Effects of Pesticide Seed Treatments on *Rhizobium japonicum* and Its Symbiotic Relationship with Soybean*

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Pesticide seed treatment is used to control insect damage and seed-borne pathogens and to promote early plant emergence. Information available on the effect of pesticides on rhizobia, and subsequent growth and nodulation on leguminous plants is limited. Some workers have demonstrated the need for fungicidal seed treatment for soybeans (HARTY and BYGOTT, 1964; SINCLAIR and SHURTLEFF, 1975) and insecticidal treatment to control early attacks by the bean fly (JONES, 1965) and seed harvesting ants (CHAMP et al., 1961). Problems arise when legume inoculants are used in conjunction with pesticide treatments. Damage to the inoculants by fungicidal seed treatment (HARTY and BYGOTT, 1964) and insecticides (BRAITHWAITE et al., 1958) has been reported.

The use of chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) as an insecticide has increased in the past few years. Chlorpyrifos is a wide spectrum organophosphorus insecticide. It is less soluble in water than diazinon (O,O-diethyl O-2-isopropyl-4-methyl-6-pyrimidinyl phosphorothioate), but volatile and existent on plant surfaces. These two insecticides had similar effects on bacteria and fungi in soil (TU, 1970). Conversely, SIVASITHAMPARAM (1970) showed that chlorpyrifos did not cause any appreciable change in the number of aerobic bacteria but appeared to have a stimulating effect on soil actinomycete populations in a submerged soil. Diazinon has been recommended as one of the insecticides in soybean seed treatment (OPAC, 1975). Chlorpyrifos was chosen in preference to diazinon in this investigation because of its promising effectiveness for insect control and effects on microbial activity.

The relative toxic effects of pesticide seed treatments on rhizobium symbiotic nitrogen fixation and subsequent growth of soybean plants under controlled environments were assessed in the work reported here.

MATERIALS AND METHODS

Soybean plants (Glycine max Merr. variety Chippewa) inoculated with a commercial soybean inoculant (The Nitragin Co., Milwaukee, Wis. 53209) were grown in white silica sand (natural grain) in 550-ml plastic cottage cheese containers in a growth chamber for

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3 to 4 weeks at temperatures programmed to vary from 23° to 28°C with 15-hr photoperiod. The sand was autoclaved at 15 lb of steam pressure at 121°C for 5 hr on three successive days then oven-dried for 3 hr at 105°C. The sand was irrigated with nitrogen-free nutrient solution (WILSON and REISENAUER, 1963) each day and once each week it was flushed with water to prevent accumulation of salts. Drain holes in the bottom of each container permitted the escape of excess nutrient solution or water.

From each of the plants, one nodule chosen on the basis of size and pigmentation was exised. Nodules were washed first with sterile distilled water then suspended and shaken for 5 min in 1:1000 $HgCl_2$ then rinsed 5 times immediately with sterile distilled water. After macerating by grinding with a glass rod, the macerate was removed by a pipette to dilution bottles for plating by the pour plating method using yeast extract mannitol agar (YEMA).

Colonies suspected of fixing N_2 were tested for purity and acetylene-reducing capacity. Each test organism was cultured in duplicate at 28°C in 10 ml of medium (HINO and WILSON, 1958). Tubes were sealed with serum stoppers, evacuated and filled with a gas mixture containing 0.4% CO_2:22% O_2:77.6% argon. Each culture received 0.2 ml of purified acetylene at time zero for the assay of C_2H_4 production by gas chromatography as a qualitative test for nitrogenase activity. Isolates were taxonomically identified to species by the accepted characteristics in Bergey's Manual of Determinative Bacteriology (BUCHANAN and GIBBONS, 1974).

Each isolate at 2 X 10^6 cells/seedling was used to inoculate 16 disinfected soybean seeds. Sixteen seedlings were also inoculated with each of the authentic strains, Rhizobium japonicum 3I1b6 and 3I1b110, at the same cell number per seedling to provide standards for comparison with the isolates. Numbers of nodules formed on taproots and laterals were counted separately.

