Inhibition of fungal colonization on the rhizoplane of the C&-producing plant, *Mimosa pudica* L.

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Abstract: Carbon disulfide (CS_2) is a colourless, volatile, foul-smelling, fungicidal liquid that is produced by some plants. We determined the ability of a model CS_2-producing plant, *Mimosa pudica*, to affect the rhizoplane colonisation'of six species of soil fungi. Tomato (*Lycopersicon esculentum*), a plant which does not produce CS_2, was the control. In plate assays, the mycelia *of Fusarium moniliforme, Pythium aphanidermatum, Phytophthora* sp., *Rhizoctonia solani, Sclerotium rolfsii* and *Trichoderma viride* showed variable responses to CS_2, but all mycelia were inhibited by *4* µg CS_2 *ml"*. Inhibition *of spore* germination of *F. moniliforme, P. aphanidermatum* and *T. viride* was similar to myceiial inhibition. When gnotobiotic tomato *or M. pudica* plants were inoculated with F. moniliforme or T. viride, spore counts were similar in the nonrhizosphere, but 10- to 1 00-fold lower *on* the rhizoplane of *M. pudica than those* of tomato. When the roots of 11 d-old gnotobiotic tomato or *M. pudica* plants were each inoculated separately with one of the six fungal species, abundant hyphae of 'all six fungal species were observed on the roots of tomato after 7 days. In contrast, *roots* of *M. pudica* showed many or abundant hyphae of *R. solani* -- and S. *rolfsii* but no or few hyphae of the remaining species. These observations were confirmed by ergosterol analysis. Plant-generated CS2 may account for this decreased fungal colonisation, although other compounds may also be responsible.

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1 . INTRODUCTION

CS_2 is a colorless, volatile, foul-smelling liquid (Peyton et al., 1978) that has been long (Home, 19 14) and widely (Meister, 1995) used as a fumigant of stored gram (to control insects) and, to a lesser extent, as a fumigant of soil to control fungi. *Although Trichoderma viride* (Martin et al., 1957), *Verticiilium albo-atrum* (Wilhelm and Ferguson, 1953) and some microfungi (Morrell, 1990) are relatively tolerant of CS_2, most fungi are eradicated by high concentrations of CS_2. High concentrations of CS_2 completely eradicated *Alternaria altemata, Cladosporium cladosporioides, Penicillium chrysogenum* and *Epicoccum purpurascens* from paddy rice (Mazen et al., 1988), and *Armillaria mellea* in an agar medium (Munnecke et al., 1973), soil (Bliss, 195 1; Thomas and Lawyer, 1939) and ponderosa pine (*Pinus ponderosu* Laws.) stumps (Filip and Roth, 1977). An application of sodium tetrathiocarbonate, which releases CS_2 when applied as an aqueous solution to soil, reduced zoospore motility, zoospore cyst viability, sporangia production and mycelial growth of *Phytophthora citrophthora* and *Phytophthora parasitica* (Materon and Matejka, 1989). Also, an application of 1 $\mu g\ CS_2\ ml^{-1}$ did not stimulate germination of sclerotia of Sclerotium cepivorum contained in soil (Coley-Smith and King, 1969).

Thirty-one plant species are known to produce CS_2 (Hartel and Haines, 1992), including all known Leucaena species (Feng and Hartel, 1996). In addition, three other plant species, alfalfa (*Medicago sativa* L.), corn (*Zea mays* L.) and white oak (*Quercus alba* L.; Westberg and Lamb, 1984) may produce CS_2, but these results are questionable because the plants were grown in nonsterile soil and some soil bacteria can produce organic sulfides (Kelly and Smith, 1990). Low concentrations of CS_2 do not inhibit the growth, photosynthesis or respiration of plants (Abeles, 1984).

Can CS_2 produced by plants affect soil fungi? Kelly and Smith (1990) suggest that the roots of CS_2-producing plants should influence soil fungi. In an abstract, Lewis and Papavizas (1969) observed that volatile organic sulfides, including CS_2, produced from a steam distillate of cabbage stems *affected* several *stages* of *the* life cycle of *Aphanomyces euteiches.* In

Australia, valuable timber *trees* of *the Eucalyptus* genus were protected from *the* soil fungus, *Phytophthora cinnamomi*, *when* grown *with Acacia pulchella* (Shea et al., 1978). CS_2 is a major volatile constituent of the roots of *Acacia pulchella.*

In this study, *we* determined *if* a model C&producing plant, *Mimosa pudica,* could affect colonisation of its rhizoplane by six common soil fungi. Because tomato *'(Lycopersicon esculentum)* does not produce CS_2 (J. Piluk, personal communication) and is readily colonised by many fungi (Jones et al., 1991), this plant was used as a negative control. All plants were grown griotobiotically to ensure CS_2 was of a plant rather than microbial origin.

