Detection and Identification of *Peronosclerospora sacchari* in Maize by DNA Hybridization

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ABSTRACT

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The causal organism of an incidence of maize downy mildew in Southern China proved difficult to classify by standard techniques. The pathogen, subsequently identified as *Peronosclerospora sacchari*, was detected by DNA hybridization in endosperm, pericarp, and pedicel tissues, but not in embryos of infected maize seeds. Plasmid pCLY83, which had been selected from a *P. maydis* DNA library, served as the probe. No evidence for hybridization was detected between the probe and DNAs extracted from ten common seedborne fungi of maize: *Colletotrichum graminicola, Acremonium strictum, Curvularia lunata, Fusarium moniliforme, Bipolaris maydis, Macrophomina phaseolina, Rhizoctonia* sp., *Rhizopus* sp., *Penicillium* sp., and *Alternaria* sp. Hybridization was also not detected with DNAs isolated from plant tissues infected with *Sclerospora graminicola* or *Sclerophthora macrospora*. The hybridizing DNA of the corn pathogen from China was readily distinguished from *P. sorghi* and *P. maydis* by differences in *Eco*RI, *Pvu*I, *Bam*HI and *Hin*dIII restriction patterns. RFLP patterns on blots of DNA from the plants showing symptoms of downy mildew in this case were the same as those for *P. philippinensis* and *P. sacchari*, now believed to be conspecific.

Additional keywords: DNA probe, downy mildew detection and identification, fungal genetics, maize disease

Downy mildew of maize, sorghum, and sugarcane can be extremely destructive diseases, particularly in the tropical and subtropical regions of the world (8). *Peronosclerospora sorghi* (Weston and Uppal) C. G. Shaw is the cause of sorghum downy mildew of both sorghum (*Sorghum bicolor* L.) and maize (*Zea mays* L.) and is the only *Peronosclerospora* downy mildew pathogen known present on a graminaceous host in the New World (8). The sorghum strain can infect maize; however, the fungus is most damaging to sorghum. In Texas, disease incidence has exceeded 30% on sorghum and can be even higher under the appropriate environmental conditions (7).

A maize strain of *P. sorghi*, perhaps existing only in Thailand, rarely infects sorghum but has caused large losses to maize in that country. Isozyme and epidemiological studies conducted at the Foreign Disease-Weed Science Research Unit (FDWSRU) in Frederick, MD, suggest that the maize strain is distinct from other downy mildew pathogens and perhaps should be considered a separate species (3,14).

P. sacchari (T. Miyake) Shirai and K. Hara has been a serious disease of both sugarcane (*Saccharum officinarum* L.) and maize, particularly in Taiwan. Although the disease is readily transmissible between maize and sugarcane, maize is usually more susceptible (21). *P. philippinensis* (Weston) C. G. Shaw, the causal organism of Philippine downy mildew of maize, has caused 40–60% losses in susceptible varieties in the Philippines (6). Morphology, symptoms, host range, and isozyme comparisons strongly suggest that *P. philippinensis* is closely related to, or the same as, *P. sacchari*.

P. maydis (Racib) C. G. Shaw, the cause of Java downy mildew, was the first *Peronosclerospora* downy mildew pathogen described on maize. Nearly 100% losses have been reported to late-planted maize due to this pathogen (19). Countries where downy mildews are not a problem have attempted to prevent their entry by the use of quarantines. This prevention depends partially on the correct identification of the pathogens. Currently, the primary characteristics used to define *Peronosclerospora* spp. are the size and shape of the conidia and conidiophores, as well as the determined host ranges and oospore production (20). However, host range and oospore production tests are not practical in most regulatory situations, and the size and appearance of the conidia and conidiophores of the *Peronosclerospora* fungi can vary significantly, depending on the host species or cultivar on which the pathogen was collected, the environmental conditions at the time of sporulation, the time of collection, the mounting fluid used during microscopic examination, and the person making the observation (1,5,13,16,23,24). These problems cause considerable confusion in classification and make identification very difficult.

Although Micales et al (14) have used isozyme analysis to identify these four *Peronosclerospora* spp., the great difficulties in collecting living *Peronosclerospora* specimens, maintaining these obligate parasites on living plants, preventing spread and mixing of cultures, and obtaining sufficient quantities of living spores for enzyme extraction limit the practicality of isozyme analysis for routine identification.

