Evaluation of *Polymyxa betae* Keskin Contaminated by Beet Necrotic Yellow Vein Virus in Soil

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The fungus *Polymyxa betae* Keskin belongs to the family *Plasmodiophoraceae* and lives in the soil as an obligatory parasite of the roots of the *Chenopodiaceae*. When contaminated by beet necrotic yellow vein virus, this viruliferous fungus causes a serious disease of sugar beet known as rhizomania, whereas the infection by the fungus alone (aviruliferous fungus) causes only slight damage to the plant with little economic consequence.

The manifestation of rhizomania in sugar beet is directly related to the concentration of infecting units of aviruliferous *P. betae* present in the soil. (One infecting unit is a group of one or more sporosori that liberate zoospores capable of visibly infecting a plant.) By using current methods of analysis, it is possible to estimate the total quantity of *P. betae* present in the soil, but one cannot distinguish quantitatively the infecting units of aviruliferous from viruliferous *P. betae*. A new method has been developed based on the technique of the most probable number and enzyme-linked immunosorbent assay to estimate the concentration of infecting units of viruliferous *P. betae* in soil. The method is suitable for the routine analysis of numerous soil samples and allows one to estimate the concentration of viable forms of the fungus *P. betae*, whether or not contaminated by beet necrotic yellow vein virus, present in a soil affected by rhizomania or presumed healthy. The analyses performed with this method are economical and use a reagent kit and equipment in wide use.

*Polymyxa betae* Keskin belongs to the family *Plasmodiophoraceae* and lives in the soil as an obligatory parasite of the roots of the *Chenopodiaceae* (3, 4, 11). A characteristic of this fungus is that it acts as a vector for beet necrotic yellow vein virus (BNYVV) (1, 8), causing a serious disease known as rhizomania in sugar beet; this is the viruliferous fungus. Infection by the uncontaminated (aviruliferous) fungus causes only slight plant damage (12, 13). Plants affected with this disease are characterized by an abnormal proliferation of rootlets, accompanied by a mild yellowing and wilting of the leaves during the hottest hours of the day. In infested fields rhizomania drastically reduces both the sugar content and the yield of beet roots, with considerable economic damage. Root hair infection occurs by means of the zoospores originating from zoosporangia or sporosori (cystosori) (10). A plasmadium forms within the host cell and then differentiates into a zoosporangium or sporosorus. The sporosori are the means by which the fungus is preserved and can survive in the soil for very long periods. During multiplication in the host cells, the fungus becomes contaminated with BNYVV, which remains viable for years in the sporosori preserved in the soil. With the ever increasing spread of rhizomania in sugar beet across vast areas of Europe and the United States, the attention of many workers has been focused on evaluating the concentration of infecting units (IU) of viruliferous *P. betae* present in infected, and presumably uninfected, soil (9, 7). The strict *P. betae*-host interdependence makes it impossible to isolate living forms of the fungus directly without the presence of the root of a member of the *Chenopodiaceae*. For this reason, the presence in soil of living forms of this fungus, whether or not contaminated by BNYVV, can be ascertained only indirectly by infecting the root of a specific test plant. We previously developed a quantitative assay method for evaluating living forms of *P. betae* in soil by using the most-probable-number (MPN) technique (5). However, there is currently no method that can distinguish in a given sample of soil the quantity of infecting units of *P. betae* contaminated by aviruliferous fungus from that contaminated by viruliferous fungus, which causes rhizomania.

In this paper, I report a quantitative analytical method for evaluating *P. betae* contaminated by BNYVV, using both the MPN technique and the enzyme-linked immunosorbent assay (ELISA). The quantitative data afforded by this method permit one to estimate the degree of infestation by viruliferous *P. betae* in soil in which rhizomania is present.

**MATERIALS AND METHODS**

The definitive version of the method for evaluating *P. betae* contaminated by BNYVV entails the following steps, which are diagrammed in Fig. 1.

**Growing the test plant culture in pans.** The commercial rhizomania-susceptible genotype Monomassa (SES, Italy) was used for all experiments.

