CALIFORNIA LETTUCE RESEARCH PROGRAM

April 1, 2007 - March 31, 2008

BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE

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SUMMARY

There were five objectives during the current funding cycle and included: a) continued monitoring of Verticillium wilt and soil inoculum density in coastal California; b) to determine the horizontal spread of *Verticillium dahliae* microsclerotia after fumigation; c) to assess seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation; d) to continue identification and development of resistance in crisphead, leaf, and other lettuce types; and e) to determine the potential of Acibenzolar-S-methyl (Blockade) to control Verticillium wilt in lettuce. Verticillium wilt of lettuce was observed in a new ranch in Salinas this year. Incidence of wilt was <30% suggesting a recent introduction of the pathogen. The number of microsclerotia in soil was >150 microsclerotia g^{-1} soil. The extensive destruction of French marigold this summer in the Lompoc area suggests a potential risk to lettuce in the Santa Maria area. The study of horizontal spread of *V. dahliae* following fumigation was concluded and confirmed that fumigation offered protection from Verticillium wilt to about three crops including strawberries following fumigation. After two lettuce crops, the concentration of microsclerotia in the field was greatest in areas where the numbers were highest prior to fumigation. Areas closer to the edge of the field had greater numbers than inland. Thus, the horizontal spread of the pathogen following fumigation depended on the initial distribution, the types of crops grown, and potentially also on the reintroduction of inoculum from contaminated farm equipment. We assayed 55 seed and 37 soil samples from companies for *V. dahliae*. The number of microsclerotia in soil samples ranged from 0-38 per gram of soil. Of the 55 seed lots evaluated, 26 yielded *V. dahliae* between 1-6%. The breeding for resistance to Verticillium wilt has taken several approaches. On the host side, many sources of resistance to race 1 have been identified and several of these sources were released to commercial seed companies over the summer by Ryan Hayes. A successful field trial evaluating resistance in breeding material, cultivars and germplasm lines against race 1 was also conducted over the summer. Identification of resistance to race 2 is also currently underway. The commercial field sites are no longer available to screen for race 1 resistance as they were all fumigated. Hence, a field at the USDA Station is being developed to conduct future trials. On the pathogen side, we are currently assessing the relative proportion of race 1 and race 2 among the >250 isolates collected from infected lettuce over the past many years. This is being accomplished not only by inoculating each isolate on both Salinas and La Brillante in the greenhouse but also by a PCR assay based on the distinctive rDNA IGS sequences between the two races. Evaluation of Actigard was completed and had little effect on Verticillium wilt of lettuce.

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PROJECT TITLE: **BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE**

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OBJECTIVES:

1. Continued monitoring of Verticillium wilt and soil inoculum density in coastal California.
2. To determine the horizontal spread of *Verticillium dahliae* microsclerotia after fumigation.
3. To assess seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation.
4. To continue identification and development of resistance in crisphead, leaf, and other lettuce types.
5. To determine the potential of Acibenzolar-S-methyl (Blockade) to control Verticillium wilt in lettuce.

PROCEDURES AND RESULTS:

Objective 1. Continued monitoring of Verticillium wilt and soil inoculum density in coastal California.

Methods. Monthly surveys were undertaken to identify new fields with Verticillium wilt on lettuce and reappearance of the disease in previously fumigated fields in coastal California. The surveyed fields included all types of lettuce. During each survey, approximately 20 fields were evaluated. In each field, an average of 100 plants were examined for symptoms typical of Verticillium wilt, and any plants showing symptoms were further examined for the characteristic vascular discoloration. A random sample of plants showing symptoms was brought to the laboratory for pathogen isolation from affected tissues on modified NP-10 medium semi-

selective for *V. dahliae*. In fields, where the disease was found, incidence data was collected by sampling plants from an average 20 sites by walking the fields in an X-pattern. At each site, the total number of plants in a 1-m x 1-m area and the number of symptomatic plants were counted. Data from the 20 sites were averaged to give the mean disease incidence for each field. Soil samples were also collected in fields with Verticillium wilt to assay for *V. dahliae* microsclerotia. Furthermore, literature was reviewed to examine the occurrence of Verticillium wilt on lettuce in other parts of the world and crops related to lettuce were also evaluated in the Santa Maria area.

