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Studies on a pathogenicity assay for screening cotton germplasms for resistance to *Fusarium oxysporum* f. sp. *vasinfectum* in the glasshouse

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Summary. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *vasinfectum*, is a new and important disease of cotton in Australia. Some factors affecting either the infection process or the subsequent development of symptoms under glasshouse conditions were examined in this study. The pathogenicity of inocula was significantly affected by the media in which they were produced. The most severe symptoms developed in the plants inoculated with the inoculum produced in Komada-Ezuka liquid medium, in which glucose and L-asparagine were used as the carbon and nitrogen source, respectively. Symptoms were significantly more severe in plants inoculated with the inocula suspended in culture filtrates than in those inoculated with the inocula suspended in distilled water, indicating that fungal metabolites played an important role in the infection process. The disease was enhanced by high conidial concentration (>1.0 x 10^6 conidia/mL), slightly acidic inoculum (pH 4.0–5.5) and longer inoculation period (5–25 min). One-week-old seedlings were most susceptible, regardless of cultivar, and the resistance of plants increased with their age. The development of symptoms was enhanced at a moderate temperature range (18–23^{o}C), but suppressed at a higher temperature range (28–33^{o}C). Based on these results, an optimised procedure of pathogenicity assay is described.

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* Schlecht. f. sp. *vasinfectum* (Atk.) Snyd. & Hans. (*Fov*), is an economically important disease of cotton (*Gossypium hirsutum* L.) in almost all cotton growing areas of the world (Hillocks 1992). The disease was first detected in Australia in 1993 and now occurs in both Queensland and New South Wales (Kochman 1995). Preliminary studies have shown that the Australian isolates of *Fov* are distinct from all the overseas races studied so far (Davis *et al*. 1996; S. Bentley pers. comm.), indicating that they perhaps originated from endemic populations of local *Fusarium oxysporum*. Some of the current Australian commercial cotton cultivars are very susceptible and significant losses have occurred where they have been grown.

Glasshouse screening is the primary means of determining the pathogenicity of isolates of *Fov* and evaluating the resistance of cotton germplasms. Root dipping of cotton into a conidial suspension of *Fov* (Miller and Cooper 1967) is the most commonly used inoculation technique because of its convenience and speed. However, little is known about how the disease is affected by the factors concerning inocula, plants and environmental conditions. Studies on similar processes in other crops indicate that the disease may be influenced by the conidial concentration (Peterson and Pound 1960), inoculum pH (Schuerger and Mitchell 1992*a*, 1992*b*) and temperature (Bhatti and Kraft 1992). In addition, the pathogenicity of many *formae speciales* of *Fusarium oxysporum* can be affected by the composition of media in which they have been grown (Ragazzi *et al*. 1994) and the resistance of plants may change with their growth and development.

This study was undertaken to evaluate and optimise some key factors which may affect a pathogenicity assay and then to develop a better and more reliable procedure to screen cotton germplasms against fusarium wilt in the glasshouse.

Materials and methods

Isolates of Fov *and preparation of inocula*

Two isolates of *Fov* obtained from the Cecil Plains area of the Darling Downs of Queensland in 1995 were used. Isolate CP 95/04

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(isolated from a symptomatic cotton plant) was provided by R. D. Davis (Queensland Department of Primary Industries) and isolate TF-1 was isolated from a soil sample collected from an infested field. The fungi were maintained on sterile filter paper at 4°C (Correll *et al*. 1986) and recovered in modified fresh potato dextrose broth (5% potato infusion and 1% glucose) at 25°C for 3 days before the preparation of inocula.

Unless otherwise specified, conidial suspensions of isolate CP 95/04 were used as inocula. The fungus was grown in 300 mL of potato dextrose broth (Difco) in 500 mL Erlenmeyer flasks by inoculating each flask with 1 mL of recovered culture and incubating at 25°C on an orbital shaker at 175 rpm for 1 week. The cultures were filtered through 8 layers of Kleenex tissue to remove hyphal fragments and the hyphae-free filtrates were centrifuged at 2500 rpm for 15 min. The pellet (consisting mainly of conidia) was suspended in a small quantity of distilled water, while the supernatant was diluted in 2 volumes of fresh water. Conidial suspensions with a concentration of 5.0 (± 0.5) x 10^6 conidia/mL, determined using a haemocytometer, were prepared by adding the conidia to the diluted supernatant. The pH values of conidial suspensions, measured using a potentiometer and thin-glass electrode, were adjusted to 5.0 (± 0.1) with 0.05 mol/L HCl. The inocula were then maintained at room temperature (15–25°C) and used within 3 h after their preparation.