(i) **Disinfection of sugar beet seeds.** The commercial sugar beet seed was freed from its coating by soaking in a beaker with tap water (1:100, wt/vol) for 2 h with magnetic stirring at 40 to 50 rpm. The seed was then placed in a 2-mm-mesh sieve and rinsed with 10 volumes of distilled water. Disinfection was achieved by suspending the seed for 5 min in 10 volumes of a sodium hypochlorite solution (Merck and Co., Rahway, N.J.) at a final concentration of 2% (vol/vol) without stirring. Finally, the seed was rinsed with sterile water eight times as reported above and partially dried on sterile blotting paper.

(ii) **Sterilization of river sand, vermiculite, glass cylinders, and pans.** The river sand was sifted through a 2-mm-mesh sieve, placed on a second 0.5-mm-mesh sieve, and washed under the tap with 10 volumes of deionized water. The washed sand was air dried and then sterilized overnight in a 250°C oven. The vermiculite was ground in a Waring blender, sifted through a 0.5-mm-mesh sieve, and sterilized as reported for the sand. The vermiculite was mixed 1:25...
with sterile river sand and used as the inert substratum to grow the sugar beet plants in "minimoist chambers" (ATIES, Bologna, Italy). These previously described assemblies (5) essentially consist of tissue culture plates covered with Plexiglas boxes provided with two adjustable openings. The glass cylinders, 16 mm in diameter and 24 mm high (ATIES), were sterilized for 20 min in a 120°C autoclave. Plexiglas pans (ATIES) were sterilized in concentrated sulfuric acid (97%; Merck) for 3 h, rinsed with sterile water, and air dried.

(ii) Germination of the sugar beet seeds. The sugar beet seeds were germinated as reported previously (5) with the following modifications. The glass cylinders were adjacent placed in the pans (Fig. 1, no. 1) and two-thirds filled with sterile river sand. Four sugar beet seeds were placed in each cylinder, which was then filled completely with sand, wet with sterile water, and incubated for 4 to 5 days in the greenhouse at 27 ± 1°C, 70 to 80% humidity, and 20,000 lx in cycles of 16 h of light and 8 h of darkness.

Growing the test plant culture in the minimoist chamber. The sugar beet plants growing in the germination pans were transplanted along with the glass cylinder into the wells of the minimoist chambers. Before receiving the plants, the wells were two-thirds filled with the sand and vermiculite mixture and inoculated as described below. Two test tubes (16 mm in diameter and 100 mm high), filled with water and containing two 8- by 80-mm strips of blotting paper to automatically maintain the humidity inside the minimoist chamber, were inverted into the two wells of each minimoist chamber (Fig. 1). The base and upper portion of the minimoist chamber were then fitted together and placed in the greenhouse for 20 to 40 days under the same conditions reported for the germination of the sugar beet seeds. During incubation, the water evaporating from the tubes was replaced by topping up the two tubes to their original level every 10 to 15 days; the air vents of the minimoist chambers were adjusted as necessary.

(i) Preparation of standard soil samples. Standard soil samples with increasing quantities of viruliferous P. betae were prepared by mixing in various proportions two types of soils from rhizomania-infected fields in two different geographical areas. The first type, from northern Italy (Bologna), contained a total of 40 to 50 IU of P. betae per ml, half of which was viruliferous; the second type was from England (Higham) and contained 30 IU of aviruliferous P. betae.

All soil was sampled at a depth of 15 cm, air dried in a dry dark place, ground with a mortar and pestle, sifted through a 1-mm-mesh sieve, and kept in dark glass containers in a cool (6 to 8°C) place. The standard samples were prepared at the time of use and analyzed by the MPN technique (5).

(ii) Preparation of soil dilutions. The dilutions were prepared in 150-ml beakers containing 10 g of each sample suspended in a final volume of 100 ml of growing solution and stirred at 60 rpm. This was the first dilution. After 5 min of magnetic stirring, the samples were diluted fourfold by transferring (without interrupting the stirring) 25 ml of the first dilution into a second beaker containing 75 ml of a suspension of noninfested soil (1:10, wt/vol), and so on, up to the fourth beaker. In this manner the infested soil to be analyzed had the following dilutions: 1:10, 1:40, 1:160, and 1:640. The suspension of noninfested soil was prepared by suspending 100 g of a silty clay loam soil (Vertic Xerorthent) (2) in 1,000 ml of plant growing solution as reported previously (5).

