

Sites of Infection of *Fusarium oxysporum* f. *pisi* Race 5 on Peas

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ABSTRACT

Propagules of *Fusarium oxysporum* f. sp. *pisi* race 5 initially infect pea cotyledons, but independent infections of roots must occur for plants to exhibit severe wilt symptoms. In 7-14 days, other soil fungi such as *F. solani*, *F. roseum*, and *F. oxysporum* types, nonpathogenic to peas, colonize both cotyledons and roots as secondary

invaders. Infection by race 5 at a single site on one root may kill a pea plant before maturity, but severity of symptoms is directly proportional to the number of infection sites. Race 5 infects through wounds or penetrates directly through the epidermis.

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From 1963 through 1970, a new and highly pathogenic race of *Fusarium oxysporum* Schlecht. emend. Snyder & Hans. f. sp. *pisi* (Lindf.) Snyder & Hans. (race 5) was found causing Fusarium wilt of peas (*Pisum sativum* L.) in northwestern Washington (6, 7). The fungus was first found on a single farm, but by 1970, it occurred throughout three counties, Skagit, Snohomish, and Whatcom.

Doling (4) emphasized the importance of wounds for infection of peas by *F. oxysporum* f. sp. *pisi* race 1, and found that infection occurred through cut surfaces at the end of roots rather than at random over the root surface. Virgin & Walker (13), working with the near-wilt fungus of peas, *F. oxysporum* f. sp. *pisi* race 2, found that penetration of pea seedlings by the fungus occurred most commonly at root tips and cotyledonary nodes. Penetration occasionally took place at various points along the root and epicotyl.

Hypae of other pathogenic form species of *F. oxysporum* penetrate through root hairs [flax and cabbage (9, 12)] and epidermal cells of roots, seed coats, and cotyledons [alfalfa and red clover (3)].

This study of the sites of infection by *F. oxysporum* f. sp. *pisi* race 5 on peas was undertaken to determine (i) which portion of the pea must be protected by placement of soil fungicides or fumigants to prevent infection; and (ii) which cultural practices would decrease populations of race 5 propagules in the soil immediately surrounding peas.

MATERIALS AND METHODS.—*Isolation of Fusarium spp. from peas.*—Pea stems and roots were cut into 2-cm lengths, surface-treated in a 1:1 solution of 1% NaOCl and 75% ethanol (stems for 30 sec and roots for 60 sec), and placed on pentachloronitrobenzene (PCNB) medium (10) adjusted to pH 4.5. Cotyledon halves were treated the

same as roots. All samples were incubated for 5-7 days at 22 C in diffuse daylight.

Identification of race 5.—Races of *F. oxysporum* f. sp. *pisi* are identified by their pathogenicity to different pea cultivars. Race 5 is pathogenic to Little Marvel, Dark-skin Perfection, New Era, and New Wales (7). Races 3, 4, and 5 were not compared directly because type cultures of race 3 and 4 are not available. Race 3 has been described as similar to race 2 in symptoms and disease expression, whereas race 4 is similar to race 1. Also, race 4 does not differ appreciably from race 5, except that race 5 is pathogenic and race 4 nonpathogenic to New Wales cultivar of peas.

We determined pathogenicity of isolates by dipping cut roots of 10-day-old seedlings into conidial suspensions and transplanting the seedlings into vermiculite (three replicates, 10 plants/replicate). Conidia were produced on Kerr's basal medium (8) for 5 days at 24 C in diffuse daylight. Inoculated seedlings were watered with Hoagland's solution at 7-day intervals. Isolates were considered to be race 5 if 8-10 plants of each of the four test cultivars displayed wilt symptoms 14-21 days after inoculation.

