Multiple-Pathogen Inoculation of Cucumber (*Cucumis sativus*) Seedlings

A. J. WYSZOGRODZKA, Postdoctoral Research Associate, ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568, and P. H. WILLIAMS, Professor, Department of Plant Pathology, University of Wisconsin-Madison, 1630 Linden Drive, and C. E. PETERSON, Professor, USDA-ARS, Department of Horticulture, University of Wisconsin-Madison, 1575 Linden Drive, Madison 53706

ABSTRACT

Wyszogrodzka, A. J., Williams, P. H., and Peterson, C. E. 1987. Multiple-pathogen inoculation of cucumber (*Cucumis sativus*) seedlings. Plant Disease 71: 275-280.

Methods were developed for simultaneous inoculation of cucumber seedlings with *Pseudomonas syringae* pv. *lachrymans* (*P. s.* pv. *lachrymans*), *Pseudoperonospora cubensis*, *Colletotrichum orbiculare* race 1, and *Cladosporium cucumerinum* and for sequential inoculation with *Corynespora cassiicola* under controlled environments. Localized inoculation of selected sites on 2-day postemergent cotyledons with *P. s.* pv. *lachrymans* (2.2×10^4 colony-forming units delivered in 1 μl of phosphate buffer into a toothpick wound), with *P. cubensis* (50 zoospores in 1 μl of water), with *Colletotrichum orbiculare* (200 spores in 5 μl of water), and with *Cladosporium cucumerinum* (2,000 spores in 10 μl of water delivered on the apical growing point), followed by 48 hr of incubation at 20 C, 100% relative humidity (RH) in the dark and 24 hr in a 20 C lighted growth chamber, permitted differentiation between resistant and susceptible cucumber cultivars GY 14, Wisconsin SMR 18, GY 3, and Straight 8. No induced resistance was observed when seedlings of GY 14 were inoculated with *Corynespora cassiicola* (1,000 spores in 5 μl of water followed by 48 hr of incubation at 24 C, 100% RH in the dark) 72 hr after inoculation with the above pathogens.

Additional key words: angular leaf spot, cucumber anthracnose, cucumber scab, disease resistance, downy mildew, multiple-disease resistance, target leaf spot

Breeding for resistance to major cucumber diseases has long been an objective of the U.S. national cucumber improvement program carried out jointly between the USDA and the Department of Plant Pathology at the University of Wisconsin-Madison. Much of the success of this program has been due to the effective use of seedling screening methodology permitting the selection of individuals resistant to cucumber scab (*Cladosporium cucumerinum* Ellis & Arth.), anthracnose (*Colletotrichum orbiculare* (Berk. & Mont.) Arx.), downy mildew (*Pseudoperonospora cubensis* (Berk. & Curt.) Rostow), angular leaf spot (*Pseudomonas syringae* pv. *lachrymans* (*P. s.* pv. *lachrymans*) (Smith & Bryan) Young, Dye, & Wilkie), target leaf spot (*Corynespora cassiicola* (Berk. & Curt.) Wei.), bacterial wilt (*Erwinia tracheiphila* (Smith) Bergey et al), Fusarium wilt (*Fusarium oxysporum* (Schlecht.) Snyd. & Hans. f. sp. *cucumerinum* (*F. o.* f. sp. *cucumerinum*) Owen), and cucumber mosaic virus (1,14). This technology permits time- and space-efficient selection of multiple-disease-resistant individuals from large, segregating seedling populations grown under controlled environments provided by growth chambers and greenhouses.

Because of the relatively large proportion of each seedling assigned as the target to receive inoculum, existing multiple-disease-resistance seedling screens are limited in the number of different pathogens or pathotypes of a given pathogen that may be evaluated on single plants. However, by reducing the target area and minimizing the volume of inoculum, it should be possible to increase the number of pathogens inoculated on a seedling, thus expanding the scope of simultaneous inoculations and intensifying the selection for multiple-disease-

Accepted for publication 5 November 1986 (submitted for electronic processing).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

resistant individuals. By increasing the number of pathogens in a screen, multiple-pathogen inoculations would help reduce the number of evaluations, thus lowering the cost involved with expensive greenhouse and growth chamber space. An additional benefit of the technique would be the reduction in number of seeds used in the screening, particularly when seeds derived from difficult-to-make crosses are limited. If a high degree of uniformity in inoculum delivery and host preparation can be achieved, multiple-inoculation methodologies should be amenable to mechanization.

Among factors considered in the development of simultaneous inoculation methods was the need for different environments for infection and incubation by different pathogens. Another factor was the different time required by each pathogen for development of symptoms. Yet another important consideration was the possibility of the interactions that may result in alteration in the expression of interaction phenotypes of the various pathogens applied to the host.

An alternative to simultaneous inoculation of various pathogens is the sequential inoculation and evaluation of interaction phenotypes over an extended time frame. In sequential inoculations, the possibility of induced resistance may limit the application of particular combinations of pathogens on particular host genotypes. The induced resistance phenomenon observed in cucumbers by Kuć and his colleagues (3,7,10–12,15) after inoculation with *Colletotrichum lagenarium* (*C. orbiculare*), *Cladosporium cucumerinum*, or *Pseudomonas syringae* pv. *lachrymans* (*P. s.* pv. *lachrymans*) was regarded as an important response to be examined in the development of a multiple-disease-resistance screening program with this species.