Staining methods have been used to detect downy mildew oospores and mycelium (15), but the use of these techniques to distinguish downy mildew mycelium from some plant tissue structures or other fungal mycelium that might be present is exceedingly difficult. Often the pathogen is missed.

DNA probes are being increasingly used for identification and detection of plant pathogenic fungi (9,10,11). This technology may be particularly important for obligate pathogens, because they cannot be cultured on artificial media. Being aware of the uncertain identity of a *Peronosclerospora* sp. that occurs in Southern China and having experienced difficulty in detecting it in infected maize seeds with staining techniques, we were prompted to apply DNA hybridization methods for detection and identification of this pathogen. This paper describes the development of DNA probes from a *P. maydis* genomic library for the detection of downy mildew pathogens in maize leaves and seeds and their use in determining the distribution of the fungus in specific anatomical parts of maize seeds. It further describes the potential use of a 1.5 kb DNA probe selected from the *P. maydis* library for the identification of *Peronosclerospora* spp., including the pathogen in China.

MATERIALS AND METHODS

Sample collection. Isolates of *P. sorghi, P. sacchari, P. philippinensis*, and *P. maydis* were obtained from different collectors; species names, isolate designations, locations and dates of collection, and the names of the collectors are listed in Table 1. The collection of fungal conidia and conidiophores followed the methods described previously (14,25). To detect the location of downy mildew inoculum in maize seeds, infected seeds from China were soaked in sterile distilled water for 24 h in a 25 C incubator. Then the pericarps, endosperms, embryos, and pedicels from 20 seeds of each sample were carefully dissected with a scalpel and tweezers under a stereomicroscope and like structures from the seeds were combined for DNA extraction. To determine the percentage of seeds that are infected when the parent plant is systemically infected, DNA was extracted separately from 50 single seeds of cultivar Local Yellow. In order to verify that the probe did not hybridize to other fungal pathogens, DNA extracts from ten common seedborne fungi of maize: *Colletotrichum graminicola* (Ces.) G. W. Wils., *Acremonium strictum* W. Gams, *Curvularia lunata* (Wakk.) Boedijn, *Fusarium moniliforme* J. Sheld., *Bipolaris maydis* (Nisikado & Miyake) Shoemaker, *Macrophomina phaseolina* (Tassi) Goidanich, *Rhizoctonia* sp., *Rhizopus* sp., *Penicillium* sp., and *Alternaria* sp. were also tested for cross-hybridization. The fungi were cultured on potato-dextrose agar (PDA) medium for 8 days at 25 C, and for each of the ten fungal species, the mycelium from four PDA plates provided the sample for DNA isolation. Pure cultures were not available for two other pathogens. For these, DNAs isolated from leaves of foxtail millet (*Setaria italica* (L.) Beauv.) infected by *Sclerospora graminicola* (Sacc.) J. Schröt., and also from both a sorghum panicle and leaves of St. Augustine grass (*Stenotaphrum secundatum* (Walt.) Kuntze) collected from plants infected by *Sclerophthora macrospora* (Sacc.) Thirumalachar, C. G. Shaw & Narasimhan were used to test for cross-hybridization.

DNA isolation and manipulation. A minipreparation method for isolating total genomic DNA developed by Lee et al (12) was used with some modification. When extracting DNA from conidia and conidiophores of *Peronosclerospora* spp., the samples were frozen directly in liquid nitrogen, and allowed to thaw at room temperature in order to break down conidia. The original procedures were followed for DNA isolation from other fungi, leaf, and seed samples. However, after DNA samples had been dissolved in TE buffer and precipitated with ethanol, they were redissolved in TE buffer and reprecipitated with 2-propanol and ethanol successively. The second precipitation greatly increased the purity of DNA extracted from leaves, seeds, and fungal mycelia. DNA was extracted successfully from relatively large amounts of plant material by proportionately increasing the components of the minipreparation procedure. Standard DNA manipulations were performed according to Sambrook et al (18), unless otherwise stated.