Cotton cultivars and preparation of seedlings

Two cotton cultivars, Siokra 1–4 (most susceptible) and CS 189+ (less susceptible), were used. Unless specified otherwise, all the experiments were conducted on the seedlings of cultivar Siokra 1–4. Seeds were placed in small cheesecloth bags, hot-water treated at 40–50°C for 30 min and incubated at 25°C overnight. Germinated seeds with 2–5 mm radicles were sown into potting mix in polystyrene seedling flats and grown in a glasshouse at 18–23°C.

General procedure of pathogenicity assay

Inoculation was usually conducted 2 weeks after the seeds were sown. For each replicate of a treatment, 9 seedlings were pulled out from the potting mix and their roots were washed to remove the potting mix debris. The roots were dipped into 80 mL of conidial suspension in a 100 mL beaker for 5 min at room temperature (15–25°C). The inoculated seedlings were then transplanted into potting mix in 15 cm plastic pots with 3 plants per pot and grown in a glasshouse at 18–23°C. Normally, there were 3 replicates for a treatment.

The effect of culture media on the disease

The following nine media, which have been reported to be suitable for growing *Fusarium oxysporum*, were selected: (A) Czapek's broth, Kappelman (1983); (B) Czapek's broth + 0.1% yeast extract, Harrison (1987); (C) potato dextrose broth (Difco), Namiki *et al*. (1994); (D) Komada-Ezuka liquid medium, Larkin (1993); (E) Kerr's liquid medium, Kerr (1963); (F) Tochinai nutrient solution, Jorge *et al*. (1992); (G) FLC broth, Starr *et al*. (1989); (H) modified Richards' solution, Miller and Cooper (1967); and (I) potato dextrose agar (Difco), Assigbetse *et al*. (1994). Cultures grown in 8 liquid media were prepared using the method described previously. Meanwhile, fifteen 9 cm Petri dishes containing potato dextrose agar (Difco) were inoculated by spreading 0.1 mL of recovered culture on each and incubated at 25°C for 1 week. The cultures were then washed off the plates into 300 mL of fresh water. Variations in the pH values of media caused by the growth of the fungus were investigated by measuring pH before inoculation and after incubation. The hyphae-free filtrates, prepared using the method described previously, were directly used as inocula in this experiment.

The effect of fungal metabolites on the disease

Cultures of isolates CP 95/04 and TF-1 grown in potato dextrose broth (Difco) and modified Richards' solution (Miller and Cooper 1967) were each filtered and their hyphae-free filtrates were centrifuged using the method described previously. For each combination of isolate and medium, the pellet was suspended in an equal volume of distilled water, washed and separated again by centrifugation. This process was repeated 3 times and then the conidia were suspended in a small quantity of distilled water. The inocula containing different levels of the ingredients of media and/or fungal metabolites were prepared by adding the conidia into the corresponding supernatants, media and distilled water.

Effects of conidial concentration, inoculum pH and inoculation period on the disease

In order to examine the effect of conidial concentration on different cotton cultivars qualitatively, seedlings of cultivars Siokra 1–4 and CS 189+ were inoculated with a series of 5 inocula with a conidial concentration of 1.0 (± 0.2) x 10^4, 10^5, 10^6, 10^7 and 10^8 conidia/mL, respectively. Eighteen seedlings of each cultivar were used for each unreplicated treatment.

Either 0.05 mol/L HCl or 0.05 mol/L NaOH were used to prepare inocula with pH values of 4.0, 5.5 and 7.0. The roots of seedlings were first dipped into fresh water with the required pH for 5 min and then into the corresponding inoculum when inoculation was conducted.

The effects of conidial concentration and inoculation period were investigated in combination. A 4 by 3 factorial design was used with 4 conidial concentrations [1.0 (± 0.2) x 10^5, 10^6, 10^7 and 10^8 conidia/mL] as 1 factor and 3 inoculation periods (1, 5 and 25 min) as the second factor.

