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Modeling the Effects of Environmental Factors on the Population of *Fusarium oxysporum* in Cucumber Continuously Cropped Soil

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The influences of soil environmental parameters (water potential [W_p], pH, and temperature) on the indigenous population of Fusarium oxysporum *(FO) over 5 years of continuously cropped cucumber (CCC) soil were studied. The use of response surface methodology to model the combined effects of environmental parameters on the soil log_{10} FO internal transcribed spacer copies (ITSC) showed that high W_p of the soil, above –20 kPa, caused increased ITSC in the soil that was independent of the temperature and pH.* Fusarium oxysporum *was able to grow at all temperatures and pH values tested, and the greatest growth rate was observed at 23–24 °C and pH 5.3–5.4. The W_p is a crucial environmental factor that affects the growth of soil FO. This study should contribute to estimating the impact of environmental factors on the soil FO proliferation rate in CCC soil.*

Keywords Fungal population, *Fusarium* wilt, real-time PCR, response surface methodology, water potential

Introduction

Cucumber (*Cucumis sativus*) is an important economic crop worldwide. *Fusarium* wilt of cucumber, caused by *Fusarium oxysporum* f. sp. cucumerinum (FOC), is one of the most serious diseases in long-term monoculture cropping. *Fusarium oxysporum* F. sp. cucumerinum infects plant roots by invading the vascular system and secreting mycotoxin, which causes cucumber stem necrotic lesions and induces cells apoptosis, followed by foliar wilting and then death within a few days or weeks (Booth 1971; Domínguez, Negrín, and Rodríguez 1996; Chen et al. 2011a, 2012a, 2012b). When cucumber plants were grown for three continuous seasons as a monoculture in the same field, the disease incidence of *Fusarium* wilt was as high as 70% and caused severe yield reduction or even complete crop failure (Chen et al. 2011a; Booth 1971; Yang et al. 2011).

It has been reported that various *Fusarium oxysporum* (FO) pathotypes can survive successfully in the soil by means of thick-walled chlamydospores that are either free or embedded in infected plant debris (Booth 1971; Vakalounakis and Chalkias 2004). Furthermore, the populations of different FO pathotypes were shown to increase as the number of crop-planting years increased (Navas-Cortés et al. 2007; Chen et al. 2011a; Yang

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et al. 2011). In general, little or no disease occurred in the soil below certain threshold population sizes of FO; typically, density that reached an average of 4.08 $\log_{10}$ CFUs (colony forming units) g^{-1} soil for *F. oxysporum* f. sp. radicis-lycopersici could cause the exhibition of disease symptoms (Yael, Shtienberg, and Katan 2001). Furthermore, 50% disease incidence was caused by 171 CFUs g^{-1} soil of *F. oxysporum* f. sp. niveum (Boughalleb and Mahjoub 2006), and propagation at 8×10^4 CFUs ml^{-1} potting mix for *F. oxysporum* f. sp. cucumerinum could cause 100% infection of cucumber (Yang et al. 2011). An increase of inoculum density or growth rate of FO accelerated the development of *Fusarium* wilt, whereas low inoculum densities or growth rate delayed the expression of wilt symptoms (Yael, Shtienberg, and Katan 2001). Similarly, in naturally FO-infected soil, the *Fusarium* wilt severity increased with an increasing soil FO proliferation rate (Boughalleb and Mahjoub 2006; Yael, Shtienberg, and Katan 2001; Yang et al. 2011).

Scientists have reported that soil environmental factors significantly affect the growth of FO (Bhatti and Kraft 1992; Fine et al. 2007; Means and Kremer 2007; Navas-Cortés et al. 2007; Palmero et al. 2008). *Fusarium oxysporum* populations and *Fusarium* wilt were increased as the soil matric potential increased from –20 kPa to –1060 kPa (Bhatti and Kraft 1992); furthermore, the favorable soil temperature for *F. oxysporum* f. sp. ciceris to infect chickpea was 22–26 °C, whereas a soil temperature of 10 °C showed no disease development (Navas-Cortés et al. 2007). In addition, an increase in soil pH, by amending with lime, was effective to reduce the soil FO population and disease incidence (Fine et al. 2007). Thus, in continuously cropped cucumber (CCC) soil, the elucidation of the soil environmental factors that govern fungus proliferation which maintains a high indigenous FO population in the soil for years of cucumber monoculture (Chen et al. 2011a, 2012b; Yang et al. 2011), is required for the control of *Fusarium* wilt of cucumber by preventing the pathogen from proliferating to the threshold value that causes the emergence of disease.

