Research

Standardization of Screening Methodology for Assessing the Susceptibility of Sweet Basil Cultivars and Lines to *Fusarium oxysporum* f. sp. *basilici*

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Accepted for publication 23 May 2022.

Abstract

Fusarium wilt (FOB), caused by *Fusarium oxysporum* f. sp. *basilici*, remains an important disease of basil because of its persistence in the soil and seed transmission. Standardization of the methodology for investigating FOB resistance of basil cultivars is necessary for meaningful results. In a seed source experiment, six commercial seed sources of the sweet basil cultivar Nufar were tested in the greenhouse for their response to a single isolate of FOB at four inoculum concentrations (0, 10^2, 10^4, 10^6) at the six-leaf stage of growth. Differential susceptibility of the Nufar seed source lines was only revealed at a concentration of 10^4, but not at the lowest (10^2) or highest (10^6) concentrations. To understand the effects of inoculum concentration (0, 10^4, 10^5, 10^6), cultivar (Caesar, Nufar, RU172), and leaf stage (two, four, six) on FOB incidence and severity, a split-split plot experiment was conducted. There was a highly significant ($P < 0.0001$) cultivar with inoculum concentration interaction effect on AUDPC, final plant height, and mortality. There were significant leaf stage with inoculum concentration interaction effects on AUDPC values ($P = 0.0093$) and percentage of live plants ($P < 0.0001$). There was a significant cultivar by leaf stage interaction effect ($P = 0.0006$) on the final plant height. These results demonstrate that inoculum concentration and leaf stage interact to influence FOB incidence and severity. Based on our results, we recommend (i) screening at an intermediate inoculum concentration of 10^4 and a range of leaf stages; and (ii) culturing stem tissue from asymptomatic plants to detect latent infections.

Keywords: FOB resistance, Fusarium wilt resistance, *Ocimum basilicum*

Sweet basil (*Ocimum basilicum* L.) is a high-value specialty crop grown in fields and greenhouses throughout the world, where it is used as a fresh culinary herb, a source of essential oil for use in fragrances and cosmetics, medicinal purposes, and as an ornamental (Reis et al. 2008; Vieira and Simon 2000). Before the introduction of the basil downy mildew pathogen *Peronospora belbahrii* in the United States and other parts of the world (Wyenandt et al. 2015), Fusarium of basil (FOB) was considered the most economically important disease (Elmer et al. 1994). FOB was first recorded in southern Russia in 1956 (Vergovskii 1956). Since that time, FOB has been reported in many other countries, including the United States (Summerell et al. 2006; Wick and Haviland 1992). In some countries, there has been a resurgence of FOB (Toussaint et al. 2008). FOB infection produces vascular wilt and foot, crown, and root rot symptoms (Gamliel et al. 1996). Initial symptoms resemble water stress (i.e., wilting), and begin at the apex of the plant, progressing to the base (Biris et al. 2004). Other symptoms can include stunting, chlorosis and necrosis of leaves, asymmetric growth, defoliation, dark longitudinal streaking on stems and petioles, twisting of stems, vascular tissue discoloration that is red or brown in color, crown and root rot, and plant death (Biris et al. 2004; Keinath 1994). The introduction and rapid spread of FOB throughout the world and its persistence as an economically damaging pathogen has been attributed to the distribution of FOB-infested seed and infected transplants coupled with intensive monoculture practices in the field and greenhouse and the prohibition of methyl bromide applications (Chaimovitsh et al. 2006; Dudai et al. 2002; Elmer et al. 1994; Reuveni et al. 1997).

Since chemical, biological, and cultural methods are either expensive or not effective, one of the most economically viable strategies of controlling FOB is to develop sweet basils with genetic resistance (Chaimovitsh et al. 2006; Garibaldi et al. 1997; Reis et al. 2004, 2008; Reuveni et al. 1997). The first FOB-resistant sweet basil germplasm was reported in 1995 in Israel, which later led to the first Fusarium resistant cultivar Nufar (Reuveni et al. 1997).

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Funding: Funding for this project in part was provided by the United States Department of Agriculture Specialty Crops Research Initiative project award no. 2018-03382 to Rutgers University (in concert with Cornell University, University of Florida, and University of Massachusetts) "Managing Downy Mildew and Fusarium in Basil with New Resistant Varieties, Improved Genetics, Seed Treatment, and Disease Occurrence Mapping." Partial funding was also provided by the New Use Agriculture and Natural Plant Products Program, the Rutgers/New Jersey Agricultural Experiment Station and Hatch Project NJ12170.

The author(s) declare no conflict of interest.

Studies confirmed that the single dominant gene (locus) that is associated with resistance in Nufar is specific to an isolate of FOB originating from Israel (Chaimovitsh et al. 2006; Dudai et al. 2002; Reis et al. 2008). Unfortunately, commercial growers in the United States have recently observed Nufar to exhibit FOB symptoms, and in our basil breeding research program we also have observed that when Nufar is exposed to a high concentration of FOB inoculum (e.g., 1×10^6 conidia/ml), the cultivar expresses symptoms (J. E. Simon, *unpublished data*).