Total genomic DNA of *P. maydis* was digested with *Eco*RI and ligated into *Eco*RI-digested plasmid pUC19, and used to transform competent cells of *Escherichia coli* (Migula) Castellani & Chalmers DH5α. White colonies containing recombinant plasmids were selected and confirmed to contain inserts by colony hybridization to *P. maydis* total genomic DNA labeled with ^{32}P-dATP by nick translation. To maximize the probability of detection when used as a probe, plasmids that provided strong signals when hybridized to *P. maydis* total genomic DNA were selected for further characterization. Plasmid DNA was isolated by a minipreparation procedure developed by Zhou et al (26) and then used to hybridize to Southern blots of *Eco*RI-, *Pvu*I-, *Bam*HI- or *Hin*dIII-digested fungal DNA or DNA extracted from fungus-infected leaf tissues. Among 10 recombinant plasmids used in Southern hybridizations, pCLY83 gave the clearest banding patterns and the strongest signal when hybridized to fungal DNA extracted from infected maize seeds. DNA of plasmid pCLY83 was purified by cesium chloride ultracentrifugation (18) for restriction site characterization.

DNA blots and hybridization. All DNA concentrations in this work were determined by DNA fluorometry (Model TKO 100, Hoefer Scientific Instruments, San Francisco, CA). Each sample used for dot-blots contained 50 ng of DNA. Solutions for dot-blots were prepared as follows: The DNA sample was made 0.4 M in NaOH and brought to a total volume of 10 μl. Samples were centrifuged for 5 sec in a microcentrifuge and allowed to stand for 10 min at room temperature before adding 10 μl of 2 M ammonium acetate. The procedures for preparing dot-blots were the same as described previously (25).

Restriction enzyme digestions were carried out according to the recommendations of the supplier (Promega, Madison, WI). Since 0.75 μg of DNA extracted directly from fungi and 1.5–2.0 μg of DNA isolated from infected leaves were observed to produce similarly strong signals when hybridized to probe pCLY83, these amounts were digested in each reaction. Digested DNA was subjected to electrophoresis in a 0.9% agarose gel in TBE buffer (18) at 40 V (3 V/cm) overnight. Alkaline blotting of DNA from the gel to Genescreen Plus hybridization membrane utilized the method developed by Reed and Mann (17). After transfer, the membrane strips were rinsed in 2X SSC for 5 min, then dried in a vacuum oven at 80 C for 2 h.

Membrane strips were prehybridized at 65 C for 12–24 h in a sealed plastic bag in a solution made by combining 12.6 ml of water, 5 ml of 20X SSC, 0.4 ml of 100X Denhardt's solution (18), 1.0 ml of 1 M Tris, 0.2 ml of 20% SDS, 0.4 ml of 0.5 M EDTA, and 0.4 ml of denatured salmon sperm DNA (10 mg/ml). Nick-translations of 100 ng of probe DNA to a specific activity of approximately 10^8 cpm/μg were made with a kit provided by BRL, using ^{32}P-dATP as label. After prehybridization, the labeled probe was added to the prehybridization solution and the bag was resealed and placed at 65 C for another 24 h. Membranes were then removed and washed with constant agitation first at 65 C for 30 min in 500 ml of 1X SSC and 1% SDS and then at room temperature for 30 min in 500 ml of 0.1X SSC. After washing, the membrane strips were exposed to Kodak Blue Brand X-ray film for 12–48 h at −70 C with intensifying screens.

RESULTS

Plasmid pCLY83 contains a 1.5 kb *P. maydis* DNA fragment. It has restriction sites for *Kpn*I, *Sph*I, and *Sma*I but not for

TABLE 1. *Peronosclerospora* spp. used in this study

Species	Isolate	Origin	Source
	pathotype 1	Texas, 1988	R. A. Frederiksen
	pathotype 3	Texas, 1988	R. A. Frederiksen
P. sorghi	Botswana	Botswana, 1989	G. N. Odvody
	Thailand 1[a]	Pak Chang, 1975	B. Renfro
	Thailand 2[a]	Pak Chang, 1985	C. DeLeon
P. maydis	Indonesia	Malang	H. Vermeulen
P. sacchari	New Guinea	Papua	
Peronosclerospora sp.	China 1	Guangxi, 1990	S. Y. Zhou
	China 2	Yunnan, 1990	S. X. Chen
P. philippinensis	Philippines	Luzon Exp. Station, 1984	J. M. Bonman

[a] Based on isozyme and other data, the species classification of these isolates is in question.