The isolate R. japonicum No. 16 was used for further study. The cell suspension of culture No. 16 was obtained by incubation in YEM broth for 3 days at 28°C on a shaker. Seeded medium for paper disc assays was prepared by adding 10 ml of the suspension to 100 ml of the YEMA at 45°C before dispensing into petri dishes. The required amounts of pesticides (Table 1) were dissolved in acetone and applied on 10-mm filter paper discs by micropipettes. Control discs were dipped into acetone. The acetone was allowed to evaporate before placing the disc onto the agar surface. Plates were incubated at 28°C for 7 days and with zones of inhibited growth surrounding the discs were measured.

Soybean seeds were disinfected by treating with 75% ethanol 3-5 min followed by $HgCl_2$ (1:1000) for 5 min. After treatment the seeds were washed five times with sterile distilled water. Seeds were disseminated in sterile petri dishes and placed in a 37°C incubator so as to affect immediate drying. To determine the effects of insecticide-fungicide seed treatments on R. japonicum and its symbiotic relationship with soybean root nodules, two insecticides, lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane), chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) and a fungicide, thiram (tetramethylthiuram disulphide) were applied. The rates of application were 14 g per 27 kg (bu) of seed for technical lindane and at 21 g for thiram (OPAC, 1975).

TABLE 1

Levels of pesticide (g/27 kg seed) applied to medium for paper disc assay on inhibiton of growth of R. japonicum No. 16

Level	Lindane	Chlorpyrifos	Thiram
0.1XR*	1.4	0.85	2.1
1XR	14.0	8.50	21.0
10XR	140.0	85.00	210.0

* R: Concentration equivalent to that used for seed treatment.

Chlorpyrifos was applied at 8.5 g which is equivalent to the amount for diazinon. Pesticides used for seed treatment were dissolved in acetone and applied to soybean seeds as a slurry suspended in 0.5% methyl cellulose** solution. Seeds were tumble-dried. Treated seeds were placed on YEMA and incubated for 48-72 hr at 28°C. During this incubation the seeds were rotated periodically on the agar to ensure that all outside portions of the seed contacted the agar surface. Eight germinated seedlings free from comtaminating microorganisms were planted in 650 g of moist sterile silica sand in a plastic container. A YEM broth culture of R. japonicum No. 16 at 2 X 10^7 cells/seedling was added to the soybean seedlings for all treatments except the uninoculated control. The experiment was conducted in a growth chamber and supplied with nitrogen free nutrient solution as in a similar manner described above.

After 3, 6, and 8 weeks, soybean plants growing in silica sand were sealed in 7000 ml polyethylene containers. The lid was fitted with a sleeve type rubber serum stopper. The sand was adjusted to 60% of moisture holding capacity (TU and HIETKAMP,1976). Acetylene gas was added to provide 0.029 atm using a 3-ml hypodermic syringe and the gas phase was sampled for C_2H_4 analysis after 60 min. At the conclusion of an incubation period, 2.5-ml gas samples were withdrawn from the container by a gas-tight syringe and chromatographed immediately to avoid storage problems. Ethylene was measured by a Varian Aerograph model 600-C gas chromatograph equipped with a hydrogen flame ionization detector and a Beckman recorder. A column 1.5 m in length by 2 mm internal diameter, was packed with Porapak T (100 - 120 mesh, 30-10 PAK, Waters Assoc. Inc., Framingham, Mass.) and maintained at an oven temperature of 60°C. Nitrogen was the carrier gas at a flow rate of 30 ml/min. Ethylene determinations were based on the average of at least five 2.5-ml sample injections. Peak areas for C_2H_4 and C_2H_2 were directly proportional to their respective concentrations over the range utilized in all assays. The retention times for C_2H_4 and C_2H_2 were approximately 45 and 120 sec respectively.

