Resistance of in Vitro-derived Cucumber Plants to *Pythium* *aphanidermatum*

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Abstract. 'Embryonic axes-derived 'Burpless Hybrid' cucumber (*Cucumis sativus* L.) plantlets germinated on Murashige and Skoog (MS) medium supplemented with 16 combinations of BAP and NAA and seedlings derived from whole seeds cultured on semi-solid agar were inoculated in vitro with two isolates (WFU3 and WFM13) of *Pythium aphanidermatum*. All axes-derived plantlets and whole seedlings inoculated with WFM13 isolates were susceptible to blight and died 2 days after inoculation. Similarly, all seedlings inoculated with WFU3 isolates were killed within 2 days after inoculation; however, the rate of development and severity of blight varied among the axes-derived plantlets. Blight on axes-derived plantlets, regenerated on MS medium supplemented with 2 mg BAP/liter and 0.2 mg NAA/liter, was significantly less than on regenerants cultured on all other amended MS media. On some media, callus developed on crowns and/or primary roots. The presence of callus influenced resistance to *Pythium*. In a second experiment, axes-derived cucumber regenerants from five genotypes, cultured on MS medium supplemented with 2 mg BAP/liter and 0.2 mg NAA/liter, were compared for their resistance to *P. aphanidermatum* isolate WFU3. Resistance was significantly greater for 'Burpless Hybrid' and 'Sweetslice' than for three other genotypes. Chemical names used: 6-benzylaminopurine (BAP); α -naphthaleneacetic acid (NAA).

Damping-off and/or root rots are incited by various soilborne pathogens, including species of *Pythium*. These are ubiquitous in agricultural soils and have a broad host range that includes cucumbers (Bedlan, 1986; Hendrix and Campbell, 1973). Most pathogenic fungi derive nutritional energy from living cells and, to obtain these, they must penetrate or degrade the cell wall (Aist, 1976; Mount, 1978). Penetration of host cell walls is a complex process that has been examined for numerous pathogens (Murray and Maxwell, 1974) and appears to involve both mechanical and enzymatic means (Hendrix and Campbell, 1973; Murray and Maxwell, 1975) controlled by the pathogen. Structural and chemical constituents in the plant epidermal wall are postulated to influence penetration by pathogens. For example, cell maturation, rapid death, and suberization generally inhibit pathogenesis (Akai, 1959). Intracellular callose generally forms between the cell wall and plasma membrane at a location ad-

jacent to the point of penetration. This material is an amorphous mass of polysaccharides and lignin. The epidermal cells usually intercept the pathogen first. The potential for a compatible interaction (i.e., disease) will be greatly affected by the physiological state of the host cell. Parenchymatous callose growing in a nutrient-rich environment represents a very different infection court than a structurally organized seedling. Hormonal amendments in callus culture media could affect the susceptibility to infection by pathogenic fungi. In addition, cell variation is

likely to occur during callus formation, which could result in a nonuniform infection court (Meinert and Delmer, 1977). In some tissue culture-derived cucumber plants, callus was observed to persist on the primary roots and/or crown regions up to the time of transplanting to soil (Msikita, 1989). The persistence of this tissue on seedlings transplanted to soil could influence pathogenesis by *P. aphanidermatum*.

This study was undertaken to determine if the observed persistence of callus on some tissue culture-derived cucumber regenerants would affect pathogenesis by *P. aphanidermatum*.

Control seedlings. Thirty seeds of 'Burpless Hybrid' cucumber (Sakata Seed Co., San Francisco) were separated from their seedcoats, surface-sterilized in 10% bleach (0.52% sodium hypochlorite, v/v), rinsed in sterile distilled water (SDW), and aseptically germinated in test tubes (25 × 150 mm) containing 10 ml of semi-solid agar [prepared by dissolving 8% (w/v) Difco-bacto agar in distilled water and autoclaving for 15 min at 1.06 kg·cm⁻²]. After 3 weeks, seedlings with three to four leaves and tissue culture-derived plants were transplanted into plastic Conetainers (Ray Leach Conetainer Corp., Canby, Ore.) and inoculated with *Pythium* 2 days later.