Race 5 (grown on PCNB medium, pH 4.5, at 22-23 C for 10 days) can also be identified with some certainty on a morphological basis compared to races 1 and 2 using the following characteristics: (i) race 5 has slow growth in culture (3-10 mm diam), races 1 and 2 (11-21 mm diam); (ii) macroconidia are normally absent with race 5, but abundant with races 1 and 2; unicellular microconidia are abundant with races 1, 2, and 5; (iii) races 1 and 5 have white aerial mycelium, whereas that of race 2 is pink; race 5 colonies have definite margins, whereas those of races 1 and 2 are indefinite; and (iv) chlamydo spores are

(terminal or intercalary) usually present with all three races.

Races 1 and 2 were chosen for comparison with race 5 because these two races were frequently isolated, especially from the soil.

Incidence of race 5 and other *F. oxysporum* types isolated from roots, cotyledons, and stems of 7-, 14-, and 21-day-old peas.—New Era peas (thinned to five/pot after emergence) were planted in 15-cm plastic pots containing field soil (Puget silty clay loam) either naturally infested or not infested with race 5. At 7-, 14-, and 21-day intervals, 50 plants/soil were removed by washing, and a 2-cm section of the taproot (2 cm below the cotyledon), half a cotyledon, and the second internode of the stem were removed from each plant, surface-treated, and placed on PCNB medium (five/petri dish) to isolate *F. oxysporum*. We then randomly selected 25 isolates of *F. oxysporum* by arbitrarily choosing five petri dishes, each with five *F. oxysporum* cultures growing in it to determine what percent of total isolates were race 5. When there were not 25 *F. oxysporum* colonies isolated from each organ per time period (as at 7 days), all available *F. oxysporum* isolates were used. Inoculum was increased on Kerr's medium, and each culture was tested for pathogenicity to the four pea cultivars previously mentioned. The experiment was repeated once, making a total of 100 plants/treatment.

Dexon (*p*-Dimethylaminobenzendiazoo sodium sulfonate) was added to soil (0.208 g actual/1.5 dm³) at planting to control damping-off organisms. Dexon had no effect on the population of race 5 in the soil or on the incidence of isolation from roots.

Inoculation of roots, cotyledons, or stems with field soil naturally infested with race 5.—Roots, cotyledons, or stems of New Era peas were infected singly by exposure to a band of infested soil layered at various depths in noninfested soil. The infested soil was a Puget silty clay loam, pH 5.8, containing about 10,500 *Fusarium* spp. propagules/g of which 8,500 were race 5. Noninfested soil was a mixture of silt loam, peat, and sand (2:2:1, v/v), pH 6.0. Pea seeds were planted 50 mm below soil surface and thinned to 10/container after emergence. Stems, cotyledons, and taproots of peas were exposed to treatments as illustrated in Fig. 1. The plastic containers (25 cm diam X 35 cm deep) were placed in a greenhouse at 20-23 C utilizing available sunlight only. Controls consisted of plants growing in noninfested soil only. Each treatment consisted of 20 plants, replicated 5 times, making a total of 100 plants/treatment.

A thicker band (125 mm) of infested soil was used to increase the number of infection sites on the roots.

Plants were subsurface-irrigated. Prior to adding soil, a plastic pipe was inserted into each container until one end touched the top of the sand layer. The sand functioned as a reservoir for water poured down the pipe, and facilitated water plants from below.

Number of nodes at plant death, dry weight (exclusive of roots and seeds), and yield (dry weight of seed) were used to determine severity of wilt. We determined dry weight after placing plant material at