Soils were air-dried in the laboratory (23 ºC ± 2 ºC), mixed thoroughly, and pulverized using mortar and pestle. From each sample, 10 g of soil were placed in snap cap vials and mixed with 2.5 ml of a dl-methionine solution (7.5 mg ml^{-1}). Vials were capped and incubated in the dark at 30 ºC for 1 week. The vials were then opened and allowed to air-dry for 1 week at 22 to 24 ºC. Samples were re-pulverized and dispensed onto Petri dishes containing modified NP-10 selective medium using the modified Anderson sampler. With the Anderson sampler, 0.5 g of pulverized soil from each sample was distributed over two replicates of six Petri dishes. Plates were incubated in the dark at 22 to 24 ºC for 3 weeks. After incubation, the surfaces of the agar media were gently washed under running tap water to dislodge and remove soil particles. Washed Petri dishes were examined for *V. dahliae* microsclerotia clusters using a dissecting microscope with transmitted light. Counts from the two replications were combined for mean values and expressed as microsclerotia g^{-1} dry soil.

Results. One newly infested ranch with several fields within this ranch developed Verticillium wilt on lettuce over the summer this year. The fields were located in the Salinas area. Disease incidence in these staggered plantings ranged between 10 – 30% suggesting a new introduction of the pathogen in these fields. Soil assays from these fields revealed between 100 and 150 microsclerotia g^{-1} soil. These were the only lettuce fields that developed Verticillium wilt during the year. However, an ominous development over the past year was the complete destruction of French Marigold seed crops in the Santa Maria area. Nearly 60 acres of seed production crops suffered total losses from Verticillium wilt. The pathogen was isolated from all tissue samples received and isolates were collected to determine their cross pathogenicity to lettuce and their genetic relationship to the lettuce isolates. While Verticillium wilt on lettuce was hitherto reported only from California and the island of Crête, that also changed over the past year. Verticillium wilt on lettuce was reported from northern and southern Italy and Greece suggesting that the disease is spreading globally. The reasons for this sudden spread across continents is unclear but the role of infested seed in the introduction of the pathogen as also the rotation of infested artichoke fields to lettuce cannot be discounted. Furthermore, the role of infested spinach seed being planted in the Salinas Valley on Verticillium wilt on lettuce requires further scrutiny.

Objective 2: To determine the horizontal spread of *Verticillium dahliae* microsclerotia after fumigation.

Methods and Results. This was the final year of study for this objective initiated in 2005 to provide information on how the soil is recolonized post-fumigation over a horizontal plane. We thank Belinda Platts for allowing us to sample these fields over multiple years. Verticillium wilt on lettuce on this ranch developed in 2004 but its distribution across the fields was not uniform.

This patchy distribution of the disease with severe disease in one edge of the fields where nearly 85% of symptomatic plants were recorded and little or no disease in the center and far end of the fields suggested the potential introduction of inoculum from contaminated equipment or personnel. Once again all fields in this ranch were fumigated and planted to strawberries in 2005. In 2006, one field sampled was planted to crisphead lettuce during spring and cauliflower during fall and the other field to crisphead lettuce during spring and romaine during fall. The same cropping pattern was followed in 2007.

Two fields within this ranch were chosen for detailed sampling. In each field, grids of 64 (8- x 8-m) contiguous quadrats (1- x 1-m) were established on the edge, middle and far end of each field. The precise location of each grid was recorded using a GPS unit to help collect future samples at or close to these sample sites. Soil samples to a depth of 3-4 inches were collected from each quadrat before fumigation in August 2004, after fumigation in September 2005, in July 2006, and in May 2007. Soil samples were assayed for *V. dahliae* microsclerotia as described in objective 1.

