Induced Resistance in Cotton Seedlings Against Fusarium Wilt by Dried Biomass of *Penicillium chrysogenum* and Its Water Extract

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Dry mycelium (DM) of killed *Penicillium chrysogenum* and its water extract (DME) were used to induce resistance in cotton plants against *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*). Results showed that the efficacy of either DM or DME in controlling the disease depends on both the concentration and the mode of application. DM amended to the soil at 0.25–2% (w/w) provided 32–75% protection against *Fov*. Soil drench with 2–5% DME (w/v) and pre-sowing seed soakage with 5–10% DME provided 51–77% and 28–35% protection against the wilt disease, respectively, whereas no protection was obtained with foliar sprays of 1–10% DME. DM and its water extract had no direct antifungal activity on growth of *Fov in vitro*, suggesting that disease control with DM or DME resulted from the induction of natural defense mechanisms in the cotton plants. Soil drench with 5% DME was as effective as 2% DM powder in inducing resistance against *Fov*, implying that the resistance-inducing substances were mostly water-soluble. Four cotton cultivars with various genetic resistance levels against *Fov* were tested at the seedling stage: two resistant 'Pima' cultivars and two susceptible 'Acala' cultivars. The level of protection achieved in the two susceptible cultivars with DME was equal to, or higher than, that of the two resistant cultivars treated with water. Innate and induced peroxidase activity in cotyledons or hypocotyls and roots coincided with the level of genetic resistance and DME-induced resistance, respectively. Based on our results, an integrated control strategy of *Fov* with both genetic resistance and induced resistance is suggested.

KEY WORDS: Genetic resistance; peroxidase activity; *Penicillium chrysogenum*; *Fusarium oxysporum* f.sp. *vasinfectum*.

INTRODUCTION

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*), is a major root disease of cotton worldwide (2,13,15). Control of root diseases is generally based on the application of fungicides (30), resistant cultivars (1,31) and some agronomic techniques (32,38). The importance of reduced pesticide levels in agricultural products and the environment, together with increasing difficulties in breeding new resistant cultivars, dictate the need for alternative methods of disease control (1,11,27). One of the potential methods of reducing the severity of fungal diseases is the induction of resistance achieved with the aid of certain chemicals (8,25). In contrast to genetic resistance, induced resistance

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can be obtained in plants without alterations of their genome and is characterized by a broad spectrum of protection against various pathogens. Induced resistance is based on multiple mechanisms, and therefore the pathogens may not readily develop resistance (25).

Induced resistance can be achieved by using biotic inducing agents or application of abiotic inducers (3,12,20). In cotton, it was reported that 2,6-dichloroisonicotic acid (INA), 3-aminobutyric acid and benzo[1,2,3]thiadiazole-7-carbothioic acid (BTH) were each able to induce resistance against Verticillium wilt and Alternaria leaf spot (9,10,26). Pre-soaking of seed in BTH (1) and application of the biological control agent *Trichoderma virens* (21) induced systemic resistance against *Fov* and *Rhizoctonia solani*, respectively, in cotton during the early stage of growth. Elicitors from *Verticillium dahliae* induced defense responses in cotton leaf disks against the same pathogen (14). At present, there is no evidence of induced resistance against Fusarium wilt in cotton plants by killed microbial biomass. Dry mycelium (DM) used in this study is made of the dried fungal biomass of *Penicillium chrysogenum* after extraction of penicillin. *P. chrysogenum* was reported to be an antagonist against *Botrytis fabae* (23). Limited information is available on the efficacy of the killed *P. chrysogenum* in disease control. When amended to the soil, DM enhanced plant growth by nutrient supplementation and improving soil fertility (19). Recently, Gao *et al.* (17) reported that DM applied to the soil protected corn against *Fusarium moniliforme*. We conducted a series of experiments with four cotton cultivars, to learn if DM or its water extract can induce resistance in cotton against Fusarium wilt. The modes of application of DM or DME as related to control of Fusarium wilt, and the involvement of peroxidase in resistance, were also investigated.

