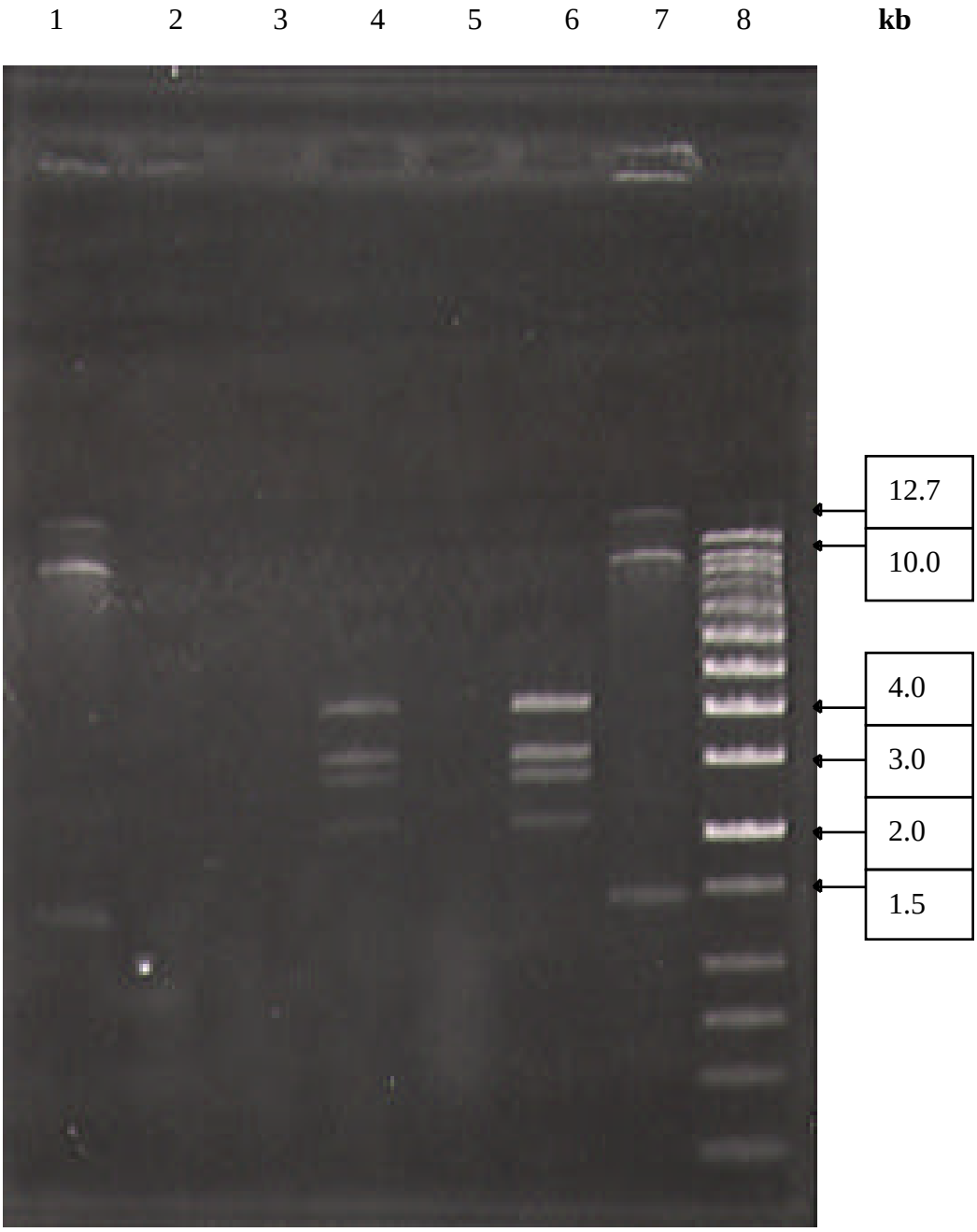


**Figure 4.6.** Double-stranded (ds) RNA segment patterns of two hypovirulent *F. oxysporum* isolates on agarose gel (0.07 %), stained with ethidium bromide, after RNase (0.01 mg/ml) treatment in 0.03 M NaCl for 30 min. Lanes 1 and 7, 1/10 concentration of dsRNA from *Cryphonectria parasitica* strain EP-713 and undiluted dsRNA from *C. parasitica* strain EP-713 (1.3, 10.0, and 12.7 kb) used as size standards. Lanes 2 and 3, dsRNA-free hypovirulent isolates *F. oxysporum* 10 and 31. Lane 5, dsRNA-free moderately pathogenic isolate *F. oxysporum* 11. Lanes 4 and 6, dsRNA from hypovirulent *F. oxysporum* 6 and *F. oxysporum* 1. Lane 8, kb DNA ladder (0.25 to 12.0 kb) used as a size standard. Kb sizes shown are for EP-713 dsRNA and kb DNA ladder.

### 4.6 Inoculation of seeds with hypovirulent isolates of *F. oxysporum*

#### 4.6.1 Effect of prior inoculation of Essex soybean seeds with hypovirulent *F. oxysporum* isolates on disease severity in naturally infested soil using plastic-doll assays

Nine selected monoconidial isolates of hypovirulent *F. oxysporum*, identified in pathogenicity trials, were used for prior inoculation of seeds. These hypovirulent *F. oxysporum* isolates, 59, 23, 61, 56, 6, 62, 58, 3, and 1, gave the lowest-ranked disease severity ratings (Table 4.2) in pathogenicity studies using artificially infested soil. Among these nine hypovirulent isolates, six isolates (59, 23, 61, 56, 62, 58) were dsRNA-free and three isolates(6, 3, 1) were dsRNA-containing isolates.

During the preliminary experiments of this study we observed that prior inoculation of seeds with hypovirulent isolates did not control *P. ultimum*, the main component of a fungal complex associated with the early phase of the Essex seedling disease**,** and a contributor to the late phase of the disease. This fungus was isolated on WA from characteristic water-soaked, brown, large lesions on cotyledons and hypocotyls (Figure 4.7) with variable frequency, resulted in the increase of disease severity index, and interfered with evaluations of disease caused by *F. oxysporum* and *F. solani* as indicated. Therefore, in addition to the prior inoculation of seeds with individual hypovirulent *F. oxysporum* isolates alone, the combination of metalaxyl (Apron$^{R}$ 25 W), a *Pythium*-specific fungicide, plus hypovirulent *F. oxysporum* isolates was applied to the seeds. Data from separate trials, conducted with each hypovirulent isolate, were combined in the analysis of variance. Treatment of seeds with the combination of individual dsRNA-free and dsRNA-containing hypovirulent *F. oxysporum* isolates plus Apron$^{R}$ significantly ($p<0.05$) reduced the disease severity index of both cotyledons and hypocotyls grown in naturally infested soil, compared to the control plants treated with the combination of Methocel plus Apron$^{R}$, with Methocel alone, or not treated with Methocel (Table 4.5 and Figures 4.8 and 4.9). Treatment of seeds with dsRNA-free hypovirulent isolates alone (no Apron$^{R}$ treatment) also significantly ($p<0.05$) reduced disease severity compared to the controls. No significant ($p>0.05$) difference was found between dsRNA-containing and dsRNA-free hypovirulent isolates for their effect on the decrease of disease severity (Table 4.5). Methocel was found to have no influence on the disease severity of plants in these studies (Table 4.5). No *R. solani* was isolated from any diseased plant tissues. No *P. ultimum* was recovered from the treated plants in which Apron$^{R}$ was used. The results for the isolation of *F. oxysporum*, *F. solani* and *P. ultimum* from lesions on cotyledons, hypocotyls, and roots of plant tissues are summarized in Table 4.6. Isolation frequencies of *F. oxysporum* and *F. solani* from

diseased plant tissues varied among different treatment groups (Table 4.6). However, *F. oxysporum* and *F. solani* were isolated less frequently from plants treated with hypovirulent isolates, either alone or as combination with metalaxyl, compared to the control plants.

**Figure 4.7.** Lesions on cotyledons, hypocotyls, and roots of 8-days old Essex soybean seedlings grown in P. Minor naturally infested soil, osing Cone-Trainers, in growth chamber at 20 C, -0.01 MPa water potential, and a photo period of 14 hr. Brown, water soaked lesions on coty;edons, hypocotyls, and roots from which *Pythium ultimum* was isolated.