Table 1. Numbers of *Verticillium dahliae* microsclerotia and associated standard errors of the mean at three sites (that were 75-m apart from each other) in two different fields pre- and post-fumigation with methyl bromide and chloropicrin between 2004 - 2007. Note the high numbers of microsclerotia prior to fumigation at the first site, which was close to the edge of the field

Field	Site 1	Site 2	Site 3
Pre-fumigation – 1	91.5 ± 13.4	22.4 ± 2.8	23.5 ± 1.2
Post-fumigation – 1 (strawberry)	2.3 ± 0.40	6.4 ± 1.2	1.0 ± 0.3
Post-fumigation – 1 (head lettuce – cauliflower)	10.1 ± 0.51	3.69 ± 0.4	4.2 ± 0.5
Post-fumigation – 1 (head lettuce – cauliflower)	162.4 ± 48.2	46.06 ± 9.0	18.1 ± 6.9
Pre-fumigation – 2	62.9 ± 4.35	37.7 ± 2.2	13.4 ± 2.2
Post-fumigation – 2 (strawberry)	3.7 ± 1.90	2.6 ± 0.5	3.3 ± 1.3
Post-fumigation – 2 (head lettuce – Romaine)	7.31 ± 0.45	5.0 ± 0.4	3.0 ± 0.4
Post-fumigation – 2 (head lettuce – Romaine)	6.81 ± 4.74	5.6 ± 3.9	4.4 ± 3.7

The numbers of microsclerotia in the different sampling grids reflected the distribution of Verticillium wilt on lettuce before fumigation. Microsclerotia in soil were significantly greater in grids close to the edge of the two fields (Table 1) and were lower in grids sampled in the center or the far end of the field. Post-fumigation, the numbers of microsclerotia in all grids were nearly uniform and lower than prior to fumigation (Table 1). Following strawberries, both fields initially were planted to crisphead lettuce and one field had cauliflower and the other romaine lettuce during the fall. As observed with the study on the recolonization of soil at different depths following fumigation, crisphead lettuce significantly increased the numbers of microsclerotia and this increase was most pronounced in areas of the field that had high numbers of microsclerotia prior to fumigation (Figs. 1 and 2). After two lettuce crops, the concentration of microsclerotia in the field was greatest in areas where the numbers were highest prior to fumigation. Areas close to the edge of the field had greater numbers than inland (Figs. 1 and 2). Thus, the horizontal spread of the pathogen following fumigation depended on the initial distribution, the crops planted post-fumigation, and potentially also on the reintroduction of inoculum from personnel and contaminated farm equipment.

Objective 3. To assess seed lots and soil from seed production fields of private companies for potential *V. dahliae* infestation.

Verticillium wilt that occurs on many dicotyledonous plants is predominantly caused by *Verticillium dahliae* that colonizes xylem tissues and causes disease on a broad array of plants. In some instances these vascular pathogens may even invade the inflorescence, and subsequently the developing fruits and seeds. The seed-borne nature of *V. dahliae* has been documented in cotton, eggplant, tomato, and spinach, and in the cultivated composites, safflower and sunflower. We reported the seed transmission of *V. dahliae* in 2005 on *L. sativa*, which was previously reported to be a new host of *V. dahliae*. The recovery of *V. dahliae* following the disinfestation of seed surfaces suggests that the fungus resides within the achene, similar to findings in safflower and sunflower. In preliminary studies, the pericarps shed from germinating lettuce seeds were colonized by *V. dahliae*. Subsequent studies employing a green fluorescent protein-transformed race 1 strain of *V. dahliae* from lettuce suggested that the fungus resides in the endosperm but never compromises the embryo. Thus, even if the pathogen resides in the seed, it may not reduce seed germination. The susceptibility of several weed species to *V. dahliae* and the infestation of seed, and subsequent infection of seedlings from these seed also has the potential to spread the pathogen in coastal California. The susceptibility of *Lactuca* species to isolates of *V. dahliae* from lettuce, in addition to the susceptibility of lettuce to several isolates of *V. dahliae* that were collected from weed species raises concerns about the potential of weed species to act as a reservoir of *V. dahliae* in California vegetable production areas. Even though no Verticillium wilt has been reported from commercial lettuce seed production fields, it was important enough to examine if commercial seed lots carried the pathogen, and if they carried the pathogen, to determine the level of infestation in these seed lots.