The aim of the present work was to elaborate the response surface methodology (RSM) models describing the combined effects of temperature, pH, and W_p on the proliferation of the indigenous FO in CCC soil. The traditional "three-factor, three-level" technique used for optimizing a multivariable system with all potential combinations not only requires many experiments but may also result in the wrong conclusions. Under these circumstances, RSM is an attractive alternative that can be used to study the effect of several factors that influence the dependent responses by varying the factors simultaneously and carrying out a limited number of experiments (Powell, Lyons, and Haby 1995; Chen et al. 2011b). This work would help to build a more rational control strategy, possibly involving drainage, temperature, and soil pH management.

Materials and Methods

Source of FO Isolates

One hundred eighty-five FO-like fungal strains were isolated from 5 years of CCC soil (Jinhua City, ZheJiang Province, China) by a series dilution technique on FO-selective medium (Komada 1975). The properties of the soil were as follows: FO 2.69 $\log_{10}$ ITS copies g^{-1} dry soil (ITSC), 40.28 mg g^{-1} soil of organic-matter content, 2.66 mg g^{-1} soil of total nitrogen (N), 2.09 mg g^{-1} soil of total phosphorus (P), soil pH 5.3, 660 mg g^{-1} of soil clay size >0.01 mm and 340 mg g^{-1} of soil clay size <0.01 mm. The isolated FO strains were maintained on potato dextrose agar (PDA; Yang et al. 2011) and stored at 4 °C for further use.

Identification and the Pathogenicity and Virulence of FO Strains on Cucumber Plants

The morphological identifications of the FO strains were performed according to the methods of Booth (1971).

For the molecular identification, FO was grown in liquid potato dextrose broth (Yang et al. 2011) at 28 °C at 170 rpm for 72 h. The mycelium was filtered with sterile Whatman No. 4 filter paper, frozen at –70 °C, and then ground in a mortar. Genomic DNA was extracted with the Fungal DNA Mini Kit (Omega Bio-Tek, Inc., Col.) according to the manufacturer's instructions, using approximately 100 mg fresh mycelium. A region of DNA, containing the internal transcribed spacers (ITS) ITS1, 5.8S and ITS2 regions, was amplified by using PCR with the primer combinations of ITS1 and ITS4 (Yang et al. 2011). The PCR was performed in a 50-μL reaction volume containing 5 μL 10× PCR buffer, 0.2 mM dNTPs, 0.35 μM of each primer, 1.0 U of Taq polymerase (Dingguo Corp., Beijing, China), and 2 μL of genomic DNA. PCR amplification was performed in a Mastercycler epgradient thermal cycler (Eppendorf, Germany). The amplification program consisted of the following: 5 min initial denaturation (94 °C), 35 cycles of amplification (1.5 min at 94 °C, annealing at 55 °C for 2 min, polymerization at 72 °C for 3 min), and a final extension period of 5 min at 72 °C. The phylogenetic analysis was performed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA) (Yang et al. 2011).

All FO strains were cultured on PDA medium and grown for 8 days at 28 °C. Conidia suspension of each FO strain was prepared by flooding the plates of the 8-day-old cultures with sterilized and distilled water, scraping with a sterile glass rod, filtering through a double layer of sterile cheesecloth, and centrifuging in a sterile tube at 12,000*g* for 10 min at 4 °C. The liquid was poured out, and the conidia at the bottom of the tube were washed by vortexing with sterile water. The CFU mL^{-1} counts of FO conidia were determined by the series dilution technique of Komada (1975) on PDA. The concentration of the conidia suspension was 4.0×10^4 CFUs ml^{-1}.

The FOC-susceptible cultivars of cucurbitaceous plants, *Citrullus lanatus* cv. Di Lei Wang (Qin City Shenhua Seed Technology Co. Ltd., Heibei, China), *Luffa cylindrica* cv. Jiang Shu No.1 (Jiangshu Seed Technology Co. Ltd., Jiangsu, China), *Lagenaria siceraria* cv. Caifulu (Beijing Jindi agriculture technology Co. Ltd., Beijing, China), *C. melo* cv. Japanese Sweet (Jinyangguang Seed Co. Ltd., Henan, China), *C. sativus* cv. Xin Xia Feng (Ningyang Agriculture Institute, Shandong, China), and an FOC-resistant cultivar of cucumber, *C. sativus* cv. Jin Chun No. 4 (Tianjing Cucumber Research institute, Tianjin, China), were used in the experiments. All seeds were surface sterilized with 1% sodium hypochlorite for 5 min and rinsed three times in sterile, distilled water. Three germinating seeds were planted in 300 g of FO-inoculated rice field soil, which was first sterilized at 121 °C for 1 h three times with an autoclave and then inoculated with FO *conidia* suspension to maintain the FO population at 10^4 CFUs g^{-1} soil, in a plastic pot (9 cm in diameter and 11 cm in height). The properties of the soil were 28.1 mg g^{-1} of organic matter, 3.0 mg g^{-1} of total N, 1.8 mg g^{-1} of total P, 12.5 mg g^{-1} of total K, and pH 7.5. Nine replicates were set for each cultivar. The seedlings were grown in a greenhouse at 20–35 °C (day) and 18–26 °C (night), with a photoperiod of 12 h and daily watering by an overhead watering system.