In this study, five cucumber pathogens were selected to develop and test a model procedure for multiple inoculations of cucumber cotyledons. *P. s.* pv. *lachrymans*, *Pseudoperonospora cubensis*, *Colletotrichum orbiculare*, and *Cladosporium cucumerinum* were selected for simultaneous inoculations because they require the same temperature for infection and infect cotyledons of young seedlings (1). *Corynespora cassiicola* was used in sequential inoculation after the simultaneous inoculation with the four pathogens listed because it requires higher temperature for infection and produced symptoms most reliably on older cotyledons.

MATERIALS AND METHODS

Inoculum preparation. Conditions for culture maintenance and inoculum preparation for *P. s.* pv. *lachrymans*, *Colletotrichum orbiculare*, *Corynespora cassiicola*, and *Cladosporium cucumerinum* are summarized in Table 1. Fungal inocula were quantified with a bright-line hemacytometer, and bacterial suspensions were quantified by measuring optical density (OD) at 625 nm, which had been standardized to colony-forming units (cfu) on tetrazolium chloride agar (17) plates at 28 C. The obligately biotrophic *Pseudoperonospora cubensis* was maintained on cotyledons of susceptible cultivar Wisconsin SMR 18 grown in vermiculite at 16 C, 12-hr photoperiod. To increase the inoculum, 3-day-old seedlings were inoculated by placing 1.2 $\times 10^3$ zoosporangia in 10 μl of distilled water in the center of the cotyledon, followed by 48 hr incubation at 20 C, 100% relative humidity (RH) in the dark. Inoculum was prepared by placing 3-wk-old seedlings in a darkened dew chamber at 20 C for 12 hr. Cotyledons with profuse sporulation on the abaxial side were then pinched off, immersed in distilled water, and the zoosporangia dislodged by gently rubbing the cotyledons against the wall of a beaker. To release the zoospores, 2×10^6 zoosporangia were incubated without agitation in 10 ml of distilled water for 2 hr at 20 C, then the motile zoospores that tended to swim to the surface were separated from the remaining zoosporangia by withdrawing the upper 5 ml with a pipette. The zoospore concentration was quantified with a bright-line hemacytometer, and dilutions were made with distilled water kept at 20 C.

Host preparation. Cucumber cultivars GY 14 (resistant to anthracnose, downy mildew, angular leaf spot, and scab; susceptible to target leaf spot), GY 3 (resistant to angular leaf spot, downy mildew, and anthracnose; susceptible to scab and target leaf spot), Wisconsin SMR 18 (resistant to scab; susceptible to angular leaf spot, downy mildew, and anthracnose; segregating for resistance to target leaf spot), and Straight 8 (susceptible to angular leaf spot, downy mildew, anthracnose and scab; segregating for resistance to target leaf spot) were used in the simultaneous inoculations with *P. s.* pv. *lachrymans*, *Pseudoperonospora cubensis*, *Colletotrichum orbiculare*, and *Cladosporium cucumerinum*. Cultivar GY 14 was also used in the

Table 1. Conditions for culture maintenance and inoculum preparation for *Pseudomonas syringae* pv. *lachrymans*, *Colletotrichum orbiculare*, *Corynespora cassiicola*, and *Cladosporium cucumerinum*

Pathogen	Storage	Retrieval	Inoculum increase	Inoculum preparation
P. s. pv. *lachrymans*	7-ml PDA[a] slant under mineral oil at 4 C in the dark	Streak a TZC[b] plate, incubate 48 hr at 28 C, then at 4 C in the dark for up to 3 mo	Transfer a single colony to 50 ml Husain-Kelman broth (8), shake 20–24 hr at 28 C on rotary shaker (130 rpm)	Adjust concentration by diluting with 0.01 M phosphate buffer, pH 7.1
Colletotrichum orbiculare	7-ml PDA slant at 4 C in the dark	7-ml GBA[c] slant at room temperature for 6–7 days	Spread loopful of spores over a GBA slant, incubate at room temperature for 5–6 days	Add 5–7 ml of distilled water, shake 10 sec, then wash 3× by centrifuging at 1,400 *g* and resuspending in distilled water
Corynespora cassiicola	7-ml PDA slant at 4 C in the dark	7-ml PDA slant, 4–6 days at 22 ± 2 C under continuous light	Spread loopful of surface hyphae and spores over 7-ml CZA[d] slant, incubate for 3–4 days at 22 ± under continuous light	Add 5–7 ml of distilled water, scrape surface hyphae, filter through double layer of nonsterile cheesecloth
Cladosporium cucumerinum	7-ml PDA slant at 4 C in the dark	7-ml PDA slant, 4–6 days at 20 C in the dark	Spread loopful of spores over 7-ml PDA slant, incubate for 3–4 days at 20 C in the dark	Add 5–6 ml of distilled water, scrape surface hyphae, filter through double layer of nonsterile cheesecloth

[a] Potato-dextrose agar (17).
[b] Tetrazolium chloride agar (17).
[c] Green bean agar (360 ml Gerber green bean baby food, 30 g agar, distilled water to 1 L).
[d] Czapek agar (17).

sequential inoculations with *Corynespora cassiicola*.