(iii) Soil inoculation. The samples of diluted standard soil were inoculated by using 1 ml for each well located in the base of the minimoist chambers. Four dilution steps were used for each sample, five wells per dilution. In two rows of

**FIG. 1. Schematic diagram showing the main steps of the method.** 1, Germination of the test plants in glass cylinders inside Plexiglas pans; 2, inoculation and transplantation of plants along with the glass cylinders into the wells of a tissue culture plate; 3, sealing of the minimoist chambers and incubation in the greenhouse; 4, numbering and sampling of test plant roots; 5, evaluation of P. betae by the MPN technique; 6, transfer of samples to microcentrifuge tubes and addition of sample buffer; 7, homogenization of samples; 8, evaluation of BNYVV by ELISA.
each mini-moist chamber, a control consisting of a well inoculated with 1 ml of noninfested soil suspension was distributed at random. The plant growing solution and the inoculation were prepared as reported previously (5). The experiment with the standard soil was repeated three times, and each sample was analyzed with five replications.

Analysis of soil and soil debris from the rhizomania-infested field. For this experiment we used 20 samples of soil from a field in northern Italy (Bologna) in which sugar beets showing symptoms of rhizomania were growing. The samples consisted of 1,500 g of fresh soil collected at a depth of 15 cm. After air drying, they were ground in a Waring laboratory blender (model 3390-D25; Thomas Scientific, Swedesboro, N.J.), sifted through a 1-mm-mesh sieve, and kept in glass containers in a cool place. For each sample, 10 g was analyzed with the same procedure used for the standard samples, using a 10-fold dilution factor, three wells per dilution, and six dilution steps (from 10^{-1} to 10^{-6}), and the remaining sample was used for the extraction of soil debris.

(i) Extraction of soil debris. In addition to analyzing the infested soil as such, I wanted to determine whether the viruliferous and aviruliferous fungus could be analyzed starting from the plant debris extracted from the infested soil and then concentrated. The soil debris was extracted by suspending, each time, 500 g of soil in 2,000 ml of distilled water in a 3,000-ml beaker. After stirring with a magnetic bar for 5 min, flocculation of the clay colloids was induced by adding 3 g of MgO (no. 5865; Merck). After another 5 min of stirring, the soil was left to settle for 20 min; the supernatant was centrifuged at 1,469 x g for 5 min (Kontron-Hermle model Centrikon H 401 centrifuge), and, finally, the debris remaining in suspension in the liquid fraction was collected by filtering through Whatman no. 1 filter paper. The debris collected on the filters corresponding to 1,000 g of each sample was resuspended in 10 ml of noninfested soil suspension (1 g of debris-enriched noninfested soil) and analyzed by the procedure reported for the soil samples.

Plant analysis. After 20 days of incubation, the mini-moist chamber assemblies were opened and the glass cylinders containing the plants were carefully removed with a metal spatula (Fig. 1, no. 4). The roots of the test plants were completely freed of inert substrate by washing under a stream of tap water, separated from the rest of the plant with a scalpel, and used to ascertain the presence of P. betae and BNYVV as described below.

(i) Evaluation of the fungus P. betae. The roots corresponding to each well of the mini-moist chamber were placed in parallel on a glass slide, marked with an identification number for their province, moistened with a few drops of phosphate buffer (pH 7; 0.1 M), covered with a cover glass, and observed at x150 with a model BH-2 microscope (Olympus Corp., Scientific Instrument Div., Stamford, Conn.). The presence of sporosori of P. betae was ascertained on the scanned roots without staining the tissue (Fig. 1, no. 5). The MPN of infecting units of P. betae per gram of soil sample was calculated on the basis of the statistical tables reported in reference 5.

(ii) Evaluation of BNYVV. After microscopic observation, the roots were partially dried on blotting paper with a forceps and placed in a 1.5-ml polypropylene microcentrifuge tube marked with the root identification number. Roots were transferred by keeping each tube on a model PM100 electronic digital top-loading balance (Thomas Scientific) and weighing the roots after calibration. Then, with the samples still on the balance, sample buffer (code 773913; Boehringer Mannheim) was added with a syringe until the final weight of the roots made up 10% (wt/wt) of the entire mass (Fig. 1, no. 6).