The development of *Fusarium* wilt on the seedlings was assessed after 30 days. Wilt development in each plant was rated by the scale of Chen et al. (2012b), as follows: 0 (the whole plant was healthy); 1 (<10% of the leaves wilted); 2 (11–20% of the leaves wilted); 3 (21–50% of the leaves wilted); 4 (50–100% of the leaves wilted); and 5 (the whole plant

died). The disease index was transformed to the percentage disease index (PDI) for the analysis of variance. The PDIs were calculated by the following formula:

$$\text{Percentage Disease Index} = \frac{\sum(\text{Rating} \times \text{Number of plants rated})}{\text{Total number of plants} \times \text{Highest rating}} \times 100\%$$

Quantification of FO in CCC Soil by Real-Time PCR

Total DNA from 1 g CCC soil samples was extracted according to the protocol provided with the EZNA soil DNA kit (Omega Bio-Tek, Inc., Colorado, USA) and dissolved in 10 μL of ultrapure water. The primer pair Fn1/Fn2, designed by Zhang et al. (2005), was used for the quantification of FO in this experiment, and it was shown to amplify the ITS region of all six FO strains isolated from the CCC soil (Figure 1). The reaction mixture (50 μL) for the real-time analysis contained 4 μL of template DNA, 25 μL of SYBR Premix EX Taq (2×, Takara), 1.0 μL of each primer (10 μM), 1.0 μL of ROX reference dye (50×), and 18 μL of sterile, distilled water. The PCR was run in an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Foster City, Calif.) using the following standard program: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 45 s, and a final melting curve from 40 to 90 °C. The standards were prepared by 10-fold dilution of ZJ-02 ITS DNA, ranging from 1.1×10^6 to 1.1×10^1 target copies.

Experimental Design and Model Development

For all experimental designs, 5-year CCC soil (composite of the 10-cm depth profile) amended with 0.15 g urea kg^{-1} soil and 0.15 g monopotassium phosphate (KH_2PO_4)

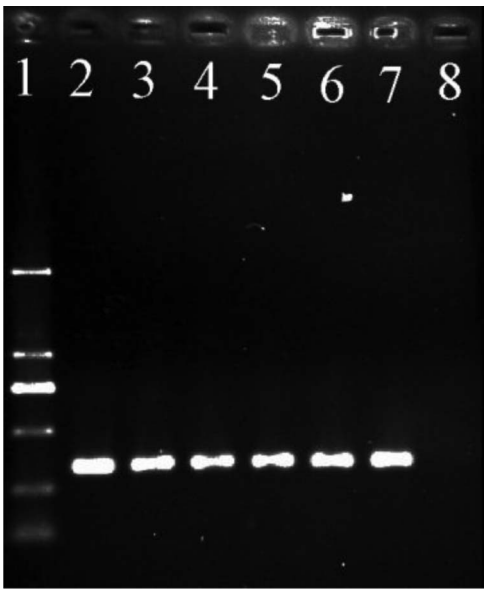


Figure 1. FO-specific primers set used to amplify genomic DNA of 6 FO strains isolated from CCC soil. Lane 1, low-mass marker (Takara, with DNA ladders of 2000, 1000, 750, 500, 250 and 100 bp); lane 2, ZJ-01; lane 3, ZJ-02; lane 4, ZJ-03; lane 5, ZJ-04; lane 6, ZJ-05; lane 7, ZJ-06; and lane 8, negative control.

kg^{-1} soil, was homogenized and sieved through a screen (2 mm). A stable soil pH was achieved by continuous regulation with the addition of sulfuric acid (H_2SO_4; 1 mol L^{-1}) or sodium hydroxide (NaOH; 1 mol L^{-1}). The soil W_p was measured with a soil SM-4 model water potential meter (HUIER, Hangzhou, China) and was regulated by adding sterile, deionized water. One hundred g of soil, screened with a 2-mm sieve, was placed in 500-mL Erlenmeyer flasks, and the flasks were sealed (Parafilm; Pechiney Plastic Packaging, Chicago, Ill.) to prevent water loss and then incubated for 30 days under different environmental conditions.

The FO population in the CCC soil as ITSC was investigated by an analysis of variance. Statistical significance was judged at the level of $P < 0.05$. Whenever the analysis revealed significant differences, Duncan′s multiple-range tests for the separation of means was performed. RSM with a 3^k factorial design was used, including three levels of temperature (15, 25, and 35 °C), soil W_p (–20, –13, and –6 kPa), and pH (4.0, 5.5, and 7.0). Data modeling was done by multiple regression analysis. The design contained 20 experiments with three replicates and was conducted twice. A second-order polynomial model was defined to fit the response:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i \chi_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=i+1} \beta_{ij} \chi_i \chi_j + \varepsilon$$

where Y is the predicted response (ITSC), β_0 coefficient is the off-set term called intercept, X_i are independent variables related to the factors, β_i are linear coefficients, β_{ii} are second-order interactions coefficients, β_{ij} are quadratic coefficients, and ε is the error of the model. The values of the coefficients were estimated by the least-squares method. The interactions between factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient). The experiments were designed using the software Design Expert Version 7.0.0 version (Stat Ease, Minneapolis, Minn.).

