Leaf Disk Immersion (LDI) Inoculation of Sunflower with *Plasmopara halstedii* for In Vitro Determination of Host-Pathogen Relationships

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ABSTRACT

Sackston, W. E., and Vimard, B. 1988. Leaf disk immersion (LDI) inoculation of sunflower with *Plasmopara halstedii* for in vitro determination of host-pathogen relationships. Plant Disease 72:227-229.

Sunflowers were successfully inoculated with downy mildew by immersing detached leaves (LI) or leaf disks (LDI) in suspensions of zoosporangia of *Plasmopara halstedii* for 3 hr at 15 C, then maintaining the leaves or leaf disks on water agar in petri dishes in an illuminated incubator at 15 C. Reactions in compatible and incompatible cultivar-race combinations were comparable to those of plants inoculated by the standard whole seedling immersion (WSI) method. LDI can be used to check the reaction of healthy "escapes" in compatible WSI inoculations, to check the race of *P. halstedii* on susceptible seedlings in incompatible combinations, and to determine the reaction of individual plants to several races of *P. halstedii* or to *P. halstedii* as well as to other pathogens.

Downy mildew (*Plasmopara halstedii* (Farl.) Berl. et de Toni) of sunflower (*Helianthus annuus* L.) normally infects through roots or hypocotyls during germination and emergence of seedlings and typically gives rise to systemic infection (9). Although zoosporangia may be produced on the leaves during periods of high humidity and may be disseminated by wind (3,4), infection of aboveground parts producing local lesions occurs only on young tissues and during protracted periods of wet weather (1,8,12). Because such secondary infection is difficult to induce by normal procedures (2), it has not been possible to make in vitro studies of downy mildew on sunflower leaves comparable to those on lettuce (11) and other crops.

Kajornchaiyakul and Brown (7) infected sunflower seedlings with *Albugo tragopogi* (Pers.) Schroet. by submerging the first pair of true leaves in a zoosporangial suspension. We report in this paper on our adaptations of their method to inoculate sunflower leaves with *P. halstedii* and on some applications of the method. A preliminary note has appeared elsewhere (10).

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Accepted for publication 9 October 1987 (submitted for electronic processing).

MATERIALS AND METHODS

Sunflowers are routinely inoculated with downy mildew by the whole seedling immersion method (WSI), in which the pregerminated seeds are soaked in a suspension of zoosporangia (in our laboratory usually 30,000 per milliliter, in the dark at 15 C, usually for 3 hr) (2). The seeds are then sown in a synthetic soil mix (Promix) and placed in controlled-environment cabinets with 16 hr day length, light/dark temperatures of 20/18 C, and light intensity of about 15,000 lx (180 μ Em^{-2} sec^{-1}) at plant level from VHO cool-white fluorescent tubes supplemented with incandescent bulbs. In this work, infection of leaves was attempted by three methods:

1. Intact seedlings with one or two pairs of true leaves (about 13 days after seeding), growing in pots, were inverted over beakers so that the leaves were immersed in suspensions of 30,000 or 100,000 zoosporangia per milliliter (LI). They were placed in an incubator at 15 C in the dark for 3 hr, then removed from the suspensions and placed in controlled-environment cabinets under the conditions used routinely for WSI inoculations. They were covered with plastic bags for the first 24 hr to maintain them at high humidity.

2. The first pair of true leaves was detached from 13-day-old sunflower plants and immersed in zoosporangial suspensions in petri dishes in the dark at 15 C for 3 hr. As the leaves were difficult to wet, particular care had to be taken to ensure they were submerged in the inoculum; to avoid an adverse effect on

the zoospores, surfactants were not used. The leaves were then transferred to the surface of 1% water agar in plastic petri dishes under controlled conditions, a method found useful in earlier work with rust (5). In the rust work, surface-sterilization of leaves before inoculation and addition of auxins, kinetin, or benzimidazole to the medium had not improved either the longevity of the leaves or infection and therefore were not tried in these experiments. On the basis of preliminary tests, detached leaves were incubated in an illuminated incubator maintained at 15 C with 12 hr day length at light intensity of about 5,000 lx (60 μ Em^{-2} sec^{-1}) from HO cool-white fluorescent tubes mounted above the shelves. Condensation on the inner surface of the petri dish cover was prevented by spraying it with an antistatic textile spray (Static Guard, Alberto-Culver Canada Ltd.) (6).