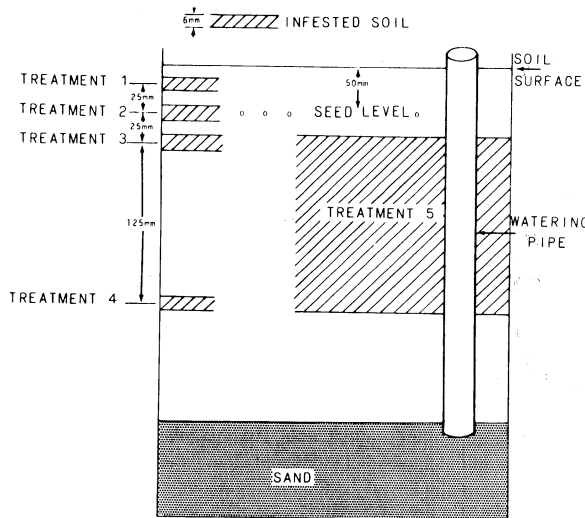


Fig. 1. Treatments used to inoculate different zones of pea plants with *Fusarium oxysporum* f. sp. *pisi* race 5 using naturally infested field soil.

43 C in a mechanical convection oven for 7 days.

Hypodermic inoculation of roots, cotyledons, and stems with race 5.—Individual plants of New Era peas were grown in 2-liter polyethylene bags filled with noninfested soil. The bags were wrapped with aluminum foil to exclude light from the roots, and inclined at a 45° angle. White, disease-free roots grew appressed to the side of the bag, and either the cotyledons or the roots, or both, could be inoculated by insertion of a hypodermic needle through the bag wall and placement of 5,000-6,000 chlamydo spores of race 5 at single sites.

The roots were inoculated 125 mm from the cotyledon with a minimum of disturbance to the plant. Plants were inoculated when roots were 20-30 cm and stems were 2-5 cm long, respectively. Half the plants in each treatment were wounded with the hypodermic needle during inoculation. Each treatment consisted of 50 plants, replicated thrice, making a total of 150 plants/treatment. Controls were plants injected with sterile distilled water.

The race 5 isolate used to produce chlamydo spores was grown in Kerr's basal medium for 5 days at 24 C in diffuse daylight. Conidia were separated from the medium by centrifugation. We then produced chlamydo spores by placing conidia into a sterile soil extract using the method of Alexander et al. (1).

Plants were grown in the greenhouse, and wilt severity was determined by plant height and dry weight of plant material as described previously.

Inoculation of seedlings by immersion in chlamydo spore suspension.—To determine on which organ germination of race 5 chlamydo spores was likely to occur first, seedlings of New Era peas were grown in vermiculite for 10 days, carefully washed free of adhering vermiculite particles to prevent excessive wounding, and inoculated by immersion of roots, cotyledons, and 5 mm of stem into a chlamydo spore suspension (6,000,000/ml) of race 5. The seedlings were replanted into vermiculite. This large inoculum dosage facilitated finding chlamydo spores on the plant surface. Every 24 hr for 3 days, 25 seedlings were stained and examined under a microscope (X 100-450) for chlamydo spore germination and penetration using the method of Weston (14). Then chlamydo spores were counted for each organ per seedling (total of 250 chlamydo spores/organ per time period). The experiment was repeated once.

RESULTS.—*Frequency of isolation of F. oxysporum and race 5 from roots, cotyledons, and stems of plants grown in field soil naturally infested with race 5.*—The percent of *F. oxysporum* among total fungal isolates from roots, cotyledons, and stems of peas grown in infested soil steadily increased up to 21 days, with the greatest increase from stems and the least from roots (Table 1). There was also a similar increase of isolates of *F. oxysporum* from plants grown in noninfested soil up to 14 days; after 21 days, however, the incidence of *F. oxysporum* actually decreased.

At 7 days, most *F. oxysporum* isolates from peas

TABLE 1. Frequency of *Fusarium oxysporum* and occurrence of race 5 among cultures from roots, cotyledons, and stems of 7-, 14-, and 21-day-old peas grown in soil infested or not infested with race 5

Time after planting (days)	Wilt-soil infested (+) or uninfested (-)		Plant organ	% ^a <i>F. oxysporum</i>	% ^b Race 5
7	+	Root	15	93	
		Cotyledon	26	100	
		Stem	20	100	
	-	Root	3	0	
		Cotyledon	15	0	
		Stem	0	0	
14	+	Root	38	71	
		Cotyledon	62	40	
		Stem	62	100	
	-	Root	43	0	
		Cotyledon	68	0	
		Stem	22	0	
21	+	Root	42	60	
		Cotyledon	80	44	
		Stem	90	91	
	-	Root	7	0	
		Cotyledon	62	0	
		Stem	18	0	

^a Based on number of *F. oxysporum* isolates amount total number of fungal isolates from 100 plants.