2. METHODS

2.1 Seeds

Seeds of *Mimosa pudica* L. were' obtained from Vaughan's Seed Company, Downers Grove, Ill., and were sterilised as described by Hartel and Haines (1992) except the NaOCl concentration was increased from 0.2 1 to 0.71 M. Tomato *seeds (Lycopersicon esculentum* Mill. cv. Marion) were obtained commercially and were sterilised according to the method of Hartel et al. (1989). All seeds were germinated on water agar (15 g agar L-I distilled water) at 30°C for 2 to 3 days.

2.2 Plant assemblies

Plants were grown in gnotobiotic plant assemblies as described by Hartel et al. (1993). Briefly, each sterile seedling was contained in a sterile packet of Versapor membrane (0.22 urn-pore size; Gelman Sciences, Inc., Ann Arbor, Mich.) which was held in place with sterile sand in a covered, 52 by 200-mm (diameter by height) Pyrex tube. Each assembly received a plant nutrient solution supplying macro- and micro-nutrients as described by McClure and Israel (1979) and Ahmed and Evans (1960), respectively. All ingredients were half-strength except NaFeEDTA and the micronutrients, which were full strength. In addition, the N content of the medium was strengthened with KNO_3 (0.72 g L^{-1}, -100 mg N L^{-1}). Because production

of plant-generated organic sulfides is S-dependent (Feng and Hartel, 1996), the plant nutrient medium was also amended with 0.88 g $CaSO_4$ L^{-1} (equivalent to 150 mg S L^{-1}). Plants were grown under metal halide, high-intensity-discharge lamps with the light filtered through 3 cm of water (to reduce infrared radiation). The average light intensity at the top of each assembly was 550 µM photons m^{-2} sec^{-1} on a 1618-h light/dark cycle. The day/night temperatures were 30 and 26°C, respectively.

2.3 Fungi

The six species of fungi, *Fusarium moniliforme* J. Sheld, *Pythium aphanidermatum* (Edson) Fitzp., *Phytophthora sp., Rhizoctonia solani* Kiihn, *Sclerotium rolfsii* Sacc. and *Trichoderma viride* Pers.: Fr., were from our culture collection. All fungi were maintained on V-8 juice agar (Tuite, 1969) at 30°C.

2.3.1 Inhibition of mycelial growth and spore germination by CS_2

All fungi were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) at 30°C for 2 to 5 days until the mycelium reached the edge of the agar plate. A 9-mm plug of mycelia was removed from the outer edge of the mycelium with a sterilised cork borer, and the plug was transferred to the centre of a fresh potato dextrose agar plate. Three 10-cm petri plates of each species were placed in a sealed bell jar with a septum in the lid. Liquid CS_2 (HPLC grade, Sigma Chemical Co., St. Louis, MO.) was added to each jar to give '0.05, 0.3, 1, 2 and 4 µg CS_2 ml" or the jar was left unamended (negative control). Each day the jar was sampled through the septum and the headspace analysed for CS_2 by gas chromatography (Hartel and Haines, 1992). After each sampling, the top of the jar was removed and the growth of the mycelium recorded as an average diameter. The top of the jar was replaced and fresh CS_2 added to maintain the appropriate concentration.

Zoospores of *P. aphanidermatum* were produced first by growing the fungus on V-8 juice agar for 2 days at 30°C. Four 9-mm plugs of new mycelial growth were removed and the plugs were placed in a sterile, 60 mmdiameter petri dish containing 10 mL of sterile, distilled water. To stimulate production of sporangia, the petri dish was incubated at 30°C overnight. The water was replaced and the petri dish was incubated for an additional 2 to 3 hours to release *the* zoospores. Spores of *F. moniliforme*

and T. ***viride*** were collected from cultures grown on V-8 juice agar at 30°C for 5 days. The plates were flooded with sterile distilled water, and the conidia were dislodged from the mycelium with a sterile glass rod. The spore suspension was poured through four layers of sterile cheesecloth to remove large mycelial fragments. Spores and zoospores were diluted to a 'density of 4 to 50 spores μL^{-1} with sterile distilled water and three 8-µl portions of each suspension were added to each water agar plate. The plates were exposed to CS_2 as previously described. Germination was determined by counting the number of spores with germ tubes under 40X magnification. A spore was considered germinated if the germ tube length exceeded the spore width. In addition, germ tube length was measured for a minimum of 10 germinated spores. Spore germination and germ tube length of ***F. moniliforme***, ***P. aphanidermatum*** and ***T. viride*** were determined after 16, 7 and 18 hours incubation at 30°C, respectively. The experiment was repeated twice.