MATERIALS AND METHODS

Preparation of extracts Dry mycelium of *P. chrysogenum* was provided by Biochemie Ltd. (Kundl, Austria). This fungal biomass was dried for 4 h at 110 °C by the manufacturer and contains no penicillin. Dry mycelium extract (DME) was prepared using the following procedure: 100 g of DM was suspended in 1000 ml of distilled water. The suspension was shaken for 2 h at 100 rpm; stored for 22 h at room temperature; and then briefly agitated and filtered through Whatman No. 1 filter paper. The filtrate was autoclaved for 30 min at 110°C and the pH measured after cooling (10% DME, pH 2.6–2.8). DME (10%) was stored as stock solution at 4°C and used within one month.

Plants and DM or DME treatment Two upland cotton cultivars (*Gossypium hirsutum* L. cvs. 'H552' and 'Vered') and two sea-island cotton cultivars (*Gossypium barbadense* L. cvs. 'P906' and 'PF15'), supplied by Hazera Genetics Ltd. (Brurim, Israel), were used in this study. Seeds were sown in 1000-ml pots containing a mixture of perlite and peat (1:1, v/v) and left to grow in the greenhouse at 18/28 °C (night/day) under a 12-h photoperiod. At 2 days after emergence (usually 8–10 days after sowing), five healthy seedlings were left in each pot. 0.5–5% DME (40 ml per pot) was drenched into each pot with a pipette. Plants drenched with an equal volume of distilled water were used as controls, unless indicated otherwise.

In the foliar spray treatment, water (control) or 1–10% DME (5 ml/pot) was sprayed with a fine glass atomizer onto the upper leaf surfaces of the 2-day-old cotton seedlings. Plants were incubated at 20°C (140 μE m^{-2} s^{-1} illumination) for 2 h and then transferred to a greenhouse.

For DM treatment, the mixture of perlite and peat was amended with a DM (0.25–2%, w/w), potted and then watered. Seeds were sown in each pot one week afterwards. For seed soakage, cotton seeds were soaked in 1–10% DME for 24 h, then sown in the potting soil mixture without DM. Seedlings were left to grow in a greenhouse under the conditions described above. After emergence, plants were watered every 2 or 3 days with an equal volume of water given to each pot.

Fungal inoculum *Fusarium oxysporum* f.sp. *vasinfectum* was grown in 9-cm petri dishes on PDA at 25°C in the dark. Cultures 10–15 days old were crushed in sterile water and the conidial concentration was adjusted to 10^7 ml^{-1} before inoculation, unless indicated otherwise.

Inoculation Cotton seedlings (5–6 days after emergence) were removed carefully from the soil and washed thoroughly with tap water. The root tips (2 cm) were cut off and then the root system was dipped in the inoculum for 5 min. Inoculated seedlings pretreated with DME were then transplanted into pots containing fresh potting mixture, while those pretreated with DM were transplanted to their original soil mixture in pots. After inoculation, plants were placed in large plastic boxes for 2 days to avoid loss of turgor and then placed in a greenhouse under controlled temperature (20/28 °C, night/day) and light regime (12-h photoperiod).

Disease assessment Our preliminary data showed that young cotton seedlings ($\leq$ 7 days after emergence) inoculated with as many as 10^7 conidia/ml of *Fov* usually exhibited wilt symptoms 4–5 days after inoculation, and most of the wilting plants died during the following 3–4 days. Some surviving plants exhibited symptoms of leaf wilt and/or vascular discoloration in hypocotyls, 3 weeks after inoculation. To simplify calculation, dead plants and plants with either leaf wilt or stem vascular discoloration were considered diseased. The percentage of diseased plants (dead and wilted) and of 'protected' plants was calculated 8 and 24 days after inoculation, respectively. Each experiment was carried out with five replicates of ten plants each, and was repeated at least twice.