The effect of plant age at the time of inoculation on the disease

Germinated seeds of cultivars Siokra 1–4 and CS 189+ were sown directly into potting mix in 15 cm plastic pots at weekly intervals and grown in a glasshouse at 18–23°C. On the 6th week seedlings of different ages from 1 to 5 weeks old were inoculated. The roots of 1- or 2-week-old seedlings were dipped into 80 mL of inoculum in a 100 mL beaker, those of 3- or 4-week-old seedlings into 300 mL in a 500 mL beaker and those of 5-week-old seedlings into 800 mL in a 1 L beaker for 5 min, respectively.

The effect of temperature on the disease

Four chambers with different night and day temperature ranges (13–18°C, 18–23°C, 23–28°C and 28–33°C) in a glasshouse were used. For each replicate of all the 4 treatments, the roots of 36 seedlings were dipped into 250 mL of inoculum in a 500 mL beaker for 5 min. The inoculated seedlings were transplanted into potting mix in 15 cm plastic pots with 3 plants per pot. They were then incubated at different temperatures with 3 pots in each of the 4 chambers.

Assessment of disease severity and data analysis

Disease assessment was conducted 4 weeks after inoculation.

Table 1. Variations in the pH values of media before inoculation and after incubation and the number of conidia produced

Medium	pH before inoculation	pH after inoculation	Inoculate (No. of conidia/mL)
A	7.2	7.5	1.1×10^6
B	7.2	7.7	3.0×10^7
C	4.9	6.5	5.5×10^7
D	7.1	6.0	1.4×10^7
E	5.3	7.2	2.1×10^7
F	6.3	6.5	1.4×10^8
G	4.4	6.1	1.4×10^6
H	7.1	7.3	2.3×10^6
I	4.8	7.8*	9.3×10^6

* The pH value of the inoculum made with fresh water (pH 7.6).

Diseased plants were identified by the appearance of typical symptoms of fusarium wilt and dark brown discolouration of the leaf bases and vascular tissues. Disease severity was assessed by rating the plants on a scale of 0–4 according to their foliar wilt symptoms, in which 0, healthy; 1, cotyledons only wilted; 2, ≤50% true leaves wilted; 3, >50% but ≤90% true leaves wilted; and 4, all leaves wilted and the plant was dead. The disease index (the mean of the ratings of individual plants within a replicate or treatment) was calculated using the formula below:

$$\text{Disease index} = \frac{\sum_{\text{Rating}=1}^{4} (\text{Rating} \times \text{number of plants with the rating})}{\text{Total number of plants investigated}}$$

Data were analysed using analysis of variance (ANOVA) in the general linear models of the MINITAB Release 11 for Windows (Minitab Inc.). Significance level of $P = 0.01$ was used. The means were compared using Duncan's multiple range test when the difference was significant.

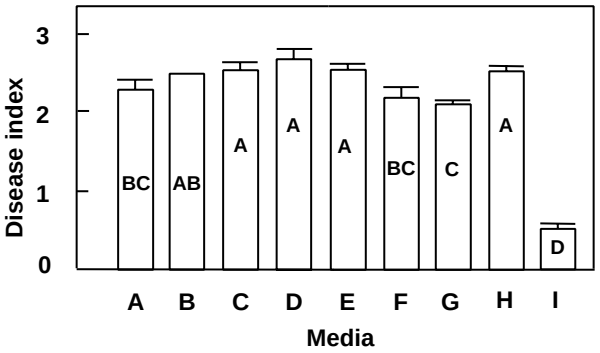

Figure 1. Disease indices of plants inoculated with the inocula produced in 9 media. Values are the means of 3 replicates. The bars show the standard errors. Different letters (inside bars) indicate differences at $P<0.01$.