**Table 4.5** Effect of prior inoculation of Essex soybean seeds with dsRNA-free and dsRNA-containing hypovirulent *Fusarium oxysporum* isolates on disease severity of plants grown in naturally infested P.Minor field soil at 20 C AND -0.01 MPa water potential in plastic-doll assays

| **Treatment**[1,2,3] | **Disease severity ratings**[4,5] | | |
|---|---|---|---|
| | **cotyledon** | **hypocotyl** | **average** |
| Control with Methocel | $2.52^{A}$ | $2.45^{A}$ | $2.49^{A}$ |
| Control without Methocel | $2.40^{AB}$ | $20.33^{AB}$ | $2.37^{AB}$ |
| Control with Apron+Methocel | $2.03^{B}$ | $1.91^{B}$ | $1.97^{B}$ |
| *F.oxysporum* $6^{6}$+Methocel | $1.23^{C}$ | $1.27^{C}$ | $1.25^{C}$ |
| *F.oxysporum* $59^{7}$+Methocel | $1.15^{C}$ | $1.07^{C}$ | $1.11^{C}$ |
| *F.oxysporum* $1^{6}$+Methocel | $1.16^{C}$ | $1.02^{C}$ | $1.09^{C}$ |
| *F.oxysporum* $3^{6}$+Methocel | $1.15^{C}$ | $1.01^{C}$ | $1.08^{C}$ |
| *F.oxysporum* $58^{7}$+Methocel | $1.06^{C}$ | $0.92^{C}$ | $0.99^{C}$ |
| *F.oxysporum* $61^{7}$+Methocel | $1.08^{C}$ | $0.82^{C}$ | $0.95^{C}$ |
| *F.oxysporum* $23^{7}$+Methocel | $0.98^{C}$ | $0.79^{C}$ | $0.89^{C}$ |
| *F.oxysporum* $62^{7}$+Methocel | $0.93^{C}$ | $0.82^{C}$ | $0.88^{C}$ |
| *F.oxysporum* $56^{7}$+Methocel | $0.72^{CD}$ | $0.62^{CD}$ | $0.67^{CD}$ |
| *F.oxysporum* $3^{6}$+Methocel+Apron | $0.50^{D}$ | $0.46^{D}$ | $0.48^{D}$ |
| *F.oxysporum* $56^{7}$+Methocel+Apron | $0.38^{D}$ | $0.35^{D}$ | $0.36^{D}$ |
| *F.oxysporum* $1^{6}$+Methocel+Apron | $0.45^{D}$ | $0.23^{D}$ | $0.34^{D}$ |
| *F.oxysporum* $6^{6}$+Methocel+Apron | $0.46^{D}$ | $0.18^{D}$ | $0.32^{D}$ |
| *F.oxysporum* $23^{7}$+Methocel+Apron | $0.32^{D}$ | $0.28^{D}$ | $0.30^{D}$ |
| *F.oxysporum* $58^{7}$+Methocel+Apron | $0.34^{D}$ | $0.24^{D}$ | $0.29^{D}$ |
| *F.oxysporum* $62^{7}$+Methocel+Apron | $0.32^{D}$ | $0.24^{D}$ | $0.28^{D}$ |
| *F.oxysporum* $61^{7}$+Methocel+Apron | $0.28^{D}$ | $0.22^{D}$ | $0.25^{D}$ |
| *F.oxysporum* $59^{7}$+Methocel+Apron | $0.23^{D}$ | $0.25^{D}$ | $0.24^{D}$ |

[1] 2 % (w/v) Methocel A4C Premium$^{R}$ was used as a sticker.
[2] Inoculum density for all *F. oxysporum* isolates = $10^{6}$ conidia/ml
[3] The rate for Apron$^{R}$ 25 W (metalaxyl) = 1.25g a.i. per kg seed.
[4] Disease severity was rated, after 6 days in the incubator, using the following disease scale; 0: healthy plant, 0.5: trace disease, 1: slight disease, 2: moderate disease, 3: extensive

disease, 4: severe disease, 5: dead plant. Values are average of three experiments with 30 plants per experiment.

[5] Values followed by the same letter are not significantly ($p>0.05$) different according to the Fisher's LSD multiple comparison procedure.

[6] DsRNA-containing hypovirulent *F.oxysporum* isolates.

[7] DsRNA-free hypovirulent *F.oxysporum* isolates

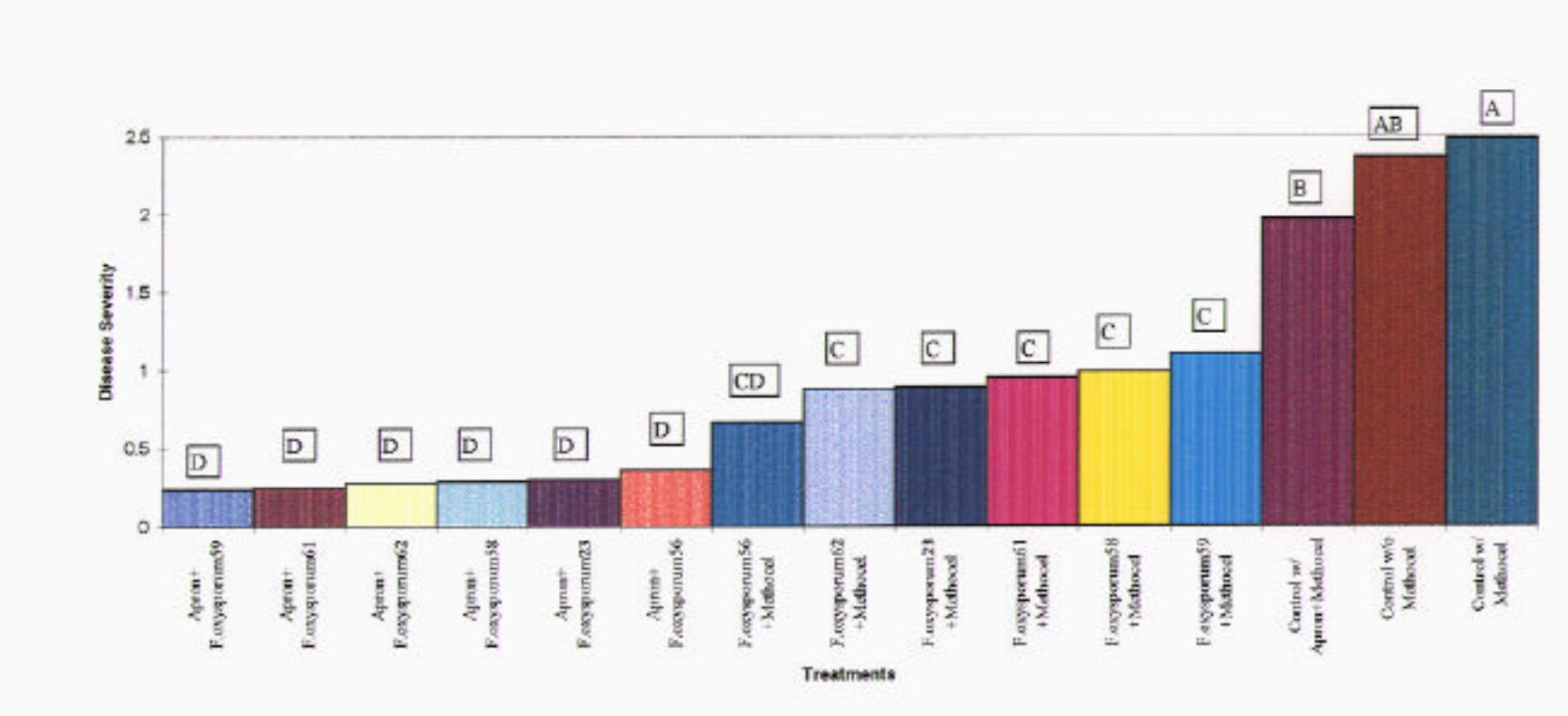


**Figure 4.8.** Effect of prior inoculation of Essex soybean seeds with dsRNA-free hypovirulent *Fusarium oxysporum* isolates on disease severity after 6 days in naturally infested soil at 20 C and -0.01 MPa water potential in plastic-doll assays. Bars with the same letter are not significantly different according to Fisher's least significant difference ($p>0.05$).