Fungitoxicity tests Czapek Dox (CZA) agar containing either 0.5–5% (v/v) DME or 0.5–2% (w/v) DM (powder) was autoclaved, poured into 9-cm petri dishes and inoculated with three mycelium plugs (3 mm) of the *Fov* pathogen per dish (three dishes per treatment). The inoculated plates were kept at 25°C in the dark and colony diameter was measured 5 days later.

Peroxidase activity The upper part (cotyledons) and lower part (roots together with hypocotyls) of either healthy or *Fov*-inoculated cotton seedlings, treated or untreated with DME, were used to assess peroxidase activity. Whole plants were removed from soil 1–3 days after DME treatment or 2 days after inoculation, washed thoroughly with water, and blotted dry. The upper part or lower part ($\sim$ 0.5 g) was ground with a mortar and pestle in 10 ml of cold 15 mM sodium phosphate buffer (pH 6.0). The suspension was poured into a 50 ml tube, held still for 30 min in an ice bath, and then centrifuged (10,000 *g* for 10 min at 4°C). Two hundred μl of the supernatant was added to a mixture of 5 ml of 15 mM sodium phosphate buffer (pH 6.0), 100 μl of 0.05 M guaiacol, and 100 μl of 2% (v/v) H_2O_2 in a 50 ml tube. After 3 min of reaction, the increase in optical density at 470 nm was recorded with a Milton Roy Spectronic Genesys 5 spectrophotometer. Peroxidase activity was expressed as the change in absorbance at 470 nm min^{-1} g $f.wt^{-1}$. Four replicate plants per treatment were used in each experiment.

RESULTS

Effect of DM and DME on fungal growth *in vitro* Various concentrations of DM (0.5–2%, w/v) or DME (1–5%, v/v) were added to CZA agar to study the possible fungitoxic activity of DM and DME against *Fov*. Fungal colony diameter recorded 5 days after inoculation did not differ significantly between DME-free (control) and either DME-amended or DM-amended plates (data not shown), indicating that $\leq$ 5% DME or 2% DM had no direct activity against the pathogen.

TABLE 1. Protection obtained in cotton plants (cv. H552) against *Fusarium oxysporum* f.sp. *vasinfectum* by various concentrations of dry mycelium extract (DME) of *Penicillium chrysogenum* as soil drench, seed soakage or foliar spray. Data represent percentage of diseased plants 8 days after inoculation, from three separate experiments. DME was drenched into soil or sprayed onto the leaves 3 days before inoculation. For seed soakage treatment, seeds were soaked in DME 1 day before sowing. Within rows, values followed by the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test.

Treatment	Concentration of DME (%)					
	0 (Ck)	0.5	1	2	5	10
Soil drench	80.5±5.4a	64.1±5.8b	52.3±5.6b	39.1±2.4c	17.2±5.2d	–
Seed soakage	80.1±6.0a	–	77.7±7.3a	74.6±6.9a	58.8±8.4b	51.8±6.5b
Foliar spray	84.1±6.0a	–	83.3±7.8a	73.8±10.5a	81.6±7.8a	82.6±5.7a

– not examined.

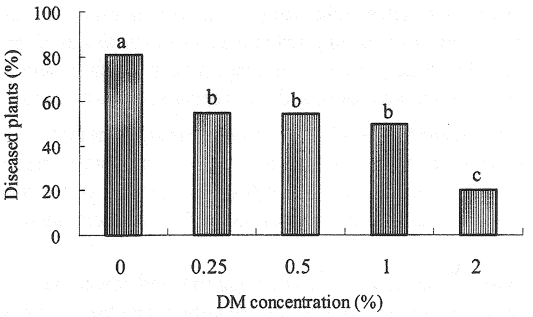

Fig. 1. Protection obtained in cotton plants (cv. H552) against *Fusarium oxysporum* f.sp. *vasinfectum (Fov)* by dry mycelium (DM) of *Penicillium chrysogenum*. 0.25–2% DM (w/w) was amended to the soil before sowing. Plants were inoculated with *Fov* 4 days after emergence. Data were recorded 8 days after inoculation. Values in columns marked with the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test.