2.3.2 Rhizoplane studies

The potential effects of plant-generated CS_2 on the growth of the six fungal species was assessed by a combination of visual observation, spore counts and ergosterol production. Fungi were grown on V-8 juice. agar at 30°C. After 5 days, a 3 by 0.5 mm (diameter by depth) circular plug from the colony margin of each fungal species was placed in the comer of the Versapor packet of a gnotobiotic assembly containing an 11 day-old tomato. or ***M. pudica*** plant. For fungal spore counts in the rhizosphere of tomato and ***M. pudica.*** only ***F. moniliforme*** and ***T. viride*** were tested; under our conditions, the other species did not sporulate. Membrane packets were removed from the gnotobiotic assemblies at 0, 3, 5, 7, 9, 11 and 14 d after inoculation for F. moniliforme and at 0, 2, 4, 7, 9 and 11 d after inoculation for ***T. viride.*** At each sampling day, the plant top was removed and the packet peeled apart and inverted. Roots (representing rhizoplane counts) and membranes (representing nonrhizosphere counts) were added separately to 20ml of sterile phosphate buffer (2.6 mM KH_2PO_4 and 3.7 mM K_2HPO_4; pH 7.0) contained in polyethylene bags and mixed for 1 min with a Stomacher blender (Seward Medical, London). The suspension was serially diluted with a Spiral Plater (Spiral Systems Instruments, Bethesda, Md.) onto potato dextrose agar plates. Plates were incubated for 2 d at 30°C before the colonies were counted. Three replicate plant assemblies were sampled on each sampling day. Plant roots and packets were dried at 50°C before weighing.

For visual observations, five replicates were used for each fungus. In addition, five assemblies of tomato and *M. pudica* were not inoculated (negative control). The experiment was repeated twice. The fungi were allowed to colonise the plants for 7 days, after which the packets were removed and cut open. The plant shoot was cut off and the roots were stained with Trypan blue (crystallised phenol, 20 g; 85% (w/w) lactic acid, 20 mL; glycerol, 40 mL,, Trypan blue, 50 mg; distilled water, 40 mL) for 1 min. The roots were observed microscopically at 40X for the presence or absence of hyphae. Colonisation was scored: –, no hyphae observed; +, few hyphae observed; ++, many hyphae observed; +++, abundant hyphae. To confirm these visual observations, plants were also assayed for ergosterol by high performance liquid chromatography according to the method of Nylund and Wallander (1992) as modified by Sung et al. (1995). Two replicates of 10 plants each were tested for each fungal species. Because of the small size of the seedlings, 10 plants were necessary for each ergosterol analysis. Tomato and *M. pudica* plants were grown and inoculated as described for the visual observation. Plants inoculated with an agar plug only were the negative control. The plant shoot was cut off and the roots were combined for each ergosterol analysis.

3. RESULTS

3.1 Inhibition'of mycelial growth and spore germination by CS_2

The six fungal species showed variable responses to different levels of CS_2 (data not shown). The mycelial growth of the six species was not inhibited by either 0.05 or 0.3 μg CS_2 ml^{-1}. At 1 μg CS_2 ml", only the mycelial- growth of *F. moniliforme* was significantly inhibited; the remaining fungi were not inhibited. At 2 μg CS_2 ml", the mycelia of *F. moniliforme*, *R solani, and S. rolfsii* were significantly inhibited. At 4 μg CS_2 ml", the mycelial growth of all the fungal species were strongly or completely inhibited.

Spore *germination* of *F. moniliforme*, *P. aphanidermatum* and T. *viride* was also inhibited by CS_2 (Table 1). As CS_2 concentration increased, spore germination for *F. moniliforme* (correlation coefficient, r = -0.83), *P. aphanidermatum* (r = -0.93) and *T. viride* (r = -0.93) decreased significantly ($p<0.05$) for all three species. As CS_2 concentration increased, the germ

tube length for *F. moniliforme* (r = -0.66; not significant, $p<0.05$), *P. aphanidermatum* (r = -0.73; significant, $p<0.05$) and *T. viride* (r = -0.76; significant, $p<0.05$) showed a similar trend.