*Eco*RI, *Bam*HI, *Pst*I, or *Hin*dIII (Fig. 1).

DNA sequences homologous to plasmid pCLY83 were detected in DNA extracts from endosperm, pericarp, and pedicel (but not in embryo extracts) (Fig. 2) of maize seeds that had been collected from plants of cultivars Guidan 16 and Local Yellow that were infected with a species of *Peronosclerospora*. Among 50 seeds from Local Yellow plants that were systemically infected by this pathogen, DNA isolated from 27 seeds hybridized with the probe. There was no evidence for hybridization between the probe and DNA extracted from ten common seedborne fungi of maize: *C. graminicola, A. strictum, C. lunata, F. moniliforme, B. maydis, M. phaseolina, Rhizoctonia* sp., *Rhizopus* sp., *Penicillium* sp., and *Alternaria* sp. (data not shown). Hybridization also was not detected with DNA extracted from a sorghum head or St. Augustine grass leaves infected by *S. macrospora* or from foxtail millet leaves infected by *S. graminicola*. Under the hybridization conditions used, DNA from the pUC19 vector did not hybridize to plant DNA or to DNA extracted from any of the *Peronosclerospora* spp. used in this study. Likewise, there was no hybridization between the fungal and plant DNAs (data not shown).

The Southern blot banding patterns of DNA extracted directly from fungal conidia and conidiophores or from leaf tissue infected by the respective fungi were identical after digestion with each restriction endonuclease. When hybridized to the pCLY83 probe, Southern blots of *Eco*RI, *Pvu*I, *Bam*HI, and *Hin*dIII digests of DNA isolated from the *Peronosclerospora* sp. causing symptoms of downy mildew on maize plants obtained from China gave the same RFLP patterns as *P. philippinensis* and *P. sacchari*, which were readily distinguished from those of *P. maydis* and *P. sorghi* (Fig. 3).

DISCUSSION

In our early work we used lactophenol-cotton blue to stain for mycelium of *Peronosclerospora* in maize seed but found the mycelium difficult to visualize and to distinguish from mycelia of other species or host tissue structures. In only a few cases could the typical coenocytic mycelium of downy mildew fungi be discerned in the pericarp of maize seeds collected from systemically infected plants.

Results obtained in this study show that DNA hybridization can provide a rapid, accurate, and sensitive means of detecting

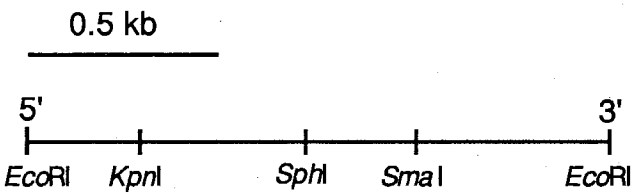


Fig. 1. Restriction enzyme map of pCLY83 insert DNA. Total length of the insert is 1.5 kb.

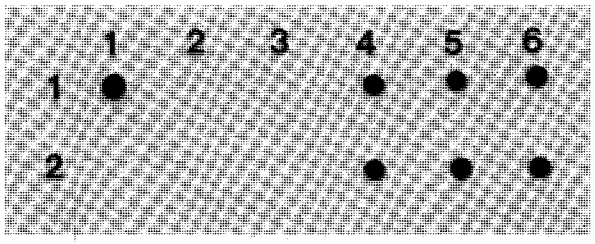
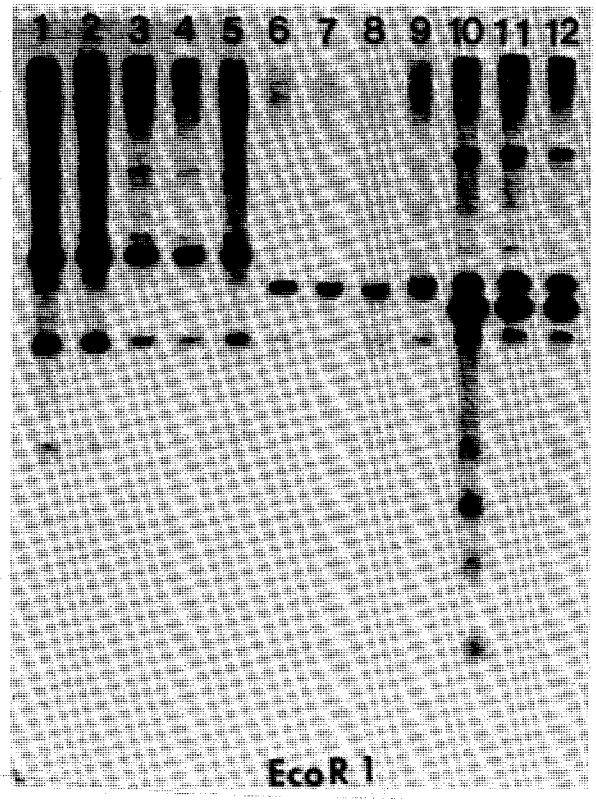