To obtain a highly uniform seedling population (which was necessary for uniformity and reproducibility of the host-pathogen interaction), seeds were germinated between moist paper towels covered with moist vermiculite at 32 C for 24 hr. Uniformly sized (3–5 mm radicle length) germinated seeds were transplanted into unsterilized, moist vermiculite in polyethylene freezer containers (10 × 10 × 7.5 cm with nine drainage holes in the bottom), in two rows, 1 cm within and 5 cm between rows, and covered with a layer of vermiculite 1 cm deep. The seedlings were grown in a growth chamber at 21 C at night and at 26 C during the 14-hr day under mixed incandescent and fluorescent lamps (Sylvania, Excel-line 60W and Cool-White 215W, no barrier). The photosynthetic photon flux density (PPFD) at plant canopy was 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$. The seedlings emerged and the cotyledons unfolded 2 days after transplanting. The plants were watered daily with tap water.

Inoculation. Based on various observations and prior experience, each cotyledon was divided into six areas for inoculation (Fig. 1). Experiments on inoculum concentration and placement were carried out so that interaction phenotype evaluation for each pathogen was confined to one of the six areas. Potential effects of the different target sites on interaction phenotype expression were studied by inoculating each pathogen in areas 1, 3, and 5. Temporal development of symptoms was studied on cotyledons ranging in age from 1 to 5 days. Inoculations were made with a Hamilton repeating dispenser (Hamilton Co., Reno, NV) with a 50-, 250-, or 500-μl syringe (Series 700, luer tip, Hamilton Co., Reno, NV) for delivery of 1-, 5-, and 10-μl droplets of inoculum, respectively. In numerous tests, inoculation of cucumber cotyledons with *Cladosporium cucumerinum* failed to produce reliable symptoms when various inoculum concentrations, methods of inoculum presentation, and seedling ages were tested. Therefore, the method of inoculating the growing point (1) was used in the multiple-pathogen inoculations.

By optimizing the conditions for inoculation with each pathogen separately and testing the range in which they were compatible, the following protocol for simultaneous inoculation of cucumber cotyledons with *P. s.* pv. *lachrymans, Pseudoperonospora cubensis, Colletotrichum orbiculare*, and *Cladosporium cucumerinum* and sequential inoculation with *Corynespora cassiicola* was developed.

Protocol for multiple inoculations of cucumber cotyledons. On the day after the cotyledons unfolded, *P. s.* pv. *lachrymans* was inoculated by pricking the tissue in area 6 with a round wooden toothpick and delivering a 1-μl droplet containing about 2.2×10^4 cfu to the wound, then 50 zoospores of *Pseudoperonospora cubensis* were applied in a 1-μl droplet in area 3 and 200 spores of *Colletotrichum orbiculare* were applied in a 5-μl droplet in area 4. *Cladosporium cucumerinum* was inoculated by placing 2,000 spores in a 10-μl droplet on the growing point. Plants were incubated for 48 hr at 20 C, 100% RH in the dark. After incubation, plants were kept for the first 24 hr in a 20 C growth chamber with 12-hr photoperiod under mixed incandescent and fluorescent lamps (Sylvania, Excel-line 60W and Cool-White 215W, with Plexiglas barrier) to permit the development of scab (1,19). Then *Corynespora cassiicola* was inoculated by placing a 5-μl droplet containing 1,000 spores in area 5, followed by 48 hr of incubation at 24 C, 100% RH in the dark. After incubation, the plants were moved to a 21/26 C growth chamber. When *Corynespora cassiicola* was not inoculated, the plants were moved from the 20 C growth chamber to the 21/26 C growth chamber after 24 hr. Symptoms were read 6–7 days after the first inoculation in both simultaneous and sequential inoculations.

To examine the variation in interaction phenotypes resulting from interactions between and among the pathogens developing on the same cotyledon, the symptoms of pathogens inoculated singly at corresponding target locations were studied concurrently with the multiple-pathogen inoculations. All tests were conducted in a randomized complete block, replicated twice with 10 seedlings per replicate in each treatment.

To evaluate the variation in interaction phenotype for each pathogen, standardized six-point scales were used in which 0–3 indicated resistant reaction and 5–9 susceptible, with the exception of target leaf spot, where only 0 and 1 were judged resistant. The even numbers were used to assess intermediate responses.

Angular leaf spot: 0 = no symptoms, 1 = olive-green ring < 1 mm wide, 3 = olive-green ring 1–3 mm wide, 5 = yellow ring >3 mm wide, 7 = brown zone around injury surrounded by yellow halo < 1 mm wide, and 9 = brown zone around injury surrounded by yellow halo > 1 mm wide.

Downy mildew: 0 = no symptoms; 1 = olive-green lesion 1–3 mm in diameter; 3 = olive-green lesion 3–5 mm in diameter; 5 = chlorosis 3–5 mm in diameter; 7 = chlorosis 5–7 mm in diameter; and 9 = chlorosis >7 mm in diameter, tissue sunken on abaxial side of cotyledon.

