A Simple Method for Field and Greenhouse Inoculation of *Polymyxa betae* and Beet Necrotic Yellow Vein Virus

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ABSTRACT

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A method of inoculating sugar beet with viruliferous *Polymyxa betae* for use in field and greenhouse studies was evaluated for two years (1991–1992). Seeds of sugar beet cultivar HH39 were coated with powdered sugar beet roots containing beet necrotic yellow vein virus (BNYVV)-infested *P. betae* cystosori. Two percent methyl cellulose was used as the carrier at rates of 1:20:20 and 1:10:10 (w/v/w) inoculum/methyl cellulose/seed. Seeds were planted in field plots and maintained by conventional agronomic practices. During 1991, plots were sampled and tested for BNYVV incidence twice between planting and harvest. The second year, plots were sampled once. For comparison, seeds also were planted in the greenhouse and assayed simultaneously with field samples. All assays employed the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for BNYVV detection. During 1991, 46 and 50% of field and greenhouse samples, respectively, were positive after 4 wk. After 6 wk, the percentage of plants infected had risen to 57 and 80. During 1992, the higher inoculum rate and longer growing season resulted in >90% infection. We concluded the technique is valuable for establishing infection with BNYVV under both field and greenhouse conditions. It is possible that the method also could be used with other viruses transmitted by *Polymyxa* spp.

Additional keywords: *Beta vulgaris*, furoviruses.

Polymyxa betae Keskin is a member of the family Plasmodiophoraceae and is a ubiquitous soil inhabitant that lives as an obligate parasite on plant roots in the families Chenopodiaceae, Amaranthaceae, and Portulacaceae (1,3,12). *P. betae* was first identified in North America in 1977 (9) and is present in most soils in which sugar beets are grown (15). Interest in *P. betae* has increased in recent years due to its role as vector of beet necrotic yellow vein virus (BNYVV). BNYVV belongs to the newly established furovirus group and is the causal agent of rhizomania, a serious disease of sugar beets worldwide. Rhizomania was first described and named by Canova in Italy in 1959 (11). The disease is now widespread throughout sugar beet-production areas of Japan and Europe. It was not detected in the United States until 1983, when it was found in California. By 1989, the disease was known to have contaminated over 70,000 acres in that state (11). During 1986, rhizomania also was discovered in Texas (8). The disease was thought to be limited to these two states until 1992 when reports were published confirming its presence in Idaho, Wyoming, and Nebraska (10,17).

Symptoms of rhizomania include a small constricted taproot, secondary proliferation of small feeder roots, and vascular necrosis. Foliar symptoms are much less pronounced. They usually are limited to chlorosis and an upright posture in the field. Occasionally the virus becomes systemic, and yellow blotches that later become necrotic appear along leaf veins. Yield reduction is caused by the lack of a well-developed taproot, and root impurities that increase as a result of infection by BNYVV and interfere with sugar extraction during processing (11).

Root infection occurs by means of zoospores that originate from zoosporangia or cystosori (6,7). As an obligate parasite, *P. betae* can multiply only within the roots of a host plant. During this multiplicative phase, a plasmodium is formed within the plant cell and can become contaminated with BNYVV (6,7). The plasmodium then differentiates into either a zoosporangium, giving rise to new zoospores, or thick-walled resting spores called cystosori. Cystosori, which are released into soil as roots senesce and die, allow the survival of both the fungus and the virus for long periods without a host (6,27). Susceptible weed species also serve as hosts in which these pathogens survive and multiply (4).

The only other *Polymyxa* species, *P. graminis* Ledingham, is morphologically indistinguishable from *P. betae* but is maintained as a separate species because of its host range (1,3). *P. graminis* is a parasite of many grasses and can transmit a number of viruses to its hosts (1,3,12). It also is unique in that it infects and transmits two distinct strains of peanut clump virus (PCV) to peanut but cannot reproduce on peanut (28,30).