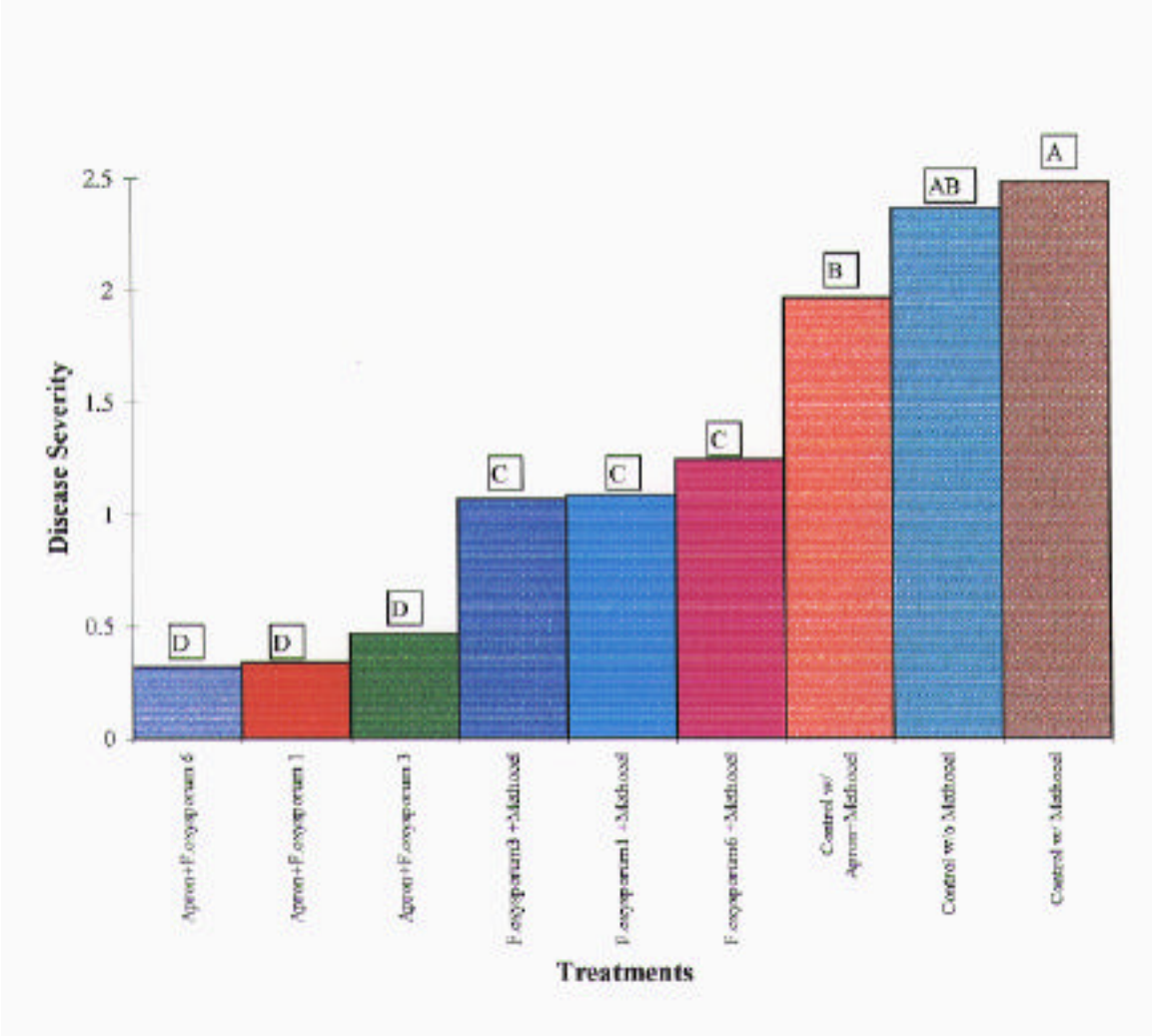


**Figure 4.9.** Effect of prior inoculation of Essex soybean seeds with dsRNA-containing hypovirulent *Fusarium oxysporum* isolates on disease severity after 6 days in naturally infested soil at 20 C and -0.01 MPa water potential in plastic-doll assays. Bars with the same letter are not significantly different according to Fisher's least significant difference ($p>0.05$).

**Table 4.6.** Frequency of isolation of *F. oxysporum*, *F. solani*, and *P. ultimum* from lesions on cotyledons, hypocotyls, and roots of Essex soybean plants grown in naturally infested P. Minor field soil at 20 C and -0.01 MPa water potential in plastic-doll assays.

| | **Isolation frequency (%)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***F.oxysporum***[1] | | | ***F.solani***[1] | | | ***P.ultimum***[1] | | |
| **Treatment** | cotyledon | hypocotyl | root | cotyledon | hypocotyl | root | cotyledon | hypocotyl | root |
| Control with Methocel | 54.3 | 56.6 | 23.1 | 14.2 | 13.3 | 32.7 | 31.5 | 30.1 | 44.2 |
| | $(127)^2$ | $(113)^2$ | $(52)^2$ | $(127)^2$ | $(113)^2$ | $(52)^2$ | $(127)^2$ | $(113)^2$ | $(52)^2$ |
| Control without Methocel | 57.9 | 45.4 | 5 | 14 | 21.6 | 20 | 28.1 | 33 | 75 |
| | $(121)^2$ | $(97)^2$ | $(40)^2$ | $(121)^2$ | $(97)^2$ | $(40)^2$ | $(121)^2$ | $(97)^2$ | $(40)^2$ |
| Control with Apron+Methocel | 85.9 | 68.9 | 25 | 14.1 | 31.1 | 75 | 0 | 0 | 0 |
| | $(92)^2$ | $(61)^2$ | $(16)^2$ | $(92)^2$ | $(61)^2$ | $(16)^2$ | $(92)^2$ | $(61)^2$ | $(16)^2$ |
| *F. oxysporum* 6+Methocel | 22.6 | 22.7 | 0 | 7.6 | 18.2 | 0 | 69.8 | 59.1 | 100 |
| | $(53)^2$ | $(44)^2$ | $(14)^2$ | $(53)^2$ | $(44)^2$ | $(14)^2$ | $(53)^2$ | $(44)^2$ | $(14)^2$ |
| *F. oxysporum* 59+Methocel | 13 | 13.2 | 0 | 8.7 | 15.8 | 0 | 78.3 | 71 | 100 |
| | $(46)^2$ | $(38)^2$ | $(16)^2$ | $(46)^2$ | $(38)^2$ | $(16)^2$ | $(46)^2$ | $(38)^2$ | $(16)^2$ |
| *F. oxysporum* 1+Methocel | 28.6 | 35.7 | 0 | 2.4 | 17.9 | 25 | 69 | 46.4 | 75 |
| | $(42)^2$ | $(28)^2$ | $(12)^2$ | $(42)^2$ | $(28)^2$ | $(12)^2$ | $(42)^2$ | $(28)^2$ | $(12)^2$ |
| *F. oxysporum* 3+Methocel | 35 | 22.6 | 0 | 10 | 19.4 | 0 | 55 | 58 | 100 |
| | $(40)^2$ | $(31)^2$ | $(11)^2$ | $(40)^2$ | $(31)^2$ | $(11)^2$ | $(40)^2$ | $(31)^2$ | $(11)^2$ |
| *F. oxysporum* 58+Methocel | 21.6 | 15.4 | 0 | 2.7 | 23.1 | 18.2 | 75.7 | 61.5 | 81.8 |
| | $(37)^2$ | $(26)^2$ | $(11)^2$ | $(37)^2$ | $(26)^2$ | $(11)^2$ | $(37)^2$ | $(26)^2$ | $(11)^2$ |
| *F. oxysporum* 61+Methocel | 38.9 | 33.3 | 0 | 8.3 | 16.7 | 0 | 52.8 | 50 | 100 |
| | $(36)^2$ | $(30)^2$ | $(8)^2$ | $(36)^2$ | $(30)^2$ | $(8)^2$ | $(36)^2$ | $(30)^2$ | $(8)^2$ |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *F. oxysporum* 23+Methocel | 39.4 (33)[2] | 23.3 (30)[2] | 0 (3)[2] | 12.1 (33)[2] | 23.3 (30)[2] | 0 (3)[2] | 48.5 (33)[2] | 53.4 (30)[2] | 100 (3)[2] |
| *F. oxysporum* 62+Methocel | 39.4 (33)[2] | 30.8 (26)[2] | 0 (8)[2] | 12.1 (33)[2] | 23 (26)[2] | 0 (8)[2] | 48.5 (33)[2] | 46.2 (26)[2] | 100 (8)[2] |
| *F. oxysporum* 56+Methocel | 48 (25)[2] | 34.5 (23)[2] | 0 (7)[2] | 4 (25)[2] | 31 (23)[2] | 42.9 (7)[2] | 48 (25)[2] | 34.5 (23)[2] | 57.1 (7)[2] |
| *F. oxysporum* 3+Apron+Methocel | 96.2 (26)[2] | 63.2 (19)[2] | - | 3.8 (26)[2] | 36.8 (19)[2] | - | 0 (26)[2] | 0 (19)[2] | - |
| *F. oxysporum* 56+Apron+Methocel | 75 (16)[2] | 64.7 (17)[2] | - | 25 (16)[2] | 35.3 (17)[2] | - | 0 (16)[2] | 0 (17)[2] | - |
| *F. oxysporum* 1+Apron+Methocel | 71.4 (21)[2] | 77.8 (9)[2] | - | 28.6 (21)[2] | 22.2 (9)[2] | - | 0 (21)[2] | 0 (9)[2] | - |
| *F. oxysporum* 6+Apron+Methocel | 72.2 (18)[2] | 60 (10)[2] | - | 27.8 (18)[2] | 40 (10)[2] | - | 0 (18)[2] | 0 (10)[2] | - |
| *F. oxysporum* 23+Apron+Methocel | 100 (11)[2] | 61.5 (13)[2] | - | 0 (11)[2] | 38.5 (13)[2] | - | 0 (11)[2] | 0 (13)[2] | - |
| *F. oxysporum* 58+Apron+Methocel | 80 (15)[2] | 60 (10)[2] | - | 20 (15)[2] | 40 (10)[2] | - | 0 (15)[2] | 0 (10)[2] | - |
| *F. oxysporum* 62+Apron+Methocel | 83.3 (12)[2] | 55.6 (9)[2] | - | 16.7 (12)[2] | 44.4 (9)[2] | - | 0 (12)[2] | 0 (9)[2] | - |
| *F. oxysporum* 61+Apron+Methocel | 88.9 (9)[2] | 50 (4)[2] | - | 11.1 (9)[2] | 50 (4)[2] | - | 0 (9)[2] | 0 (4)[2] | - |
| *F. oxysporum* 59+Apron+Methocel | 85.7 (7)[2] | 50 (6)[2] | - | 14.3 (7)[2] | 50 (6)[2] | - | 0 (7)[2] | 0 (6)[2] | - |

[1] Komada's selective medium and APDA were used for *Fusarium* isolations. *Pythium* was isolated on WA.