3. In preliminary experiments, disks 17, 13, and 10 mm in diameter were cut from the first and second pairs of leaves of plants grown at the same time and under the same conditions as those for the experiments with intact plants and detached leaves. The disks were immersed in suspensions containing 100, 1,000, 30,000, or 100,000 zoosporangia per milliliter of races 1 or 3, in the various compatible and incompatible combinations, for 3 hr at 15 C in the dark, then transferred onto water agar in petri dishes and placed in the illuminated incubator at 15 C (LDI). Some disks from each combination were placed with the abaxial leaf surface on the agar, others with the adaxial surface on the agar. As no consistent differences in infection were observed among the disk sizes or between the surfaces exposed, 10 mm diameter and the adaxial surface up were arbitrarily used as the standard in subsequent work.

In an ad hoc test of the reaction of old leaves, disks cut from the lowest, middle, and upper surviving leaves of uninoculated sunflowers about 3 mo old, of eight different genotypes and left over in the greenhouse from rust studies, were inoculated with downy mildew race 3 by LDI. The concentration was 30,000 zoosporangia per milliliter; one-half of each group of disks was immersed for 3 hr in a suspension that had been kept at 15 C for 2 hr to pregerminate the spores, and the other half was immersed in the suspension for 18 hr.

The sunflower cultivars used in all other experiments were Peredovik, which is susceptible to all available races of downy mildew, and 894, a hybrid resistant to races 1 and 2 but susceptible to race 3. The races of pathogen used were 1, which is unable to attack plants with any known resistance genes, and 3, which can infect plants carrying either or both resistance genes Pl_1 and Pl_2.

Inoculum was prepared by making suspensions of zoosporangia harvested from infected sunflower seedlings or from mildewed leaves stored in an ultralow-temperature freezer at −75 C. Spore concentrations were determined using a hemacytometer and diluting as necessary with sterile distilled water.

Infection was determined on intact plants 10 or more days after inoculation by placing them in a saturated atmosphere overnight, then examining them for sporulation with the naked eye. Detached leaves and leaf disks were examined 13 or 14 days after inoculation, using a dissecting stereomicroscope.

All experiments included uninoculated controls immersed in water and plants inoculated by the WSI method.

As the space available in the illuminated incubator was limited, most experiments were done only twice, although some were done three or four times. The experiments were considered to be qualitative rather than quantitative; no statistical design was employed, so statistical analyses were not appropriate.

RESULTS AND DISCUSSION

No disease developed in any of the uninoculated control plants. The pathogen sporulated profusely on cotyledons and the first pair of true leaves of seedlings inoculated by WSI with downy mildew races to which they were susceptible. Seedlings of the hybrid cultivar 894 inoculated with race 1 by WSI remained healthy, although some seedlings were somewhat shorter than uninoculated plants. Occasional seedlings (up to 10%) in this incompatible combination were completely susceptible; these were usually assumed to be accidental, from selfed seeds of the susceptible female parent. It would have been preferable to use an inbred line with the Pl_2 gene to avoid such segregation. As all seed used in our laboratory is provided through the courtesy of plant breeders at other institutions, we tend to use what is most readily available.

Intact plants. In compatible combinations inoculated with 30,000 zoosporangia per milliliter by LI, localized chlorotic areas bearing sporangiophores and zoosporangia developed on the leaves of all plants (about 10 plants per combination). Lesions on plants inoculated with 100,000 zoosporangia per milliliter tended to be necrotic, with less profuse sporulation. In incompatible combinations at both spore concentrations, about 90% of the seedlings remained free from mildew, although slightly shorter than uninoculated plants; about 10% developed localized chlorotic spots bearing zoosporangia.

In a preliminary experiment, plants inoculated in compatible combinations by LI before the second pair of true leaves had expanded developed chlorotic spots with sporulation on the first pair of true leaves. Eight of 10 affected plants kept in a growth cabinet for an additional 35 days developed symmetrical chlorotic patterns with sporulation, characteristic of systemic infection, on the upper four to eight leaves.

Detached leaves. Sporulation developed on four of 10 and three of 10 leaves of Peredovik inoculated with 30,000 and 100,000 zoosporangia per milliliter, respectively, of race 1 and on six of 10 and nine of 10, respectively, of those inoculated with race 3. Sporulation was more profuse on the leaves inoculated with race 3 and on those inoculated with 100,000 zoosporangia per milliliter. Sporulation was not observed on leaves of 894 inoculated with race 1 but did occur on six of 10 leaves of 894 inoculated with each concentration of race 3; sporulation was slightly more abundant on those inoculated with 100,000 zoosporangia per milliliter. Most of the sporulation was observed around the edges of the leaves, but it occurred on the leaf surface in some instances. No chlorotic symptoms developed on any of the leaves.