^b Based on number of race 5 isolates confirmed from 25 randomly selected isolates of *F. oxysporum*. When there was not a total of 25 *F. oxysporum* colonies per time period, all available *F. oxysporum* isolates were used.

grown in infested soil were race 5. The percent of race 5 isolates within the total *F. oxysporum* isolates from roots declined up to 21 days, and from cotyledons declined rapidly from 7 to 14 days. There was little change in percent of race 5 from stems, even though 90% of total fungal isolates were *F. oxysporum* at 21 days. Other Fusaria, particularly *F. solani* (not known whether f. sp. *pisi*), *F. roseum*, and *F. oxysporum* (other than race 5) were isolated with increasing frequency from cotyledons and roots up to 21 days. None of the isolates from plants grown in noninfested soil was race 5. Sequential isolations from plants growing in fields infested or not infested with race 5 gave similar results.

In general, the roots of plants grown in infested soil were shorter and more deformed than those grown in noninfested soil. By 21 days, cotyledons of all plants grown in infested soil had been invaded by bacteria, yeasts, and other fungi. Cotyledons from noninfested soil were relatively free of bacteria but were invaded by *F. oxysporum* types nonpathogenic to peas. None of the *F. oxysporum* isolates from plants grown in noninfested soil was identified as race 5. Most of these cultures were either *F. oxysporum* f. sp. *pisi* races 1 or 2, or were nonpathogenic to peas.

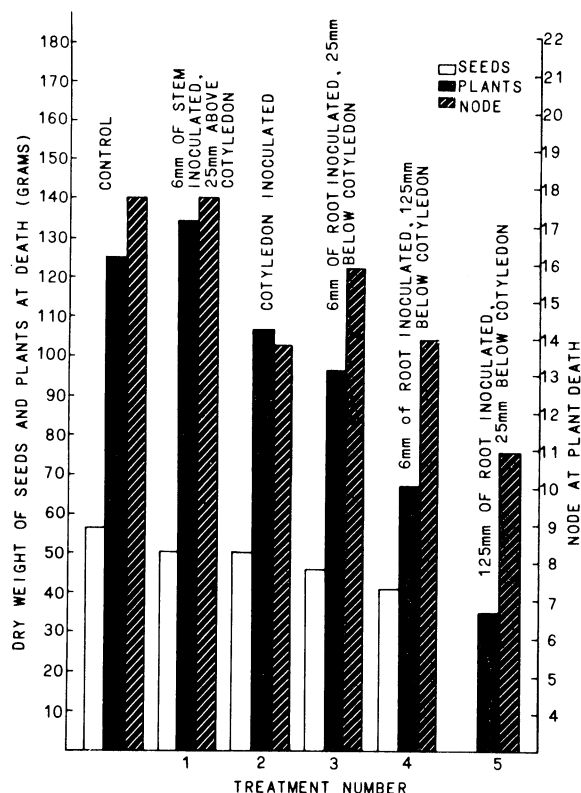


Fig. 2. Severity of *Fusarium* wilt to peas (New Era) as affected by inoculation of roots, cotyledons, and stems by placement of bands of soil (naturally infested with race 5) of different thicknesses in noninfested soil at different distances from seed. Results based on total of 100 plants/treatment.