Anthracnose: 0 = no symptoms; 1 = separate, tan necrotic lesions (0.5–1 mm in diameter), affected area 1–3 mm in diameter; 3 =separate, tan necrotic lesions (0.5–1 mm in diameter) with light green halos, affected area 1–3 mm in diameter; 5 = separate, brown necrotic lesions (0.5–1 mm in diameter) with yellow halos, affected area 1–3 mm in diameter; 7 = coalescing, brown necrotic lesions (1–2 mm in diameter) with yellow halos, affected area 3–5 mm in diameter; and 9 = brown, necrotic lesion with yellow halo and collapsed tissue, affected area > 5 mm in diameter.

Target leaf spot: 0 = no symptoms; 1 = brown necrosis 1–2 mm in diameter on adaxial side, no symptoms on abaxial side; 3 = brown necrosis 1–2 mm in diameter on adaxial side, tissue sunken on abaxial side; 5 = brown necrosis 2–3 mm in diameter on adaxial side, tissue sunken on abaxial side; 7 = brown necrosis 3–5 mm in diameter on adaxial side, tissue sunken on abaxial side; and 9 = brown necrosis > 5 mm in diameter on adaxial side, tissue sunken on abaxial side.

RESULTS

Highly localized and reproducible symptoms were obtained in inoculations with *P. s.* pv. *lachrymans*, *Pseudoperonospora cubensis*, *Colletotrichum orbiculare*, and *Corynespora cassiicola*. On the cotyledons susceptible to *P. s.* pv. *lachrymans*, the site of injury was surrounded by brown necrotic tissue

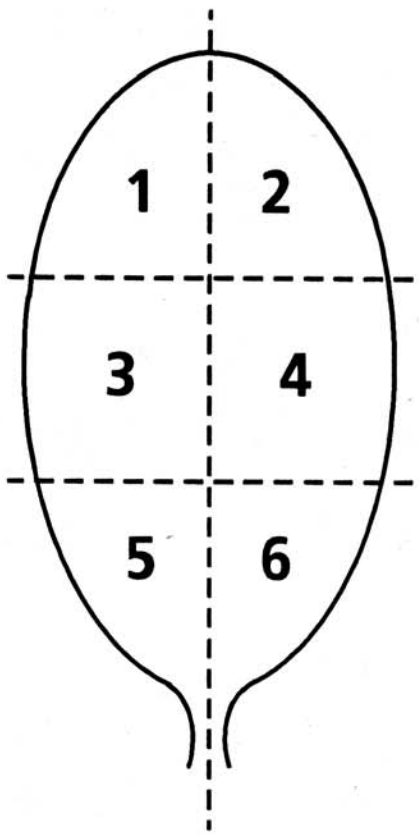

Fig. 1. Diagram of the division of a cucumber cotyledon into six areas of inoculation. Areas 1 and 2 constitute the distal region, areas 3 and 4 constitute the middle region, and areas 5 and 6 constitute the proximal region of the cotyledon.

expanding into a concentric zone (1–2 mm wide) of conspicuous chlorosis. The resistant reaction consisted of a narrow (1–3 mm) ring of olive-green tissue surrounding the tan necrotic rim of the toothpick injury. With *Pseudoperonospora cubensis*, the susceptible reaction consisted of a bright, diffuse chlorosis (3–7 mm in diameter), whereas no symptoms or small (1–5 mm in diameter) olive-green lesions were observed on the cotyledons of resistant seedlings. With *Colletotrichum orbiculare*, the susceptible reaction consisted of coalescing necrotic lesions (1–2 mm in diameter) with conspicuous chlorotic boundaries and was easily distinguished from separate, minute (0.5–1 mm in diameter) necrotic lesions on the resistant host. For *Corynespora cassiicola*, water-soaked lesions beneath the inoculum droplet were observed 48 hr after inoculation of the susceptible cotyledons. These lesions became sunken 3–4 hr after removing plants from 100% RH and turned necrotic and brown after 3–4 days. Resistant cotyledons showed no water-soaking or lesion development, though occasionally slight necrosis of the cotyledon epidermis was observed.

In multiple inoculations, the resistant or susceptible reaction to one pathogen did not alter the phenotypic reaction to another pathogen (Fig. 2). Disease ratings in multiple-pathogen inoculations did not differ from single-pathogen inoculations on the susceptible Wisconsin SMR 18 or the resistant GY 14 (Table 2.). All plants susceptible to *Cladosporium cucumerinum* were killed in both multiple and single inoculations with the pathogen. Occasionally, one of the cotyledons of the inoculated with *Cladosporium cucumerinum* seedlings remained viable for a period of time sufficient for the development of symptoms produced by *P. s.* pv. *lachrymans*, *Pseudoperonospora cubensis*, and *Colletotrichum orbiculare*, in which case no alterations in the expression of symptoms of the pathogens were observed (Fig. 3). In sequential inoculation of GY 14, which is known to be susceptible to *Corynespora cassiicola* and resistant to the other four pathogens, corresponding interaction phenotypes were produced as expected (Table 3).

