A Rapid Method for Inoculating Pea Seedlings with *Fusarium oxysporum* f. sp. *pisi*

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ABSTRACT

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The standard and the newly described method of inoculation of pea seedlings were used to determine the pathogenicity of 92 unknown cultures of *Fusarium oxysporum* isolated from diseased pea stems and cultures of four known races of *F. o.* f. sp. *pisi*. Use of either method resulted in nearly identical characterization of unknown cultures and known races. The new method involves growing pea seedlings in Styrofoam trays, which allows six pea seedlings to be inoculated simultaneously. The new method is approximately four times faster than the standard method and is less injurious to the transplanted seedlings.

Additional keywords: Fusarium wilt, *Pisum sativum*

Several methods have been used to evaluate pea seedlings (*Pisum sativum* L.) for resistance to Fusarium wilt (*Fusarium oxysporum* Schlect. emend. Snyd. & Hans. f. sp. *pisi* (van Hall) Snyd. & Hans.) and to determine virulence of *F. o.* f. sp. *pisi* isolates (1–4,6,9–11). With the most common standard method, seedlings are uprooted and the roots are washed. They are then submerged in a spore suspension and cut. Inoculated seedlings are then transplanted into a growing medium (2–4,8). The new method described here was developed to test large numbers of breeding lines for resistance to *F. o.* f. sp. *pisi* and to determine the pathogenicity of *F. oxysporum* isolates.

MATERIALS AND METHODS

Inoculum preparation. Conidial or bud cell inoculum of *F. oxysporum* was prepared by sprinkling sterilized dry soil infested with bud cells of the test culture on acidified potato-dextrose agar (APDA), pH 4.5. Inoculated plates were incubated for 3–4 days or until resultant colonies were 4–6 mm in diameter. One agar block (2 mm^2) was removed from the colony margin and placed in a 250-ml Erlenmeyer flask containing 50 ml of Kerr's liquid medium (7). A second agar block was placed on APDA. Inoculated flasks were placed on a reciprocal shaker oscillating at 60–70 strokes per minute. The petri dishes were incubated under diffused natural light and the flasks on the shaker under constant cool-white fluorescent light at approximately 60 $\mu mol \cdot s^{-1} \cdot m^{-2}$. After 5–6 days, the inoculated APDA plates were examined for colonies characteristic of *F. o.* f. sp. *pisi*. Corresponding liquid cultures were selected and strained through a plastic screen with a pore size of 0.3–0.4 mm^2. The spore concentration was adjusted to 1×10^6 cells per milliliter using a hemacytometer.

Growth of seedlings for inoculation. Trays (Speedling Corp., Orlando, FL) containing 72 cells $5 \times 5 \times 7.2$ cm deep, with the shape of an inverted pyramid, were filled with vermiculite (Terra-lite No. 2, W. R. Grace & Co., Cambridge, MA). Six seeds were planted in each cell 4–4.5 cm deep. As an alternative, eight seeds can be planted in each cell of trays that contain 32 cells. Seed, vermiculite, and trays are disinfested with 0.1% sodium hypochlorite in the initial watering. Only seeds not treated with fungicide were planted. Sufficient water-sodium hypochlorite solution was applied to leach from all cells. After 2 hr, the medium was leached with normal greenhouse water to remove excess sodium hypochlorite. Seedlings were grown for 16–20 days at 16–18 C or until peas were in the 4–5 node stage of development (cotyledonary node = 0). Root systems at this time had formed a cohesive plug and were removed from the cell as a single unit. Trays were suspended 5–10 cm above the bench after the first watering to allow the roots growing from the bottom of the tray to be air-pruned.

Inoculation procedure. Plants were removed from individual cells using a gentle pulling motion. Care was taken to prevent breaking the stems of the seedlings. After the seedlings were removed from the cell, excess vermiculite above the cotyledons was removed by gentle shaking. Approximately one-third of the root plugs were cut off as shown in Figure 1. The remaining roots and vermiculite were submerged into the inoculum to cover the cotyledons and were allowed to remain in inoculum for 3–5 sec. The plugs were removed, excess inoculum was drained from the plugs, and the plugs were transplanted into 10-cm-diameter plastic pots. Number 2 horticultural vermiculite was used as the growing medium.

Postinoculation care. Inoculated plants were watered daily for 3 days after planting with sufficient water to keep the vermiculite wet, but not so wet as to cause leaching. After the initial three days, seedlings were watered as needed with a constant feed nutrient solution. In these studies, Peters 20-20-20 soluble fertilizer (W. R. Grace & Co., Fogelsville, PA) was used at 50 ppm of nitrogen along with S.T.E.M. micronutrient (Robert B. Peters, Allentown, PA) at a concentration of one part S.T.E.M. to 200 parts of the 20-20-20 fertilizer.

Seven differential cultivars of peas

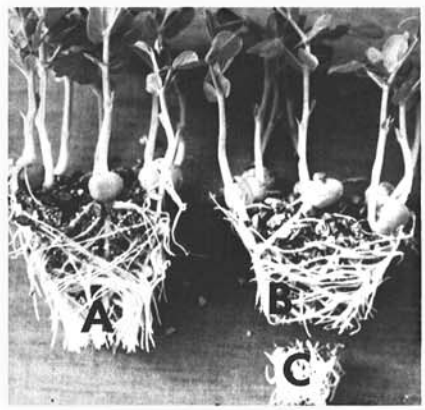

Fig. 1. Degree of root pruning when using the tray system of inoculation. **(A)** Development of the root systems of six pea seedlings in a single $5 \times 5 \times 7.2$ cm tray plug. **(B)** Root systems of six pea seedlings after root pruning and before inoculation with *F. o.* f. sp. *pisi*. **(C)** Root systems of the six seedlings removed by pruning before inoculation.