Before transplanting, the Conetainers were thoroughly washed in 10% sodium hypochlorite solution (0.52%, v/v), rinsed two times with SDW, and dried in a bio-free environment. A nonsterile cotton ball was placed in the bottom of each Conetainer that was then filled with fine-textured vermiculite (which had been autoclave twice for 40 min at 1.06 kg·cm⁻² on two separate days) to within 1 cm of the Conetainer's upper lip.

Tissue culture-derived plantlets. Forty-five seeds of 'Burpless Hybrid' cucumber were separated from their seedcoats and cut into pieces consisting of an embryonic axis and

Table 1. Ex vitro score distribution for *Pythium aphanidermatum* blight isolate WFU3 on 'Burpless Hybrid' cucumber embryonic axes germinated on Murashige and Skoog medium supplemented with BAP and NAA.^a

BAP/NAA (mg·liter ⁻¹)	Disease severity ^b					Callused plants (%) ^c
	1	2	3	4	5	
	No. of plants					
2.0/0.0	0	2	0	10	18	55
2.0/0.1	0	0	0	0	30	0
2.0/0.2	12	8	5	0	4	76
2.0/0.3	4	0	0	6	24	33
3.0/0.0	0	0	0	0	30	0
3.0/0.1	0	0	0	0	29	0
3.0/0.2	0	0	0	0	30	0
3.0/0.3	0	0	0	0	30	0
4.0/0.0	0	0	0	0	30	0
4.0/0.1	0	0	0	6	24	33
4.0/0.2	1	0	2	4	23	57
4.0/0.3	0	1	1	5	22	45
5.0/0.0	0	0	0	0	30	0
5.0/0.1	0	0	0	0	30	0
5.0/0.2	0	0	0	0	30	0
5.0/0.3	0	0	0	0	30	0

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^aCallus on primary roots and/or crown region.

^b1 = no blight on plants, 2 = 25% blight, 3 = 50% blight, 4 = 75% blight, 5 = 100% blight (plants dead).

^cn = 29 or 30.

Fig. 1. Embryonic axes-derived seedlings that were germinated in vitro on Murashige and Skoog medium supplemented with (mg-liter⁻¹) (left) 2 BAP and 0.2 NAA and (right) 3 BAP and 0.2 NAA. Note callus growth at the base of the plantlet on the left.

Table 2. Ex vitro mean scores of *Pythium aphanidermatum* (isolate WFU3) blight on embryonic axes-derived cucumber cultivars and lines cultured on Murashige and Skoog medium supplemented with BAP (2.0 mg-liter⁻¹) and NAA (0.2 mg-liter⁻¹) (n = 15).

Cultivars/lines	Disease severity*	Seed source
VGP 5058	4.5 a	Asgrow
VGP 5049	5.0 a	Asgrow
VGD 254	4.7 a	Asgrow
Burpless Hybrid	2.9 b	T. Sakata
Sweetslice	2.8 b	Peto Seed
LSD _{0.05}	2.0	

*1 = no blight on plants, 2 = 25% blight, 3 = 50% blight, 4 = 75% blight, 5 = 100% blight (plants dead).

two cotyledons. To obtain some callused plantlets, a method-described by Msikita et al. (1990) was used. The axes were tied in a square of cheesecloth ($\approx 2 \times 2$ cm) and surface-sterilized (10 rein) in 10% commercial bleach to which 40 mg Alconox powder had been added as a surfactant. The pieces were later rinsed five times (30 sec/rinse) with SDW. The bags were untied and each piece was aseptically transferred to a culture tube (25 $\times$ 150 mm) containing 10 ml of modified Murashige and Skoog (MS) (1962) salt medium amended with 16 combinations of BAP and NAA (see Table 1). Before autoclaving (1.06 kg-cm⁻², 15 min), Staba (1969) vitamins were added, the pH of the medium was adjusted to 5.7, and then Difco-bacto agar (7% w/v) was added. Axes were placed on the media vertically, with the tip-end positioned ≈ 1 to 2 mm beneath the medium surface.