Statistical Analysis

The means and standard deviation of height, dry weight, and root fresh weight of the cucumber plants were calculated and statistically analyzed using analysis of variance (ANOVA) and Duncan′s multiple-range tests if a significant difference was detected ($P < 0.05$). SPSS, version 13.0, was used for statistical analysis (SPSS Inc., Chicago, Ill.).

Results

Pathogenicity, Virulence, and Identification of FOs Isolated from the CCC Soil

A PCR product (530 bp) was amplified from the genomic DNA by using the primer pair of ITS1 and ITS4. Among the 185 strains isolated, six strains were selected based upon sequence similarities with the DNA fragment (ITS1 + 5.8S + ITS2) and morphological characteristics similar to the type species of FO (Booth 1971) for the phylogenetic analysis. The isolated six strains were coded as ZJ-01, ZJ-02, ZJ-03, ZJ-04, ZJ-05, and ZJ-06 with the percentages of the total 185 FO strains being 13.5, 74.6, 4.9, 2.7, 2.7, and 1.6%, respectively. The accession numbers of the DNA sequences registered in the National Center for Biotechnology Information (NCBI) were HM179529 for ZJ-01, HM179530 for ZJ-02, HM179531 for ZJ-03, HM179532 for ZJ-04, HM179533 for ZJ-05, and HM179534 for

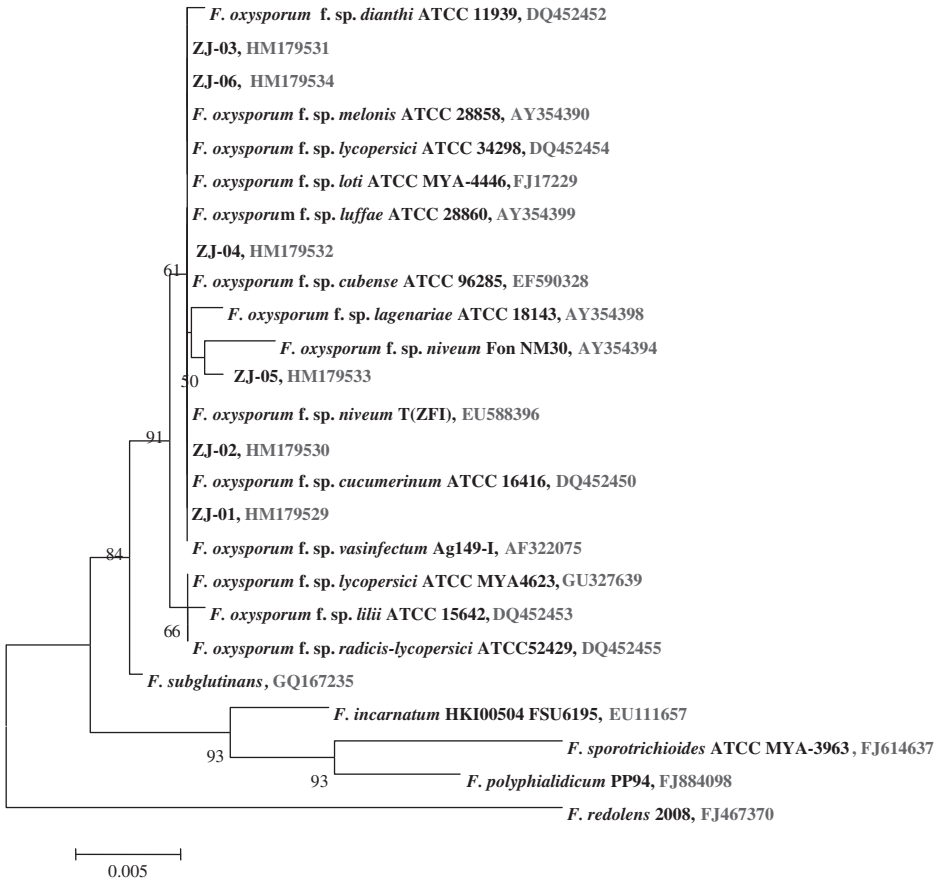


Figure 2. Phylogenetic tree for the partial sequences of cloned ITS regions, and the most closely related fungi. The clones are indicated by their code and accession number (NCBI), respectively.

ZJ-06. The phylogenetic analysis demonstrated that the six strains constituted different FO formae speciales (Figure 2).