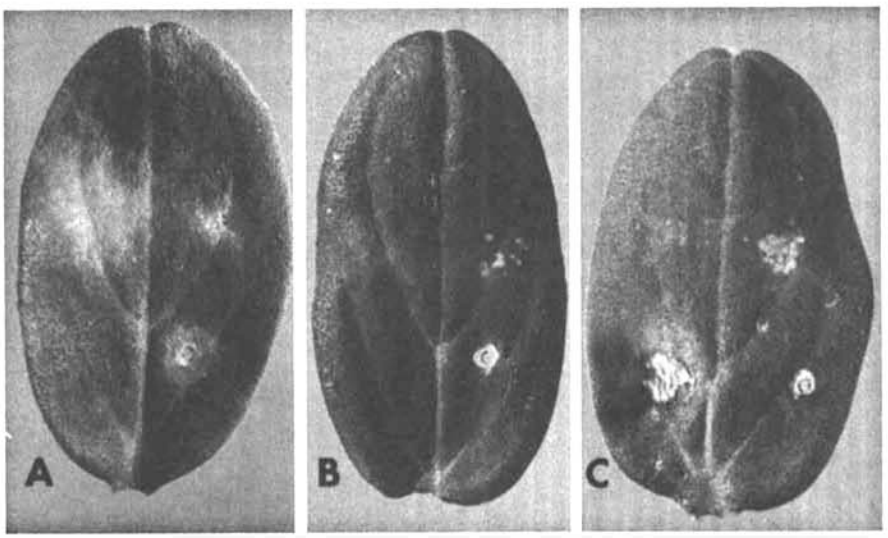

Fig. 2. Interaction phenotypes of cotyledons of cucumbers resistant to scab 7 days after inoculation with *Pseudomonas syringae* pv. *lachrymans* (Psl), *Pseudoperonospora cubensis* (Pc), *Colletotrichum orbiculare* race 1 (Co 1), *Cladosporium cucumerinum* (Ccu), and *Corynespora cassiicola* (Cca). **(A)** Cultivar Wisconsin SMR 18, susceptible reactions to: Pc (middle-left), Co 1 (middle-right), and Psl (proximal-right). **(B)** Cultivar GY 14, resistant reactions to Pc (middle-left), Col (middle-right), and Psl (proximal-right). **(C)** Cultivar GY 14, resistant reaction to Pc, Co 1, and Psl as in B and susceptible reaction to sequentially inoculated Cca (proximal-left). In A, B, and C, Ccu was inoculated on the growing point of the seedling.

Table 2. Comparison of interaction phenotypes (IP) in multiple- and single-pathogen inoculations in seedlings[a] of cucumber cultivars Wisconsin SMR 18 and GY 14 inoculated with *Pseudomonas syringae* pv. *lachrymans*, *Pseudoperonospora cubensis*, *Colletotrichum orbiculare* race 1, and *Cladosporium cucumerinum*

		Mean IP rating[b]			
		Wisconsin SMR 18		GY 14	
Pathogen	Test	Multiple inoculation	Single inoculation	Multiple inoculation	Single inoculation
P. s. pv. *lachrymans*	1	8.2	8.0	2.7	1.8*[c]
	2	8.3	8.1	2.1	2.0
Pseudoperonospora cubensis	1	7.3	6.6	2.5	1.9
	2	5.7	6.4	0.9	1.1
Colletotrichum orbiculare race 1	1	6.3	5.9	2.6	3.5*
	2	4.7	5.6	1.9	1.9
Cladosporium cucumerinum	1	0.0	0.0	0.0	0.0
	2	0.0	0.0	0.0	0.0

[a] Seedlings grown in moist, nonsterile vermiculite (21 C at night and 26 C during the day), 14-hr photoperiod, PPFD 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$; both cotyledons inoculated 2 days after emergence, followed by 48-hr incubation at 20 C, 100% RH in the dark.

[b] Average of one replicate with 20 cotyledons and two replicates each with 20 cotyledons in tests 1 and 2, respectively; symptoms assessed 7 days after inoculation on scales of 0–9.

[c] IP ratings in multiple and single inoculations are significantly different at $P = 0.05$ based on Mann-Whitney test (5) within a cultivar and a test.

DISCUSSION

The development of the multiple-pathogen inoculation methodology illustrates the potential for improvement of existing screening procedures and demonstrates that a high level of precision can be achieved in multiple-disease resistance screening. By careful manipulation of the host, the pathogen, and the environment, it is possible to combine single-pathogen inoculations onto a limited tissue area without adversely affecting or altering the symptom expression observed when each pathogen is inoculated singly.