Inoculation of roots, cotyledons, and stems with soil infested with race 5.—Wilt severity was directly related to the surface area of the root exposed to soil infested with race 5 (Fig. 2). Wilt symptoms were most severe when a large area of root was exposed to infested soil (125 mm, treatment 5) and no seed was produced by plants. Wilt symptoms were observed when a small area of the root (6 mm) was exposed to infested soil, but symptoms were more severe when inoculum was placed closer to the seed, and infection occurred soon after seed germination [inoculum placed 25 mm (treatment 3) versus 125 mm from cotyledon (treatment 4)].

When only cotyledons were inoculated (treatment 2), plants appeared less vigorous and plant height and dry weight were reduced, but there was only a small decrease in yield (dry weight of seed) as compared to the control. If inoculum was placed around the stem only (treatment 1), no wilt was observed.

Inoculation of roots, cotyledons, and stems by syringe with a pure culture of race 5.—Inoculation of cotyledons produced the greatest wilt symptoms as compared to controls. Although height was not reduced, inoculated plants were "spindly" and less vigorous (Table 2). There was no difference between wounding and nonwounding of cotyledons, although

wounding, compared to nonwounding, reduced dry weight in all other treatments.

Single point inoculation of roots 25 mm from the cotyledon caused more severe wilting than 125 mm from the cotyledon. Plants displayed wilt symptoms 2-5 days sooner, and in general were less thrifty.

When inoculum was placed on the stem, no wilt was observed, and there was no penetration by hyphae through the epidermis, based on microscopic observation. When stems were wounded, plants were less vigorous and wilting of foliage occurred. However, plants apparently recovered from this injury, and only a small reduction in dry weight was observed.

Multiple inoculation sites on roots versus single inoculation of cotyledon on wilt symptoms.—The effect of multiple infection sites on wilt symptoms was studied using the same technique as that described for inoculation studies, but instead of one infection site, multiple sites (0-35) were spaced equidistantly on the roots only.

Wilt severity was directly proportional to the number of inoculation sites on roots. A noninoculated plant grew to 16 nodes by maturity. The difference in plant nodes at death or maturity between one and five inoculation sites was small (12 and 11, respectively); however, ingress at 10 and 35 inoculation sites killed peas at the eighth and sixth

TABLE 2. Wilt symptoms of peas inoculated at one site on roots, cotyledons, or stems with a pure culture of *Fusarium oxysporum* f. sp. *pisi* race 5

Treatment	Wounded and un-wounded plants ^a	Average no. of nodes ^b	Avg total dry wt of plants per replicate (g) ^c
Root inoculated 25 mm below cotyledon	—	15	86
	+	11	30
Root inoculated 125 mm below cotyledon	—	14	81
	+	16	68
Cotyledons inoculated	—	14	65
	+	14	66
Stem inoculated 25 mm from cotyledon	—	15	104
	+	14	80
Control A ^d	—	14	106
	+	14	101
Control B ^e	—	14	101
	+	14	97

^a Half of peas in each treatment were wounded with a hypodermic syringe at time of inoculation. + = Wounded; — = unwounded.

^b Node used as measure of plant height; based on average of 150 plants.

^c Based on total dry wt of average replicate, tops only.

^d Inoculated 25 mm above cotyledon with sterile distilled water.

^e Inoculated 25 mm below cotyledon with sterile distilled water.

nodes of development, respectively. Plants died at the 11th node when the cotyledon was inoculated.

Chlamydospore germination on plant surfaces.—No germination of chlamydospores was evident on any organ 24 hr after inoculation. At 48 hr, 1% of chlamydospores in the cotyledonary region had germinated, and less than 1% of chlamydospores had germinated on the roots. After 72 hr, approximately 50% of the chlamydospores had germinated and the germ tubes had penetrated cotyledons and roots. Infrequently, a chlamydospore appeared to have germinated on the stem but no penetration was evident.

DISCUSSION.—Race 5 invades the cotyledons initially because this is the first organ exposed to the fungus in soil; however, this infection is less significant than subsequent infections of the root system. Race 5 must infect at several sites on the roots before peas will be killed. As the roots grow, providing a larger surface area, new sites are continuously being exposed to further infection by race 5. However, the number of infection sites on the cotyledon remain constant.