[2] The numbers in parenthesis indicate total number of cotyledon, hypocotyl, and root tissue pieces plated on media.

### 4.6.2 Effect of prior inoculation of Essex soybean seeds with selected hypovirulent *F. oxysporum* isolates on seedling emergence and disease severity in naturally infested soil using Cone-Tainers

*Fusarium oxysporum* 59 and *F. oxysporum* 61, the best hypovirulent isolates according to the results of the plastic-doll, seed-treatment studies, were used in growth chamber tests on seedling emergence and disease severity in naturally infested soil. The results are presented in Tables 4.7 and 4.8, and Figures 4.10 and 4.11. At day 4, 100 % of the plants, treated with the combination of the mixture of *F. oxysporum* 59, *F. oxysporum* 61 plus Apron$^{R}$, had emerged, whereas emergence for control plants ranged from 32.5% to 52.5 % (Table 4.7). This combination treatment resulted in a significantly ($p<0.05$) higher emergence at 4 days than when individual hypovirulent isolates were used with or without Apron$^{R}$ (Table 4.7). However, seedling emergence at 8 days after planting was not significantly ($p>0.05$) different among all treatments (Table 4.7). Disease severity ratings of plants were significantly ($p<0.05$) lower when hypovirulent isolates, either alone or as a mixture, were used with or without Apron$^{R}$, compared to the control plants (Table 4.8). Seedling disease severity was not significantly ($p>0.05$) different among the treatments when either individual or a mixture of hypovirulent isolates were used with or without Apron$^{R}$.

No *P. ultimum* was recovered from the plants treated with Apron$^{R}$. However, in other treatments in which Apron$^{R}$ was not used, all water-soaked type lesions on cotyledon, hypocotyl, and root tissues yielded in *P. ultimum* on WA. In addition, *F. oxysporum* and *F. solani* were found in association with *P. ultimum* from brown, water-soaked cotyledon tissues of control plants not treated with Methocel. No *R. solani* was isolated from any diseased plant tissues. The results for the isolation of *F. oxysporum*, *F. solani* and *P. ultimum* from lesions on cotyledons, hypocotyls, and roots of Essex soybean plant tissues are presented in Table 4.9. Isolation frequencies of *F. oxysporum* and *F. solani* from diseased plant tissues varied among different treatment groups, but generally *F. oxysporum* was the predominant fungus.

**Table 4.7.** Effect of prior inoculation of Essex soybean seeds with dsRNA-free hypovirulent *Fusarium oxysporum* isolates on the percentage seedling emergence of plants grown in naturally infested P.Minor field soil in Cone-Tainers at 20 C and a photo period of 14 hours[1]

| **Treatment**[2,3,4] | **Days after planting** | |
|---|---|---|
| | **4** | **8** |
| | **Seedling Emergence (%)**[5,6] | |
| *F. oxysporum* 59+ *F. oxysporum* 61+Methocel+Apron | $100^{A}$ | $100^{A}$ |
| *F. oxysporum* 61+Methocel+Apron | $80^{BCD}$ | $100^{A}$ |
| *F. oxysporum* 61+ Methocel | $77.5^{CD}$ | $97.5^{A}$ |
| *F. oxysporum* 59+Methocel+Apron | $77.5^{CD}$ | $100^{A}$ |
| *F. oxysporum* 59+ Methocel | $70^{D}$ | $100^{A}$ |
| Control without Methocel | $52.5^{E}$ | $97.5^{A}$ |
| Control with Methocel | $42.5^{EF}$ | $92.5^{ABC}$ |
| Control with Apron+Methocel | $32.5^{F}$ | $95^{AB}$ |

[1] Soil water was adjusted daily to -0.01 MPa water potential.
[2] Inoculum density for all *F. oxysporum* isolates = $10^{6}$ conidia/ml.
[3] 2 % (w/v) Methocel A4C Premium$^{R}$ was used as a sticker.
[4] The rate for Apron$^{R}$ 25 W (metalaxyl) = 1.25g a.i. per kg seed.
[5] Values are average of two experiments with 20 plants per experiment.
[6] Values followed by the same letter within a column are not significantly ($p>0.05$) different according to the Fisher's LSD multiple comparison procedure.

**Table 4.8.** Effect of prior inoculation of Essex soybean seeds with dsRNA-free hypovirulent *Fusarium oxysporum* isolates on disease severity of plants grown in naturally infested P. Minor field soil in Cone-Tainers at 20 C and a photo period of 14 hours[1]

| **Treatment**[2,3,4] | **Disease severity ratings**[5,6] | | |
|---|---|---|---|
| | **cotyledon** | **hypocotyl** | **average** |
| Control with Apron+Methocel | 1.52[A] | 1.38[A] | 1.45[A] |
| Control with Methocel | 1.44[A] | 1.25[A] | 1.35[A] |
| Control without Methocel | 0.98[B] | 1.11[B] | 1.05[B] |
| *F. oxysporum* 61+Methocel | 0.20[C] | 0.25[C] | 0.23[C] |
| *F. oxysporum* 59+Methocel | 0.11[C] | 0.10[C] | 0.11[C] |
| *F. oxysporum* 61+Methocel+Apron | 0.08[C] | 0.11[C] | 0.10[C] |
| *F. oxysporum* 59+Methocel+Apron | 0.09[C] | 0.03[C] | 0.06[C] |
| *F. oxysporum* 59+ *F. oxysporum* 61+Methocel+Apron | 0.04[C] | 0.04[C] | 0.04[C] |

[1] Soil water was adjusted daily to -0.01 MPa water potential.

[2] Inoculum density for all *F. oxysporum* isolates = $10^6$ conidia/ml.

[3] 2 % (w/v) Methocel A4C Premium[R] was used as a sticker.

[4] The rate for Apron[R] 25 W (metalaxyl) = 1.25g a.i. per kg seed.

[5] Disease severity was rated after 8 days in the growth chamber, using the following disease scale; 0: healthy plant, 0.5: trace disease, 1: slight disease, 2: moderate disease, 3: extensive disease, 4: severe disease, 5: dead plant. Values are average of two experiments with 20 plants per experiment.

[6] Values followed by the same letter are not significantly ($p>0.05$) different according to the Fisher's LSD multiple comparison procedure.