Because of the limited area of the tissue assigned to receive inoculum of each pathogen, the most critical aspects in the development of the multiple-pathogen inoculation method were the reduction of the inoculum volume and the method of inoculation. The necessity to reduce the inoculum volume was most important with *Pseudoperonospora cubensis*, where the smallest volume of inoculum feasible (1 μl) was needed. This problem arises from the fact that colonization of tissue by *Pseudoperonospora cubensis* is enhanced by low temperature (15–20 C) (4), and if *Pseudoperonospora cubensis* was inoculated simultaneously with *Cladosporium cucumerinum* (which requires incubation at 20 C) (1,19), droplets of *Pseudoperonospora cubensis* larger than 1 μl produced lesions that expanded into other target areas. For inoculation with *Colletotrichum orbiculare*, the use of 5-μl droplets was critical for accurate differentiation of the interaction phenotypes. The 5-μl volume of inoculum permitted separation of the infection sites, which facilitated full

expression of the differential lesion development of resistant and susceptible interaction phenotypes. In preliminary experiments, we observed that with a 1-μl droplet, a single, large necrotic lesion was produced on both resistant and susceptible cotyledons, and with 10-μl droplets, the infected area of the cotyledon was too large. *P. s.* pv. *lachrymans* normally invades plants through stomata, hydathodes, or wounds (6), though in preliminary studies, the inconsistency of establishing infection in intact tissue necessitated the wounding method of inoculation.

More work is needed to develop methods of inoculation with *Cladosporium cucumerinum* that would allow expression of susceptibility without killing the seedling. In this study, the inability to obtain reliable symptoms on cucumber cotyledons was partially related to the fact that only very young cotyledonary tissue was susceptible to *Cladosporium cucumerinum* (1). Because of rapid physiological development of the tissue at 21/26 C, the cotyledons were susceptible for a very short time and the critical period of susceptibility could easily be missed. Because the period of susceptibility can be extended by growing seedlings at low temperature before inoculation, such a treatment should be evaluated. In preliminary experiments (unreported), we observed that inoculation of the regions close to the veins is more reliable. Thus, by changing the target location and the preinfectional environment, reliable readings of susceptibility to *Cladosporium cucumerinum* on cucumber cotyledons may be possible.

Evaluations of the target locations indicated that despite apparent uniformity of the cotyledonary tissue, not all the regions were equally suited for inoculation with particular pathogens. Some of the differences were probably due to the slight curvature of the proximal and distal regions and the tendency of the larger (5-μl) droplets with *Colletotrichum orbiculare* and *Corynespora cassiicola* spores to run off curved surfaces, resulting in escapes. In cotyledons susceptible to *Corynespora cassiicola*, collapsed tissue, which was sometimes observed in proximal regions of the cotyledon when the fungus was inoculated either in distal or middle regions, could interfere with the development of symptoms of other pathogens. This occurrence was minimized when inoculum was applied in the proximal region. The evaluation of target location also showed that different pathotypes of the same pathogen can be evaluated on a single cotyledon, because usually more than one region of the cotyledon was suitable for satisfactory symptom expression of a particular pathogen.

An important aspect of the multiple-pathogen inoculations is the possibility of the host-pathogen interactions that may alter the expression of interaction phenotypes among various pathogens. Abul-Hayja (1) observed decreased numbers of anthracnose lesions on cucumber cotyledons when inoculated simultaneously with *Cladosporium cucumerinum*. Synergistic effects resulting in increased disease have been reported for bean plants inoculated with mixed