The BNYVV was extracted by homogenizing the roots directly in the microcentrifuge tubes with a ground-tip glass rod driven by a Masterflex electric motor (ATIES) (Fig. 1, no. 7). The ground tip of the glass rod was cleaned between samples by immersing it for 30 s in a beaker with 75% ethyl alcohol followed by 30 s in sterile water and drying with blotting paper. After homogenization, the samples were immediately stored in the freezer at -40°C and, after 2 to 3 days, analyzed serially by ELISA (6), using the reagent kits from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany. The reagent stocks in the kit were diluted according to the instructions included, whereas the evaluation of BNYVV was as follows: 0.2 ml of Boehringer coating solution (code 726559, 814296) was placed in the wells of the microplates, which were placed in a plastic bag and incubated at 37°C for 2 h. Then the solution was removed by tapping on filter paper, the wells were washed three times with the addition of 0.2 ml of Boehringer wash buffer (code 726915, 726923), and the buffer was removed by tapping on filter paper. Then 0.2 ml of homogenized sample root was placed in each well of the microplate, which was incubated at 4°C overnight in sealed plastic bags. After incubation, the samples were removed by tapping on filter paper and the plates were washed three times with wash buffer as reported above. Then 0.2 ml of Boehringer antibody-alkaline phosphatase conjugate solution (code 814300) was placed in the wells and the plates were again sealed in plastic bags and incubated for 4 h at 37°C. After incubation, the solution was removed by tapping on filter paper and the wells were washed three times with wash buffer as reported above. Finally, 0.2 ml of substrate solution (code 726915, 726923) was placed in each well and the plates were incubated for 60 min at room temperature in the dark. The formation of a yellow color in the wells evaluated visually or the A_{405} read with a model MR590 micro-ELISA minireader (Dynatec Instruments, Inc., Santa Monica, Calif.) was recorded. The values were compared with some negative controls represented by wells containing samples of healthy sugar beet and with other positive samples represented by wells that had received pure BNYVV or sugar beet plants with rhizomania. The MPN of the infecting units of viruliferous P. betae per gram of soil was calculated by using the statistical tables reported previously for evaluating the fungus, the only difference being that, in this case, only the wells in which the sugar beet had both P. betae and BNYVV were considered.

Statistical analysis. The data for the MPN of the infecting units of viruliferous P. betae obtained from the analysis of the sample of standard soil were statistically evaluated for analysis of variance, and the means were compared with Duncan’s test.

RESULTS

Evaluation of viruliferous P. betae in the standard soil sample. The result of the analysis of the soil samples analyzed by the MPN technique and ELISA showed a positive correlation (r = 0.98) between the concentration of soil infested with viruliferous P. betae and the MPN of the infecting units of P. betae contaminated with BNYVV (Fig. 2). From the analysis of standard soil, a significant (P < 0.05) increase in the infecting units of viruliferous P. betae was observed as the fraction of soil contaminated with P. betae BNYVV was increased.

On the average, the total MPN of P. betae calculated in
the various standard soil samples remained constant at 40 to 50 IU/g, whereas the MPN of viruliferous *P. betae* varied progressively from 5 IU/g in the samples with 20% soil contamination by both the fungus and virus to 20 IU in samples with 100% contamination by *P. betae* and BNYVV. Consistently negative BNYVV ELISA results were found for the control samples of soil contaminated with *P. betae* without BNYVV.

**Evaluation of viruliferous *P. betae* in soil samples from the rhizomania-affected field.** The experiments conducted on soil from the rhizomania-affected field demonstrated the presence of viruliferous *P. betae* in both the soil samples analyzed as such and the plant debris recovered from the same samples. For the soil samples, we calculated a total MPN of 71 ± 9.2 IU (mean ± standard error) of *P. betae* per g of sample, of which 20 ± 3.2 IU/g consisted of viruliferous fungus. In the debris extracted from 1,000 g of soil and concentrated in 1 g of noninfested soil, we recovered a total MPN of *P. betae* about 500 times lower than that for the same original soil samples, 124 ± 15, of which 25 ± 2.1 IU/g consisted of viruliferous fungus. The average ratio of infecting units of viruliferous to aviruliferous *P. betae* was 0.39 for the soil samples and 0.25 for the soil debris.