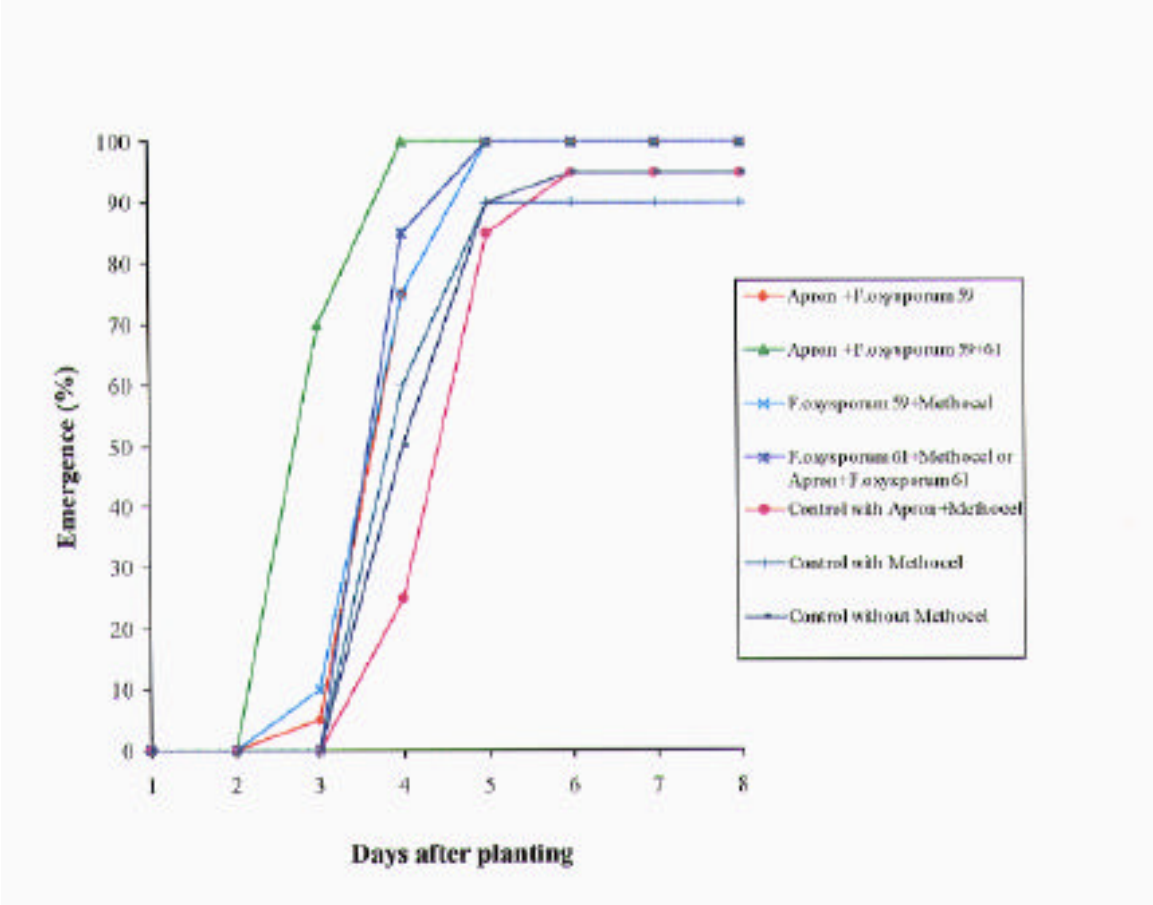


**Figure 4.10.** Speed of emergence of Essex soybean priorly inoculated with two dsRNA-free hypovirulent *Fusarium oxysporum* isolates in naturally infested P. Minor field soil in Cone-Tainers in a growth chamber at 20 C, -0.01 MPa water potential and a photo period of 14 hr. First experiment.

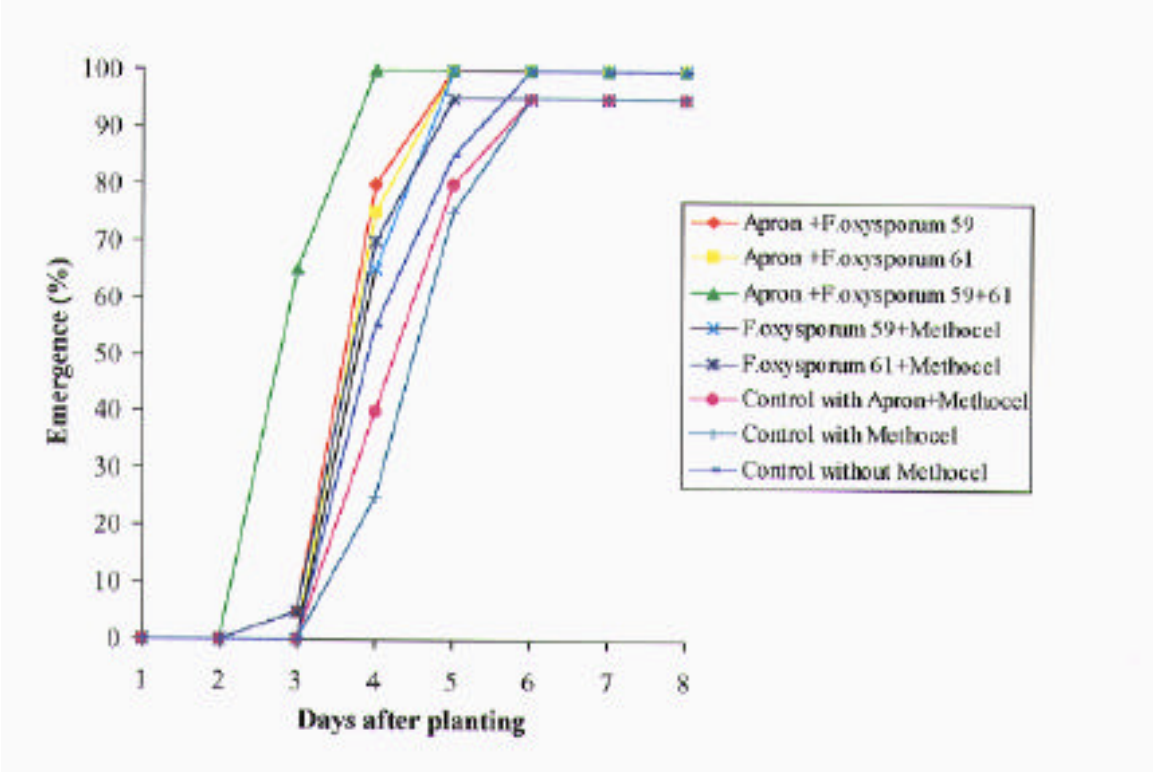


**Figure 4.11.** Speed of emergence of Essex soybean priorly inoculated with two dsRNA-free hypovirulent *Fusarium oxysporum* isolates in naturally infested P. Minor field soil in Cone-Tainers in a growth chamber at 20 C, -0.01 MPa water potential and a photo period of 14 hr. Second experiment

**Table 4.9**. Frequency of isolation of *F. oxysporum*, *F. solani* and *P. ultimum* from lesions on cotyledons, hypocotyls and roots of Essex soybean plants grown in naturally infested P. Minor field soil in Cone-Tainers at 20 C, -0.01 MPa water potential and a photo period of 14 hr.

| | **Isolation frequency (%)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***F.oxysporum***[1] | | | ***F.solani***[1] | | | ***P.ultimum***[1] | | |
| **Treatment** | cotyledon | hypocotyl | root | cotyledon | hypocotyl | root | cotyledon | hypocotyl | root |
| *F. oxysporum* 59+*F. oxysporum* 61+ Methocel+Apron | 100 | 66.6 | - | 0 | 33.3 | - | 0 | 0 | - |
| | (1)[2] | (3)[2] | | (1)[2] | (3)[2] | | (1)[2] | (3)[2] | |
| *F. oxysporum* 61+Methocel+Apron | 100 | 75 | - | 0 | 25 | - | 0 | 0 | - |
| | (2)[2] | (4)[2] | | (2)[2] | (4)[2] | | (2)[2] | (4)[2] | |
| *F. oxysporum* 61+Methocel | 66.6 | 0 | 0 | 0 | 50 | 0 | 33.3 | 50 | 100 |
| | (3)[2] | (2)[2] | (1)[2] | (3)[2] | (2)[2] | (1)[2] | (3)[2] | (2)[2] | (1)[2] |
| *F. oxysporum* 59+Methocel+Apron | 100 | - | - | 0 | - | - | 0 | - | - |
| | (2)[2] | | | (2)[2] | | | (2)[2] | | |
| *F. oxysporum* 59+Methocel | 60 | 60 | - | 40 | 40 | - | 0 | 0 | - |
| | (5)[2] | (5)[2] | | (5)[2] | (5)[2] | | (5)[2] | (5)[2] | |
| Control without Methocel | 83.3 | 50 | 0 | 33.3 | 41.6 | 0 | 16.6 | 8.3 | 100 |
| | (12)[2] | (12)[2] | (3)[2] | (12)[2] | (12)[2] | (3)[2] | (12)[2] | (12)[2] | (3)[2] |
| Control with Methocel | 35 | 57.1 | 0 | 35 | 28.6 | 0 | 40 | 14.3 | 100 |
| | (20)[2] | (7)[2] | (2)[2] | (20)[2] | (7)[2] | (2)[2] | (20)[2] | (7)[2] | (2)[2] |
| Control with Apron+Methocel | 96 | 61.5 | 0 | 12 | 61.5 | 100 | 0 | 0 | 0 |
| | (25)[2] | (13)[2] | (5)[2] | (25)[2] | (13)[2] | (5)[2] | (25)[2] | (13)[2] | (5)[2] |

[1]Komada's selective medium and APDA were used for *Fusarium* isolations. *Pythium* was isolated on WA

[2]The numbers in parenthesis indicate total number of cotyledon, hypocotyl, and root tissue pieces plated on media

### 4.7 Curing of dsRNA-containing hypovirulent *F. oxysporum* isolates

Three dsRNA-containing hypovirulent *F. oxysporum* isolates (1, 3, 6) were cultured on PDA amended with cycloheximide at 0, 15, 20, 40, 60, 140, and 280 µg/ml to cure these isolates of dsRNA. However, attempts to cure of dsRNA from these cultures using the range of concentrations of cycloheximide, indicated above, were unsuccessful. When lower concentrations of cycloheximide (15, 20, 40, 60 µg/ml) failed to cause curing of dsRNA, 140 and 280 µg/ml were tried but these higher concentrations also failed to cure dsRNA-containing isolates. Isolates treated with higher concentrations grew slower than isolates treated with lower concentrations and than untreated isolates. At day 18, colony diameters of cultures treated with 280 µg/ml cycloheximide were only 10 mm and colony diameters of cultures treated with 140 µg/ml cycloheximide were 35 mm. The untreated cultures or cultures treated with lower concentrations covered the plates within 1 week. Colony colors of *F. oxysporum* 1 hyphal tip subcultures, obtained from 140 and 280 µg/ml cycloheximide treatments, were ranged from white to light pink, while colony colors of untreated control cultures of *F. oxysporum* 1 were purple. No differences on colony colors were observed between the hyphal tip subcultures of *F. oxysporum* 3 and *F. oxysporum* 6 and untreated control cultures of this isolates.