There are several theories for the observed variability of FOB resistance in Nufar. These include quality control issues in seed production resulting in loss of genetic uniformity, possible development of new races of the pathogen, and breakdown in resistance due to high inoculum density coupled with environmental conditions favorable to the pathogen (Reis et al. 2008). There is no published information about the spectrum or stability of resistance in Nufar. However, the variability of resistance observed in Nufar and other sweet basil cultivars may also be a result of differences in the pathogenicity of FOB isolates (Reis et al. 2008). Other potential factors include the age and condition of the plant material, the inoculation procedure, environmental conditions, disease rating systems, and the concentration of the inoculum. These can result in variable data (Biris et al. 2004; Chaimovitsh et al. 2006; Elmer et al. 1994; Gamliel et al. 1996; Keinath 1994; Moya et al. 2004; Reis et al. 2008; Reuveni et al. 1997; Vannacci et al. 1999). These are all important factors that can influence the results of a pathogenicity test. However, standardization of the methodology for investigating FOB resistance of basil cultivars is necessary for the production of meaningful results.

The objectives of this paper were: (i) to determine the effect of FOB inoculum concentration on the expression of resistance in six accessions of Nufar seed, and (ii) to evaluate the effects of inoculum concentration and plant growth stage on known FOB-susceptible and -resistant basil cultivars and lines. If a specific inoculum concentration and plant growth stage combination are known to effectively and consistently result in FOB disease symptoms, then this combination can be used to standardize an FOB pathogenicity method among *Ocimum* spp. This experiment is the first known attempt to standardize an FOB pathogenicity method to obtain reproducible results for an FOB-resistance sweet basil breeding program.

Greenhouse Nufar Seed Source Experiment

On 30 July 2016, one 72-cell flat of each seed source of Nufar was hand seeded in Redi-Earth Coir Mix Sun Gro Redi-Earth Plug and Seedling Mix Series Growing Medium. Seed sources included Basil-Seeds Seed Company (Montecito, CA; coded NufarF1BSSC, seed source 1), Johnny's Selected Seeds (Winslow, ME; coded Nufar OG, seed source 2), Park Seed (Hodges, SC; coded Nufar hybrid, seed source 3), Richters (Goodwood, ON; coded NufarF1Richters, seed source 4), Stokes Seeds (Buffalo, NY; coded Nufar YR, seed source 5), Seeds of Change (Rancho Dominquez, CA; NufarF1SOC, seed source 6), and Harris Seeds (Rochester, NY; coded Caesar, seed source 7). Two 72-cell flats of Caesar were seeded as an FOB-susceptible control. The flats were then placed in the Rutgers SEBS greenhouse complex and gently watered once per day for 4 weeks. Maintenance fertilizer (385 ppm total N of Jack's Professional 20-20-20 General Purpose Fertilizer; JR Peters, Allentown, PA) was applied bi-weekly. One application of each insecticide including a tank mixture of bifenthrin (Talstar at 1.6 ml/liter) and pyriproxyfen (Distance at 1.6 ml/liter) on 1 September, a tank mixture of methiocarb (Mesurol at 1 ml/liter) and thiamethoxam (Flagship at 0.3 ml/liter) on 13 September, a tank mixture of spirotetramat (Kontos at 0.3 ml/liter) and potassium salts of fatty acids (M-pede at 0.02 kg/liter) on 6 October were applied to manage thrips and whiteflies. The average temperature of the greenhouse was 24°C. On 12 August, flats were thinned to one plant per cell.

On 27 August (28 days after seeding), when plants reached the six-leaf stage of growth, separate zero (control), 1×10^2, 1×10^4, and 1×10^6 conidia/ml suspensions were prepared from a 1-month-old virulent single spore colony of FOB (obtained from UMass-Amherst) growing on half strength PDA. Plants were treated using the cut and dip method as described by Reis et al. (2008). This method was selected based on a preliminary experiment (data not shown) comparing the cut and dip method to a soil injection method where the most consistent results were obtained using the cut and dip method (Keinath 1994; Reis et al. 2008). This consisted of washing the roots clean of media, pruning the roots, inoculating for 1 min, and replanting in flats. Treated plants were inoculated from the lowest inoculum concentration to the highest inoculum concentration level using the same method. Control plants received sterile water only. The inoculated plants were replanted into 24-cell trays containing Redi-Earth Coir Mix Sun Gro Redi-Earth Plug and Seedling Mix Series Growing Medium. The experiment was a randomized complete block design with three blocks, and subsamples of 12 plants per experimental unit. The inoculated plants were maintained in the Rutgers SEBS greenhouse complex. Beginning 1 day after the inoculation, plants were gently watered once daily. Maintenance insecticides were applied as needed. The average temperature of the greenhouse remained at 24°C.

Data collection and statistical analysis

Initial plant heights were recorded following each inoculation treatment on 27 August (day 0). Ratings of each plant began 2 weeks after the initial inoculation and consisted of a standard disease severity rating scale as described by Reis et al. (2004), where 1 = no symptoms; 2 = no wilt symptoms, stem browning; 3 = wilting and stem browning; 4 = severe wilting, foliar necrosis, and chlorosis; and 5 = plant death. Plants were rated for disease severity once per week for 40 days. At the conclusion of the experiment on 6 October, final plant heights were recorded for each plant of each treatment of each block.