Effect of DM on protection of cotton plants against *Fov* Cotton plants (cv. H552) grown in soil containing 0.25–2% DM showed significant protection against the disease 8 days after inoculation (Fig. 1). The level of protection ranged from 32% to 75% with 0.25–2% DM. 2% DM provided the highest level of protection (75%) relative to plants grown in DM-free soil.

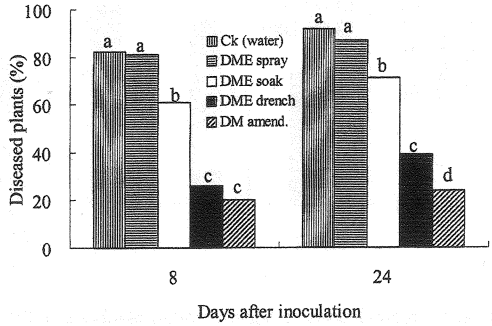

Fig. 2. The effect of mode of application of dry mycelium (DM) of *Penicillium chrysogenum* or DM extract (DME) on the percentage of cotton plants (cv. H552) affected by *Fusarium oxysporum* f.sp. *vasinfectum (Fov)* 8 days after inoculation. DM was applied as soil amendment (2%, w/w) before sowing. DME was applied either as seed soakage (5%, v/v) 1 day before sowing, or soil drench (5%, v/v) or foliar spray (10%, v/v) 2 days after emergence. Plants were inoculated with *Fov* 2 days after DME treatment or 4 days after emergence. Values in columns marked with the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test.

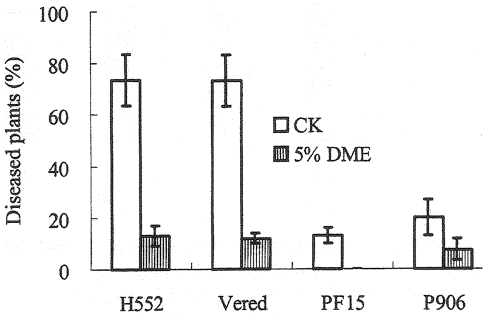

Fig. 3. Protection obtained by 5% dry mycelium extract (DME) of *Penicillium chrysogenum* in cotton plants of four cultivars expressing various genetic resistance. 5% DME was applied as soil drench 2 days after emergence. Data recorded 8 days after inoculation showed that two 'Pima' cultivars (PF15 and P906) were more resistant to *Fusarium oxysporum* f.sp. *vasinfectum* than two 'Acala' cultivars (H552 and Vered). Bars indicate ± SD of the means.

Effect of mode of DME application on protection against *Fov* Table 1 provides data on percentage of diseased plants in three separate experiments in which DME was applied as soil drench, seed soakage or foliar spray. Soil drench treatments with 0.5–5% DME protected the cotton seedlings significantly against *Fov* compared with water-treated, challenged plants (Ck). Application of 5% DME provided the highest level of protection (77%). In seed soakage experiments, as little as 5–10% DME provided

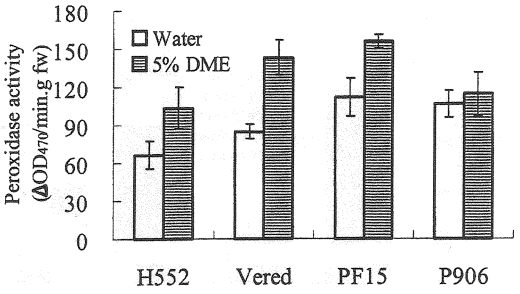

Fig. 4. Peroxidase activity in cotyledons of healthy cotton plants treated with either water or 5% dry mycelium extract (DME) of *Penicillium chrysogenum* as soil drench. Peroxidase activity was examined in plants of four cultivars (H552, Vered, PF15 and P906) with various genetic resistance against *Fusarium oxysporum* f.sp. *vasinfectum*. Bars indicate ± SD of the means.

