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Effect of Fungicide Application on Soybean-Rhizobia Symbiosis and Isolation of Fungicide-Resistant Strains of *Rhizobia japonicum*

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Rhizobial inoculant is commonly applied to seeds of soybean and other leguminous crops. This inoculant is often used along with one or more fungicides to protect seedlings from soil borne pathogens. Adverse effect of fungicides on rhizobia in vitro has been reported (Mallik and Tesfai 1983; Tu 1980). Chamber and Montes (1982) found that captan and thiram (TMTD) adversely affected nodulation in soybean inoculated with peat inoculant but PCNB and benomyl were relatively harmless. Carboxin, oxycarboxin and tridemorph, applied at higher concentrations, were found to reduce nitrogenase activity (C_2H_2 reduction) in the clover (Trifolium repens)-rhizobia symbiosis, but not at their recommended concentrations (Fisher and Hays 1981). Similar adverse effect of fungicide application on nitrogenase activity was also reported (Fisher et al. 1978). To overcome this incompatibility between fungicide and rhizobia, Hossain and Alexander (1984) used a fungicide resistant strain of rhizobia as inoculant. The objectives of the present investigation were (1) to assess compatibility of Rhozobium japonicum with commonly used fungicides in soybean cultivation and (2) to isolate fungicide resistant strains of Rhizobium japonicum.

MATERIALS AND METHODS

Of 10 strains of Rhizobium japonicum tested for sensitivity against selected pesticides in agar plate tests, R. japonicum 3Ilb110 was moderately sensitive (Mallik and Tesfai 1983). Therefore, this strain was used in this investigation. The culture was grown on yeast extract-mannitol broth (YMB) (Vincent 1970) for 4 days in an aerated fermenter. The cells were harvested by centrifugation and suspended in fresh YMB; 5 ml of this suspension were injected into a plastic bag containing 50 g autoclaved finely ground peat which was previously neutralized to pH 7 by adding ($Ca(OH)_2$. The peat inoculant contained $4x10^8$ cells/g peat and the moisture content was ca. 45% of water holding capacity of this peat.

The following fungicides of commercial formulations were used: captan (Captan 50 WP), captafol (Difolatan), carboxin (Vitavax), fenaminsulf (Lesan), and PCNB (Terraclor).

Soybean (Glycine max (L.) Merrill) seeds cv. Forrest of uniform size were surface sterilized by treating with ethanol (95%) for 2 min followed by Clorox (50%) for 10 min. The seeds were rinsed five times with sterile water to wash off the Clorox, and were then dried for 2 h in a "Laminar flow" cabinet. The surface sterilized seeds were treated with fungicides at concentrations recommended by Oklahoma State University Cooperative Extension.* An appropriate quantity of the fungicide was made into a slurry with a few drops of water in a beaker; seeds were placed in the beaker, thoroughly mixed and tumble dried. The fungicide treated or untreated seeds (as control) were inoculated with slurry of peat inoculant (2 g of peat inoculant/100 g of seeds) made by mixing 1 g peat inoculant with 5 ml of 40% gum arabic hydrosol and then air dried.

Ten seeds were planted per pot containing 2 kg sandy loam soil (sand 79%, silt 14%, clay 7%, pH 6.1, organic carbon 1.2%, total nitrogen 0.03%). The pots were arranged in a randomized Latin square design with one line of untreated and uninoculated plants as the border row. The pots were sunk in sand boxes and the sand was watered by drip irrigation. The soil in the pots received water by capillary action through the bottom holes in the pots. Six replications of each treatment were used. After germination the plants were thinned to one per pot.

The plants were harvested 35 days after planting. At harvest, which took place between 8-9 A.M., the shoot was cut off at soil level, placed in a paper bag, dried at 75 C for 24 h in a forced draft oven, and the dry weight noted. The root-nodule system, carefully taken out from the pots, was transferred along with adhering soil to a "Mason jar" for determination of nitrogenase activity by the acetylene reduction technique (Hardy et al. 1968). This jar was fitted with a serum cap through a hole cut in the metal cap. Thirty ml of acetylene gas (99.9% purity) at 1 atm. pressure were injected into the "Mason jar" con-

*Extension Agent's Handbook of Insect, plant diseases and weed control, E-832, Cooperative Extension, Oklahoma State University, 1979.

taining roots with nodules. After incubation for 90 min at 25 C an aliquot of 5 ml gas from this jar was transferred to a 25 ml bottle with a serum cap. One ml of this gas sample was injected into a Gas Chromatograph (Packard, Model #417, fitted with a stainless steel column, 18.3 m x 3.2 mm, packed with Porapak N 80/200 mesh, and Flame Ionization Detector). Soon after the withdrawal of the gas sample from a "Mason jar" containing roots, the nodules were counted and weighed. The roots were oven dried for 24 h at 75 C and weighed.