** Methyl cellulose is "Methocel" of Dow Chemical Co.

TABLE 2

Production of ethylene and number of nodules on roots of 16 plants by *Rhizobium japonicum*

Inoculant Strain	Nodule number per plant		C_2H_4 production
	Taproot	Laterals	µM/culture/day
1	6 ef*	2 ef	9 ab
2	5 ef	7 ab	12 a
3	7 de	7 ab	4 b
4	5 ef	5 abcdef	6 ab
5	7 de	6 abcd	4 b
6	10 abcd	7 ab	3 b
7	8 cde	6 abcd	6 ab
8	9 bcde	6 abcd	5 ab
9	8 cde	5 abcdef	6 ab
10	5 ef	5 abcdef	5 ab
11	7 de	4 bcdef	9 ab
12	10 abcd	5 abcdef	5 ab
13	9 bcde	8 a	4 b
14	9 bcde	3 def	7 ab
15	12 ab	6 abcd	5 ab
16	14 a	8 a	10 a
17	11 abc	3 def	6 ab
Rhizobium japonicum 3I1b6	3 f	6 abcd	7 ab
Rhizobium japonicum 3I1b110	7 de	5 abcdef	9 ab

* Values within each column indicated by the same letter are not significantly different at the 5% level determined by Duncan's multiple range test.

At the end of the experiment, plants were separated into leaves, stems, roots and nodules and oven-dried for dry weight determinations.

RESULTS AND DISCUSSION

Nodule formation on soybean roots after inoculation with *R. japonicum* is shown in Table 2. With the exception of *R. japonicum* 3I1b6 and Nos. 1, 2, 4, and 10, most isolates showed good formation of nodules on taproots. The taproot nodulation of soybeans is a qualitative characteristic, which appears useful as an index of the level of adequacy and activity of bacterial inoculum at planting. For nodules to be formed on the upper taproot of soybeans, they must be initiated 6 days after planting (VEST *et al.*, 1973). WEAVER and FREDERICK (1972) reported that total nodule mass on lateral roots was not increased by inoculation, but inoculation increased total nodule mass on taproots. Plants with only lateral root nodulation had an extended delay in setting out of nitrogen fixation.

Nineteen *R. japonicum* cultures reduced C_2H_2 and produced respectively 3 to 12 µM of C_2H_4 per 10 ml medium per day (Table 2). The capacities of organisms to reduce C_2H_2 to C_2H_4 provide presumptive evidence of N_2 fixation.

The effects of pesticides on growth of the rhizobia are summarized in Table 3. The data are averages of 6 replicates.

TABLE 3

Effect of pesticides on growth of R. japonicum No. 16 determined by a paper disc inhibition method (width of inhibition zones in mm)

Level	Lindane	Chlorpyrifos	Thiram	Lindane + Chlorpyrifos	Lindane + Thiram	Chlorpyrifos + Thiram	Lindane + Chlorpyrifos + Thiram
0.1XR*	1.7 b**	1.0 b	7.2 c	2.0 b	10.0 c	6.5 c	8.3 c
1XR	4.1 a	1.0 b	9.5 b	4.9 a	11.3 b	8.0 b	10.0 b
10XR	4.3 a	5.2 a	20.3 a	6.7 a	25.0 a	25.0 a	25.0 a

* R: Concentration equivalent to that used for seed treatment.
** Within each column, values accompanied by the same letter are not significantly different at the 5% level with Duncan's multiple range test.