Results

The effect of culture media on the disease

Disease indices of plants inoculated with the inocula produced in 8 liquid media showed some significant differences (Fig. 1). The highest disease index was observed on the plants inoculated with the inoculum produced in Komada-Ezuka liquid medium, and the lowest on those inoculated with the inoculum produced in FLC broth. No significant differences were observed among the disease indices of plants inoculated with the inocula produced in Komada-Ezuka liquid medium, Kerr's liquid medium, modified Richards' solution and potato dextrose broth, which were all significantly higher than those of plants inoculated with the inocula produced in Czapek's broth, Tochinai nutrient solution and FLC broth. Little disease was observed on the plants inoculated with the inoculum produced on potato dextrose agar where the disease index was only 0.48.

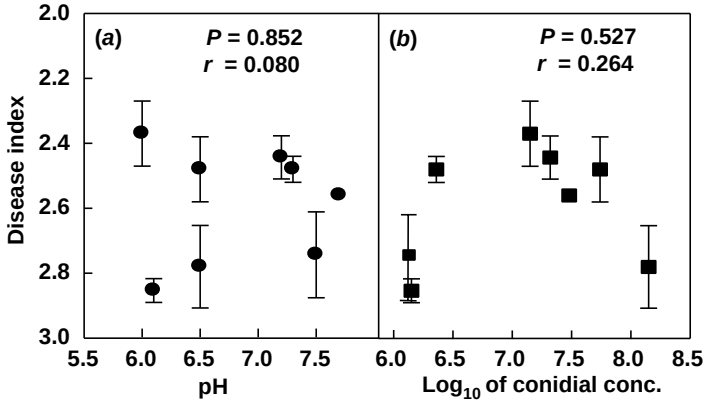

Figure 2. Correlations between the disease indices of plants and either (*a*) the pH values or (*b*) the conidial concentrations of inocula produced in 8 liquid media. Values are the means of 3 replicates. Vertical bars indicate the standard errors.

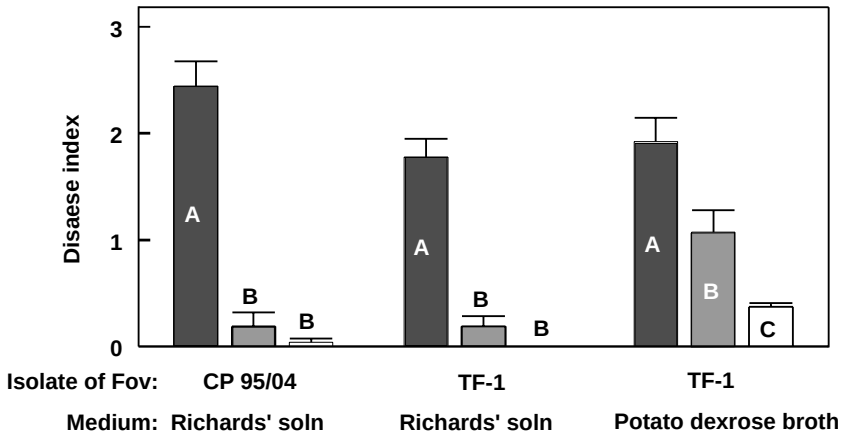

Figure 3. Disease indices of plants inoculated with the inocula of isolates CP 95/04 or TF-1 which were made with the corresponding supernatant (solid bars), media (shaded bars) or distilled water (open bars). Values are the means of 3 replicates. Vertical bars show the standard errors. Different letters indicate significant differences among the 3 inocula in each combination of isolate and medium at $P<0.01$.

Variations in the pH values and conidial concentrations of inocula produced in those media were observed (Table 1). However, no significant correlation was found between the disease indices and either the pH values (Fig. 2*a*) or the conidial concentrations (Fig. 2*b*) of inocula produced in 8 liquid media in the ranges tested.

Effect of fungal metabolites on the disease

Significant differences were observed among the disease indices of plants inoculated with the inocula suspended in supernatants, media and distilled water, respectively (Fig. 3). The disease indices of plants inoculated with the inocula suspended in supernatants were all significantly higher than those of plants inoculated with the inocula suspended in either the corresponding media or distilled water, regardless of isolate and medium.