Data from this experiment were analyzed by fitting a generalized linear mixed model (using the GLIMMIX procedure of the SAS System ver. 9.4; SAS Institute, Cary, NC) appropriate for a randomized complete block experiment with blocks as a random effect and subsampling. The model was used for testing main effects and interaction effects of seed source and inoculum concentration on AUDPC, final plant height, and mortality (as final proportion of live plants). The zero-inoculum concentration (control) was excluded from the AUDPC analysis, as all disease ratings were zero for this treatment.

Seedling Growth Chamber Experiment

Basil cultivars and lines of known susceptibility to FOB were selected. These included Caesar, a sweet basil cultivar that is FOB susceptible, Nufar, a sweet basil cultivar that is resistant to FOB, and an advanced Rutgers breeding line, RU172, that is also FOB resistant. The disease classification of each cultivar or line was determined based on previous experiments conducted at the Rutgers SEBS greenhouse complex (data not shown). Nufar was selected based on observations and results from the seed source study described above. Nufar and Caesar seed were both obtained from Johnny's Selected Seeds, while the advanced Rutgers breeding line

RU172 was selected as an additional FOB-resistant comparative check.

Before initiating the experiment, a preliminary test was conducted to determine the time needed to achieve proper leaf stages for each cultivar and the experimental line before inoculation. Eighteen days were required to reach the two-leaf stage for each cultivar and experimental line; 31 days were required to reach the four-leaf stage for Caesar and Nufar, while 25 days were required to reach the four-leaf stage for RU172; 38 days was required for Caesar and Nufar to reach the six-leaf stage and 31 days were required for RU172 to reach the six-leaf stage.

The experiment was initiated by hand seeding three 72-cell flats of each basil cultivar or line as described above. All seedlings at the two-, four- and six-leaf stage of growth were inoculated on 7 May 2016 for the first replicated experiment and 15 July 2017 for the second replicated experiment. A 1-month-old virulent single spore colony of FOB (obtained from UMass-Amherst) growing on half-strength PDA was used for the inoculation procedure. For the inoculum preparation in both 2016 and 2017, 20 plates were washed with 10 ml each of deionized water. Separate spore suspensions of 1×10^4, 1×10^5, and 1×10^6 conidia/ml concentration were prepared. Plants were inoculated at zero (control, water only) or at the inoculum concentration levels according to treatment using the cut and dip method according to Reis et al. (2008). The inoculated plants were transplanted into 24-cell trays containing Redi-Earth Coir Mix Sun Gro Redi-Earth Plug and Seedling Mix Series Growing Medium. Control plants were treated the same as treated plants except that each control plant was dipped in sterile deionized water. In each of the two years, the experiment was conducted as a split-split plot experimental design with four blocks and eight plants per block. Consistent environmental parameters were maintained (28°C day temperature, 20°C night temperature, 60% relative humidity, 450 μE/m^2/s radiation, 12-h photoperiod). Beginning 1 day after the inoculation, flats were gently watered once daily. Flowers were removed as necessary.

Data collection and statistical analysis

Initial plant heights were recorded for all plants 1 day after inoculation during both years. Ratings of each treatment began 2 weeks after the initial inoculation using the standard disease severity rating scale as previously described (Reis et al. 2004). Plants were rated for disease severity once per week for 40 days. At the conclusion of the experiment on 16 June 2016 and 26 August 2017, final plant heights were recorded for each plant of each treatment of each block.

At the conclusion of the seedling growth chamber experiment on 16 June 2016, to determine if FOB inoculated asymptomatic plants were infected with FOB, 40 whole plant stems (9 stems of Caesar, 19 of Nufar, and 12 of RU172) were randomly sampled from various treatments and blocks from the 24-cell trays by cutting stems 2 cm above the media line, removing all leaves, and placing them into Ziploc bags. Scissors were cleaned before each plant sample with isopropyl alcohol. Additionally, 117 randomly selected plants (28 Caesar and 94 Nufar) that were rated as symptomatic and two control plants of each cultivar or line not treated with FOB were also randomly selected. Stem samples were transported to the Rutgers Foran Hall biology lab where they were cut into approximately 2 cm sections from the top, middle, and bottom sections of each plant and then surface sterilized in a 10% sodium hypochlorite solution for 5 min. Following this, stem segments of each plant were plated on half strength PDA, labeled, and monitored for presence or absence of FOB for 1 week.

Combined data from 2016 and 2017 of this experiment were analyzed by fitting a generalized linear mixed model (using the GLIMMIX procedure of the SAS System ver. 9.4; SAS Institute, Cary, NC) appropriate for a randomized complete block experiment with blocks as a random effect and subsampling. The split-split plot experiment consisted of inoculum concentration as the whole plot, cultivar as the subplot, and leaf stage as the sub-subplot. The year effect was included in the model as a fixed main effect after testing interactions with year and removing them from the model as they were nonsignificant. The model tested for main effects and interaction effects of inoculum concentration, cultivar, and leaf stage on final plant height, percentage live plants, and AUDPC. RU172 was excluded from the AUDPC and percentage live plant analyses, as all disease ratings were zero for this treatment.

Effect of Inoculum Concentration and Nufar Seed Source on Disease

In the seed source experiment, six commercial sources of the sweet basil cultivar Nufar were tested for their response to a single isolate of FOB.