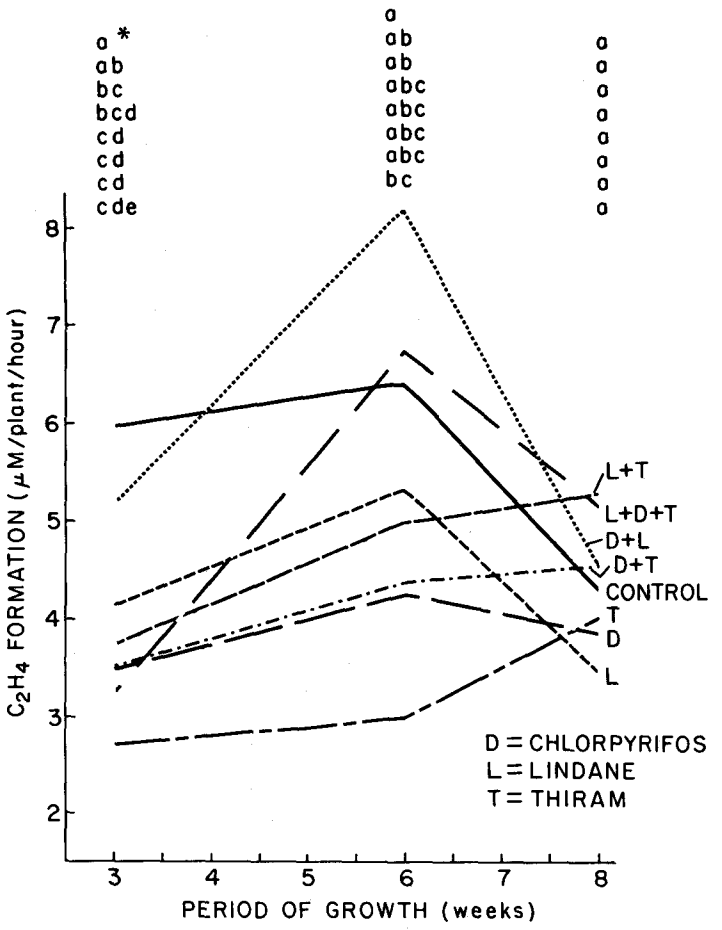

FIGURE 1. Acetylene reduction by soybean plants grown in silica sand in relation to insecticide-fungicide seed treatments (*Duncan's multiple range P=0.05).

Results obtained in the replicated experiments were similar. Chlorpyrifos had a small inhibition zone with the organism at 0.1 X R and 1 X R levels, but showed greater inhibition at 10 X R. Lindane produced moderately toxic effects on bacteria at the R and 10 times higher levels. Thiram was more toxic than the insecticides singly or in combination. It is interesting to note that the Rhizobium was stimulated at the periphery of the toxic zone. This was especially noticeable when thiram was used.

The acetylene reduction assay can be applied to identify the more efficient soybean-rhizobia associations. Figure 1 shows results of the treatments on the acetylene reduction of the soybean plants. There was a significant depression in the formation of C_2H_4 after 3 weeks with treatments of lindane, lindane +thiram, chlorpyrifos + thiram, chlorpyrifos, lindane + chlorpyrifos + thiram and with thiram alone. Plants showed a steady increase in activity with time. After 6 weeks' growth, however, the nitrogenase activity in the reduction of C_2H_2 was, with the exception of thiram, equal to or greater than that of the control plants. No significant difference in the acetylene-reducing activity of the soybean plants was noted after 8 weeks.

Typical growth of the soybean plants 3 weeks after seed treatment with lindane, chlorpyrifos and thiram is shown in Figure 2. Increased dry weights of leaves, stem, root, and nodule were taken as the criteria of early root infection. A high degree of compatibility between R. japonicum and insecticides was evident in these experiments as shown in Table 4. There was some reduction in nodulation following fungicide treatment. Thiram singly or in combination with insecticides, was harmful to nodule formulation 3 weeks after treatments. The weights of leaves and stems also responded similarly as the nodules. However, no significant effects of the pesticide treatments were observed after 8 weeks in all samples.

There is evidently some factor determining pesticide-Rhizobium compatibility in these studies. It is possible that, under a good moisture condition in the free draining sand, the potentially toxic doses were mitigated by the dilution of the pesticides (ISAAC and HEALE, 1961), or by bacterial migration away from the toxic zones. Bacterial movement in soils is normally enhanced by higher moisture contents (ALEXANDER, 1961). Although the exact mechanism through which the moist sand or soil enables the rhizobia to survive is not known, the instances where crown root nodulation was suppressed, but later nodulation appeared on the lateral roots were found in the present study.