The selected strains were inoculated in the soil with the cucumber plants, and *Fusarium* wilt was then documented on the plants. Initially, in the ZJ-01- and ZJ-02-inoculated treatments, the leaf wilt was found in the midday and then a brownish discoloration and water-soaked appearance was observed on the base of the stem. After 3–5 days, the wilted leaves could not recover and the brownish discoloration turned to black, which indicated rotting of the stem and root tissues. Strain ZJ-03 did not cause death but midday wilt of cucumber plants during the experiment. Strains ZJ-01 and ZJ-02 were successfully recovered from the root and stem of infected plants, which indicated that these two FO strains were able to cause root and stem rot of the cucumber plants.

For the infection of the cucumber root, the ZJ-02 strain showed the greatest infection rate, with a PDI of 95.8% in Xin Xia Feng and 81.5% in Jin Chun No. 4. We did not find *Fusarium* wilt by ZJ-01 in Jin Chun No. 4; however, a 28.5% and 10.1% PDI appeared in Xin Xia Feng and *C. melo* cv. Japanese Sweet, respectively. ZJ-03 caused an 8.1% PDI of *Fusarium* wilt in Xin Xia Feng. Of the total 185 FO strains, 93.0% could infect Xin Xia

Feng, and 74.6% could infect Jin Chun No. 4. Only three pathogenic strains, ZJ-01, ZJ-02, and ZJ-03, of the six FOs caused a significant ($P < 0.05$) decrease in height, dry weight, and root fresh weight in Jin Chun No. 4 (Table 1). However, all of the FO strains showed significant ($P < 0.05$) negative effects on Xin Xia Feng. Throughout the experiments, none of the six FOs caused *Fusarium* wilt in other susceptible cultivars of cucurbitaceous plants, such as *C. lanatus* cv. Di Lei Wang, *L. cylindrica* cv. Jiang Shu and *L. siceraria* cv. Cai Fu Lu, used in the experiment.

Based on the results of the phylogenetic analysis and pathogenic specificity of the six strains for cucurbitaceous plants, the strains ZJ-02 and ZJ-03 were identified as *F. oxysporum* f. sp. cucumerinum with a different virulence to cucumber.

Modeling the Growth Rate of FO Strains in the CCC Soil

A real-time PCR method was developed to quantify the total soil FO population. A standard curve was developed by the known concentration of pure ZJ-02 DNA, plotting the cycle thresholds against the logarithm of the initial number of target copies. The cycle threshold values detected from the soil samples were plotted onto this curve, and the inferred concentration of FO was calculated.

Response of ITSC to the environmental variables was quantified using polynomial RSM. Table 2 lists the mean ITSC obtained with the model for the experimental test conditions, along with the corresponding predicted experimental values. Based on the analysis of variance, the effects of W_p, T, and pH and the effect of their interactions on the ITSC were significant (Table 3). The results of the multiple regression analysis provided the estimated regression coefficients of the model. The value of the coefficient of determination R^2 was 0.9494, and the adjusted R^2 was 0.9039. Using the Design Expert Software, a second-order polynomial following for the soil ITSC ($\log_{10}$ ITSC g^{-1} dry soil) was obtained from regression analysis of the results from the central composite design experiments.

$$\text{ITSC } (\log_{10}\text{ITSC g}^{-1}\text{dry soil}) = -1.3685 + 1.2991\chi_1 + 0.0803\chi_2 - 0.0241\chi_3$$
$$+ 0.0023\chi_1\chi_2 + 0.0012\chi_1\chi_3 + 0.0000\chi_2\chi_3 - 0.1264\chi_1^2 - 0.0017\chi_2^2 - 0.0012\chi_3^2$$

The negative value of the regression coefficients suggested an antagonistic effect on the soil ITSC. It appeared that as the absolute value of the linear coefficient (β_i) increased so did the influence of the corresponding factor on the soil ITSC. The pH had more influence than temperature or W_p on the soil ITSC. The linear effects of pH on the soil ITSC appeared to be positive. In contrast, the W_p and T had negative linear effects.

Modeling showed that the ITSC differed significantly between the soil W_p, temperature, and pH (Figure 3). The ITSCs were plotted as three-dimensional graphs showing the mean values of three replicates of this experiment as affected by interactions of two of the environmental factors [Figures 3(a)–3(c)]. The FO had its high ITSC, of 3.30, across a fairly broad range of temperatures (25.3–29.6 °C) and W_p (–10.7 to –6.0) but a narrow range of pH (5.1–5.6). The use of the lack of fit test enabled us to evaluate the quality of the model and whether the model provided a very good fit with the actual soil ITSC. The model showed that the lack of fit was not significant statistically for the soil ITSC. Therefore, the model was found to be adequate for the prediction of conditions under which the soil FO maintained the greatest ITSC value of 3.31 when the W_p was –8.12 kPa, the temperature was 27.43 °C, and the pH was 5.35.