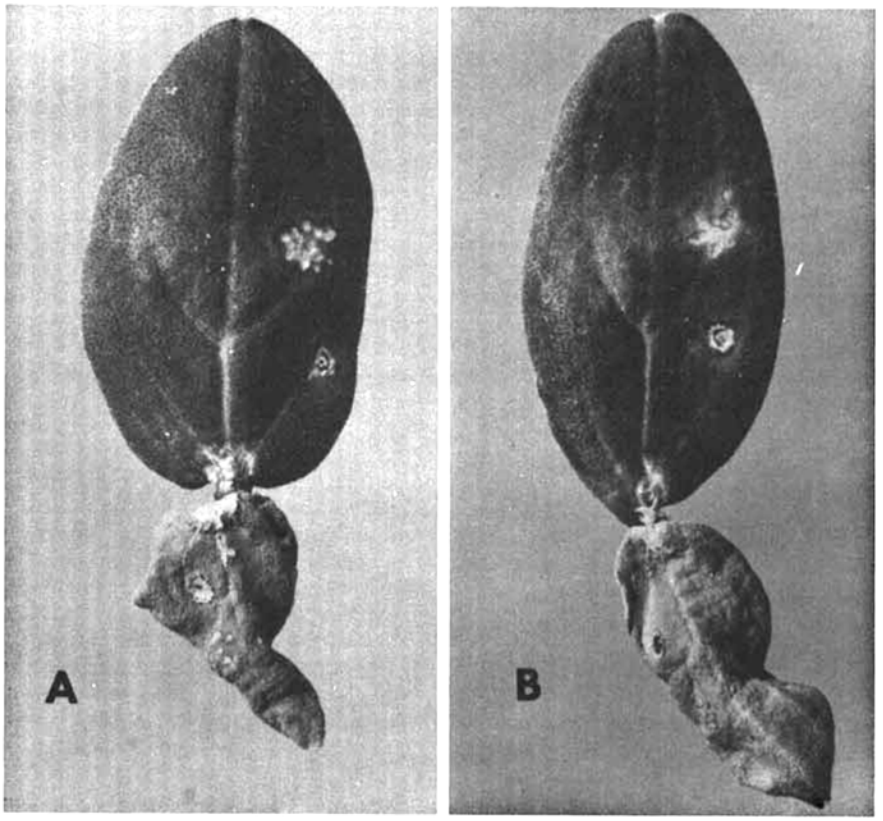

Fig. 3. Interaction phenotypes on cotyledons of cucumbers susceptible to scab 7 days after inoculation with *Pseudomonas syringae* pv. *lachrymans* (Psl), *Pseudoperonospora cubensis* (Pc), *Colletotrichum orbiculare* race 1 (Co 1), and *Cladosporium cucumerinum* (Ccu). **(A)** Cultivar GY 3, resistant reaction in the upper cotyledon to: Pc (middle-left), Co 1 (middle-right), and Psl (proximal-right). **(B)** Cultivar Straight 8, susceptible reaction in the upper cotyledon to: Pc (middle-left), Co 1 (middle-right), and Psl (proximal-right). In both cultivars, necrotic lower cotyledon indicates susceptibility to Ccu, which was inoculated on the growing point of the seedling.

Table 3. Comparison of interaction phenotypes (IP) in multiple-sequential and single-pathogen inoculations in seedlings[a] of cucumber cultivar GY 14 inoculated with *Pseudomonas syringae* pv. *lachrymans, Pseudoperonospora cubensis, Colletotrichum orbiculare* race 1, *Cladosporium cucumerinum*, and *Cornyespora cassiicola*

		Mean IP rating[b]	
Pathogen	Test	Multiple inoculations	Single inoculations
P. s. pv. *lachrymans*	1	1.1	1.3
	2	1.6	1.8
Pseudoperonospora cubensis	1	1.0	1.5*[c]
	2	1.1	1.7*
Colletotrichum orbiculare race 1	1	2.0	1.5
	2	1.6	1.9
Corynespora cassiicola	1	8.3	8.0
	2	6.3	5.9

[a] Seedlings grown in moist, nonsterile vermiculite (21 C at night and 26 C during the day), 14-hr photoperiod, PPFD 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$; both cotyledons inoculated 2 days after emergence with *P. s.* pv. *lachrymans, P. cubensis, C. orbiculare*, and *C. cucumerinum*, followed by 48-hr incubation at 20 C, 100% RH in the dark and 24 hr at 20 C, 12-hr photoperiod, then inoculated with *C. cassiicola*, followed by 48-hr incubation at 24 C, 100% RH in the dark.

[b] Average of two replicates each with 20 cotyledons; symptoms assessed 7 days after the first inoculation on scales of 0–9.

[c] IP ratings in multiple and single inoculations are significantly different at $P = 0.05$ based on Mann-Whitney test (5) within a cultivar and a test.

cultures of *Achromobacter* sp. and *Pseudomonas phaseolicola* (13). Kilpatrick et al (9) reported lowered symptom ratings in multiple inoculations compared with single inoculations of barley seedlings with three fungi. Interactions were not observed by Stavely (16), who reported independence of reactions of pathotypes of *Uromyces phaseoli* studied on the same or different leaves of a bean plant. Based on the independence of reaction to *Cladosporium cucumerinum* from reactions produced by the other pathogens in cultivars GY 3 and Straight 8 as well as the lack of significant differences between multiple and single inoculations in cultivars Wisconsin SMR 18 and GY 14, we conclude that in our study, the intrahost-interpathogen interactions were not present.

Induced resistance may occur in sequential inoculations and is a potential problem in the multiple-pathogen inoculation methodology. Prior inoculation of cucumbers with *Cladosporium cucumerinum*, *Colletotrichum orbiculare*, or *P. s.* pv. *lachrymans* was reported to result in induced resistance when the plant was challenged with the same pathogen or a different pathogen (3,7,10,12,15). In our study, resistance against *Corynespora cassiicola* was not induced, because there was no alteration of the symptoms produced by *Corynespora cassiicola* in sequential inoculation compared with concurrent single inoculation. It cannot be concluded, however, that induced resistance is not operating against this fungus, because it is possible that more than 72 hr between inoculations is required for the protection to be effective or measurable. Caruso and Kuć (3) and Kuć and Richmond (12) indicated that 96 hr were necessary for effective protection to be elicited. Another possibility is that induced resistance is not observed in GY 14 but may be operating in other genotypes of cucumbers. Staub and Kuć (15) reported that in the case of *Colletotrichum orbiculare* and *Cladosporium cucumerinum*, effective protection against *Cladosporium cucumerinum* was elicited only when tested cultivars were susceptible to some degree to *Colletotrichum orbiculare*. Because GY 14 was resistant to all four pathogens, the possibility of induced resistance should also be evaluated on susceptible genotypes.

A slight but significant ($P = 0.05$) reduction in downy mildew symptoms occurred in cultivar GY 14 when the cotyledons were sequentially inoculated with *Corynespora cassiicola*. However, this reduction did not affect the classification of interaction phenotypes. More research is needed to determine the cause of this interaction and to test how it may affect the symptom expression in genotypes susceptible to *Pseudoperonospora cubensis*.