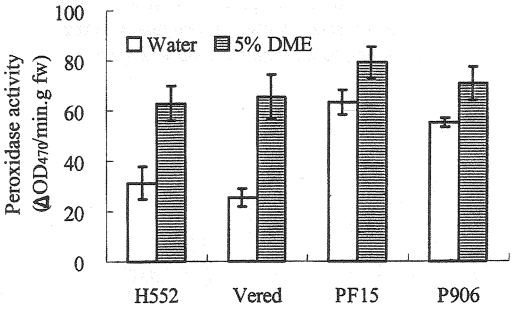

Fig. 5. Peroxidase activity in hypocotyls and roots of healthy cotton plants treated with either water or 5% dry mycelium extract (DME) of *Penicillium chrysogenum* as soil drench. Peroxidase activity was examined in four cultivars (H552, Vered, PF15 and P906) with various genetic resistance against *Fusarium oxysporum* f.sp. *vasinfectum*. Bars indicate ± SD of the means.

significant protection (27–35%) against *Fov*. A foliar spray with up to 10% DME did not result in significant protection against the disease.

In order further to compare the efficacy of different application methods, DM and DME were applied at a single optimal dose. Data recorded 8 days after inoculation showed that soil amendment with 2% (w/w) DM and soil drench with 5% (v/v) DME provided 76% and 70% protection, respectively, whereas seed soakage with 5% DME provided a much lower, though significant, level of protection of 26%. Foliar spray with 10% DME did not provide significant control of the disease (Fig. 2). Records taken 24 days after inoculation showed a similar pattern, namely, percentage of protection by amended DM and drenched DME was 74% and 58%, respectively, and significantly higher than that of seed soakage (23%). However, a higher percentage of protection was obtained with DM than with DME.

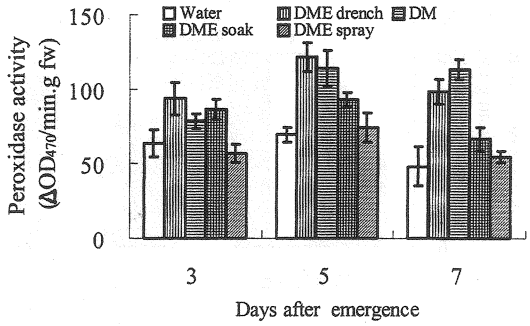

Fig. 6. Peroxidase activity in cotyledons of healthy cotton plants (cv. H552) treated with dry mycelium (DM) amendment or DM extract (DME) of *Penicillium chrysogenum* by various methods. DM was applied as soil amendment (2%, w/w) before sowing. DME was applied as seed soakage (5%, v/v) 1 day before sowing; or as soil drench (5%, v/v) or foliar spray (10%, v/v) 2 days after emergence. Bars indicate ± SD of the means.

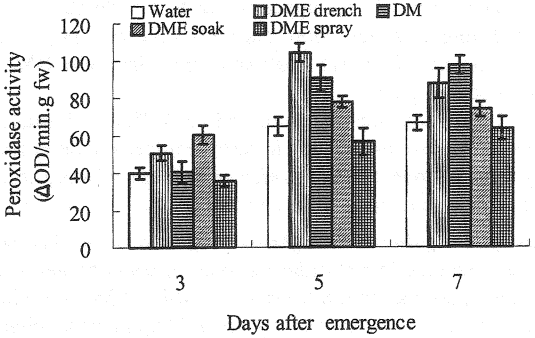

Fig. 7. Peroxidase activity in hypocotyls and roots of healthy cotton plants (cv. H552) treated with dry mycelium (DM) amendment or DM extract (DME) of *Penicillium chrysogenum* by various methods. DM was applied as soil amendment (2%, w/w) before sowing. DME was applied as seed soakage (5%, v/v) 1 day before sowing; or as soil drench (5%, v/v) or foliar spray (10%, v/v) 2 days after emergence. Bars indicate ± SD of the means.