Effects of conidial concentration, inoculum pH and inoculation period on the disease

The effect of conidial concentration alone varied with the resistance of cotton cultivars (Fig. 4). No disease was

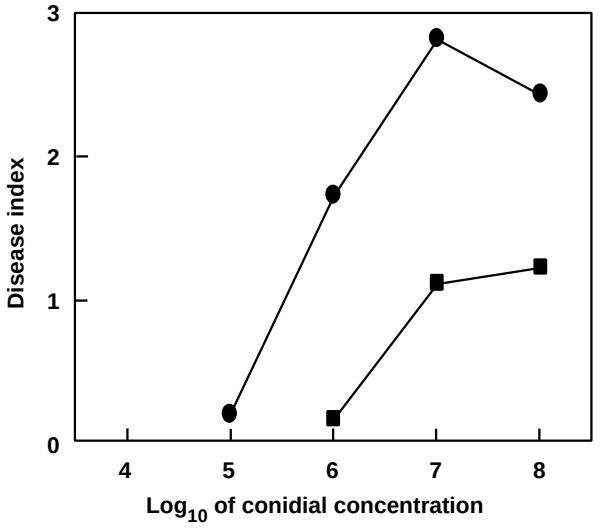

Figure 4. Difference between the disease indices of plants of cultivars Siokra 1-4 and CS 189+ which were inoculated with the inocula with different conidial concentrations.

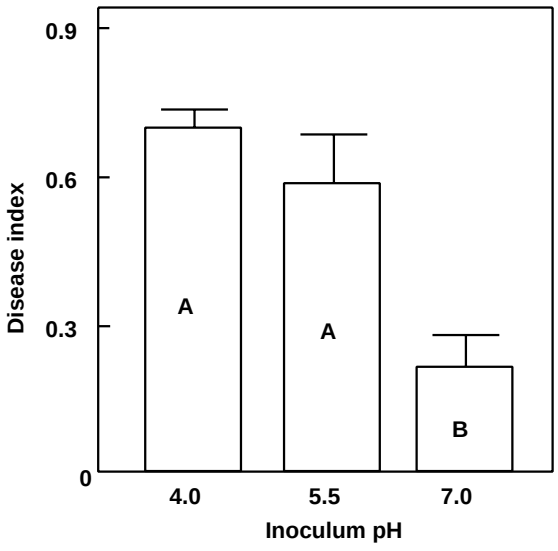

Figure 5. Disease indices of plants inoculated with the inocula of different pH values. Values are the means of 3 replicates. Vertical bars show the standard errors. Different letters indicate significant differences at $P<0.01$.

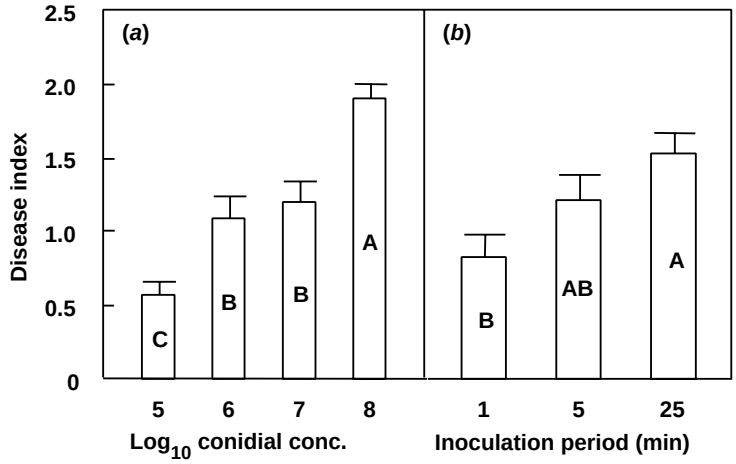

Figure 6. Effects of conidial concentration (*a*) and inoculation period (*b*) on disease index. Values are the means of 9 replicates for part *a* and 12 replicates for part *b*. Vertical bars show the standard errors. Different letters indicate significant differences in part *a* or among inoculation period in part *b* at $P<0.01$.

observed in plants of cultivar Siokra 1–4 when the conidial concentration was below 1.0 (± 0.2) x 10^5 conidia/mL, nor in plants of cultivar CS 189+ when it was below 1.0 (± 0.2) x 10^6 conidia/mL. Therefore, the minimal or critical conidial concentrations for successful infection appeared to be lower for the susceptible cultivars than that for the less susceptible cultivar.