Fig. 2. Dot blot autoradiograph of DNA extracted from Row 1: (1) maize leaves of cv. Guidan 16 infected by *Peronosclerospora* sp. of Chinese origin, (2) healthy seeds of cv. Guidan 16, (3) to (6) embryo, endosperm, pericarp, and pedicel of cv. Guidan 16 infected by *Peronosclerospora* sp.; Row 2: (1) healthy maize leaves of cv. Guidan 16, (2) healthy seeds of cv. Local Yellow, (3) to (6) embryo, endosperm, pericarp, and pedicel of cv. Local Yellow infected by *Peronosclerospora* sp. The probe was plasmid pCLY83.



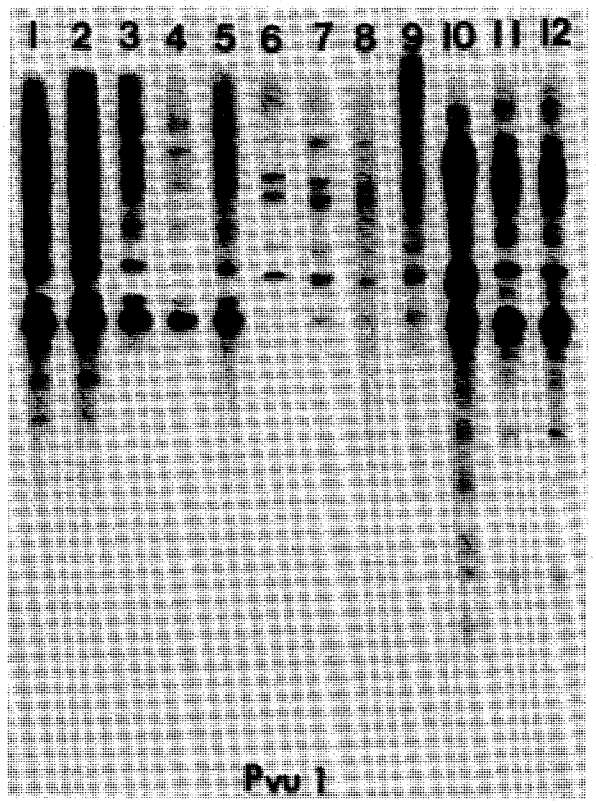


Fig. 3. Southern-blot autoradiographs of DNA extracted from lanes 1 and 2: *P. sorghi* pathotype 1 and 3, respectively; lanes 3 and 4: sorghum leaves infected by *P. sorghi* pathotype 1 and 3, respectively; lane 5: maize leaves infected by *P. sorghi* Botswana isolate; lanes 6 and 7: sugarcane leaves infected by *P. sacchari* and *P. philippinensis*, respectively; lanes 8 and 9: maize leaves infected by *Peronosclerospora* sp. Yunnan and Guangxi isolate, respectively; lane 10: *P. maydis*; lanes 11 and 12: *P. sorghi* Thailand isolate 1 and 2, respectively, digested with restriction enzyme a) *Eco*RI or b) *Pvu*I, hybridized to ^{32}P-labeled DNA of plasmid pCLY83.

