

# Inhibition of fungal colonization on the rhizoplane of the CS<sub>2</sub>-producing plant, *Mimosa pudica* L.

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**Abstract:** Carbon disulfide (CS<sub>2</sub>) is a colourless, volatile, foul-smelling, fungicidal liquid that is produced by some plants. We determined the ability of a model CS<sub>2</sub>-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<sub>2</sub>, 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<sub>2</sub>, but all mycelia were inhibited by 4 µg CS<sub>2</sub> ml<sup>-1</sup>. Inhibition of spore germination of *F. moniliforme*, *P. aphanidermatum* and *T. viride* was similar to mycelial 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 100-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 CS<sub>2</sub> may account for this decreased fungal colonisation, although other compounds may also be responsible.

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## 1. INTRODUCTION

CS<sub>2</sub> is a colorless, volatile, foul-smelling liquid (Peyton et al., 1978) that has been long (Home, 1914) and widely (Meister, 1995) used as a fumigant of stored grain (to control insects) and, to a lesser extent, as a fumigant of soil to control fungi. Although *Trichoderma viride* (Martin et al., 1957), *Verticillium albo-atrum* (Wilhelm and Ferguson, 1953) and some microfungi (Morrell, 1990) are relatively tolerant of CS<sub>2</sub>, most fungi are eradicated by high concentrations of CS<sub>2</sub>. High concentrations of CS<sub>2</sub> completely eradicated *Alternaria alternata*, *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, 1951; Thomas and Lawyer, 1939) and ponderosa pine (*Pinus ponderosa* Laws.) stumps (Filip and Roth, 1977). An application of sodium tetrathiocarbonate, which releases CS<sub>2</sub> 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 µg CS<sub>2</sub> ml<sup>-1</sup> 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<sub>2</sub> (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<sub>2</sub>, 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<sub>2</sub> do not inhibit the growth, photosynthesis or respiration of plants (Abeles, 1984).

Can CS<sub>2</sub> produced by plants affect soil fungi? Kelly and Smith (1990) suggest that the roots of CS<sub>2</sub>-producing plants should influence soil fungi. In an abstract, Lewis and Papavizas (1969) observed that volatile organic sulfides, including CS<sub>2</sub>, 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<sub>2</sub> is a major volatile constituent of the roots of *Acacia pulchella*.

In this study, we determined if a model CS<sub>2</sub>-producing plant, *Mimosa pudica*, could affect colonisation of its rhizoplane by six common soil fungi. Because tomato (*Lycopersicon esculentum*) does not produce CS<sub>2</sub> (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 gnotobiotically to ensure CS<sub>2</sub> 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 0.21 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<sup>-1</sup> 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 µm-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<sub>3</sub> (0.72 g L<sup>-1</sup>, ~100 mg N L<sup>-1</sup>). Because production

of plant-generated organic sulfides is S-dependent (Feng and Hartel, 1996), the plant nutrient medium was also amended with  $0.88 \text{ g CaSO}_4 \text{ L}^{-1}$  (equivalent to  $150 \text{ mg S L}^{-1}$ ). 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  $550 \mu\text{M photons m}^{-2} \text{ sec}^{-1}$  on a 16/8-h light/dark cycle. The day/night temperatures were 30 and  $26^\circ\text{C}$ , respectively.

## 2.3 Fungi

The six species of fungi, *Fusarium moniliforme* J. Sheld, *Pythium aphanidermatum* (Edson) Fitzp., *Phytophthora* sp., *Rhizoctonia solani* Kühn, *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  $30^\circ\text{C}$ .

### 2.3.1 Inhibition of mycelial growth and spore germination by $\text{CS}_2$

All fungi were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) at  $30^\circ\text{C}$  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  $\text{CS}_2$  (HPLC grade, Sigma Chemical Co., St. Louis, Mo.) was added to each jar to give 0.05, 0.3, 1, 2 and  $4 \mu\text{g CS}_2 \text{ ml}^{-1}$  or the jar was left unamended (negative control). Each day the jar was sampled through the septum and the headspace analysed for  $\text{CS}_2$  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  $\text{CS}_2$  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  $30^\circ\text{C}$ . Four 9-mm plugs of new mycelial growth were removed and the plugs were placed in a sterile, 60 mm-diameter petri dish containing 10 mL of sterile, distilled water. To stimulate production of sporangia, the petri dish was incubated at  $30^\circ\text{C}$  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  $\mu\text{L}^{-1}$  with sterile distilled water and three 8- $\mu\text{l}$  portions of each suspension were added to each water agar plate. The plates were exposed to  $\text{CS}_2$  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  $\text{CS}_2$  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 corner 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 20 ml of sterile phosphate buffer (2.6 mM  $\text{KH}_2\text{PO}_4$  and 3.7 mM  $\text{K}_2\text{HPO}_4$ ; 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<sub>2</sub>

The six fungal species showed variable responses to different levels of CS<sub>2</sub> (data not shown). The mycelial growth of the six species was not inhibited by either 0.05 or 0.3 µg CS<sub>2</sub> ml<sup>-1</sup>. At 1 µg CS<sub>2</sub> ml<sup>-1</sup>, only the mycelial growth of *F. moniliforme* was significantly inhibited; the remaining fungi were not inhibited. At 2 µg CS<sub>2</sub> ml<sup>-1</sup>, the mycelia of *F. moniliforme*, *R. solani*, and *S. rolfsii* were significantly inhibited. At 4 µg CS<sub>2</sub> ml<sup>-1</sup>, 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<sub>2</sub> (Table 1). As CS<sub>2</sub> 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<sub>2</sub> 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.