Methods. A letter of request was sent out by Mary Zischke to more than 15 seed companies requesting samples of seed lots and soil from seed production fields to be sent to Krishna Subbarao for evaluation of *V. dahliae* infestation. Eight seed producers submitted seed lots and soil for evaluation. From each seed lot, 200 seeds were plated onto *V. dahliae* semi-selective

NP-10 medium and incubated at room temperature (22 $\pm$ 1ºC) for 10 days and fungal colonies that emerged from individual seeds were examined under a stereomicroscope. Number of seeds yielding *V. dahliae* colonies were counted and expressed as the percentage of seeds that yielded *V. dahliae*. These colonies were transferred to fresh plates of NP-10 medium to confirm identity and also for isolate collection. After purification, single spore colonies were obtained and stored for future studies evaluating the populations.

Soil samples were processed as described in objective 1 and the number of microsclerotia from each soil sample was expressed as the number per gram dry soil.

Results. Of the 55 seed lots evaluated from eight seed companies, nearly 50% yielded at least one seed with *V. dahliae* (Fig. 3). The level of infestation among seed lots that yielded *V. dahliae* varied between 1-6%. Of the 37 soil samples assayed, 33 yielded at least 2 microsclerotia per gram dry soil with a range of 2-38 (Table 2).

Table 2. Summary of seed lots and soil samples from seed production fields evaluated during 2007

Seed companies approached	Number responded	Seed lots with *V. dahliae*/total assayed	Soil samples with *V. dahliae*/total assayed
16	8	26/55 (0-6%)	33/37 (0-38 ms/g)

Judging from historical trends, the level of microsclerotia in the soil samples from seed production fields was not high enough to cause Verticillium wilt on lettuce and yet, the seed infestation levels suggest high incidence of the disease. Our recent studies have shown a potential airborne phase for the pathogen during seed production. Perhaps this plays a greater role spreading the pathogen on seed crops than we have so far realized. We will be pursuing this angle over the coming year in addition to continuing the seed and soil assays from seed companies.

Objective 4. To continue identification and development of resistance in crisphead, leaf, and other lettuce types.

Methods and Results. The breeding for resistance to Verticillium wilt has taken several approaches. On the host side, many sources of resistance to race 1 have been identified and several of these sources were released to commercial seed companies over the summer by Ryan Hayes. A diverse set of 12 crisphead, romaine, latin, and red leaf cultivars were identified with resistance to race 1, but susceptible to race 2. None of this germplasm is suitable for direct commercial production, but has been used as parents in the breeding program. The identification of resistance in diverse lettuce types is beneficial to the breeding process, and sufficient genetic

diversity is present to develop new cultivars of each market type with resistance to race 1. Consequently, breeding program has been broadened to include introgression of resistance into iceberg, romaine, green leaf, and red leaf types. The existence of race 2 isolates in California lettuce production fields is certain. Moreover, it is highly probable that widespread use of race 1-resistant germplasm in lettuce production will select for and increase the economic damage caused by race 2 isolates. A similar situation occurred with the release of tomato germplasm with the *Ve* locus, the only other example of race-specific resistance to *V. dahliae*. Therefore, the development of lettuce cultivars with resistance to race 2 is imperative for sustaining the lettuce industry. All of the germplasm we have tested to this point is susceptible to race 2 isolates. This strongly indicates the need for a large scale comprehensive screening effort targeting race 2 isolates. We are currently screening the *Lactuca* collection for resistance to race 2 of *V. dahliae*. Our strategy uses greenhouse testing to screen up to eight plants of 160 accessions per year in unreplicated plots to identify candidate sources of resistance. This is followed by replicated greenhouse and field-micro-plot experiments to confirm resistance. Verticillium wilt disease development in lettuce is dependant on plant development, and in some genotypes symptoms are not expressed until the plant reaches flowering. We maintain the plants in the greenhouse until flowering begins, at which time disease evaluations are conducted. This substantially lengthens the duration of each experiment, but is necessary to reduce the number of false positives. At all stages of testing, crown sections of asymptomatic plants are plated on NP10 media to determine the presence / absence of *V. dahliae* stem infection. To date, we have screened 152 accessions using a race 2 isolate of *V. dahliae*; 38 accessions were selected as candidate sources of resistance and are currently being tested in replicated greenhouse experiments. Crosses will be made between resistant accessions and adapted cultivars to initiate breeding and genetics research. Details of this work are in Ryan Hayes' report.