Genetic resistance and induced protection Figure 3 provides data from an experiment in which the efficacy of DME in inducing resistance against *Fov* was tested in two Pima cultivars and two Acala cultivars. The former (PF15 and P906) were more resistant to *Fov* than the latter (H552 and Vered). Nevertheless, all cultivars, when treated by soil drench with 5% DME, showed significant protection against *Fov* (82%, 82%, 100% and 62%, respectively), regardless of their genetic resistance to the pathogen.

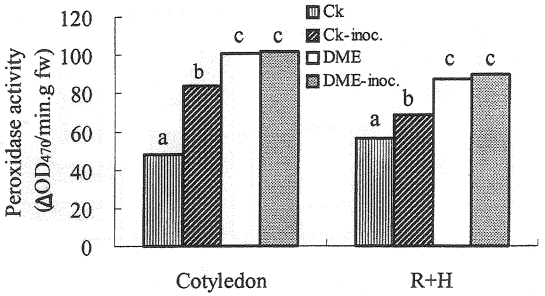

Fig. 8. Peroxidase activity in healthy or inoculated (inoc.) cotton plants (cv. H552) treated with either water (Ck) or 5% dry mycelium extract (DME) of *Penicillium chrysogenum*. R+H indicates roots and hypocotyls. Values in columns marked with the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test.

Peroxidase activity in healthy plants Data in Figures 4 and 5 show that peroxidase activity in both the upper part (Fig. 4) and the lower part (Fig. 5) of the two Pima cotton cultivars was much higher than that of the two Acala cotton cultivars; the difference was more pronounced in the lower than in the upper part. Peroxidase activity thus coincided well with the level of genetic resistance. Soil drench with 5% DME resulted in a significant increase in peroxidase activity in the upper part of H552, Vered and PF15, as well as in the lower part of all four cultivars. More than 100% increase in peroxidase activity was measured in the lower parts of the two Acala cultivars 3 days after DME treatment relative to the water-treated control plants.

Figures 6 and 7 show that application of DM or DME as soil drench or seed soakage significantly increased peroxidase activity in both the upper and lower parts of cotton plants (cv. H552) 3 days after treatment or 5 days after emergence, whereas no significant increase was found in the plants treated with foliar spray of DME.

Peroxidase activity in *Fov*-inoculated plants Figure 8 shows that inoculation with *Fov* caused a significant increase in peroxidase activity in water-treated plants (H552), more increase in the upper part than in the lower part. DME induced significant elevations in peroxidase activity, but no further increments occurred upon inoculation with *Fov*.

DISCUSSION

Induced resistance has been considered a potential strategy for disease control (1,24). Its practical application in plant protection requires both detailed knowledge of the induction mechanisms and development of more effective and safer inducing agents (22). During the last decade a number of inducers were reported as effective inducers of resistance in various plants against attack by different pathogens (4-8,24). In cotton, chemicals such as INA, 3-aminobutyric acid and BTH were able to induce resistance against Verticillium wilt and Alternaria leaf spot with either soil drench or foliar spray (9,10,26). Recently, a preliminary field study (1) showed that pre-sowing seed soakage with BTH resulted in induced resistance in cotton plants against Fusarium wilt.

Results in the present paper indicated that DM mixed into soil and DME either drenched to roots or applied to seeds significantly reduced the percentage of cotton plants infected by *Fov*. Since no inhibitory effect on the mycelial growth of the pathogen was observed *in vitro*, control of the wilt disease with DM or DME is suggested to result from the induction of the natural defense mechanisms in cotton plants. Data from several experiments revealed that a soil mixture containing 2% DM, soil drench with 5% DME, or a pre-sowing seed soakage with 10% DME, provided, respectively, up to 75%, 70% and 30% protection against Fusarium wilt 8 days after inoculation, relative to water-treated control plants, whereas foliar spray with 1–10% DME did not provide any control of the wilt disease. It is suggested that cotton plants can differentially express induced resistance depending on the concentration of DM or DME, application method, and the tissue treated. The fact that soil drench with 5% DME was as effective as 2% DM in inducing resistance against *Fov* at 8 days post-inoculation, implied that the resistance-inducing substance is mostly water-soluble.