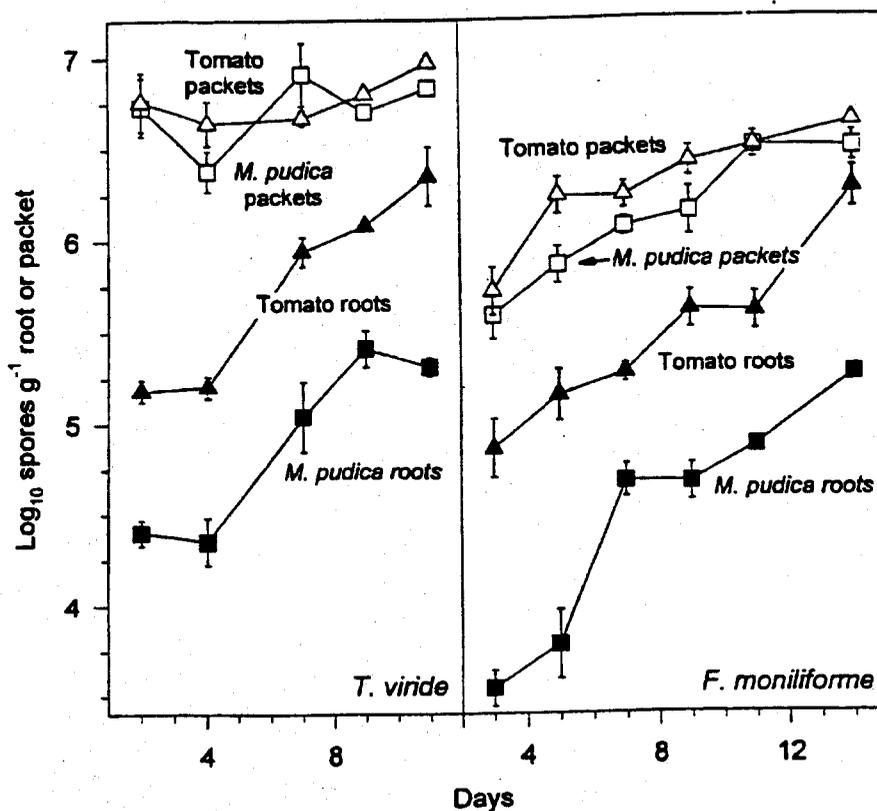


Figure 1. Numbers of spores of *Fusarium moniliforme* or *Trichoderma viride* per plant on roots (representing rhizoplane counts; closed symbols) or on packet membrane (representing nonrhizosphere counts; open symbols) containing *Mimosa pudica* (squares) or tomato (*Lycopersicon esculentum*; triangles).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

### 3.2 Rhizoplane studies

When 11-d-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*, *Phytophthora 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. In 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. aphanidermatum* and *T. viride* 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<sub>2</sub> from a CS<sub>2</sub>-producing plant can inhibit soil fungi on the rhizoplane is only circumstantial. However, several facts tend to support this hypothesis. First, CS<sub>2</sub> 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<sub>2</sub> (1 to 4 µg of CS<sub>2</sub> ml<sup>-1</sup>). Some differences were observed between the growth of germ tubes and mycelia of *F. moniliforme*, *P. aphanidermatum* and *T. viride* under different CS<sub>2</sub> 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<sub>2</sub>. Under the same conditions described here, gnotobiotic *M. pudica* produced an average of 9.5 ng CS<sub>2</sub> mg<sup>-1</sup> root dry weight (Feng and Hartel, 1996). Although it is methodologically difficult to measure CS<sub>2</sub> concentrations in the rhizosphere, CS<sub>2</sub> 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<sub>2</sub> 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 CS<sub>2</sub> on spore germination and germ tube length of *Fusarium moniliforme*, *Pythium aphanidermatum* and *Trichoderma viride*. After treatment with CS<sub>2</sub>, spores of *F. moniliforme*, *P. aphanidermatum* and *T. viride* were incubated at 30° C and observed after 16, 7 and 18 hours, respectively. Error, ±1 SE.

Fungus	CS <sub>2</sub>	Germinated spores	Germ tube length
	µg ml <sup>-1</sup>		µm
<i>F. moniliforme</i>	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
<i>P. aphanidermatum</i>	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
	1.3	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<sub>2</sub> release. CS<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>; 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<sub>2</sub> were inhibited on the rhizoplane of *M. pudica*, and

the spore counts of *T. viride* and *F. moniliforme* were lower on the rhizoplane of *M. pudica* than on tomato. These results would be expected because the roots are being disturbed and CS<sub>2</sub> 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*, *Phytophthora sp.*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Trichoderma viride*) on the roots of 11-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 <sup>1</sup>	Ergosterol <sup>2</sup> µg plant <sup>-1</sup>	Presence of hyphae	Ergosterol µg plant <sup>-1</sup>
<i>F. moniliforme</i>	-	ND	+++	0.127
<i>P. aphanidermatum</i>	+	ND	+++	ND
<i>Phytophthora sp.</i>	-	0.277	+++	0.263
<i>R. solani</i>	+++	0.186	+++	1.889
<i>S. rolfsii</i>	++	ND	+++	ND
<i>T. viride</i>	+	ND	-	ND
Control	-			

<sup>1</sup>-, no hyphae observed; +, few hyphae observed; ++, many hyphae observed; +++, abundant hyphae observed. Average of two replicates of five plants each.  
<sup>2</sup>Average of two replicates of 10 plants each. ND, not detected.

In summary, it seems less reasonable that *M. pudica* would release CS<sub>2</sub>, a known fungicide, for no purpose, and more reasonable that *M. pudica* would release CS<sub>2</sub> 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<sub>2</sub> production, would be necessary to prove this conclusively.

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