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Table 1. Survival of pea seedlings 28 days after inoculation with spores of *Fusarium oxysporum* f. sp. *pisi* using two methods of inoculation[a]

Inoculation method[b]	No. of surviving seedlings out of 18 inoculated[c]						
	LM	DSP	NE	NS	WSU 23	WSU 28	WSU 31
Race 1							
Expected[c]	0	18	18	18	18	18	18
Dip and cut	1	16	17	18	17	16	18
Tray	2	16	18	18	18	18	18
Race 2							
Expected	0	0	18	18	18	0	18
Dip and cut	2	0	16	17	18	4	16
Tray	0	1	18	17	17	2	18
Race 5							
Expected	0	0	0	0	18	18	18
Dip and cut	1	0	0	0	17	18	17
Tray	0	0	0	0	18	17	18
Race 6							
Expected	0	0	0	18	0	18	18
Dip and cut	1	2	1	16	1	15	18
Tray	0	0	0	17	0	18	18

[a] Total of three tests with six seedlings inoculated with each race in each test. Spore concentration of 1×10^6 cells per milliliter.

[b] Expected = expected survival of seedlings based on the genetic resistance in the differential cultivars. Dip and cut = standard method used to inoculate the differential cultivars; tray = new method.

[c] LM = Little Marvel; DSP = Darkskin Perfection; NE = New Era; NS = New Season; WSU 23, WSU 28, and WSU 31 = Washington State University breeding lines resistant to races 1,2,6; 1,5,6; and 1,2,5,6, respectively, of *Fusarium oxysporum* f. sp. *pisi*.

Table 2. Identification of unknown cultures of *Fusarium oxysporum* isolated from diseased pea stems using two methods of seedling inoculation

Isolates tested no.	Culture type[a]	Pathotype[b]	Race identification[c]	
			Dip and cut	Tray
8	1	1	4	4
	1	NP[d]	4	4
33	2	2	15	15
	2	NP	18	17
26	5	5	25	25
	5	NP	1	1
25	6	6	21	21
	6	NP	4	4

[a] *F. oxysporum* cultures isolated from diseased pea stems and classified to type when observed on potato-dextrose agar (adjusted to pH 4.5). Cultures 6 days old, 20–30 mm in diameter.

[b] Pathotype based on the reaction of the seven differential cultivars inoculated by the dip and cut method.

[c] Inoculum for tests of the two methods was from the same liquid culture.

[d] NP = cultures that were nonpathogenic by the dip and cut method.

were used to determine pathogenicity of *F. o.* f. sp. *pisi* cultures (5). Seven cultivars (Little Marvel, Darkskin Perfection, New Era, New Season, WSU 23, WSU 28, and WSU 31) differentiate races 1, 2, 5, and 6 of *F. o.* f. sp. *pisi*.

RESULTS

The tray method of growing and inoculating pea seedlings gave results comparable to the standard root dip and cut method. Table 1 lists the reaction of seven differential cultivars of peas to four races of *F. o.* f. sp. *pisi* inoculated with the dip and cut and the tray methods. The data presented in Table 1 established the efficacy of the tray method with four races of *F. o.* f. sp. *pisi*. The results of both the tray and the dip and cut methods were nearly identical to the expected survival. The data in Table 1 are the total of three tests with six seedlings of each cultivar inoculated in each test.

Table 2 compares the two methods of inoculation for identification of 92 unknown cultures of *F. oxysporum*. The results obtained with the tray method were nearly identical to the dip and cut method in identifying races of *F. o.* f. sp. *pisi* and/or for characterizing pathogenicity of unknown cultures.

DISCUSSION

The advantage of the tray method of inoculation is in improved efficiency, not improved accuracy. Greenhouse tests using the standard dip and cut method, inoculating six seedlings of each differential cultivar, require approximately 20 min per culture. With the tray method, four cultures can be inoculated during the same period of time. The efficiency of this method occurs because six seedlings are pulled, cut, and inoculated at one time. Also, during the inoculation procedure all of the seedlings' roots are cut to the same length and are immersed into the inoculum to the same depth. The uniformity of root pruning and depth of immersion into the inoculum is determined by the uniformity of seeding depth in the trays. Also, damage to the stems of the seedlings in the area of cotyledon attachment is reduced when seedlings are removed from individual cells as compared with uprooting and washing with the dip and cut method.

This method of inoculation can be used for testing pea seedlings for resistance to *F. o.* f. sp. *pisi* as well as evaluation of isolates of *F. o.* f. sp. *pisi* for pathogenicity. The tray method has been used at Washington State University, Northwestern Washington Research Center, for the past 2 yr. During this period of time, it has been observed that death of seedlings caused by mechanical damage during inoculation and transplanting was reduced. Also, the stunting of seedlings due to inoculation and transplant shock was less when the tray method was used. The tray method is now used in all routine testing. The final characterization of individual isolates of *F. o.* f. sp. *pisi* and the final determination of genetic resistance within specific lines of peas will be confirmed with the standard dip and cut method.

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