Studies of the mechanisms of induced resistance have been performed in various plant species during the last decade. In general, defense responses in either biotically or abiotically activated plants include processes of oxygen burst, lignification of host cell walls (33), formation of cell wall apposition at sites of attempted penetration of fungal pathogens (34), accumulation of pathogenesis-related proteins (PRs) (36), and other biochemical and physiological changes in the host plants (24). PRs comprise four families of chitinases (PR-3, -4, -8 and –11), one of β-1,3-glucanases (PR-2), one of proteinase inhibitors *Betv 1*-related (PR-6), and one specific peroxidase (PR-9), as well as the PR-1 family with unknown biological properties, the thaumatin-like PR-5 family, and the birch allergen *Betv l*-related PR-10 family (36). Among the PR proteins, chitinases and glucanases possess potential antifungal activities, while the PR-9 peroxidase is of the lignin-forming type and could be involved in the strengthening of cell walls (36,37). It has been reported (27-29,35) that infection with pathogens or application of inorganic chemicals enhanced peroxidase activity, which was very often associated with resistance. Among different genotypes of muskmelon, those with high peroxidase activity often had higher levels of genetic resistance (29,30). Peroxidase catalyzes the final polymerization step of lignin synthesis, and may therefore be directly associated with the increased ability of systemically protected tissue to lignify (18). Peroxidase activity could also be a biochemical marker for genetic resistance (27-29). However, very little is known about the effect of inducers of microbial origin on the activity of this enzyme and its relationship to induced resistance.

Vascular wilts of cotton are caused by both *V. dahliae* and *Fov*. The foliar symptoms and progress of these diseases are similar (16). These pathogens penetrate cotton plants through the roots and spread systemically through the xylem, leading to the appearance of wilt disease symptoms (15,36). In this paper we showed that peroxidase activity in hypocotyls or roots and cotyledons of two Pima cultivars was significantly higher than that of two Acala cultivars. As Pima cultivars possess greater genetic resistance to *Fov* than Acala cultivars, it is suggested that peroxidase activity is involved in the genetic resistance of untreated cotton plants. Further experiments showed that soil amendment with DM and soil drench with DME also led to significantly increased peroxidase activity in both cotyledons and lower parts of cotton plants, whereas no enhancement of peroxidase activity occurred in plants treated with DME spray. The increase in peroxidase activity in cotyledons resulting from treatment of the root with either DME or DM is indicative of a systemic effect. The

level of peroxidase activity coincides with the level of induced resistance in seedlings of the four cotton genotypes, indicating that resistance induced by DM powder or DME was closely associated with enhanced levels of peroxidase activity.

Previous reports by others (9,10,25) have shown that susceptible cotton cultivars could be significantly protected against Fusarium diseases by chemical inducers. In our experiments, it was found that two susceptible and two resistant cultivars, when treated with DME, exhibited significant resistance against Fusarium wilt relative to their corresponding water controls, regardless of their genetic resistance. Moreover, PF-15, the most resistant cultivar, showed up to 100% protection after treatment with DME. It seems that control of Fusarium wilt by both genetic resistance and induced resistance was more effective than by only one of these two factors. The use of resistant cultivars and induced resistance may make a significant contribution to effective disease control.

Based on previous studies (19) and our present research, it is concluded that DM is not only an organic fertilizer providing nutrients to crop plants and improving humus in soil, but also an inducer of resistance. DME- and DM-induced resistance depends on concentration and application mode of DM or DME.

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