The FOB-susceptible Caesar had the numerically highest AUDPC values at all inoculum concentrations (Fig. 1). Caesar exhibited significantly higher AUDPC than all Nufar seed sources at 10^2 and 10^4 inoculum concentrations, but at the 10^6 inoculum concentration AUDPC of NufarF1SOC was not different from Caesar (Fig. 1). There was a significant ($P < 0.0001$) interaction of inoculum concentration with cultivar for AUDPC (Table 1). There was an overall increase in disease response as the inoculum concentration was increased; however, the Nufar seed sources differed from one another at the intermediate inoculum concentration of 10^4, but not at the lowest or highest concentrations. Differential susceptibility of the Nufar lines was only revealed clearly at the intermediate concentration of 10^4 (Fig. 1). The mean AUDPCs for NufarF1BSSC and Nufar YR were significantly lower than all other Nufar seed sources at the 10^4 concentration (Fig. 1).

Caesar final plant height was less than all Nufar seed sources inoculated at 10^2, 10^4, and 10^6 concentrations (Fig. 2). There was a significant ($P < 0.0001$) interaction of inoculum concentration with cultivar for the final height values as final plant

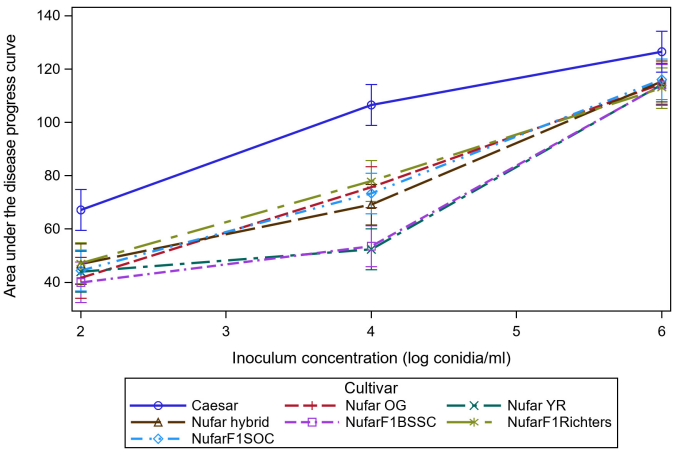

FIGURE 1

Mean AUDPC values for basil cultivars at three inoculum concentrations (10^2, 10^4, 10^6) from six different sources of FOB-resistant Nufar and FOB-susceptible Caesar.

height decreased with increasing inoculum concentrations, but not consistently across cultivars (Table 1; Fig. 2). There were significant differences in final plant height among Nufar seed sources at the 10^2 and 10^4 inoculum concentrations, but at 10^6 there were no significant differences among cultivars (Fig. 2). The 10^2 and 10^4 inoculum concentrations allowed detection of differential susceptibility of Nufar seed sources, in contrast to the 0 and 10^6 concentrations which did not (Fig. 2).

Caesar had the highest mortality at all inoculum concentrations (Fig. 3). Caesar showed an increase in mortality as the inoculum concentration was increased from 10^2 to 10^6. There was a highly significant ($P < 0.0001$) interaction of inoculum concentration with cultivar for mortality (Table 1; Fig. 3). Except for Nufar YR (seed source 5), mortality in Nufar seed sources at the 10^6 concentration was higher compared to plants inoculated at the 10^2 and 10^4 concentrations (Fig. 3).

There was a significant ($P < 0.0001$) interaction of inoculum concentration with cultivar for the AUDPC, final plant height values, and mortality (Table 1). These results indicate that seed source and inoculum concentration influence FOB severity and that host resistance decreases with increasing inoculum concentration. Although seed from different sources responded in a similar manner, AUDPC for NufarF1BSSC and Nufar YR at the 10^4 inoculum concentration was significantly lower than that of other seed sources (Fig. 1). At the 10^2 or 10^6 inoculum concentrations, there was either a very low or very high disease response that did not allow for observations in differences among the various commercial seed sources. This suggests that it is important to test the same cultivar or line from different seed sources at an intermediate inoculum concentration of 10^4 to fully evaluate FOB resistance.

Previous studies have observed that the same basil cultivars tested in several countries differed in the degree of susceptibility (Biris et al. 2004; Reuveni et al. 1997). There are several theories for the differential response or loss of FOB resistance in Nufar in certain geographical regions including quality control issues in seed production, high inoculum levels, and pathogen evolution including the development of new races or virulence factors, such as what has been observed with *Fusarium* in tomato (Ma et al. 2010). Previous research concluded that this may be due either to the presence of different races of FOB in different countries or to the inoculation method used (Biris et al. 2004). Pathogenicity and vegetative compatibility tests confirmed that FOB isolates from countries throughout the world belong to a single vegetative compatibility group (VCG 0200) (Chiocchetti et al. 2001; Elmer et al. 1994; Gamliel et al. 1996; Katan et al. 1991; Kistler et al. 1998; T. Katan, *personal communication*). However, more recently, researchers are beginning to consider that there could be physiological races in the *Ocimum*-FOB pathosystem (Reis et al. 2008). This has been observed in other subspecies of the pathogen and may explain the difference in resistance of Nufar in the United States and Israel (Fravel et al. 2005). There are still many gaps in our understanding of the variability of virulence in FOB, which makes the characterization of virulence of FOB isolates an important factor in the development of resistant breeding lines. The breakdown in FOB resistance observed in Nufar is possible since resistance is characterized as a monogenic dominant trait (Chaimovitsh et al. 2006; Dudai et al. 2002; Reuveni et al. 1997). The length of time that the resistance will remain functional in Nufar is undetermined, as it is still widely used by growers as a source of Fusarium resistance (Reuveni et al. 1997). However, there have been continuing efforts