Single-sporing of dsRNA-containing hypovirulent *F. oxysporum* isolates to cure dsRNA was not successful. No morphological differences were found between the single-spore subcultures and parent cultures. All single spore-subcultures of each dsRNA-containing hypovirulent isolate showed the presence of dsRNA segments.

## CHAPTER 5: DISCUSSION

We observed that prior inoculation of Essex soybean seeds with dsRNA-containing and dsRNA-free hypovirulent *F. oxysporum* isolates from soybean plus Apron$^R$ significantly reduced disease severity in naturally infested soil at 20 C and -0.01 MPa water potential, compared to the controls. All dsRNA-free and dsRNA-containing hypovirulent *F. oxysporum* isolates used showed similar effects on the reduction of disease severity. Protection against Fusarium wilts by application of nonpathogenic strains of *F. oxysporum* or formae speciales not pathogenic to the challenged host is a well-studied phenomenon (6, 16, 17, 29, 37, 41, 46, 48, 49). Protection also was achieved by inoculation with weakly pathogenic *Fusarium* species such as *F. solani* (50). However, to our knowledge no information exists on the reduction of Fusarium seedling disease or Fusarium root rot by using nonpathogenic or hypovirulent strains of *Fusarium* spp. Our results showed that prior inoculation of seeds with hypovirulent *F. oxysporum* isolates reduced the severity of symptoms typical of those caused by *F. oxysporum* and *F. solani*. In addition to cotyledon disease reduction, we also observed hypocotyl disease reduction even though this structure was not inoculated with hypovirulent isolates. For example, hypocotyl disease severity was 0.25 within the plants treated with the combination of hypovirulent isolate, *F. oxysporum* 59, plus Apron$^R$. In contrast, hypocotyl disease severity on control plants, treated with only Apron$^R$, was 1.91. It is possible that hypovirulent isolates spread from cotyledons and reduced the disease severity on hypocotyls or that an induced resistance mechanism may be involved. Both mechanisms have been shown to be involved in biocontrol of Fusarium wilt diseases by nonpathogenic *Fusarium* strains (1, 6, 16, 29, 37, 43, 46, 48, 49, 61).

We observed that *P. ultimum*, frequently the main component of a fungal complex associated with the early phase of the Essex seedling disease, and a contributor to the late phase of the disease, was not affected by the prior inoculation of seeds with individual hypovirulent isolates. This fungus often caused severe, water-soaked lesions and killed soybean plants within 6 days. The extent of *P. ultimum* interference with evaluation of Fusarium disease severity was greatly reduced when metalaxyl seed treatment was used. The lowest disease severity ratings were observed in plastic-doll assays when the combination of metalaxyl (Apron$^R$ 25 W), a *Pythium*-specific fungicide, and individual hypovirulent *F. oxysporum* isolates were applied to the seeds. Results of growth chamber studies on seedling emergence in naturally infested P. Minor field soil indicated that mixture of two hypovirulent isolates combined with Apron$^R$ resulted in greatest rate of seedling emergence. Faster emergence suggests that those plants are more vigorous. For example, at day 4, 100 % of the plants had emerged when treated with the combination of

the mixture of hypovirulent isolates, *F. oxysporum* 59 and *F. oxysporum* 61, plus Apron$^{R}$. Seedling emergence for controls ranged from 32.5 to 52.5 %. It is possible that an interaction of the two hypovirulent isolates or a greater inoculum density is involved in the increased seedling emergence. This interaction may involve different mechanisms of hypovirulence or induced resistance. Hervas et al. (37) found that prior inoculation of chickpea seedlings with nonpathogenic isolates of *F. oxysporum* or nonpathogenic races of *F. oxysporum* f. sp. *ciceris* reduced disease severity after challenge inoculation with the highly pathogenic *F. oxysporum* f. sp. *ciceris* race 5. It was indicated that the extent of protection decreased when the inoculum density of the pathogen was close to that of the nonpathogenic isolates. In this present study, there was no change in inoculum density when two hypovirulent isolates were used in the mixture.

We observed various degrees of delayed emergence, pre-emergence damping-off and high disease severity for control plants treated with Apron$^{R}$ plus Methocel, with Methocel, and not treated with Methocel, respectively. Metalaxyl was found to have no interference with *Fusarium* spp. We observed high isolation frequencies of *F. oxysporum* and *F. solani* from diseased plants within the treatments in which metalaxyl was used. Utkhede and Smith (65) described the fungicidal and fungistatic activity of metalaxyl *in vitro* against various root rot fungi of young apple trees. They found that metalaxyl was fungicidal to *P. ultimum* and fungistatic to *Phytophthora cactorum* and *P. cambivora.* Metalaxyl inhibited the growth of species of *Phytophthora* and *Pythium* but not of species of *Fusarium*, including *F. oxysporum* and *F. solani.* There are no fungicides presently recommended for control of Fusarium seedling disease and root rot of soybean (62). Testing of fungicides and concentrations is beyond the scope of the present study. Overall, we concluded that reduction of the seedling phase of Essex disease of soybeans can be obtained by the combination treatment of seeds with *Pythium*-specific fungicide, metalaxyl, and hypovirulent *F. oxysporum* isolates, either alone or as a mixture.

The mechanisms and ecological processes involved in reduction of the Fusarium seedling disease phase of Essex disease, by prior inoculation of seeds with hypovirulent *F. oxysporum* isolates, are yet unknown. Antagonistic interactions between hypovirulent and pathogenic *Fusarium* spp., such as competition for nutrients in the rhizosphere (1, 48), and competition for infection sites at the root surface (48, 61), or induced host resistance (6, 16, 29, 37, 43, 46, 49) may explain the mechanism of disease reduction obtained against Essex seedling disease in this present study. However, this aspect requires further study. Study of population structures and spatial pattern or distribution of pathogens in a field may provide a better understanding of the ecology of Essex disease. For example, areas of soybean fields with high and low levels of Essex disease may have different proportions or densities

of highly pathogenic, moderately pathogenic and hypovirulent isolates of *F. oxysporum* and *F. solani.* Griffin and Baker (31) indicated that information on the specific regions of root stimulation and colonization by fungi, and on inoculum efficiency in the rhizosphere, are important to biocontrol. They suggested also that enhancement of biocontrol mechanisms depends on knowledge of specifically where antagonists and biocontrol occur in the rhizosphere, rhizoplane, or root tissue. Further studies will be necessary to ascertain the nature of the protective response described here, and whether the hypovirulent isolates inoculated on seeds are able to colonize soybean roots and reduce the late phase (stunting) of Essex disease.

Results of isolations from diseased and healthy Essex soybean plants grown in naturally infested soil, coupled with pathogenicity tests, indicated that highly pathogenic, moderately pathogenic and hypovirulent isolates of *F. oxysporum* were primary and frequent colonizers of soybean tissues. Less frequently obtained *F. solani* isolates, primarily isolated from necrotic cotyledon and root lesions, showed moderate pathogenicity. While *P. ultimum* was isolated in high frequencies, no *R. solani* was isolated from any diseased plant tissues in our studies. Although many isolates of *F. oxysporum* and *F. solani* were found to be nonpathogenic colonizers of soybean plants cotyledons, hypocotyls and roots in previous studies (22, 23, 24), none of the *F. oxysporum* and *F. solani* isolates tested appeared to be nonpathogenic (no lesion development) in this present study. However, many hypovirulent *F. oxysporum* isolates were recovered from both healthy and less severely necrotic soybean seedling tissues. Farias and Griffin (24) indicated that some plant-surface colonizing strains of *F. oxysporum* and *F. solani* were involved in seedling disease of soybean plants in field soil. It was suggested also that colonization of soybean tissues by these fungal species was much more extensive than lesion development over the surfaces of hypocotyls and roots. Such colonization of soybean plant surfaces by hypovirulent *F. oxysporum* isolates in field soils may result in a natural biological control of Essex disease.