seedlots infected with *Peronosclerospora*. A probe made from recombinant plasmid pCLY83 hybridized only to extracts of samples of maize infected by *Peronosclerospora* spp. and not to extracts of samples infected by *S. macrospora, S. graminicola*, or to the DNA from ten other common non-*Peronosclerospora* seedborne fungi of maize. The findings in our work show that this pathogen is actually distributed in the endosperm, pericarp, and pedicel but not in the embryo of seeds collected from cultivars Guidan 16 and Local Yellow plants systemically infected by this pathogen. Since segments of the pericarp and pedicels often end up in the chaff produced during grain harvest and handling, it may be possible to develop a sampling procedure based on DNA extraction of chaff for quarantine purposes when large seed lots are involved. Likewise, it should be possible to verify that individual seeds to be shipped for breeding purposes are uninfected without destruction of the seed.

Numerous DNA fragments from *Peronosclerospora* spp. DNA were present on Southern blots after digestion with each enzyme as revealed by hybridization to pCLY83, indicating that all the *Peronosclerospora* spp. genomes have repetitive sequences homologous to this DNA probe, which also accounts for the strong signal obtained with dot-blots. This provides an obvious advantage for detecting very low amounts of mycelium in host tissues.

The *Peronosclerospora* sp. from China examined in this study was tentatively identified as *P. maydis* (*Sclerospora maydis* (Recib.) Butl.) (22). Much uncertainty and controversy still remains concerning the identity of some *Peronosclerospora* spp.; however, several unique characteristics of *P. maydis*, including relatively stable ovoid-to-round conidial shape and only one natural host (maize), usually make it relatively easy to discern *P. maydis* from other *Peronosclerospora* spp. Observations showing that conidial lengths of the fungus that occurred in China are highly variable under different temperatures and that the fungus can attack the alternative host *Saccharum spontaneum* L. (22) cast doubt on the original identification as *P. maydis*.

The DNA banding patterns of this Chinese isolate on Southern blots were different from those of the Indonesian isolate of *P. maydis* we tested; however, the patterns were identical for all four enzymes to those for *P. philippinensis* and *P. sacchari*. These results suggest that the China isolate belongs to the *P. philippinensis-sacchari* group. The identical RFLP patterns of the *P. philippinensis* and *P. sacchari* isolates revealed by pCLY83 are not surprising, because these organisms do not have significant differences in morphology (1), host range (2,4), or symptom expression (4). Their isozyme banding patterns are so similar, it has been proposed that *P. philippinensis* and *P. sacchari* are conspecific (14). This contention is supported by the similarity of Southern hybridization patterns seen here, as well as with the patterns produced by 30 other DNA probes selected from a *P. sorghi* pathotype 3 DNA library (*unpublished data*, C. L. Yao et al). Clearly, RFLP analysis of a larger group of independent isolates could help resolve this question.

DNA of both American and an African isolate of *P. sorghi* share identical RFLP patterns, but, on all Southern blots, the maize strain of *P. sorghi* from Thailand produced strikingly different banding patterns. In fact, the banding patterns of the Thailand isolates were similar to those of the *P. maydis* isolate. Both Thai *P. sorghi* and *P. maydis* gave identical banding patterns on Southern blots of DNA digested with *Eco*RI, *Bam*HI or *Hin*dIII, respectively, but differences were seen following digestion with *Pvu*I. As with *P. sacchari* and *P. philippinensis*, the correct classification of the Thailand organism is in question. Isolates of *P. sorghi* from Thailand have been found to differ from other *P. sorghi* isolates in many ways, including host range (4), symptom expression (4), oospore production (1), isozyme patterns (14), and other RFLP patterns not included here (*unpublished data*, C. L. Yao et al). All these data suggest that the Thailand isolates differ from "true" *P. sorghi* sufficiently to be treated as a separate species.