The differences in toxicity between seed protecting chemicals observed in this study seem to indicate that some pesticides are safer to use in conjunction with the legume inoculant than others. Rhizobium sp. may well differ in their relative sensitivity to pesticides in soil. The pesticidal treatments for legume seed protection are still of prime importance although some delaying in emergence of nodulation of the plants was observed.

These experiments give an explanation of the evidence obtained in laboratory studies on the compatability of rhizobia with various chemicals. The number of viable rhizobia in the inoculant-chemical

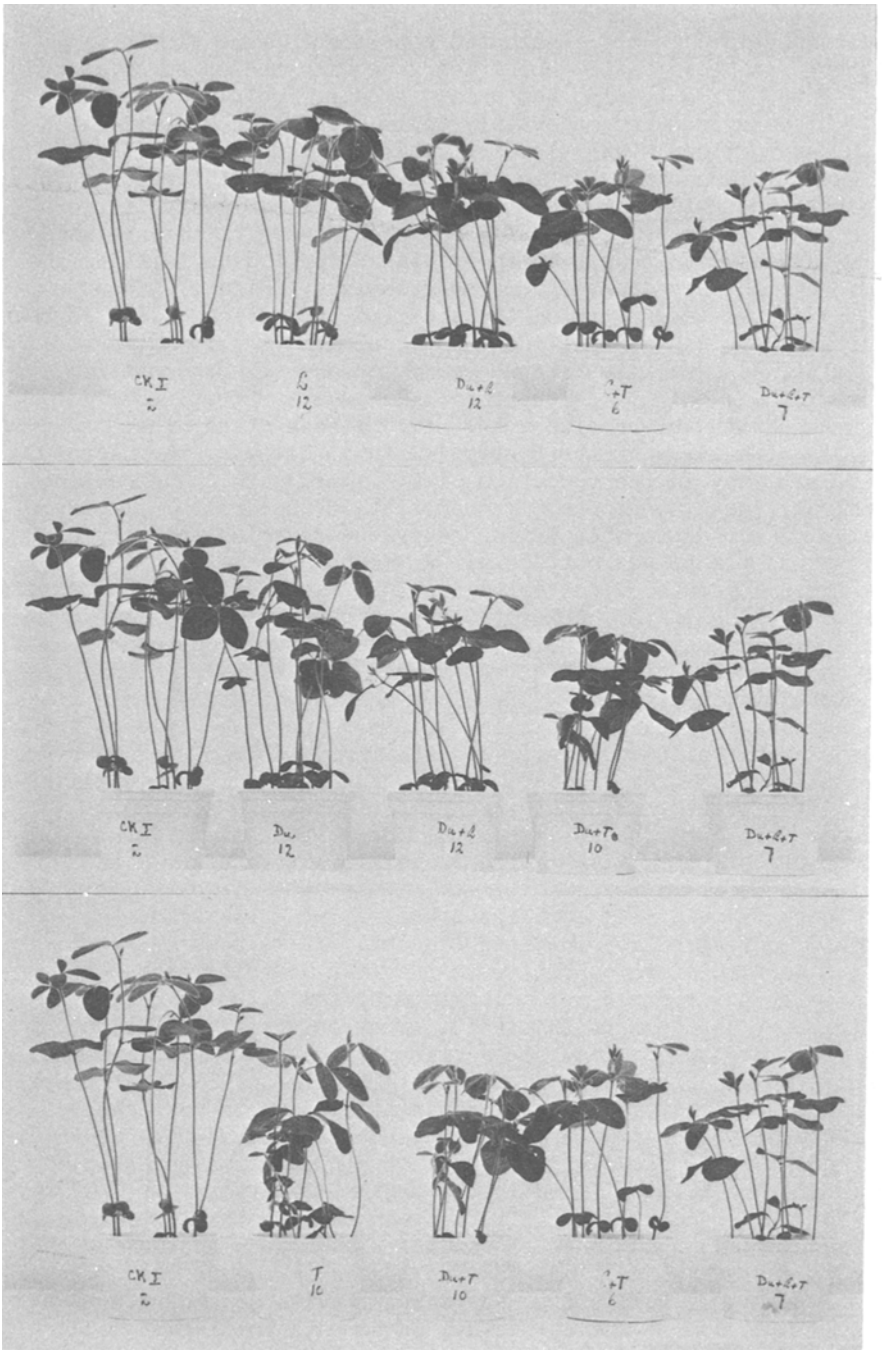

FIGURE 2. Effect of insecticide-fungicide seed treatment on soybean growth; (A) Lindane alone and in combination, (B) Chlorpyrifos alone and in combination, (C) Thiram alone and in combination. CK: Control, L: Lindane, Du: Chlorpyrifos, T: Thiram.

TABLE 4

Effect of insecticide-fungicide seed treatments on soybean growth measured at the two sampling times. Values are averages of 48 plants oven dry weight (g)

Treatment	Leaves		Stems		Roots		Nodules	
	Period of growth (weeks)							
	3	8	3	8	3	8	3	8
	Container number							
	1 - 6	7 - 12	1 - 6	7 - 12	1 - 6	7 - 12	1 - 6	7 - 12
Control	0.20 a*	0.32 a	0.15 a	0.24 bc	0.12 ab	0.09 ab	0.0179 a	0.0198 a
Lindane	0.19 ab	0.30 ab	0.22 a	0.24 bc	0.08 b	0.10 ab	0.0160 ab	0.0181 a
Chlorpyrifos	0.17 abcd	0.30 ab	0.13 abc	0.27 ab	0.09 b	0.08 b	0.0150 abc	0.0169 ab
Thiram	0.16 bcd	0.29 ab	0.11 c	0.21 cd	0.10 ab	0.11 ab	0.0133 bcd	0.0186 a
Lindane+chlorpyrifos	0.18 abc	0.30 ab	0.14 ab	0.24 bc	0.10 ab	0.10 ab	0.0153 ab	0.0162 ab
Lindane+thiram	0.13 cd	0.30 ab	0.10 cd	0.23 cd	0.08 b	0.11 ab	0.0100 de	0.0147 ab