Although hypovirulence associated with the presence of dsRNA has been reported for numerous plant pathogenic fungi (Table 1) (7, 9, 13, 14, 18, 34, 51, 57, 69), our results of dsRNA assays in *F. oxysporum* indicated that the presence of dsRNA did not appear to be associated with hypovirulence in *F. oxysporum*. We observed that some hypovirulent isolates contained dsRNA while other hypovirulent isolates did not. Furthermore, we found one highly pathogenic, and two moderately pathogenic *F. oxysporum* isolates that also contained dsRNA. Evidence for the existence of dsRNA-containing, virulent *C. parasitica* strains in nature has been previously reported (3, 20, 33).

In our studies, dsRNA was detected in only six of a total of 66 isolates tested. Four segments of dsRNA, with sizes of 4.0, 3.1, 2.7, and 2.2 kb, were detected in extracts from all six isolates of *F. oxysporum*. These four dsRNA segments were resistant to digestion by DNase and to RNase at high ionic strength (0.3 M NaCl). Thus, these segments were confirmed to be dsRNA. The colony colors of dsRNA-containing *F. oxysporum* isolates ranged from white to purple showing no morphological differences between dsRNA-containing and dsRNA-free *F. oxysporum* isolates. Detection of virus-like particles and dsRNA elements in some *Fusarium* spp. has been reported (15, 25, 44, 45, 55, 56, 65). Recently, Woo et al. (65) reported the presence of dsRNA and virus-like particles in several isolates of *F. oxysporum* f. sp. *phaseoli*, the causal agent of bean wilt disease. They found different dsRNA profiles in virulent *F. oxysporum* f. sp. *phaseoli* isolates from Italy and Colombia, and isolates from Poland that were no longer virulent. In addition, morphological differences were not detected between dsRNA-containing and dsRNA-free isolates. In our studies, attempts to cure dsRNA-containing hypovirulent *F. oxysporum* isolates by either single-sporing or by using a range of concentrations of cycloheximide, 15, 20, 40, 60, 140, and 280 μg/ml, were unsuccessful. It might be possible that these hypovirulent isolates were resistant to curing at these concentrations of cycloheximide, and higher concentrations of cycloheximide may be required for curing of these dsRNA-containing hypovirulent *F. oxysporum* isolates. Fulbright (28) cured dsRNA from one hypovirulent isolate of *C. parasitica*, GHU4, obtained from a recovering America chestnut tree. Two other hypovirulent isolates, GH2 and RC-1, representing different dsRNA banding patterns, were not cured. He explained the inability to cure dsRNA in two of the three native hypovirulent strains, obtained from the field, as an indication of high dsRNA titer or nuclear factors governing general stability of the dsRNA molecules in these particular genetic backgrounds. The mechanism of curing of a killer factor, a toxin encoded by a specific segment of the viral dsRNA, in *Saccharomyces cerevisiae* with cycloheximide was explained by Fink and Styles (26). Killer strains of *S. cerevisiae* treated with cycloheximide lost the ability to inhibit sensitive strains of yeast. They postulated that since cycloheximide inhibits eukaryotic protein synthesis, cytoplasmic ribosomal protein synthesis must be necessary for the replication of dsRNA, the genetic determinant. This action could limit the genetic determinant and may cause it to be diluted as cell division progresses. Further studies are needed on the association between the presence of dsRNA and virulence in *F. oxysporum* isolates from soybean plants. Certain segments or base sequences of dsRNA may be associated with hypovirulence.

## BIBLIOGRAPHY


1. Alabouvette, C. 1990. Biological control of Fusarium wilt pathogens in suppressive soils. Pages 27-43 in: Biological Control of Soil-borne Plant Pathogens. D. Hornby, ed. CAB International, Wallingford, England. 275 pp.
2. Anagnostakis, S. L. 1982. Biological control of chestnut blight. Science 215:466-71.
3. Anagnostakis, S. L. 1990. Improved chestnut tree condition maintained in two Connecticut plots after treatments with hypovirulent strains of the chestnut blight fungus. For. Sci. 36: 113-124.
4. Bharathan, N., and Tavantzis, S. M. 1990. Genetic diversity of double-stranded RNA from *Rhizoctonia solani.* Phytopathology 80: 631-635.
5. Bharathan, N., and Tavantzis, S. M. 1991. Assessment of genetic relatedness among double-stranded RNAs from isolates of *Rhizoctonia solani* from diverse geographic origins. Phytopathology 81: 411-415.
6. Biles, C. L., and Martyn, R. D. 1989. Local and systemic resistance induced in watermelons by formae speciales of *F. oxysporum.* Phytopathology 79:856-860.
7. Boland, G. J. 1992. Hypovirulence and double-stranded RNA in *Sclerotinia sclerotiorum.* Can. J. Plant Pathol. 14: 10-17.
8. Booth, C. 1971. The Genus Fusarium. C.M.I. Kew, Surrey, England. 273 pp.
9. Bottacin, A. M., Levesque, C. A., and Punja, Z. K. 1994. Characterization of dsRNA in *Chalara elegans* and effects on growth and virulence. Phytopathology 84: 303-312.
10. Brazier, C. M. 1986. The d-factor in *Ceratocystis ulmi*- its biological charactesistics and implications for Dutch elm. In Fungal Virology, ed. K. W. Buck, pp. 177-208. CRC Press, Boca Raton, FL. 305 pp.
11. Buck, K. W., Ackermann, H. W., Bozarth, R. F., Bruenn, J. A., Koltin, Y., Rawlinson, C. J., Ushiyama, R., and Wood, H. A. 1984. Six groups of double-stranded RNA mycoviruses. Intervirology 22:17.
12. Buss, G. R. 1994. Field evaluation of varieties for reaction to “Essex Disease”. Page 4 in: Soybean Research In Virginia-1994. Tidewater Agricultural Research and Extension Center Information Series No. 359. April 1995. Virginia Polytechnic Institute and State University with Virginia State University.
13. Castanho, B., and Butler, E. E. 1978. Rhizoctonia decline: studies on hypovirulence and potential use in biological control. Phytopathology 68: 1511-1514.
14. Castanho, B., Butler, E. E., and Shepherd, R. J. 1978. The association of double-stranded RNA with Rhizoctonia decline. Phytopathology 68: 1515-1519.

**15.** Chosson, J. F., Lapierre, H., Kusiak, C., and Molin, G. 1973. Presence of virus-like particles in fungi of the genus *Fusarium.* Ann. Phytopathologie 5:324.

**16.** Davis, D. 1967. Cross-protection in Fusarium wilt diseases. Phytopathology 57:311-314.

**17.** Davis, D. 1968. Partial control of Fusarium wilt in tomato by formae of *F. oxysporum.* Phytopathology 58: 121-122.

**18.** Day, P. R., Dodds, J. A., Elliston, J. E., Jaynes, R. A., Anagnostakis, S. L. 1977. Double-stranded RNA in *Endothia parasitica.* Phytopathology 67: 1393-96.

**19.** Dodds, J. A., and Elliston, J. E. 1978. Association between double-stranded RNA and hypovirulence in an American strain of *Endothia parasitica* (Abstr.) Proc. 3rd Int. Cong. Plant Pathol., Munich, W. Germany.

**20.** Dunn, M., McKeen, C., and Boland, G. 1994. Chestnut blight in Canada: Hypovirulence and biological control. Pages 147-155 in: Proceedings of the International Chestnut Conference, Morgantown, West Virginia, July 10-14, 1992. Mark L. Double, William L. MacDonald, eds. West Virginia University Press, Morgantown, W.Va. 215 pp.

**21.** Enebak,S. A., MacDonald, W. L., and Hillman, B. I. 1994. Effect of dsRNA associated with isolates of *Cryphonectria parasitica* from the central Appalachians and their relatedness to other dsRNAs from North America and Europe. Phytopathology 84: 528-534.

**22.** Farias, G. M. 1987. Quantitative investigations of *Fusarium oxysporum* and *F. solani* colonization and rot of *Glycine max* cv. Essex seedlings. M.S. Thesis. VPI&SU, Blacksburg, VA, 102 pp.

**23.** Farias, G. M., and Griffin, G. J. 1989. Roles of *Fusarium oxysporum* and *F. solani* in Essex disease of soybean in Virginia. Plant Disease 73: 38-42.

**24.** Farias, G. M., and Griffin, G. J. 1990. Extent and pattern of early soybean seedling colonization by *Fusarium oxysporum* and *F. solani* in naturally infested soil. Plant and Soil 123: 59-65.

**25.** Fekete, C., Giczey, G., Papp, I., Scabo, L., and Hornok, L. 1995. High-frequency occurrence of virus-like particles with double-stranded RNA genome in *Fusarium poae.* FEMS Microbiol. Lett. 131: 295-299.

**26.** Fink, G. R., and Styles, C. A. 1972. Curing of a killer factor in *Saccharomyces cerevisiae*. Proc. Nat. Acad. Sci. USA 69: 2846-2849.

**27.** Frank, Z. R. 1972. *Pythium myriotylum* and *Fusarium solani* as cofactor in a pod-rot complex of peanut. Phytopathology 62: 1331-1334.