A successful field trial evaluating resistance in breeding material, cultivars and germplasm lines against race 1 was also conducted over the summer. The commercial field where screening against race 1 resistance was conducted successfully over the past three years will not be available in the future as it is being fumigated. Hence, a field at the USDA Station is being developed to conduct future trials. The first crop of lettuce from infected seedlings was produced this fall and Verticillium wilt incidence was high. The crop was incorporated in November 2007. Another crop from infected seedlings will be produced and incorporated in the spring of 2008. The field will then be ready to conduct screening trials either during the fall of 2008 or spring of 2009.

On the pathogen side, we are currently assessing the relative proportion of race 1 and race 2 among the >250 isolates collected from infected lettuce over the past many years. This is being accomplished not only by inoculating each isolate on both Salinas and La Brillante in the greenhouse but also by a PCR assay. Isolates that are only pathogenic on Salinas are characterized as race 1 and those pathogenic on both cultivars are considered race 2. A PCR assay based on the distinctive rDNA IGS sequences between the two races developed in collaboration with Steve Klosterman is also being employed to identify the two races among the isolates. If the PCR assay turns out to be as successful as the initial results reveal, it will obviate the need for the labor and resource-intensive virulence assays in the greenhouse. In any case, when completed, the work will provide a comprehensive map of the relative distribution of individual races. When resistant cultivars are available against the two races, they can be deployed in the corresponding fields.

5. To determine the potential of Acibenzolar-S-methyl (Blockade) to control Verticillium wilt in lettuce.

The potential use of plant defense elicitors such as Blockade that is already registered for downy mildew control was tested for the control of Verticillium wilt in lettuce. While resistance to *V. dahliae* has been identified in lettuce germplasm, several field isolates of *V. dahliae* able to compromise resistance have also been identified. If the use of a plant defense elicitor is effective at controlling Verticillium wilt, then it could be immediately integrated into the current production practices on commercial lettuce varieties. Last year, we tested two rates of Blockade sprayed at 2, 4, and 6 wk after transplanting but it had no effect on Verticillium wilt. Over the past year, studies focused on the evaluation of earlier sprays on Verticillium wilt in lettuce.

Methods. Experiments were conducted in the greenhouse to evaluate two rates of Blockade on two cultivars of lettuce. Five wk-old seedlings of cultivars, Salinas and Pacific were inoculated with *V. dahliae* and transplanted to individual pots. Ten plants of each cultivar were sprayed with 800 µM and 1600 µM Blockade at 1, 2, 3, 4, 5, and 6 wk after transplanting.

Seven wk after transplanting, the plants were rated weekly for visual Verticillium wilt symptoms and at 12 wk, plants were destructively rated for wilt severity. Plants were uprooted, roots were cleaned of soil and cut longitudinally to evaluate disease severity by rating root discoloration on a 1 – 5 scale where 0 = no vascular discoloration, 1 = 1 to 25% of the vascular tissue exhibiting discoloration; 2 = 26% to 50%; 3 = 51% to 75%; and 4 = 76% to 100% discoloration in the absence of foliar symptoms; and 5 = 100% discoloration and the presence of foliar symptoms typical of Verticillium wilt. Disease severity ratings were analyzed using analysis of variance type statistics of ranked data using the PROC Mixed procedure in SAS, and the LD_CI macro to generate relative marginal effects (RME) for each treatment and 95% confidence intervals for detection of statistical difference between treatments.

Results. The rate of Blockade, application time and their interactions did not have any effect on Verticillium wilt incidence. The cultivars differed significantly in their reaction to Verticillium wilt. While the treatment has been shown to be effective on other host systems, it was not effective in lettuce. Since it is already a registered material on lettuce, it was worth the evaluation but it also showed that Verticillium wilt cannot be controlled in season that easily.