**Effects of soil sample dilution on BNYVV detection by ELISA.** In general, in all experiments we observed a lower BNYVV concentration in the dilutions at the end point of positive virus infection (Table 1). However, these values were sufficiently high to distinguish unequivocally the BNYVV ELISA-positive wells from the negative ones. In one of the preliminary experiments we demonstrated that, by analyzing the plants after a longer incubation period (40 days), the differences in BNYVV concentration between dilution steps tended to disappear; however, the MPN of infecting units of viruliferous *P. betae* did not increase significantly as the incubation period was increased (data not shown).

**DISCUSSION**

My evaluation of the MPN of infecting units of contaminated or uncontaminated *P. betae* is a refinement of the method published previously (5). It permits one to estimate distinctly the viable forms of aviruliferous *P. betae* fungus from the viruliferous ones, which are actually implicated in sugar beet rhizomania. Sugar beet infected by *P. betae* alone (aviruliferous fungus) does not show serious symptoms of rhizomania, in contrast to when the plant is infected by *P. betae* and BNYVV (viruliferous fungus). For this reason, the manifestation of rhizomania is directly related to the concentration of viruliferous *P. betae* in the soil.

The infection caused by viruliferous *P. betae* does not appear to be affected by the quantity of aviruliferous fungus present in the samples. In fact, Fig. 2 shows that the total MPN of *P. betae* remains nearly constant, whereas the MPN of viruliferous fungus increases as the percentage of soil infected with viruliferous *P. betae* increases. The experiments performed on rhizomania-affected soil demonstrated that soil debris is a source of viruliferous and aviruliferous *P. betae* and can be used as a material for analysis (see Results). The lower recovery of fungus in the debris compared with the original soil probably depends on the smaller particles in the debris, which are easily lost during the extraction process.

In all experiments the BNYVV ELISA-positive samples of sugar beet root were always positive for *P. betae* sporoisiri. The greater concentration of BNYVV seen in the roots grown in the wells of the mini-moist chambers inoculated with the less diluted sample can be explained by considering the larger number of infecting units of viruliferous *P. betae* that infect the plant with a greater initial quantity of virus compared with the virus transferred by the viruliferous fungus in higher dilutions of the same sample. However, the method described here does not take into account the concentration of virus in the samples of beet root, but only its presence or absence; thus, in 20 days of incubation one observes that virus replication reaches known thresholds unequivocally with ELISA, even at the end point dilutions for the presence of BNYVV. With this method it is possible to follow the dynamics of the relationship between the viable forms of viruliferous and aviruliferous fungus in soils affected with rhizomania that experience a reduction in inoculum potential during rotation with crops that are not infected with *P. betae* and BNYVV.

From the operational viewpoint, the method has been improved compared with that published previously: specifically, the test plants no longer require attention during incubation. The two test tubes containing water inside the mini-moist chamber (Fig. 1, no. 2) automatically ensure a high humidity inside the chambers, thus limiting the evaporation of water from the wells, which stay wet. Finally, the method entails low-cost, commercially available equipment and can be used for routine analysis in any laboratory.

**TABLE 1.** Mean values of A$_{405}$ in BNYVV ELISA-positive wells

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<tr>
<th>Dilution step</th>
<th>Soil$^a$</th>
<th>Soil debris</th>
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<tbody>
<tr>
<td>1</td>
<td>0.63 ± 0.16</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>0.42 ± 0.05</td>
<td>0.38 ± 0.05</td>
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<tr>
<td>3</td>
<td>0.38 ± 0.05</td>
<td>0.30 ± 0.02</td>
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<td>4</td>
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<td>0.28 ± 0.03</td>
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$^a$ The background obtained for the wells with healthy sugar beet samples, which on the average ranged from 0.01 to 0.03, was subtracted from the means.
ACKNOWLEDGMENT

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REFERENCES