A second experiment was conducted using

embryonic axes from 'Burpless Hybrid', 'Sweetslice' (Peto Seed Co., Saticoy, Calif.), VGP 5058, VGP 5049, and VGD 254 (Asgrow Seed Co., Kalamazoo, Mich.) cultured on MS supplemented with 2 mg BAP/liter and 0.2 mg NAA/liter. Explants were cultured for 8 weeks, and 30 uniform seedlings were selected and transplanted into Cone-tainers filled with vermiculite and incubated (30C, 16 hr light for 48 hr) before inoculation with *P. aphanidermatum* isolate WFU3.

Fungal cultures. *Pythium aphanidermatum* was isolated from infected *Agrostis palustris* Huds collected from two locations in Champaign, Ill. Grass leaves were surface-sterilized by immersing them in 10% bleach for ≈ 1 min and rinsing three times in SDW. Small pieces (≈ 5 mm²) of surface-sterilized leaf were seeded into potato dextrose agar (PDA) amended with 25 ppm rifampin (Sigma Chemical Co., St. Louis) (PDA-R). Hyphal tips growing from the leaf tissue were indi-

vidually transferred to fresh PDA-R, cultured for 3 to 4 days on a laboratory bench, and transferred again to PDA. PDA was prepared as described by Langeran (1945). Rifampin was dissolved in methanol and filter-sterilized (millipore filter 0.22 μ m) before being added to sterilized PDA cooled to 45C. *Pythium* cultured on water agar (17 g Difco-bacto agar/liter water) was stored in sterile water on a laboratory bench for up to 6 months.

Pythium inoculum was produced by transferring mycelia from water agar onto PDA in a petri plate. The cultures were sealed with parafilm and incubated for 24 hr in a growth chamber (30C, 90% RH). *Pythium* had colonized the entire surface of PDA 24 hr after incubation, and then was cut into small culture blocks (≈ 3 mm³) using a sterile scalpel. A single culture block was placed against the base of each cucumber seedling in Containers. Care was taken to ensure that the fungus was in direct contact with the plant surface. The inoculated plants were supported in a rack, and the whole assembly enclosed in a clear plastic bag and incubated in a growth chamber at 30C, 100% RH.

The first experiment consisted of ≈ 30 seedlings (10 each inoculated with either *Pythium* isolate WFU3 or WFM13 or uninoculated) from each of the 16 media and seedlings germinated on agar.

The second experiment consisted of 15 seedlings from each of the five cultivars, all inoculated with WFU3. The experiments were repeated three times, and results were analyzed in a completely randomized design with each seedling as a replicate. Data were analyzed using the General Linear Model procedure of SAS (1982). Disease severity was assessed by using a severity rating of 1 to 5 (1 = unaffected, 2 = 25% blight, 3 = 50% blight, 4 = 75% blight, and 5 = 100% blight).

Comparison of media. The growth of axes-derived plantlets on the various media is described elsewhere (Msikita et al., 1990). More than 90% of the seedlings and plantlets inoculated with WFM13 were killed after 2 days and all were dead within 4 days. The plants appeared water-soaked and macerated and were damped-off at the soil surface.

Plants inoculated with WFU3 developed blight symptoms more slowly than those inoculated with WFM13. After 5 days, WFU3-inoculated plants developed blight symptoms comparable to those observed 2 days after inoculation with WFM13. However, some tissue culture-derived plants with callus on their primary roots had lower disease severity when inoculated with WFU3 than with WFM13. Particularly notable were seedlings from medium supplemented with 2 mg BAP/liter and 0.2 mg NAA/liter. On this medium, most plants formed callus on the primary roots and/or the crown and, therefore, had a lower disease-severity rating (2.1) than the rest (4.6-5.0).