Although resistance to anthracnose, downy mildew, and target leaf spot is monogenic (2,18), and the two categories of interaction phenotypes, resistant and susceptible, generally are used, scales of 0–9 were adopted in the symptom assessment in this study. The scales were based on the phenotypic variation in symptom expression and were used to facilitate detection of intrahost-interpathogen interactions. Though direct methods, i.e., quantification of sporulation or multiplication of pathogenic propagules in the host tissue, would better characterize the interaction, the use of scales was preferred because of the speed of assessment.

Although the multiple inoculations were performed with great care, some variability in symptom development and escapes was observed. Because in preliminary experiments we observed independent disease reactions on the cotyledons of a single seedling, the inoculations were replicated on both cotyledons to minimize errors in interpretation. Such replication was sufficient to obtain clear symptoms from all pathogens and correct for escapes. The observed variation was probably caused by the difficulty of handling individual seedlings in high density and overlapping of the neighboring seedlings. To remove this source of variation, new approaches to growing seedlings should be considered. For example, a system permitting individual handling of each seedling would be desirable, because it would permit alignment of the cotyledons. Additionally, such a system would permit inoculating seedlings germinating at the same time without eliminating the late-germinating individuals, as was done in this study. That would be particularly valuable in screening segregating populations in which the seed germination may not be uniform. High uniformity of the seedling population could also permit mechanized delivery of inoculum.

The development of methodology for inoculating single cotyledons with up to five pathogens provides new possibilities for screening for disease resistance in cucumbers. However, this study served mainly to illustrate the concept of multiple-pathogen inoculations, and more research is required before this methodology is used in multiple-disease-resistance screening. Most important, the developed methodology should be tested on a number of cucumber lines differing in resistance and susceptibility to the tested pathogens. Although no interactions were observed in this study, such phenomena may occur in other genotypes.

LITERATURE CITED

1. Abul-Hayja, Z. M. 1975. Multiple disease screening and genetics of resistance in cucumber. Ph. D. thesis. University of Wisconsin, Madison. 149 pp.
2. Abul-Hayja, Z. M., Williams, P. H., and Peterson, C. E. 1978. Inheritance of resistance to anthracnose and target leaf spot in cucumbers. Plant Dis. Rep. 62:43-45.
3. Caruso, F. L., and Kuć, J. 1979. Induced resistance of cucumber to anthracnose and angular leaf spot by *Pseudomonas lachrymans* and *Colletotrichum lagenarium*. Physiol. Plant Pathol. 14:191-201.
4. Cohen, Y. 1981. Downy mildew of cucurbits. Pages 341-354 in: The Downy Mildews. D. M. Spencer, ed. Academic Press, New York. 636 pp.
5. Conover, W. J. 1980. Practical Nonparametric Statistics. 2nd ed. John Wiley & Sons, New York. 493 pp.
6. Dixon, G. R. 1982. Vegetable Crop Diseases. AVI, Westport, CT. 404 pp.
7. Hammerschmidt, R., Acres, S., and Kuć, J. 1976. Protection of cucumber against *Colletotrichum lagenarium* and *Cladosporium cucumerinum*. Phytopathology 66:790-793.
8. Husain, A., and Kelman, A. 1958. Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. Phytopathology 48:155-165.
9. Kilpatrick, R. A., Baenziger, P. S., and Moseman, J. G. 1981. Multiple inoculation technique for evaluating resistance of single barley seedlings to three fungi. Plant Dis. 65:504-506.
10. Kuć, J., and Hammerschmidt, R. 1978. Acquired resistance to bacterial and fungal infections. Ann. Appl. Biol. 89:313-317.
11. Kuć, J., Shockley, G., and Kearney, K. 1975. Protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. Physiol. Plant Pathol. 7:195-199.
12. Kuć, J., and Richmond, S. 1977. Aspects of the protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. Phytopathology 67:533-536.
13. Maino, A. L., Schroth, M. N., and Vitanza, V. B. 1974. Synergy between *Achromobacter* sp. and *Pseudomonas phaseolicola* resulting in increased disease. Phytopathology 64:277-283.
14. Palmer, M. J., and Williams, P. H. 1981. A seedling evaluation method for Fusarium wilt of cucumber incited by *Fusarium oxysporum* f.sp. *cucumerinum*. (Abstr.) Phytopathology 71:247.
15. Staub, T. H., and Kuć, J. 1980. Systemic protection of cucumber plants against disease caused by *Cladosporium cucumerinum* and *Colletotrichum lagenarium* by prior infection with either fungus. Physiol. Plant Pathol. 17:389-393.
16. Stavely, J. R. 1983. A rapid technique for inoculation of *Phaseolus vulgaris* with multiple pathotypes of *Uromyces phaseoli*. Phytopathology 73:676-679.
17. Tuite, J. C. 1969. Plant Pathological Methods. Burgess Publishing, Minneapolis, MN. 239 pp.
18. van Vliet, G. J. A., and Meysing, D. 1974. Inheritance of resistance to *Pseudoperonospora cubensis* Rost. in cucumber (*Cucumis sativus* L.). Euphytica 23:251-255.
19. Walker, J. C. 1950. Environment and host resistance in relation to cucumber scab. Phytopathology 40:1094-1102.