Miller et al reported the difficulty of accurately assessing the effect of infection by wheat spindle streak mosaic virus (WSSMV), a *P. graminis*-transmitted virus, on wheat yields (26). One problem they encountered was conducting controlled field experiments. Because *P. graminis* is an obligate parasite and the viruses it vectors are difficult to transmit mechanically, most yield studies have used naturally infested fields with asymptomatic plants as controls (26).

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A common inoculum source for *Polymyxa* spp. in greenhouse studies is zoospores; however, production of zoospores by *Polymyxa* spp. can be more difficult than production of other zoosporic fungi (12). It also is necessary to maintain actively growing cultures to obtain optimum zoospore production (12). Thus, zoospore inoculum production, even for small-scale greenhouse studies, remains problematic. Using *Polymyxa* spp., zoospores for inoculum in a large-scale field study is impractical if not impossible. Hence, it is necessary to rely on naturally infested soils for field studies.

There are at least 12 separate viruses in two taxonomic groups known to be vectored by *Polymyxa* spp. worldwide (1). Although this is a small number compared to known arthropod-transmitted viruses, these viruses cause serious disease of several economically important crops, and their importance is increasing.

Until very recently, all the research on rhizomania in the United States has been done in California. Because of its rapid spread throughout that state, research there has primarily focused on the biology of *P. betae* and its distribution within beet fields (13–16,18,23), screening for existing sources of resistance (2,5, 19,32), and soil fumigation (2,13,24). These studies necessitate the use of naturally infested fields. This type of research is difficult to carry out using soilborne pathogens, particularly obligates such as *P. betae* and BNYVV. Thus, a need exists for a reliable technique for infesting test plots. This information would be especially timely now because of the new reports of rhizomania during 1992. Based on this premise, a 2-yr study was conducted to test a method of infesting field and greenhouse sites with viruliferous *P. betae* for investigating rhizomania without relying on naturally infested soils or producing zoospores as inoculum.

MATERIALS AND METHODS

Inoculum for the test was obtained by growing BNYVV-infected sugar beet plants in Ray Leach Cone-Tainers supplied by Stuewe and Sons, Inc. (Corvallis, OR). After 8–12 wk in the greenhouse, randomly chosen plants were checked microscopically for infection by *P. betae* and assayed by enzyme-linked immunosorbent assay (ELISA) for BNYVV infection. Roots from infected plants were thoroughly washed and allowed to air-dry. The dried root tissue was pulverized and separated from soil and other foreign materials by a series of fine-meshed sieves. The resulting product consisted of powdered roots infested with viruliferous cystosori. Seeds of sugar beet cultivar HH39 were coated with powdered roots with 2% methyl cellulose as the carrier. Two different inoculum rates were employed. In the first year of the study (1991), a 1:20:20 (w/v/w) inoculum/methyl cellulose/seeds was used. The second year, the inoculum ratio was effectively doubled to 1:10:10 (w/v/w) by reducing the amount of seeds treated with a given amount of root inoculum. The same batch of treated seeds was used for both greenhouse and field studies.

Greenhouse evaluation. During August 1991, two to three seeds were planted in each container with a mixture of sand and commercial topsoil. Fifty tubes were planted with BNYVV-infected seeds as described above, and 50 were planted with untreated seeds as healthy controls. Tubes were watered heavily the first several weeks after planting to initiate early infection. After the plants became established, they were watered daily to maintain plant turgor. One-half the tubes were harvested on 18 September 1991 and assayed by ELISA for incidence of BNYVV infection. A second assay was performed on 25 October 1991 with the remainder of the tubes. Ambient greenhouse temperatures ranged from 20 to 30 C.

During July 1992, the study was repeated in the same manner except that the inoculum level was increased as indicated. Only one harvest and ELISA were conducted during November 1992.