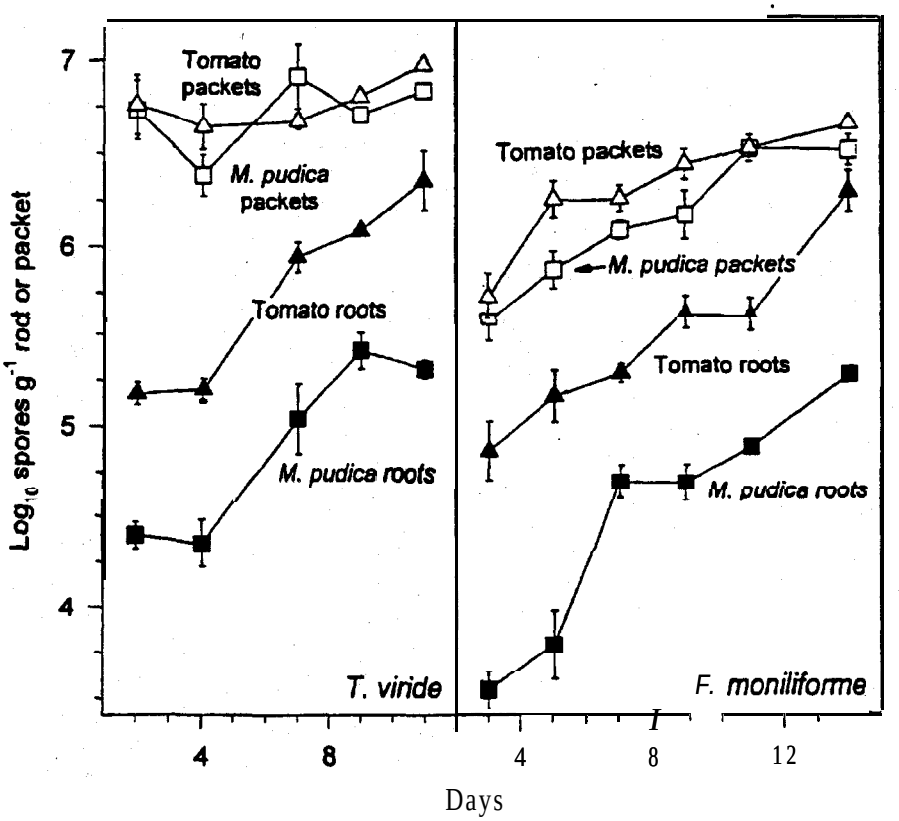

Figure I. Numbers of spores of Fusarium momiliforme or Trichoderma viride per plant on roots (representing rhizoplane counts; closed symbols) or on packet membrane (representing nonrhizosphere counts; open symbols) containing Mimosa pudica (squares) or tomato (Lycopersicon esculentum; triangles). Error, ±1 SE.

3.2 Rhizoplane studies

When lld-old gnotobiotic tomato or M. pudica plants were inoculated *with F. moniliforme* or *T. viride*, spore counts were similar in the Versapor

packet (representing nonrhizosphere counts), but 10- to 100-fold lower on the rhizoplane of *M. pudica* than those of tomato regardless of sampling time (Fig. 1).

Visual observation of mycelial growth of F. moniliforme, *P. aphanidermatum, Phytophthom sp., R. solani, S. rolfsii* and *T. viride* on the roots of gnotobiotic tomato plants showed abundant hyphae 7 days after inoculation (Table 2). No hyphae were observed on the uninoculated control. ln contrast, abundant or many hyphae were only observed for *R. solani* and S. *rolfsii* on the roots of 18-day-old gnotobiotic *M. pudica; few* hyphae of *P. aphanidennatum and T. vin'de* and no hyphae of *F. moniliforme* and *Phytophthora sp. were* observed. Therefore, with the exception of *R solani*, all the fungi were inhibited to a varying degree in the rhizosphere of *M. pudica.* Observations for *F. moniliforme, R solani* and *S. rolfsii* were confirmed by ergosterol analysis. No detectable level of ergosterol production was observed for *P. aphanidermatum, Phytophthora sp., T. viride* or the negative control for either *M. pudica* or tomato.

4. DISCUSSION

Evidence that CS_2 from a CS_2-producing plant can inhibit soil fungi on the rhizoplane is only circumstantial. However, several facts tend to support this hypothesis. First, CS_2 is fungicidal (Meister, 1995). This was confirmed here with the inhibition of spore formation of *F. moniliforme, P. aphanidermatum* and *T. viride,* and the inhibition of mycelia of the six fungal species by low concentrations of CS_2 (1 to 4 μg of CS_2 ml-'). Some differences were observed between the growth of germ tubes and mycelia of *F. moniliforme, P. aphanidermatum* and *T. viride* under different CS_2 conditions, but this may be age-related because the germ tubes represent nascent rather than mature mycelia.