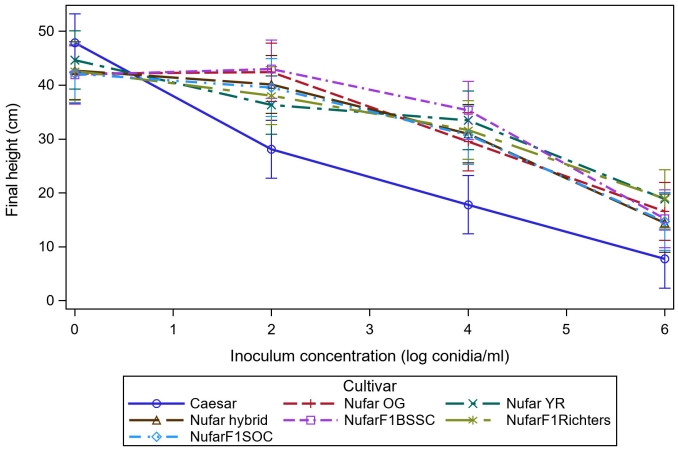

FIGURE 2
Mean final plant height for basil cultivars at four inoculum concentrations (0, 10^2, 10^4, 10^6) from six different sources of FOB-resistant Nufar and FOB-susceptible Caesar.

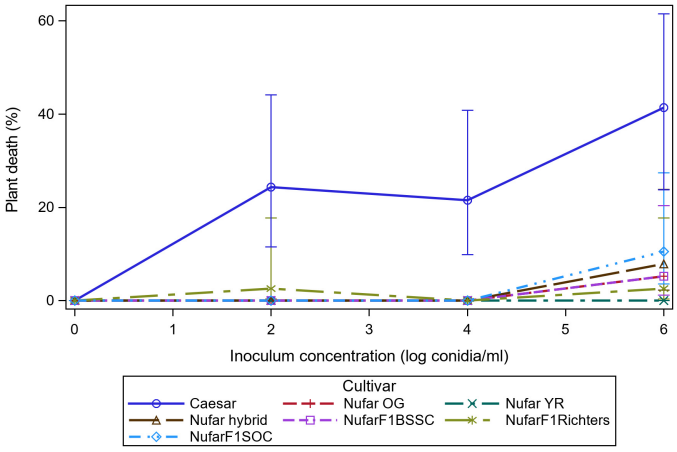

FIGURE 3
Percentage plant death for basil cultivars at four inoculum concentrations (0, 10^2, 10^4, 10^6) from six different sources of FOB-resistant Nufar and FOB-susceptible Caesar.

TABLE 1
ANOVA *P*-values for FOB-susceptible Caesar and FOB-resistant Nufar from six different commercial sources of seed at three different FOB inoculum concentrations using the cut and dip inoculation method

Effect	AUDPC	Final height (cm)	Live plants (%)
Inoculum concentration	<0.0001	<0.0001	<0.0001
Cultivar	<0.0001	<0.0001	<0.0001
Inoculum concentration × cultivar	<0.0001	<0.0001	<0.0001

to search for additional sources of resistance genes for development of new FOB-resistant cultivars (Reis et al. 2008).

In the current study, a single FOB isolate was obtained from UMass-Amherst. Our results confirmed that the breakdown of resistance was due to a high inoculum concentration of 1×10^6 conidia/ml concentration and the highly invasive inoculation method of root cutting, rather than seed source as demonstrated by the AUDPC values. Thus, it is important to screen any new FOB-resistant *Ocimum* breeding lines against the most virulent of FOB isolates at an intermediate inoculum concentration of 10^4 from a multitude of geographical regions to determine if resistance is universally stable.

Effect of Cultivar, Inoculum Concentration, and Leaf Stage on Disease

In the seedling growth chamber experiment, FOB-resistant RU172, FOB-resistant Nufar, and FOB-susceptible Caesar were tested at four different inoculum concentrations (0, 10^4, 10^5, 10^6) and three different leaf stages (two, four, six) for their response to a single isolate of FOB. Means of AUDPC, final plant height, and live plants for the full three-way factorial treatment structure are presented with the ANOVA testing summary to display the magnitude of treatment effects (Table 2).