Table 1
Effects of inoculation of the six FO strains on the height, dry weight, and fresh root weight of the cucumber with two different cultivars, the susceptible cultivar Xin Xia Feng and the resistant cultivar Jin Chun No. 4

	Height (cm)		Dry weight (g)		Root fresh weight (g)	
	Xin Xia Feng	Jin Chun	Xin Xia Feng	Jin Chun	Xin Xia Feng	Jin Chun
CK	26.87 ± 1.64a	26.7 ± 1.54ab	0.96 ± 0.02a	1.00 ± 0.04a	1.22 ± 0.04a	1.23 ± 0.05a
ZJ-01	17.5 ± 2.86d	24.67 ± 2.81bc	0.74 ± 0.08cd	0.87 ± 0.03bc	0.72 ± 0.08cd	0.94 ± 0.05cd
ZJ-02	16.63 ± 0.85d	22.33 ± 0.74c	0.53 ± 0.06e	0.82 ± 0.06c	0.62 ± 0.09d	0.84 ± 0.07d
ZJ-03	21.07 ± 1.82c	27.17 ± 1.42ab	0.77 ± 0.08d	0.90 ± 0.05bc	0.74 ± 0.09c	1.05 ± 0.09bc
ZJ-04	22.1b ± 2.04c	28.00 ± 0.46a	0.83 ± 0.04bc	0.94 ± 0.04ab	1.03 ± 0.02b	1.10 ± 0.10ab
ZJ-05	23.07 ± 2.30bc	27.17 ± 1.62ab	0.84 ± 0.04bc	0.92 ± 0.04ab	1.03 ± 0.02b	1.15 ± 0.06ab
ZJ-06	24.93 ± 1.88ab	26.57 ± 1.25ab	0.85 ± 0.04b	0.93 ± 0.05ab	1.02 ± 0.03b	1.12 ± 0.07ab

Note. Data with the same letters are not significantly different by Duncan's multiple-range test ($P < 0.05$).

Table 2
Factorial design arrangement and responses

	Environmental factors						Log_{10} ITSC g^{-1} dry soil	
	Code values			Actual values				
	X_1	X_2	X_3	χ_1	χ_2	χ_3	Observed	Predicted
1	−1	−1	−1	4.0	15.0	−20.0	2.78	2.70
2	1	−1	−1	7.0	15.0	−20.0	2.51	2.46
3	−1	1	−1	4.0	35.0	−20.0	2.85	2.81
4	1	1	−1	7.0	35.0	−20.0	2.83	2.71
5	−1	−1	1	4.0	15.0	−6.0	2.82	2.85
6	1	−1	1	7.0	15.0	−6.0	2.71	2.65
7	−1	1	1	4.0	35.0	−6.0	2.99	2.95
8	1	1	1	7.0	35.0	−6.0	2.91	2.89
9	−1.68	0	0	2.98	25.0	−13.0	2.55	2.58
10	1.68	0	0	8.02	25.0	−13.0	2.24	2.34
11	0	−1.68	0	5.5	8.18	−13.0	2.59	2.64
12	0	1.68	0	5.5	41.82	−13.0	2.85	2.93
13	0	0	−1.68	5.5	25.0	−24.77	2.85	2.97
14	0	0	1.68	5.5	25.0	−1.23	3.23	3.24
15	0	0	0	5.5	25.0	−13.0	3.31	3.26
16	0	0	0	5.5	25.0	−13.0	3.25	3.26
17	0	0	0	5.5	25.0	−13.0	3.31	3.26
18	0	0	0	5.5	25.0	−13.0	3.26	3.26
19	0	0	0	5.5	25.0	−13.0	3.13	3.26
20	0	0	0	5.5	25.0	−13.0	3.35	3.26

Discussion

The objective of this work was to determine the influence of the principal environmental factors on the population of indigenous soil FO, most of which caused severe *Fusarium* wilt in the CCC soil. The inclusion of in vivo experiments was intended to provide a baseline evaluation of the behavior of FO under practical agricultural conditions, although these are certainly not found under field conditions. We focused on the population of FO as a possible indicator related to the degree of *Fusarium* wilt disease.

Few data are available in the literature on the virulence and pathogenicity to cucumber of the different FO strains in the CCC soil. Our results showed that in the 5-year CCC soil with a more than 70% disease index of *Fusarium* wilt (Chen et al. 2011b), as much as 74.6–93.0% of the isolated strains of FO were pathogenic to *C. sativus* cultivars with different resistances; this indicated that most of the FOs in the CCC soil with high *Fusarium* wilt disease index were pathogenic rather than competitors (e.g., of nutrients, infection niche) of the pathogenic forms in a naturally suppressive soil (Forsyth et al. 2006; Mazurier et al. 2009). Compared with the *Fusarium* wilt-suppressive soil, either FO pathogenic forms or all FOs were unable to establish an ecosystem, so quantifying the FOs in the high-disease index CCC soil is valuable work (Forsyth, Smith, and Aitken 2006; Mazurier et al. 2009).