**28.** Fulbright, D. W. 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology 74: 722-724.

**29.** Gessler, C., and Kuc, J. 1982. Induction of resistance to Fusarium wilt in cucumber by root and foliar pathogens. Phytopathology 72: 1439-1441.

**30.** Griffin, G. J. 1990. Importance of *Pythium ultimum* in a disease syndrome of cv. Essex soybean. Can. J. Plant Pathol. 12: 135-140.

**31.** Griffin, G. J., and Baker, R. 1990. Population dynamics of soilborne pathogens in relation to infectious inoculum. Pages 3-21 in: Soil Solarization. Y. Katan and J. Devay, eds. CRC Press, Boca Raton, FL. 256 pp.

**32.** Griffin, G. J., Hebard, F. V., Wendt, R., Elkins, J. R. 1983. Survival of American chestnut trees: evaluation of blight resistance and virulence in *Endothia parasitica.* Phytopathology 73: 1084-1092.

**33.** Griffin, G. J., Khan, M. A., and Griffin, S. L. 1993. Superficial canker instability during winter and virulence of *Endothia parasitica* associated with managed forest clearcut and plantation American chestnut trees. Can. J. Plant Pathol. 15: 159-167.

**34.** Hammar, S., Fulbright, D. W., and Adams, G. C. 1989. Association of double-stranded RNA with low virulence in an isolate of *Leucostoma persoonii*. Phytopathology 79: 568-572.

**35.** Harman, G. E., Chet, I., and Baker, R. 1980. *Trichoderma hamatum* effects on seed and seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani.* Phytopathology 70: 1167-1172.

**36.** Harman, G. E., Chet, I., and Baker, R. 1981. Factors affecting *Trichoderma hamatum* applied to seeds as a biocontrol agent. Phytopathology 71: 569-572.

**37.** Hervas, A., Trapero-Casas, J. L., and Jimenez-Diaz, R. M. 1995. Induced resistance against Fusarium wilt of chickpea by nonpathogenic races of *Fusarium oxysporum* f. sp. *ciceris* and nonpathogenic isolates of *F. oxysporum*. Plant Dis. 79: 1110-1116.

**38.** Hillman, B. I., Fulbright, D. W., Nuss, D. L., and van Alfen, N. K. 1995. Hypoviridae. Pages 261-264 in: Virus Taxonomy: Sixth Report of International Committee for the Taxonomy of Viruses. F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarwis, G. P. Martel, M. P. Mayo, and M. D. Summers, eds. Springer-Verlag, New York.

**39.** Hunst, P. L., Latterell, F. M., and Rossi, A. E. 1986. Variation in double-stranded RNA from isolates of *Pyricularia oryzae*. Phytopathology 76: 674-678.

**40.** Komada, A. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev. Plant. Prot. Res. (Tokyo) 8: 114-125.

**41.** Komada, H. 1990. Biological control of Fusarium wilts in Japan. Pages 65-69 in: Biological Control of Soil-borne Plant Pathogens. D. Hornby, ed. CAB International, Wallingford, England. 275 pp.

**42.** Kousik, C. S., Snow, J. P., and Valverde, R. A. 1994. Comparison of double-stranded RNA components and Virulence among isolates of *Rhizoctonia solani* AG-1 IA and AG-1 IB. Pyhtopathology 84: 44-49.

**43.** Kroon, B. A. M., Scheffer, R. J., and Elgersma, D. M. 1991. Induced resistance in tomato plants against Fusarium wilt invoked by *Fusarium oxysporum* f. sp. *dianthi*. Neth. J. Pl. Path. 97: 401-408.

**44.** Lapierre, H., Kusiak, C., and Molin, G. 1974. Some properties of viral particles of *Fusarium culmorum* Smith. Ann. Pyhtopathologie 6: 500-501.

**45.** Lapierre, H., Lecoq, H., Spire, D., Boissonnet, M., and Chevaugeon, J. 1975. Les Mycovirus. Ann. Pyhtopathologie 7: 236-239.

**46.** Larkin, R. P., Hopkins, D. L., and Martin, F. N. 1996. Suppression of Fusarium wilt of watermelon by nonpathogenic *F. oxysporum* and other microorganisms recovered from a disease-suppressive soil. Phytopathology 86: 812-819.

**47.** Louter, J. H., and Edgington, L. V. 1990. Indications of cross-protection against Fusarium crown and root rot of tomato. Can. J. Plant Pathology 12: 283-288.

**48.** Mandeel, Q., and Baker, R. 1991. Mechanisms involved in biological control of Fusarium wilt of cucumber with strains of nonpathogenic *Fusarium oxysporum*. Phytopathology 81: 462-469.

**49.** Martyn, R. D., Biles, C. L., and Dillard, E. A., III. 1991. Induced resistance to Fusarium wilt of watermelon under stimulated field conditions. Plant Dis. 75: 874-877.

**50.** McClure, T. T. 1951. Fusarium foot rot of sweet potato sprouts. Phytopathology 41: 72-77.

**51.** Melzer, M. S., and Boland, G. J. 1996. Transmissible hypovirulence in *Sclerotinia minor*. Can. J. Plant Pathol. 18: 19-28.

**52.** Morris, T. J., and Dodds, J. A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69: 854-858.

**53.** Nelson, P. E., Tousson, T. A., and Marasas, W. F. O. 1983. Fusarium species. An illustrated Manual for Identification. The Pennsylvania State University Press, University Park and London. 193 pp.

**54.** Newhouse, J. R., Tooley, P. W., Smith, O. P., and Fishel, R. A. 1992. Characterization of double-stranded RNA in isolates of *Phytophthora infestans* from Mexico, the Netherlands, and Peru. Phytopathology 82: 164-169.

**55.** Nogawa, M., Nakatani, A., Gonda, K., Shimosaka, M., and Okazaki, M. 1996. Replication of double-stranded RNA in mycovirus from the plant pathogenic fungus, *Fusarium solani*. FEMS Microbiol. Lett. 137: 45-49.

**56.** Nogawa, M., Shimosaka, M., Kageyama, T., and Okazaki, M. 1993. A double-stranded RNA mycovirus from the plant pathogenic fungus, *Fusarium solani* f. sp. *robiniae.* FEMS Microbiol. Lett. 110: 153-158.

**57.** Nuss, D. L., Koltin, Y. 1990. Significance of dsRNA genetic elements in plant pathogenic fungi. Annu. Rev. Phytopathology 28: 37-58.

**58.** Paul, C. P., and Fulbright, D. W. 1988. Double-stranded RNA molecules from Michigan hypovirulent isolates of *Endothia parasitica* vary in size and sequence homology. Phytopathology 78: 751-755.

**59.** Pieczarka, D. J., and Abawi, G. S. 1978. Effect of interaction between *Fusarium, Pythium,* and *Rhizoctonia* on severity of bean root rot. Phytopathology 68: 403-408.

**60.** Pryor, A., and Boelen, M. G. 1987. A double-stranded RNA mycovirus from the maize rust *Puccinia sorghi.* Can. J. Bot. 65: 2380-2383.

**61.** Schneider, R. W. 1984. Effects of nonpathogenic strains of *Fusarium oxysporum* on celery root infection by *Fusarium oxysporum* f. sp. *apii* and a novel use of the Lineweaver-Burk double reciprocal plot technique. Phytopathology 74: 646-653.

**62.** Sinclair, J. B., and Backman, P. A. 1989. Compendium of Soybean Diseases, Third Edition. APS Press, St. Paul, MN. 116 pp.

**63.** Sokal, R. R., and Rohlf, F. J. 1995. Biometry. W. H. Freeman and Company, New York. 887 pp.

**64.** Tooley, P. W., Hewings, A. D., and Falkenstein, K. F. 1989. Detection of double-stranded RNA in *Phytophthora infestans.* Phytopathology 79: 470-474.

**65.** Utkhede, R. S., and Smith, E. M. 1991. *Phytophthora* and *Pythium* species associated with root rot of young apple trees and their control. Soil Biol. Biochem. 23: 1059-1063.

**66.** Woo, S. L., Lorito, M., Muccifora, S., Scala, F., Gori, P., and Noviello, C. 1997. Presence of double-stranded RNA and virus-like particles in isoaltes of *Fusarium oxysporum* f. sp. *phaseoli* (Abstract). Phytopathology 87: S104

**67.** Wymore, L. A., and Baker, R. 1982. Factors affecting cross-protection in control of Fusarium wilt of tomato. Plant Dis. 66: 908-910.

**68.** Zhang, R., Dickinson, M. J., and Pryor, A. 1994. Double-stranded RNAs in the rust fungi. Annu. Rev. Phytopathology 32: 115-133.

**69.** Zhou, T., and Boland, G. J. 1997. Hypovirulence and double-stranded RNA in *Sclerotinia homoeocarpa*. Phytopathology 87: 147-153.

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