Significant difference was observed among the disease indices of plants inoculated at different pH. Disease symptoms in plants inoculated with an acidic inoculum (pH 4.0 and pH 5.5) were significantly more severe than those developed in plants inoculated with a neutral inoculum (pH 7.0) (Fig. 5).

Positive correlations were observed between the disease indices and both the conidial concentrations and the inoculation periods. The Disease Index increased as the conidial concentration increased from 1.0 (± 0.2) x 10^5 to 1.0 (± 0.2) x 10^8 conidia/mL or as the inoculation period increased from 1 to 25 min (Fig. 6*a*, *b*).

Effect of plant age at the time of inoculation on the disease

Regardless of cultivar, the highest disease index was always observed on plants which were 1 week old when inoculated. However, the disease indices associated with

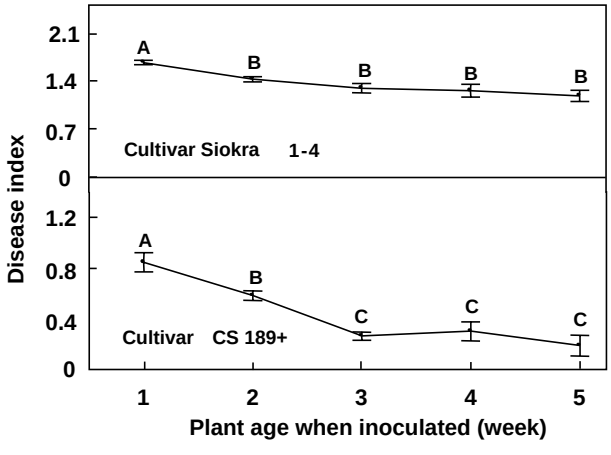

Figure 7. Disease indices of plants (cultivars Siokra 1-4 and CS 189+) which were inoculated at different ages from 1 to 5 weeks old. Values are the means of 3 replicates. Vertical bars show the standard errors. Different letters indicate significant differences at $P<0.01$.

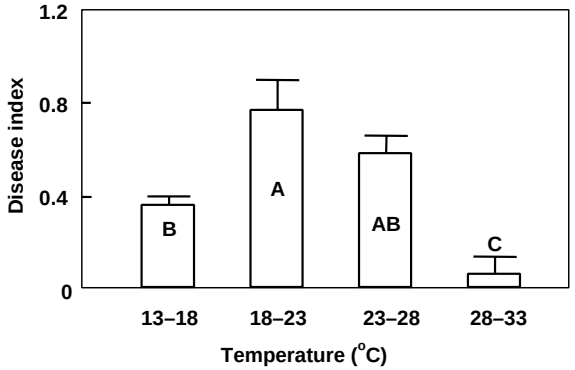

Figure 8. Disease indices of plants which were incubated at different temperatures after inoculation. Values are the means of 3 replicates. Vertical bars indicate the standard errors. Different letters indicate significant differences at $P<0.01$.

cultivar Siokra 1–4 remained high and did not vary significantly, while those associated with cultivar CS 189+ decreased significantly for plants that were 1–3 weeks old when inoculated (Fig. 7).

Effect of temperature on symptom development

The highest disease index was observed on plants which were grown at 18–23°C after inoculation and the lowest on those grown at 28–33°C (Fig. 8).

Discussion

The growth and sporulation of *Fusarium oxysporum* are different when grown in different media (Osman *et al.* 1992). In our study, the amount of conidia produced in Czapek's solution appeared to be slightly lower than that in Czapek's solution + 0.1% yeast extract. However, no significant difference was observed between the disease indices of plants inoculated with the inocula produced in them, respectively. Therefore, the addition of yeast extract at the level of 0.1% improved the production of conidia, but did not affect the pathogenicity of *Fov*. However, the latter may be enhanced when the fungus is grown on a carbon–nitrogen source of glucose and L-asparagine combination as disease symptoms were significantly more severe on the plants inoculated with the inoculum produced in Komada-Ezuka liquid medium, compared with those developed on the plants inoculated with the inocula produced in the media containing other carbon–nitrogen sources.