Field study. The field study was planted in soil that had never been cropped with sugar beets. The test consisted of two 9- × 30-m plots enclosed on all four sides by a border dike. Each plot contained eight beds with 76 cm spacing. Each bed was considered one replication. On 15 August 1991, a single row of untreated seeds was planted on the four inside beds of each border. A rate of 15–20 seeds per meter was used to plant HH39 sugar beet seed at a depth of 2 cm. The two outside beds on each side of the plot were sown in the same manner with BNYVV-coated seeds.

The study was irrigated the same day, followed by three successive irrigations during the season (Fig. 1). Irrigation was accomplished by quickly filling the furrows of the enclosed plots until the water reached to just below the top of the beds. The water was allowed time to soak into the beds, then plots were flooded once more. Each bed and furrow was blocked at the end so the irrigation water was contained within each furrow and not allowed access to an adjacent furrow or bed. Each irrigation added ~6–8 cm of water.

ELISAs were conducted twice during the season (September and October) simultaneously with the greenhouse samples. Ten samples were collected at 3-m intervals from each of the eight rows planted with BNYVV-infested seeds. Four to five beets were bulked from one location and constituted a sample. Two random samples were assayed from each of the eight control rows.

The field study was repeated during July 1992. All parameters were the same as the previous year, except a higher rate of inoculum was used and one less irrigation event was attempted. The 1992 study consisted of only one field harvest and ELISA, which was done concurrently with greenhouse samples.

ELISA assays. Double antibody sandwich (DAS)-ELISA was employed during both years of this study, and commercially available antisera and enzyme conjugates obtained from Bioreba Ag (Chapel Hill, NC) and Agdia (Elkhart, IN) were used in all assays. Field and greenhouse samples were washed free of soil, and 0.25–0.30 g of root tissue was collected for assaying. In addition to test samples, each 96-well ELISA plate included four separate healthy checks, four BNYVV positive checks, and four buffer blanks. Optical density (OD) values were determined with a Dynatech MR300 ELISA reader (Chantilly, VA). A sample was considered positive if its OD value was at least three times greater than the mean of the four healthy checks.

Environmental data during both years was recorded daily by a CR-21 weather station ~2 km from the test site. Data collected included ambient air temperatures, 10-cm soil temperatures, and precipitation. At the time of planting, the 10-cm soil temperatures for both years were within 22–27 C (Figs. 1 and 2). During the first year of the field study, 4 cm of rain fell between planting

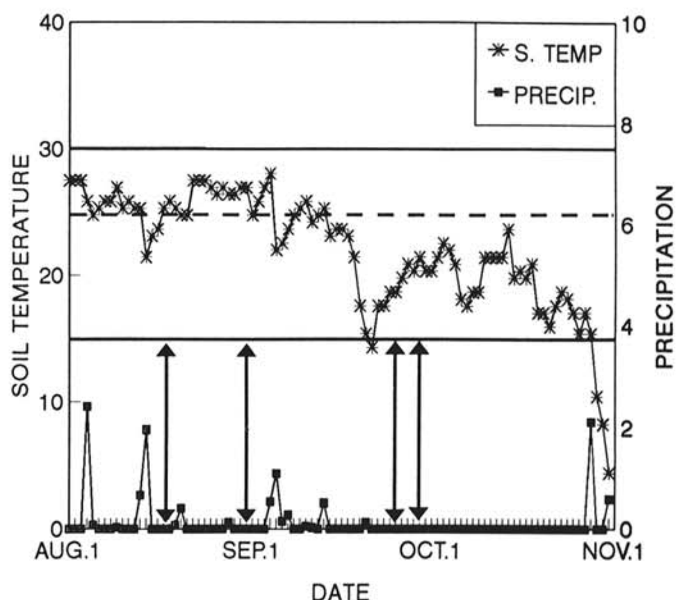

Fig. 1. Environmental data collected during the 1991 season. Two solid horizontal lines represent temperature extremes for infection by *Polymyxa betae*. The dotted horizontal line represents the optimum temperature for infection by *P. betae*. Vertical lines with arrows depict irrigation dates. Each irrigation applied 6–8 cm of water.

and final harvest (Fig. 1). The second year of the test received much more rainfall than the first—almost 15 cm (Fig. 2).