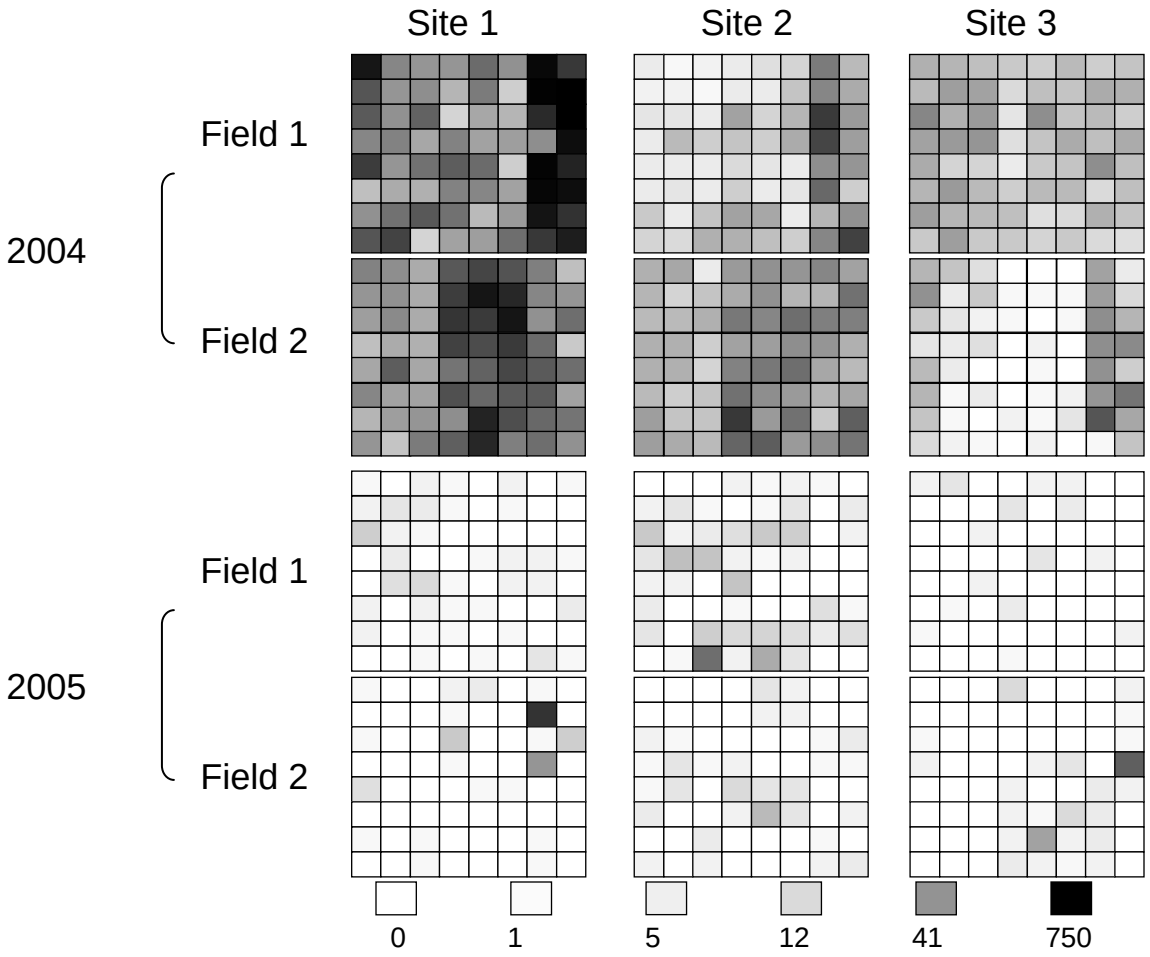

Fig. 1. Distribution of *Verticillium dahliae* microsclerotia in two fields in a 8 x 8-m grid at three sites within each field prior to fumigation in 2004 and following fumigation in 2005. Different shades of gray represent different numbers of microsclerotia.