There were significant ($P < 0.0001$) two-way interactions of inoculum concentration with cultivar, and of inoculum concentration with leaf stage ($P = 0.0093$) for AUDPC, indicating that both factors are important in influencing disease severity (Table 2). The FOB-susceptible Caesar had numerically the highest AUDPC values at all inoculum concentrations (Fig. 4) and leaf stages (Fig. 5). Caesar exhibited higher AUDPC as the inoculum concentration increased from 10^4 to 10^5 (Fig. 4). However, there was no greater disease response when Caesar was inoculated at the 10^5 to 10^6 concentrations, as there was no significant difference ($P = 0.4364$) between the 10^5 and 10^6 inoculum concentrations (Fig. 4). The FOB-resistant cultivar Nufar exhibited FOB symptoms, but at lower AUDPC values compared to Caesar at all inoculum concentrations (Fig. 4) and leaf stages (Fig. 5). In Nufar, there was an increase in the disease response (AUDPC) with increasing inoculum concentration (Fig. 4). There was a significant increase ($P < 0.0001$) in AUDPC between 10^5 and 10^6 inoculum concentrations in Nufar. The FOB-resistant experimental line RU172 had the lowest AUDPC values, irrespective of inoculum concentration or leaf stage, as there was no disease response. Therefore, RU172 was not included in the statistical analysis for AUDPC.

The final height of each plant was measured (in cm) to reflect disease response (stunting). There was a highly significant ($P < 0.0001$) interaction of cultivar with inoculum concentration and a significant ($P = 0.0006$) interaction of cultivar with leaf stage for the final height values (Table 2). However, the interactions of leaf stage with inoculum concentration and cultivar with leaf stage with inoculum concentration were not significant, demonstrating

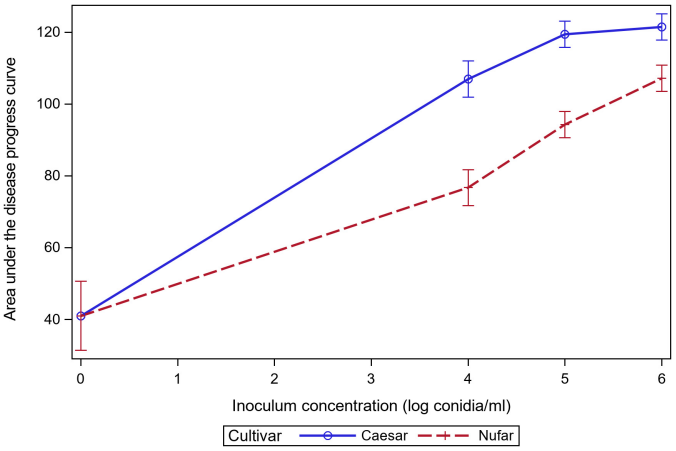

FIGURE 4
Mean AUDPC values for basil cultivars at four inoculum concentrations (0, 10^4, 10^5, 10^6) from FOB-resistant Nufar and FOB-susceptible Caesar.

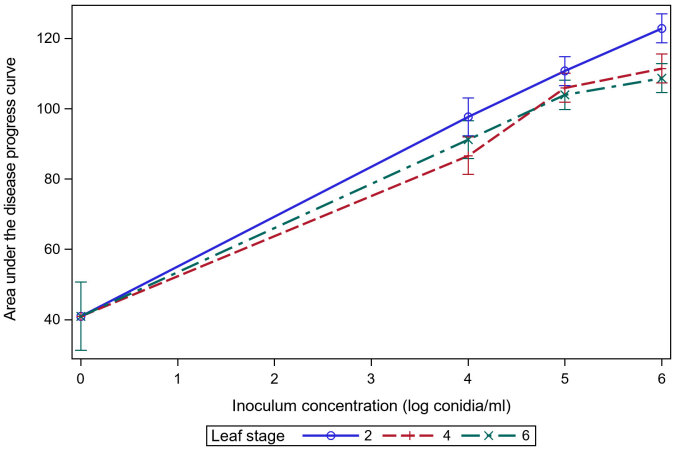

FIGURE 5
Mean AUDPC values for basil cultivars at four inoculum concentrations (0, 10^4, 10^5, 10^6) and three leaf stages (two, four, six) from FOB-resistant Nufar and FOB-susceptible Caesar.

TABLE 2
ANOVA *P*-values for FOB-susceptible Caesar and FOB-resistant Nufar in a seedling growth chamber experiment using a cut and dip inoculation method

Effect	AUDPC	Final height (cm)	Live plants (%)
Year	<0.0001	0.1955	0.0002
Inoculum concentration	<0.0001	<0.0001	<0.0001
Cultivar	<0.0001	<0.0001	<0.0001
Cultivar × inoculum concentration	<0.0001	<0.0001	<0.0001
Leaf stage	<0.0001	0.954	<0.0001
Leaf stage × inoculum concentration	0.0093	0.4166	<0.0001
Cultivar × leaf stage	0.1088	0.0006	0.1390
Leaf stage × inoculum concentration × cultivar	0.3413	0.5052	0.8023

the difference in FOB susceptibility between Caesar, Nufar, and RU172 (Table 2). Final plant height decreased in Caesar and Nufar at all leaf stages (Figure 6) and inoculum concentrations (Fig. 7). However, Caesar plants did not show greater stunting when inoculated at the 10^5 and 10^6 concentrations (Fig. 7). There was a more gradual increase in stunting in Caesar plants at the four- and six-leaf stages (Fig. 6). In Nufar, there was also an increase in stunting with increasing inoculum concentration (Fig. 7). RU172 had the lowest stunting, irrespective of inoculum concentration or leaf stage. However, at all leaf stages (Fig. 6) and inoculum concentrations (Fig. 7), there was a slight reduction in plant height.