Our observation that identical RFLP patterns were obtained from DNA extracted either directly from conidia or from *Peronosclerospora*-infected leaves (Fig. 3) is important, because it shows that identification of these biotrophic fungi can be made directly from infected leaf tissues without isolation of the pathogen. The conventional identification method for members of this group of fungi is based on microscopic observation, which is affected by many uncontrolled factors. We found that DNA extracted from severely infected young leaves can provide an alternative identification method if the leaves are appropriately handled. Infected leaves can be detached, air-dried for 2 days at room temperature, and then mailed without further treatment to an appropriate laboratory for identification. This is vastly simpler than attempting to send infected seedlings, the method currently being used by the FDWSRU, Frederick, Maryland. From many areas of the world, the mail takes too long for the seedlings to survive the trip. In many cases the seedlings have been damaged to the extent that they cannot be transplanted when they arrive. Dried, infected leaves received via mail at Texas A&M have been stored for several months at −70 C without any apparent deterioration in the quality of DNA hybridization. This technique will be extremely valuable in making future pathogen comparisons and identifications for a global effort in determining the distribution of specific downy mildew diseases.

The DNA isolated from infected leaves provides consistently good results when used for Southern blots. The plant DNA does not seem to interfere with probe hybridization. Even on blots where the bands are distorted, presumably as a result of overloading with plant DNA, pathogen RFLP patterns characteristic of the downy mildew fungus were clear. DNA hybridization with appropriate probes may provide a very powerful and convenient means for identification and classification of this group of obligate plant pathogenic fungi, since the fungal genomes are not likely to be influenced by the factors mentioned above.

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Ecology and Epidemiology

Effects of Spore Concentration, Temperature, and Dew Period on Disease of Field Bindweed Caused by *Phoma proboscis*

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ABSTRACT

Heiny, D. K., and Templeton, G. E. 1991. Effects of spore concentration, temperature, and dew period on disease of field bindweed caused by *Phoma proboscis*. Phytopathology 81:905-909.

Spore germination of *Phoma proboscis* in vitro and resulting disease development were evaluated over a range of spore concentrations and after incubation at nine dew periods at each of five temperatures. Reduced percent germination on water agar occurred at spore concentrations above 10^7 spores ml^{-1}. Spore germination on agar was optimal at 24 C. High levels of disease occurred on plants that received 12 h or more of dew at all temperatures tested, except 32 C. Fresh weight reduction in shoots and roots correlated well with disease ratings. Disease was enhanced relative to constant temperature treatments when plants from dew period temperatures of 16 and 20 C were maintained at a postdew temperature of 24 C. The results of these studies suggest that *P. proboscis* has potential for use as a mycoherbicide.

Additional keywords: biological control, *Calystegia sepium, Convolvulus arvensis*, epidemiology, mycoherbicides

Field bindweed (*Convolvulus arvensis* L.), a perennial, herbaceous vine ranked as the twelfth worst weed in the world (6), is distributed throughout most of the United States (2). Heavy infestations cause yield reductions ranging from 33% for winter wheat to 75% for summer-growing crops (15). In 1980, nearly 810,000 ha of crop land in 30 California counties was reported to be infested by field bindweed, resulting in a loss of about $25 million (11). Although tillage and herbicides are frequently effective in controlling seedling and first-year bindweed plants, destruction of shoots of older plants stimulates vigorous regeneration of shoots from the root buds (16). Complete control of field bindweed sometimes requires up to 5 yr of herbicide applications (16).

Recently, attention has been directed toward the potential of fungi as biocontrol agents of field bindweed (7,8,9,12). Ormeno-Nuñez et al (8) described blighting of field bindweed seedlings and shoots by *Phomopsis convolvulus* Ormeno over a range of environmental conditions. *Phoma proboscis* Heiny causes symptoms similar to those caused by *P. convolvulus* (5). Here we report an assessment of the impact of disease on field bindweed populations treated with *P. proboscis* under controlled parameters of spore concentration, dew period, and temperature.

MATERIALS AND METHODS

General. Seeds of field bindweed were purchased from Valley Seed Service (Fresno, CA). Seeds were surface sterilized in 0.26% sodium hypochlorite for 1 min, washed repeatedly, presoaked between wet paper towels for 24 h at 22 C, and planted about 1 cm deep in plastic pots 8 cm in diameter, containing vermiculite. After 2 wk in the greenhouse (at 24–32 C), seedlings had one to three true leaves and were thinned to 15 plants per pot unless otherwise stated. Pots were watered daily and fertilized weekly with Peters Professional water soluble fertilizer (N-P-K:20-20-20) at 470 ppm. Excepting spore concentration tests, plants were 2 wk old when inoculated and were held at the inoculation temperature for 1 day before use. Experiments were repeated