RESULTS

During both years of the study, 10-cm soil temperatures were within the range for *P. betae* infection (6,23). With the additive effect of rainfall and irrigation, over 30 cm for both years, there also was adequate moisture for *P. betae* to parasitize sugar beet plants. All environmental conditions were seemingly conducive for infection by *P. betae* and transmission of BNYVV.

The first harvest and ELISA during 1991 occurred about 1 mo after planting. At this time, only 46% of the field samples and 50% of the greenhouse samples were positive for BNYVV (Table 1). After the second assay during October, 6 wk after planting, the percentage of BNYVV positive plants had risen to 57 and 80% for the field and greenhouse, respectively (Table 1). During 1992, the test was allowed to continue for 3.5 mo before plants were harvested and assayed. The incidence for both greenhouse and field assays was over 90% (Table 2). During both years, all controls included in both field and greenhouse assays were negative for BNYVV infection.

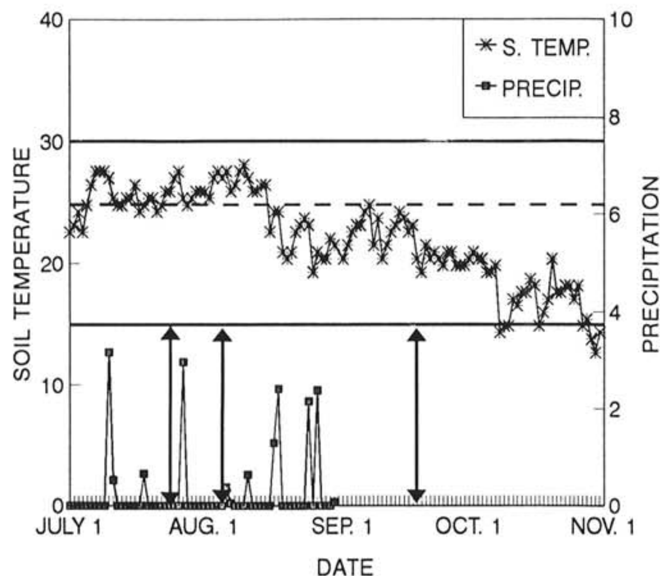

Fig. 2. Environmental data collected during the 1992 season. Two solid horizontal lines represent temperature extremes for infection by *Polymyxa betae*. The dotted horizontal line represents the optimum temperature for infection by *P. betae*. Vertical lines with arrows depict irrigation dates. Each irrigation applied 6–8 cm of water.

TABLE 1. Results of 1991 study to determine efficacy of inoculating sugar beets with beet necrotic yellow vein virus (BNYVV)

Studies	Planting date	Harvest date	Positive for BNYVV[x] (%)
Field[y]	August 15	September 18	46 ± 18.5
		October 25	57 ± 10.5
Greenhouse[z]	August 14	September 18	50
		October 25	80

[x] All assays employed double antibody sandwich enzyme-linked immunosorbent assay.
[y] Values represent mean ±2 standard errors of 80 samples.
[z] Values represent means of 25 samples.

TABLE 2. Results of 1992 study to determine efficacy of inoculating sugar beets with beet necrotic yellow vein virus (BNYVV)

Studies	Planting date	Harvest date	Positive for BNYVV[x] (%)
Field[y]	July 22	November 2	92 ± 3.3
Greenhouse[z]	July 17	November 3	90

[x] All assays employed double antibody sandwich enzyme-linked immunosorbent assay.
[y] Values represent mean ±2 standard errors of 80 samples.
[z] Values represent means of 25 samples.