No mortality was observed in any treatment of RU172; therefore, it was excluded from the statistical analysis. The three-way interaction effect of cultivar by leaf stage by inoculum concentration was not significant, nor was the two-way interaction effect of cultivar by leaf stage (Table 2). However, mortality of the two cultivars responded differently to inoculum concentrations (interaction P-value < 0.0001). Caesar had the lower percentage of live plants at all inoculum concentrations (Fig. 8). Mortality of Caesar plants at the 10^4 inoculum concentration was much greater than Nufar plants, suggesting a much greater susceptibility. Nufar mortality increased as inoculum concentration increased from 10^5 to 10^6, whereas Caesar mortality had reached its nadir by 10^5 (Fig. 8). Averaged across cultivars, the inoculum concentration effect was different at the different leaf stages (interaction P-value < 0.0001). Inoculation at the two-leaf stage caused greater mortality than inoculation at the four- or six-leaf stages at all concentrations (Fig. 9). Mortality of plants inoculated at the two-leaf stage increased with increasing inoculum concentrations, but inoculation at the four- and six-leaf stages had similar mortality at 10^4, 10^5, and 10^6 concentrations (Fig. 9).

In the seedling growth chamber experiment, the susceptibility of the cultivar or line to FOB depended on the inoculum concentration and the leaf stage. This was evident in Nufar where the percentage of live plants decreased as inoculum concentration increased, with the highest mortality at the two-leaf stage and the 10^6 concentration.

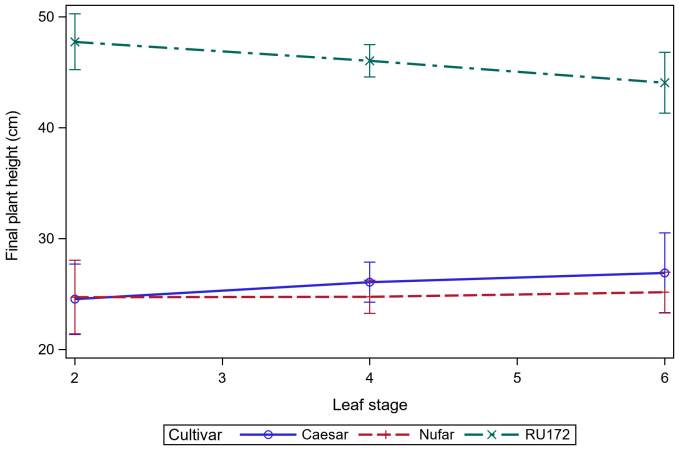

FIGURE 6
Mean final plant height for basil cultivars at three different leaf stages (two, four, six) from FOB-resistant RU172, FOB-resistant Nufar, and FOB-susceptible Caesar.

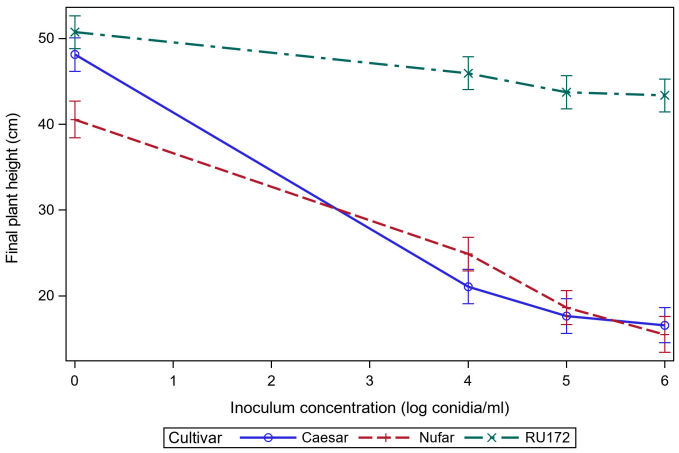

FIGURE 7
Mean final plant height for basil cultivars at four inoculum concentrations (0, 10^4, 10^5, 10^6) from FOB-resistant RU172, FOB-resistant Nufar, and FOB-susceptible Caesar.

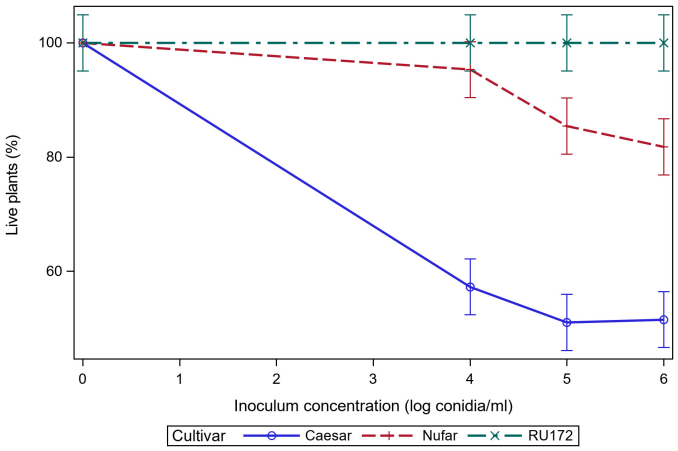

FIGURE 8
Percent live plants for basil cultivars at four inoculum concentrations (0, 10^4, 10^5, 10^6) from FOB-resistant RU172, FOB-resistant Nufar, and FOB-susceptible Caesar.