Chlorpyrifos+thiram	0.15 bcd	0.26 ab	0.12 bc	0.20 cd	0.09 b	0.08 b	0.0107 cde	0.0125 abc
Lindane+chlorpyrifos+thiram	0.15 bcd	0.30 ab	0.12 bc	0.25 abc	0.08 b	0.10 ab	0.0102 de	0.0170 ab

* Within each column, mean values accompanied by the same letter are not significantly different at 5% level determined by Duncan's multiple range test.

mixture was reduced, however, decreases in the number of rhizobia were much smaller when the mixtures were planted in moist sand. It appears highly probable that these chemicals will have no permanent deleterious effects on the rhizobia and their activities important to the symbiotic association with soybeans and on the growth of plants.

SUMMARY

Seventeen Rhizobium japonicum cultures isolated from soybean nodules induced formation of nodules on taproots of soybean plants. All isolates reduced acetylene to ethylene to different extents in vitro. Paper disc assay indicated that two insecticides, lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane), chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), and a fungicide, thiram (tetramethylthiuram disulphide) individually or in combination caused significant inhibition of the growth of R. japonicum No. 16.

The effects of insecticide-fungicide seed treatments on the nitrogenase activity of soybean plants in nitrogen-fixing capacity, weights of leaves, stems, and nodules were determined. Thiram, singly or in combination with lindane and/or chlorpyrifos, significantly delayed growth of the plants and affected the activity of nitrogenase in the fixation of nitrogen 3 weeks after treatments. No drastic effect of any of the pesticide treatments on soybean plant growth was observed after 8 weeks.

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