DISCUSSION

The lower percentage of infection during 1991, compared with those during 1992, can be explained in several ways. First, during 1991, the study was planted later and harvested earlier than during 1992. Our lab routinely bioassays soil samples in the greenhouse to detect BNYVV. The plants grown in these soils are normally allowed an incubation period of 8–10 wk before harvesting and testing by ELISA for BNYVV infection. The 1991 study was harvested at 4 and 6 wk after planting, whereas the 1992 study was harvested after 14 wk. Second, although the irrigations were similar between the two years, the second year of the study received greater rainfall. This could have possibly contributed to the higher percentages of infection. Third, the 1991 study used one-half the inoculum concentration the second year used. Although all three of these probably affected disease incidence, we feel that length of incubation was the most important variable influencing differences between the two years. Environmental conditions during both years were adequate for infection to occur. Therefore, the lower inoculum rate used during the 1991 study would presumably have produced results similar to the rate during 1992 if enough time had been allowed for virus titer to increase in the plants to a detectable level.

Because *Polymyxa* spp. and the viruses they transmit are difficult to increase, many studies have relied on naturally occurring pathogens in fields. There are several inherent problems involved with conducting field research using land naturally infested with *Polymyxa* spp. One of the most important of these is the need to rely on asymptomatic plants as controls. Infection is more likely to occur in low-lying, poorly drained areas (25,26). Symptoms of affected plants could be confused with abiotic water-related problems. Researchers have attempted to counteract this problem by selecting symptomatic and asymptomatic plants within a similar soil environment (29) or by fumigation and reintroduction with nonfumigated soil (26,31). Further complicating this issue is the fact that BNYVV is normally restricted to root tissue, and affected plants rarely show systemic symptoms. An asymptomatic plant may, in reality, be infected and, therefore, unacceptable as a control. Correct diagnosis often requires destructive sampling, further depleting plants from test plots.

The method described here has several advantages over the use of naturally infested soils or producing zoospores as inoculum. By planting BNYVV-infested seeds, the inoculum is placed wherever it is wanted, and the process is much simpler and easier than fumigation or moving large quantities of infested soil. Controls can be established by planting untreated, uninfested seeds in the same manner as the inoculated seeds. The inoculum density for each seed is approximately equal, and each plant has the same chance to become infected. There is no need to be concerned with pathogen distribution within the field. We also are certain that the *P. betae* inoculated onto the seeds is infected with BNYVV. Many indigenous populations of *P. betae* are aviruliferous (15).

It has been difficult to determine the extent of damage to sugar beets due to rhizomania under field conditions. In Texas, *P. betae* and BNYVV often are found in a disease complex with other organisms (20). Our technique allows us to directly measure effects of BNYVV without other soilborne pathogens interacting and confusing the evaluation.

Our technique provides one other major advantage—convenience. The quantity of seeds that is inoculated can be as little or as much as needed. We have treated as few as 50–75 seeds or as much as 400–500 g. Once the seeds are inoculated, they can be stored for long periods and used whenever convenient. Timing is not a factor with our technique as it is with the pro-

duction and use of zoospores as inoculum. The inoculum on seeds remains functional for as long as the cystosori are viable. We have seed treated ~2 yr ago with inoculum that is still infectious today.

Because this method has proven to be effective, it is currently being used to study the epidemiology of rhizomania under field conditions (22). Several other applications in the laboratory and greenhouse also are being utilized (21). It is very effective for maintenance and storage of various viral and/or fungal isolates without fear of cross-contamination. It has been used to screen *Beta* spp. germ plasm for tolerance to BNYVV and *P. betae* in the greenhouse. Finally, by planting infested seeds in the greenhouse every 3–4 mo, a constant supply of BNYVV-infected fresh plants is available for use as positive controls for routine ELISAs.

The purpose of this study was to develop an easy and reliable method of inoculating sugar beets with BNYVV, both in the field and in the greenhouse. The technique described here appears to meet these requirements if environmental conditions are conducive and the pathogens are allowed enough time to become established. This technique could presumably be applied to study other *Polymyxa*-transmitted viruses.

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