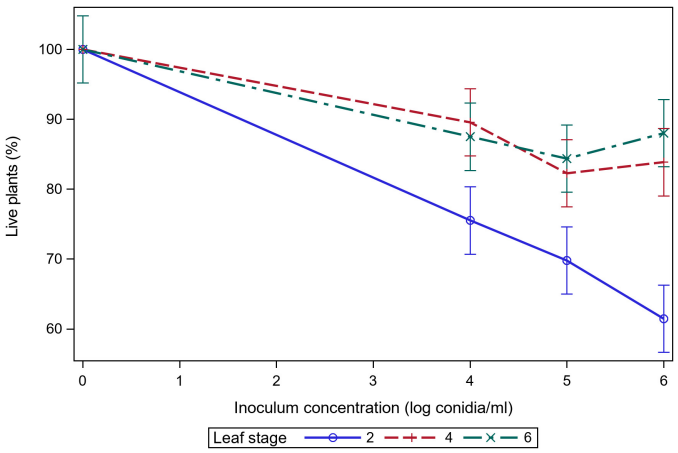

FIGURE 9
Percent live plants for basil cultivars at four concentrations (0, 10^4, 10^5, 10^6), and three leaf stages (two, four, six) averaged across cultivars (FOB-resistant Nufar and FOB-susceptible Caesar).

Similar results for how plant age affects resistance to Fusarium wilt were found in celery (Hart and Endo 1981).

At lower inoculation levels, Caesar had higher AUDPC and mortality and was more stunted than Nufar; however, at higher inoculation levels, mortality and stunting were not statistically different between cultivars regardless of leaf stage. This suggests that in FOB-susceptible cultivars, lower levels of inoculum can cause infections regardless of plant age compared to cultivars that have some level of FOB resistance.

At the conclusion of the seedling growth chamber experiment, there were a total of 204 plants (56 plants from the first block, 46 plants from the second block, 48 plants from the third block, and 54 plants from the fourth block) that remained asymptomatic after being inoculated with FOB. After surface sterilization, plating the top, middle, and bottom stem segments of control and FOB-treated plants from the seedling growth chamber experiment resulted in interesting observations. FOB was not recovered from any of the non-inoculated control plants, but it was recovered from most stems of each cultivar and line, regardless of leaf stage and inoculum concentration, that were rated as healthy throughout the experiment. Importantly, FOB was recovered from most stems of RU172 plants treated at the two-, four-, and six-leaf stages at the 1×10^6 conidia/ml concentration. These plants were infected by FOB but remained asymptomatic during the course of the experiment.

Our results determined that even when plants appear healthy, such as in the FOB-resistant advanced Rutgers breeding line RU172 following FOB inoculation, the plants may be infected but remain asymptomatic. RU172 had the lowest AUDPC values and no plant mortality, irrespective of inoculum concentration or leaf stage. However, at all leaf stages and inoculum concentrations, there was a slight reduction in plant height in RU172 suggesting infection. Importantly, FOB was recovered from most stems of each cultivar and line, regardless of leaf stage and inoculum concentration, that were rated as healthy (asymptomatic) throughout the experiment. A study at UMass-Amherst discovered similar results when testing sweet basil with resistance to FOB. Plants appeared to remain healthy after the inoculation, but Fusarium was cultured from the crowns (R. Wick, *personal communication*). This led to the suggestion that in FOB-resistant cultivars, Fusarium may have the ability to move from the roots, through the cortex, and up into the crown and stem, but it may be unable to enter the vascular system and cause symptoms (R. Wick, *personal communication*). Thus, experiments to determine FOB resistance using the above method should include reisolations to determine if the pathogen can be recovered from asymptomatic plants in FOB breeding programs and studies. Our finding also confirms that asymptomatic plants can harbor the pathogen, which could have important impacts in field production where resistant varieties are grown alongside susceptible ones (Gamliel et al. 1996; Keinath 1994; Trueman and Wick 1995; Wick and Haviland 1992).

An important aspect of verifying FOB resistance in existing commercial sweet basil cultivars and testing new FOB-resistant lines is to standardize the pathogenicity test for FOB resistance determination. In this study, the cut and dip method was used to inoculate plants because it had been shown to produce reliable, uniform, and reproducible results (Reis et al. 2008). This consisted of washing the roots clean of media, cutting the roots, inoculating for 1 min, and replanting in flats. As a soilborne pathogen, FOB primarily infects through injured roots (Kvartskhava 1957). Wounding the roots enhances FOB invasion and colonization of the xylem tissue compared to performing an inoculation in naturally infested soil (Rekah et al. 2000; Reuveni et al. 1997). Although this method is extremely time-consuming and invasive, we found it to be consistent and reliable.

Conclusions and Recommendations

The results of these experiments highlight the importance of developing an experimental design that includes: (i) screening at an intermediate inoculum concentration of 10^4 and a range of leaf stages; and (ii) culturing stem tissue from asymptomatic plants to detect latent infections. Our results also suggest that it would be valuable to screen new *Ocimum* breeding lines against the most virulent of FOB isolates from a multitude of geographical regions to determine if FOB resistance is universally stable.

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