The cotyledon performs its primary function in a relatively short period of time as compared to the continuous function of the roots throughout the life of the plant. Therefore, in spite of infection of the cotyledon by race 5 and subsequent invasion by other microorganisms, it is still able to provide food until the pea seedling becomes established.

More severe wilt symptoms were displayed by peas when cotyledons were inoculated by syringe with a pure culture of race 5 than when a single site on the roots was inoculated. However, when a larger number of sites on the roots were inoculated, wilt symptoms became proportionately more severe.

Although race 5 grows slowly in culture, in naturally infested soil it infects pea roots and cotyledons more rapidly than other *Fusarium* spp., including other races of *F. oxysporum* f. sp. *pisi*, *F. roseum*, and *F. solani*. It was not determined whether the *F. solani* isolates were pathogenic to peas, but the *F. oxysporum* f. sp. *pisi* isolates included race 1 and 2 as well as nonpathogenic, but parasitic, *F. oxysporum* isolates. In contrast, Buxton & Perry (2) and Perry (11) found that *F. solani* f. sp. *pisi* invaded pea roots earlier than did *F. oxysporum* f. sp. *pisi*. Race 5 appears to be more aggressive than other races of *Fusarium* pea wilt, at least on the pea cultivars currently grown in northwest Washington.

Race 5 constituted a lower percentage of all *F. oxysporum* isolates from cotyledons and roots of peas grown in infested soil after 14 or 21 days than after 7 days (Table 1). Other *Fusaria* such as *F. solani*, *F. roseum*, and *F. oxysporum* (nonpathogenic to peas) were isolated with increased frequency. The latter behaved as secondary colonizers, possibly competing for and cohabiting substrate with race 5, and after 2 weeks replacing race 5 in the primary infection sites. Likewise, when we inoculated 10-day-old pea seedlings by dipping into a chlamydospore suspension, mycelium infrequently grew out of the cotyledon region into the vascular

tissue; usually mycelium grew throughout and seemed confined to the two seed halves and the cotyledonary node. The possibility of two genotypes of race 5 was considered with the assumption that the type of race 5 infecting the cotyledon was incapable of causing wilt symptoms when infecting roots. This assumption proved false. Isolates from the cotyledon induced severe wilt symptoms when only roots were inoculated. Similarly, isolates from the roots also appeared confined to the seed halves when only the cotyledons were inoculated. Race 5 tends to decompose the cotyledon, thereby allowing less pathogenic but faster growing secondary fungi to cohabit the invaded substrate. This may reduce the incidence of race 5, and explains why it does not move out of vascular tissue.

This fungal succession is similar to that described by Garrett (5) in which living roots are invaded by root-infecting fungi that in turn are succeeded by a sequence of other fungi (p. 35). However, percent occurrence of race 5 in stems was not substantially reduced even at 21 days, indicating that at least in the absence of direct competition from other systemic microorganisms, race 5 is not eliminated.

Although wilt symptoms are displayed when only the cotyledon or a relatively small area of root is infected, symptoms are more severe when a larger root area becomes infected, even without wounding. Doling (4) found the rate of development of *Fusarium* wilt (race 1) associated directly with severity of root damage. Infection was found to occur through cut surfaces at the ends of roots rather than at random sites over the root surface. Thus, it seems that the more infection sites that occur on a growing, expanding root surface, the more severe the wilt symptoms, which suggests a cumulative effect.

Future work with the use of soil fumigants and fungicides to control *Fusarium* wilt caused by race 5 will have to concentrate on protecting the root region below the cotyledon rather than a relatively narrow band of soil around the see. A cultural practice to reduce the population of race 5 propagules in the soil zone around the root would be difficult to implement because of the practice of growing peas in the same soil in successive years. Pea cultivars resistant to infection by race 5 will perhaps be the only feasible control method.

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