Some plantlets developed callus on their crowns and/or primary root (Fig. 1). Some of these calli persisted ex vitro. More than 90% of the plants that had callus on their

primary roots or crown had a lower disease severity when inoculated with WFU3 than with WFM13 (mean disease severity = 2.1). The control plants were all susceptible (disease severity = 5).

Disease severity distribution (three experiments) for isolate WFU3 on ex vitro seedlings varied for different growth regulator treatments. Plants from the treatment 2 mg BAP/liter and 0.2 mg NAA/liter showed the lowest disease severity, as noted before, and had the highest proportion with callus (Table 1).

Comparison of genotypes. Cucumber genotypes differed in the rate and severity of disease development. Symptoms were similar on all genotypes, and 2 days after inoculation; all genotypes showed initial symptoms of blight. Overall mean disease severity was about equal on seedlings of VGP 5058, VGP 5049, and VGD 254 and significantly lower and about equal on 'Burple Hybrid' and 'Sweetslice' (Table 2).

Cucumber seedlings germinated on agar and regenerants from various tissue culture media reacted differently ex vitro to the two *P. aphanidermatum* isolates. The isolate WFM13 was more aggressive, attacking all inoculated seedlings and regenerants within 2 days, while WFU3 did not infect plants from some of the treatments. Variation in disease severity between isolates of a single fungus has been reported for numerous other pathogens (Asian Vegetable Research Center, 1985; Ingram, 1987; White et al., 1987). Differences in disease severity among *P. aphanidermatum* isolates has been attributed to dissimilar production of toxin and pectolytic and cellulolytic enzymes (Sadik et al., 1983).

Plantlets cultured on different media reacted dissimilarly to WFU3. Plantlets with callus on their crowns and/or primary roots exhibited greater resistance to the pathogen (i.e., lower disease severity) than noncallused plants (Table 1). *Pythium* kills plant cells primarily by penetrating epidermal tissues, destroying membranes, and colonizing intracellularly (Winstead and McCombs, 1961). The observed resistance may be a consequence of the physical change in the morphology of callused cells. For example, callus formation is associated with signification of the outer cell walls (Hammerschmidt et al., 1984), and lignin is a resistant cell wall component. It is unlikely that the mass of callus simply slows fungal colonization, because pathogenic *Pythium* isolates are generally rapid colonizers, and, therefore, would have attacked both callus and plantlet.

Cucumber resistance to *Pythium* blight appears to be a genetically controlled reaction.

Genotypes differed in their resistance to the two *Pythium* isolates. Resistance can result from both active or passive defensive mechanisms. Formation of callus as an active defensive mechanism has been reported in several crop species (Allen and Friend, 1983; Bonhoff et al., 1987; Lazarovits et al., 1976).

Successful pathogenesis involves a complex interaction of factors involving the plant, pathogen, and environment. Inoculum density, temperature, and relative humidity must be favorable for successful pathogenesis to occur (Ogura, 1984). Differences in plant reaction to the two isolates of *P. aphanidermatum* could not have originated from these factors, as they were consistent in all experiments. In the second experiment, where several cucumber genotypes and one growth regulator combination was used, differences in disease severity could have resulted from differences in the genetic makeup of the plants.

This study shows that cucumber genotypes react differently to inoculation with *Pythium* isolates and that it is possible to alter the disease severity on cucumber regenerants from tissue culture using various growth regulator combinations. The presence of callus on tissue culture-derived regenerants increased resistance to infection by *P. aphanidermatum* ex vitro. The observed resistance may be a consequence of morphological changes in the outer cells of the primary roots and/or crowns or possibly of physiological mechanisms for resistance of the seedlings. This resistance could be overcome by more aggressive *Pythium* isolates, such as WFM13.

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