Of the six FO strains, ZJ-01, ZJ-02, and ZJ-03 showed pathogenic effects on *C. sativus* cv. Xin Xia Feng and ZJ-01 infected *C. melo* cv. Japanese Sweet with a PDI of 10.1%.

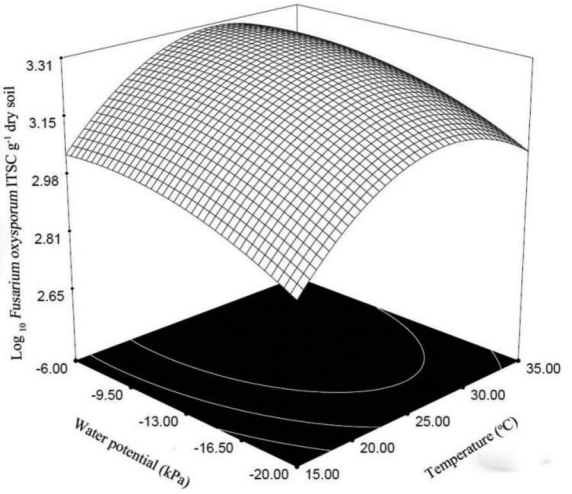
Table 3
Variance analysis of the effects of the environmental factors and the interactions on the $\log_{10}$ ITSC in the CCC soil

Source	SS	df	MS	F value	Prob > F
Model	1.74	9	0.19	20.85	<0.0001**
X_1	0.073	1	0.073	7.92	0.0183*
X_2	0.10	1	0.10	11.33	0.0072**
X_3	0.088	1	0.088	9.55	0.0114*
$X_1 \times X_2$	9.8E-003	1	9.8E-003	1.06	0.3280
$X_1 \times X_3$	1.25E-003	1	1.25E-003	0.13	0.7211
$X_2 \times X_3$	5.0E-005	1	5.0E-005	5.396E-003	0.9429
X_1^2	1.17	1	1.17	125.89	<0.0001**
X_2^2	0.41	1	0.41	44.73	<0.0001**
X_3^2	0.046	1	0.046	4.96	0.0501
Residual	0.093	10	9.266E-003		
Lack of fit	0.063	5	0.013	2.12	0.2143 n.s.
Pure error	0.030	5	5.937E-003		
Cor total	1.83	19			

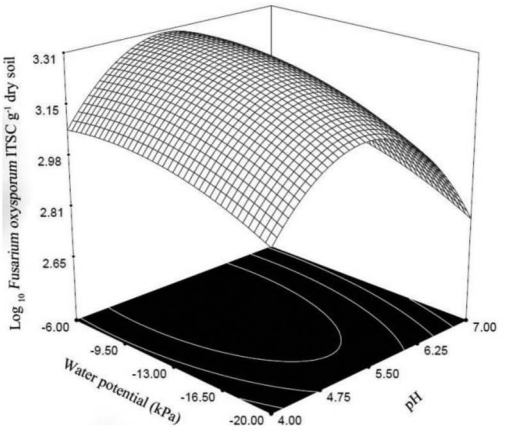
Notes. SS, sum of squares; df, degrees of freedom; MS, mean square. $R^2 = 0.9494$, adj. $R^2 = 0.9039$, pred. $R^2 = 0.7114$, adeq. precision = 13.634; n.s., not significant ($P < 0.05$).
*Significant ($P < 0.05$).
**Highly significant ($P < 0.01$).

According to reports, FO formae speciales of *C. sativus* (*F. oxysporum* f. sp. *cucumerinum*) could not infect *C. melo*, but *F. oxysporum* f. sp. *radicis-cucumerinum* casually infected *C. sativus, C. melo, Cucurbita pepo, C. vulgaris*, and *L. aegyptiaca* (Booth 1971; Punja and Parker 2000); therefore, further identification of ZJ-01 should be carried out in our future studies. At an FO inoculum density of 10^4 cfu g^{-1} soil under artificial conditions, all of the treatments showed negative effects on *C. sativus* cv. Xin Xia Feng, including the strains that did not cause *Fusarium* wilt but decreased the dry weight and fresh root weight, which could have been the result of phytotoxic metabolites (Chen et al. 2012b). The primers Fn1/Fn2 could amplify the ITS regions of the genomic DNA of all of the six FO strains in the experiment; therefore, we quantified the ITS copies of FO in the soil as an indicator of environmental effects on the soil FO population. The application of the real-time PCR method was superior to a CFU count on plates for detecting the population change of FO, because an inaccurate selective medium may cause the growth of other *Fusarium* spp. and, especially in an insignificant population change experiment, a difficulty in the identification of CFUs from a single, asexual propagule (i.e., conidia or chlamydospore) or proliferating mycelia (Chen et al. 2011b).