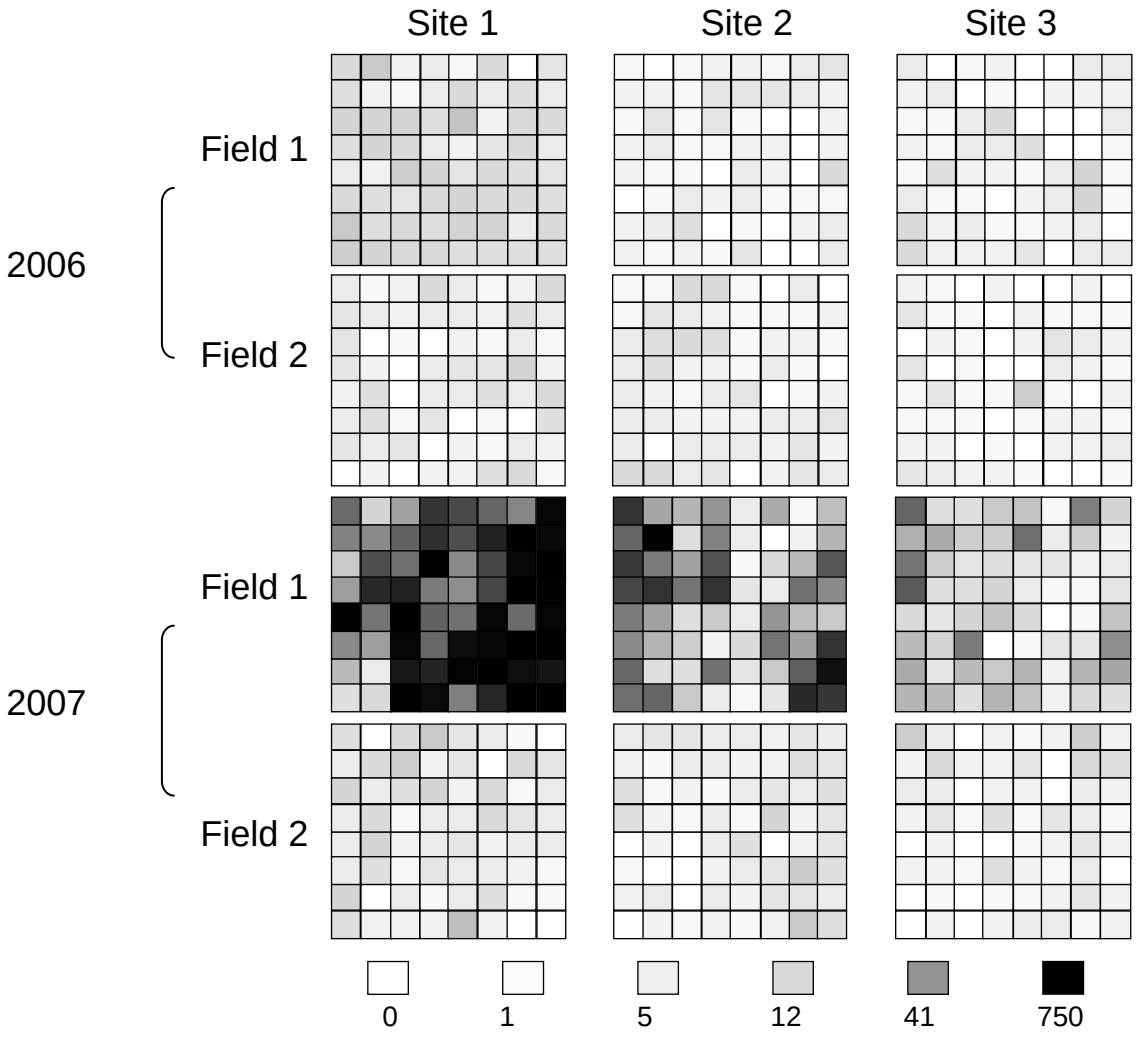

Fig. 2. Distribution of *Verticillium dahliae* microsclerotia in two fields in a 8 x 8-m grid at three sites within each field following two crisphead crops. Notice the dramatic increase in the numbers of microsclerotia at the three sites in field 1 in 2007. Different shades of gray represent different numbers of microsclerotia.

Fig. 3. *Verticillium dahliae* colonies from infested seed from a commercial seed lot 10 days after plating seed on the NP-10 medium.