Leaf disks. No sporulation was observed on any of the control leaf disks immersed in water. Sporulation was observed on only one disk of 894 inoculated with race 1 and on only one disk of each cultivar inoculated with 100 zoosporangia per milliliter of race 3. The results are given in Table 1. Although the proportion of disks with sporulation varied somewhat from experiment to experiment, it was consistently higher with 30,000 than with 1,000 zoosporangia per milliliter and sometimes higher and usually more profuse with 100,000 than with 30,000 (Table 1).

Bacteria developed on and around many of the leaf disks but usually did not prevent the sporulation of *P. halstedii*. *Alternaria alternata* (Fr.) Keissler and other saprophytic fungi were present on some of the disks; they sometimes obscured, but usually did not prevent, sporulation of the pathogen. Suitable methods of sterilizing or disinfesting the surface of leaves before disks are cut for inoculation, without affecting their reaction to downy mildew, will have to be worked out, particularly if LDI inoculation proves useful for field-grown plants.

Effect of leaf age. From 10 to 20 disks were inoculated per leaf position per genotype (3-mo-old plants). Sporulation was observed on 0–90% of the disks from various genotypes; none occurred on control disks immersed in water. Sporulation occurred on a higher proportion of the disks from lower and middle leaves than on those from upper leaves. There were no consistent differences in infection of individual genotypes attributable to immersion in inoculum for 18 as opposed to 3 hr. As the experiment was ad hoc, using leftover plants of limited populations from a rust

Table 1. Reaction of sunflower leaf disks inoculated by immersion in various concentrations of zoosporangia of *Plasmopara halstedii*[a]

Cultivar	Downy mildew race	Concentration of zoosporangia per milliliter	Number of disks	
			Examined	With sporulation
Peredovik	···	Water control	40[b]	0
894	···	Water control	40	0
Peredovik	1	30,000	20[b]	20
		100,000	20	20
894	1	30,000	20[b]	1
		100,000	20	0
Peredovik	3	100	24[c]	1
		1,000	24	11
		30,000	44	38
		100,000	20	20
894	3	100	24[c]	1
		1,000	24	5
		30,000	44	39
		100,000	20	20

[a] The experiments were not designed for statistical analyses.
[b] Ten disks per test.
[c] Eight to 12 disks per test.

study, the reaction of the various genotypes to inoculation with downy mildew by WSI was not known. The results indicate merely that disks from old leaves as well as those from seedlings can be infected using LDI inoculation.

Applications of LDI. *Checking reaction of disease escapes.* Disks were cut from several apparently healthy Peredovik seedlings inoculated with race 1 by WSI in a test in which 90% of the seedlings were stunted, with profuse sporulation of the pathogen. Some of the disks were immersed in water and the rest in a suspension of spores from the diseased plants, then all were incubated on water agar for 14 days under the usual conditions. No sporulation developed on any of the disks immersed in water; the fungus sporulated on all the inoculated disks, indicating that the plants were disease escapes rather than resistant.

Checking race identity from infected plants in presumably incompatible combinations. About 10% of seedlings of the hybrid cultivar 894 inoculated with race 1 in a test were severely stunted and bore abundant sporulation. Disks cut from the apparently healthy plants and from uninoculated healthy seedlings of Peredovik were immersed in water (controls) or in a suspension of spores from the diseased seedlings in the standard way, then incubated for 14 days. Sporulation was profuse on inoculated disks of Peredovik; no sporulation developed on the control disks or on the disks of 894 inoculated by LDI, indicating that the affected 894 seedlings were susceptible segregants, not indicators of the presence of a new race of the pathogen.

Conclusions. The ability to infect sunflower leaves and leaf disks with *P. halstedii* using LI and LDI inoculation makes it possible to study host-pathogen relationships that could not be investigated previously. As the susceptibility or resistance of leaf disks inoculated with specific races of downy mildew by LDI was the same as that of intact plants inoculated by WSI, LDI can be used to check the reaction of healthy plants in compatible host-race combinations. Similarly, LDI can be used to check the inoculum when infected seedlings occur in a presumably incompatible combination. As a number of disks can be taken from one plant, the reaction of individual plants to various races of downy mildew can be determined at the same time. The reaction to a race or races of downy mildew can also be determined for individual plants that have been inoculated previously with other pathogens such as rust (*Puccinia helianthi* Schw.) or wilt (*Verticillium dahliae* Kleb.).

Such multiple race and multiple pathogen reactions of individual plants are of obvious interest in studies on the genetics of disease resistance. Plants with a number of small disks removed from the expanded leaves continued to grow, flowered, and set seed. LDI inoculation therefore can be used to select individual sunflower plants with proven reaction to various races of downy mildew, as well as to other pathogens, for use as parents in a breeding program.

ACKNOWLEDGMENT

We are grateful for financial assistance from the Natural Sciences and Engineering Research Council of Canada in the form of an operating grant to the first author.

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