Potato dextrose agar is one of the best media for the growth of *Fusarium oxysporum* (Osman *et al.* 1992). However, in our study the disease index of plants inoculated with the inoculum produced on it was significantly lower than those of plants inoculated with the inocula produced in liquid media. The ingredients of media and/or fungal metabolites produced during the growth of the fungus probably contributed to the difference. All the ingredients of media and fungal metabolites remained in the inocula prepared in those liquid media, while neither existed in the inoculum produced on potato dextrose agar as it was prepared by washing the cultures off the plates with fresh water. Similarly, the disease was more severe in the plants inoculated with the inocula suspended in culture filtrates than in the plants inoculated with the inocula suspended in either the corresponding media or distilled water. These results strongly suggested that fungal metabolites played an important role in the infection process occurring in conidial suspensions. Previous studies indicated that adhesive materials, such as high molecular-weight glycoproteins and mucilage secreted by mycelia or conidia, and conidial metabolic processes involved in respiration and protein synthesis, were required in the infection process (Jones and Epstein 1989). Perhaps fungal metabolites may mediate the secretion of these products or affect the conidial metabolism. Further research is needed to confirm this hypothesis.

Fusarium wilt of cotton is usually enhanced by high inoculum density and acidic soil pH in the field (Tharp and Wadleigh 1939). In our study, the disease was enhanced by higher conidial concentration, slightly acidic inoculum pH and longer inoculation period. These results are in agreement with many previous studies. For instance, the number of macroconidia of *Fusarium solani* f. sp. *phaseoli* adhering to the roots of hydroponically-grown mung bean seedlings was greatest when the pH of the nutrient solution was 4–5 (Schuerger and Mitchell 1992*a*, 1992*b*). Jones and Epstein (1989) found that the proportion of macroconidia of *Nectria haematococca* adhering to polystyrene increased with the inoculation period. It is likely that the adhesion of conidia of *Fov* to roots of cotton is the only event that can occur during the short inoculation period. Adhesion has been suggested to be a crucial factor in the infection process (Jones and Epstein 1990). Since the severity of fusarium wilt is determined by the extent of multiple penetrations following adhesion (Bugbee and Sappenfield 1968) and is directly proportional to the number of infection sites on the roots (Nyvall and Haglund 1972), factors which can affect adhesion and other events occurring in conidial suspensions must influence subsequent disease. The mechanism involved in adhesion is still unknown, although there have been several hypotheses described for similar processes in other phytopathogen–host interactions (Hinch and Clarke 1980; Longman and Callow 1987). The effect of inoculum pH has been attributed to the fact that protein synthesis is important in the adhesion process and both stoichiometry of the protein and surface charge of the macromolecule can be affected by pH (Jones and Epstein 1989).

The resistance of cotton plants under field conditions depends primarily on their performance at the most susceptible stage, because the consistent attack of pathogenic propagules to the root system is maintained by the stability of the soil matrix. Our results indicated that 1-week-old seedlings, regardless of cultivar, were most susceptible, having the highest Disease Indices following inoculation during this period. In general, the

resistance appears to increase with the age at which inoculation occurs. When the resistance of a cultivar is evaluated, it is important to inoculate seedlings at the most susceptible stage of plants. According to our results, 2-week-old seedlings were the most suitable plants for inoculation because of their relatively high and stable susceptibility, well-developed root systems and ease of handling.

The optimal temperature range for fusarium wilt of cotton was 18–23°C in our study. The finding that relatively high temperatures (>33°C) tended to suppress the full symptom expression might have some epidemiological significance. Even the most susceptible cultivar Siokra 1–4 was less susceptible at higher temperatures. The difference between the relative growth rates of pathogen and host may be one of the explanations for the influence of temperature on the disease.

Overall, based on the results obtained from the current study, the following guidelines are recommended for a pathogenicity assay. Conidia of *Fov* should be grown in potato dextrose broth (Difco) because it is one of the most easily prepared media and a relatively high number of conidia are produced. Inoculation of cotton seedlings with conidial suspensions produced in this medium subsequently results in a high disease index. The metabolites of *Fov* should be retained in the conidial suspensions. The recommended procedure for the root dipping inoculation technique is to dip the roots of 2-week-old cotton seedlings for 5 min into a conidial suspension with a concentration of 5.0×10^6 conidia/mL and a pH value of 5.0. The inoculated plants should then be incubated in a glasshouse at 18–23°C.

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