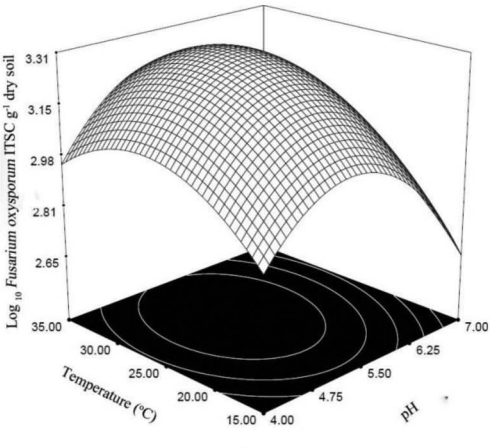
Modeling of environmental factors on soil FO ITSC showed that environmental factors significantly affected by soil FO population (Figure 3), a result that has also been demonstrated by much research both in vivo and in field experiments. Hyang and Naresh (2010) reported that *F. oxysporum* f. sp. cepae grew fast at a high water activity during in vivo experiments. Similarly, a decrease in soil water activity decreased the *F. oxysporum* f. sp. cepae growth and simultaneously decreased the virulence to garlic. A low soil W_p was responsible for the low propagule germination and mycelia elongation of FOs, and FO in CCC soil increased as the W_p increased, even when the W_p increased to –6.00 kPa. This was in accordance with the practical experience that during wet, summer days or in



a



b



c

Figure 3. Three-dimensional response surface plots of the $\log_{10}$ FO ITS copies g^{-1} dry soil showing the interaction between the W_p and temperature (a), W_p and pH (b), and pH and temperature (c) of the CCC soil.

greenhouses with a high air humidity and water-saturated soil, a large range of *Fusarium* wilt was easily observed (Hyang and Naresh 2010; Scott et al. 2010). Furthermore, soil W_p not only directly increased the growth rate of *F. oxysporum* but also changed soil physical features promoting growth of *F. oxysporum* (Chen et al. 2011a; Jackson, Whipps, and Lynch 1991). Severe *Fusarium* wilt in soil with high W_p resulted from anaerobic or near-anaerobic conditions that was injurious to roots and predisposed plants to root diseases but also increased the FO growth rate (Scott et al. 2010). Therefore, a greenhouse with a low soil W_p or a field with convenient water drainage is recommended to decrease the burst of *Fusarium* wilt.

The FO grew faster in the CCC soil at a soil pH of 5.1–5.6 [Figure 3(c)]. The maximum ITSC in the soil was predicted at a pH of 5.35, and this trend was similar with types of *Fusarium* wilt caused by FO that were in severe acidic conditions and alleviated when the soil pH was increased (Fine et al. 2007). The continuous monoculture and chemical fertilizer application acidified the soils, which was one reason that a low pH favored the *F. oxysporum* f. sp. cucumerinum accumulation in the continuously cropped soil. A change in soil pH by amending chemicals such as lime or compost in the continuously cropped soil or monoculture soil has been effective in suppressing *Fusarium* wilt (Fine et al. 2007). Therefore, the application of organic fertilizer, as part of the substitute for chemical fertilizer, not only changed the soil microbiological parameters (Chen et al. 2012a; Hyang and Naresh 2010) but also the physical properties, including the soil pH (Fine et al. 2007), which would be highly effective in controlling *Fusarium* wilt of cucumber in intensive agriculture.

Severe *Fusarium* wilt symptoms emerge at appropriate soil temperatures and FO maintains a high rate of chlamydospore germination and mycelia growth (Jackson, Whipps, and Lynch 1991; Navas-Cortés et al. 2007). In our results, soil temperature of 25.3–29.6 °C strongly influenced the FO growth, which maintained a soil ITSC greater than 3.30. Temperature significantly affects soil microbial and chemical properties (Jackson, Whipps, and Lynch 1991; Navas-Cortés et al. 2007). This was one reason that the *Fusarium* wilt of cucumber exhibited a proliferative burst at an environmental temperature of 24–28 °C (Booth 1971).

The model fits the experimental data closely; in particular, the greatest predicted soil ITSC values occurred at a soil pH of 5.35, a temperature of 27.43 °C, and a W_p of –8.12kPa. This finding was in accordance with the practical field conditions. The model, based on soil temperature, pH, and W_p, was used to construct risk threshold charts that can be used to estimate the soil FO proliferation rate and the potential risk of *Fusarium* wilt epidemics in CCC soils. The model suggested decreases in the pathogen proliferation rate could be accomplished by affecting the environmental factors with agricultural management, which may protect the environment and improve sustainable development in the production of economic crop plants.

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