Second, *M. pudica* is capable of producing CS_2. Under the same conditions described here, gnotobiotic *M. pudica* produced an average of 9.5 ng CS_2 mg^{-1} root dry weight (Feng and Hartel, 1996). Although it is methodologically difficult to measure CS_2 concentrations in the rhizosphere, CS_2 is likely to persist for some time because its lifetime in the atmosphere is approximately 12 days (Khalil and Rasmussen, 1984) and the number of organisms capable of using CS_2 as an energy source is restricted to a only few strains of thiobacilli (Jordan et al., 1995; Plas et al.; 1993; Smith and Kelly, 1988) and *Thiothrix ramosa* (Odintsova *et* al., 1993).

Table 1. Effect of different concentrations of CS2 on spore germination and germ tube length of *Fusarium moniliforme*, *Pythium aphanidetmaturm* and *Trichoderma viride*. After treatment *with* CS_2, spores of *F. moniliforme*, *P. aphanidermatum* and *T. viride* were incubated at 30^0 C and observed after 16, 7 and 18 hours, respectively. Error, ± 1 SE.

Fungus	CS_2	Germinated spores	Genn tube length
	µg ml-1		µm
F. monilifonne	0.0	385±4	200±18
	0.7	347± 12	110±17
	1.0	213±8	52±7
	3.0	157±3	24±3
	4.4	83±5	20±5
	6.4	53±7	12±2
	14.4	0	0
P. aphanidermatum	0.0	27±2	258±43
	0.5	22±3	166±17
	1.2	22±1	158±22
	2.5	18±2	102± 13
	3.0	18±2	85±10
	6.7	5±1	50±8
	12.4	0	0
	0.0	76±3	337±26
	0.4	69±6	117±23
	I.3	35±7	44±4
	2.3	6±1	17±3
	4.1	0	0

Third, the response of the fungi correspond well with the conditions necessary for CS_2 release. CS_2 is released from *the* roots of *M. pudica only* under conditions of root injury or rewetting the roots after drought stress (Hartel and Reeder, 1993). Since the *M. pudica* plants were not drought stressed, CS_2 would only be released if the fungi disturbed the roots. Here, all six fungal species grew on the roots of tomato, and no differences were observed *in* spore *counts* for either *F. moniliforme* or *T. viride* in *the* nonrhizosphere of tomato and *M. pudica*. This result would be expected on the rhizoplane and in the nonrhizosphere of tomato because this plant does not produce CS_2; this result would be expected in nonrhizosphere of M. pudica because the roots are not being disturbed. In contrast, with the exception of *R. solani*, visual observation showed that the same fungi inhibited by applied CS_2 were inhibited on *the* rhizoplane of *M. pudica*, and

the spore counts of *T. viride* and *F. monilifvrme* were lower on' the rhizoplane of *M. pudica than on tomato. These results* would be expected because the roots are being disturbed and CS_2 is being released. The reason *why R solani* was not inhibited on the rhizoplane is unclear.

Table 2. Presence of hyphae and level of ergosterol production of six fungal species (***Fusarium moniliforme, Pythium aphanidermatum, Phytophthom sp., Rhizoctonia solani,*** *Sclerotium* ***rolfsii*** and *Trichoderma viride)* on the roots of 1 l-day-old *Mimosa pudica* or tomato. The presence of fungi was observed on roots 7 days after inoculation.

Fungus	*M. pudica*		Tomato	
	Presence of hyphae[1]	Ergosterol[2]	Presence of hyphae	Ergosterol
		µg plant^{-1}		µg plant^{-1}
F. moniliforme	-	N D	+++	0.127
P. aphanidermatum	+	N D	+++	N D
Phyrophthora sp.	-	0.277	+++	0.263
R. solani	+++	0.186	+++	1.889
S. rolfsii	++	N D	+++	N D
T. viride	+	ND	-	ND
Control				

[1]–, no hyphae observed; +, few hyphae observed; ++, many hyphae observed, +++, abundant hyphae observed. Average of two replicates of five plants each.
'Average of two replicates of 10 plants each. ND, not detected.

In summary, it seems less reasonable that *M. pudica* would release CS_2, a known fungicide, for no purpose, and more reasonable *that M. pudica* would release CS_2 on the rhizoplane to inhibit fungi, at least partially. It is still possible that other compounds may be responsible. A mutant *M. pudica* plant, deficient in CS_2 production, would be necessary to prove this conclusively.

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