The oven dried shoot was pulverized by Wiley Mill to pass through a 0.25 mm sieve. For determination of total N by Kjeldahl method one gram of oven dried plant material was digested in a "Buchi" block digester (Model #430). Total nitrogen was determined by titration using a "Metrohm titrator" (Model #526). Seed yield was determined from plants grown for 90 days. The seeds, separated from pods, were oven dried at 70 C for 48 h, and weighed.

The experiments were conducted in two consecutive growing seasons --1983 and 1984; however, the total N and seed yield were determined only in 1984.

For isolation of strains of R. japonicum resistant to a fungicide, the method of gradual acclimatization was followed (Odeyemi and Alexander 1978). Strains 3Ilb136, 3Ilb110 and ATCC 10324 were grown in YMB supplemented with 25 µg/ml of one of the fungicides at 27-28 C as shake cultures for 10 days. Concentration of each fungicide was incremented by 25 µg/ml every 10 days. At 250 µg/ml of YMB the culture was grown for 20 more days with change of old YMB with fresh YMB after 10 days.

To check purity, infectivity and effectivity of the fungicide resistant strain, viable cell counts were compared by two methods: 1) dilution plate method and 2) plant infection method (Vincent 1970) in combination with most probable number (MPN) method (Alexander 1965). Pregerminated soybean seeds on water agar were transferred to "Dispo Growth Pouches". Sterilized tap water was added to the pouches when needed. The plants were held in a greenhouse.

Appropriate dilutions of the fungicide-resistant strain were plated out on yeast extract-mannitol agar (YMA) supplemented with Congo Red dye. The same set of dilutions were used for inoculation of seedlings. Five replicates for both YMA plates and pouches were used for each

dilution. Seedlings were checked for nodulation 25 days after inoculation.

RESULTS AND DISCUSSION

None of the fungicides at recommended concentrations had an adverse effect on growth of soybean in 1983, but captafol and captan reduced shoot growth in 1984 (Table 1). Captan proved detrimental to nodulation and nitrogen fixation in both years (Fig. 1). Nodulation and nitrogenase activity were unaffected by fenaminsulf and PCNB. The behavior of captafol in the 1984 experiment was bizarre and could not be explained. Total N content of shoot was not affected by any of the fungicides although nitrogen fixation was reduced by captafol, captan and carboxin. This indicates that these three fungicides inhibited nodulation, but did not interfere with nitrogen uptake from soil to meet the nitrogen requirement of the plants. Seed yield was reduced by captan and captafol treatment, and was correlated with nitrogen fixation.

The results indicate that there are differences in the effect of different fungicides on soybean-rhizobia symbiosis. Similar differences were also reported by others (Fisher and Hayes 1981; Staphorst and Strijdom 1976; Tu 1977). Our results corroborate with those of Chamber and Montes (1982) who found PCNB to be innocuous and

Table 1. Effect of fungicide application on growth of soybean during 1983, 1984

Treatment	Rate (g/100g seed)	Shoot dry wt. (g/plant) '83	'84	Root dry wt. (g/plant) '83	'84
Control	0	5.55a*	4.14a	1.60a	1.59a
Captafol	0.83	5.82a	3.06b	1.62a	1.04ab
Captan	0.25	5.20a	3.06b	1.56a	1.04ab
Carboxin	0.30	5.66a	3.26ab	1.99a	1.05ab
Fenaminsulf	0.18	5.52a	3.36ab	1.71a	1.11ab
PCNB	0.90	5.32a	3.71ab	1.68a	0.97b

* Data followed by a common letter in the same column are not significantly different at 5% level by Duncan's New Multiple Range Test.

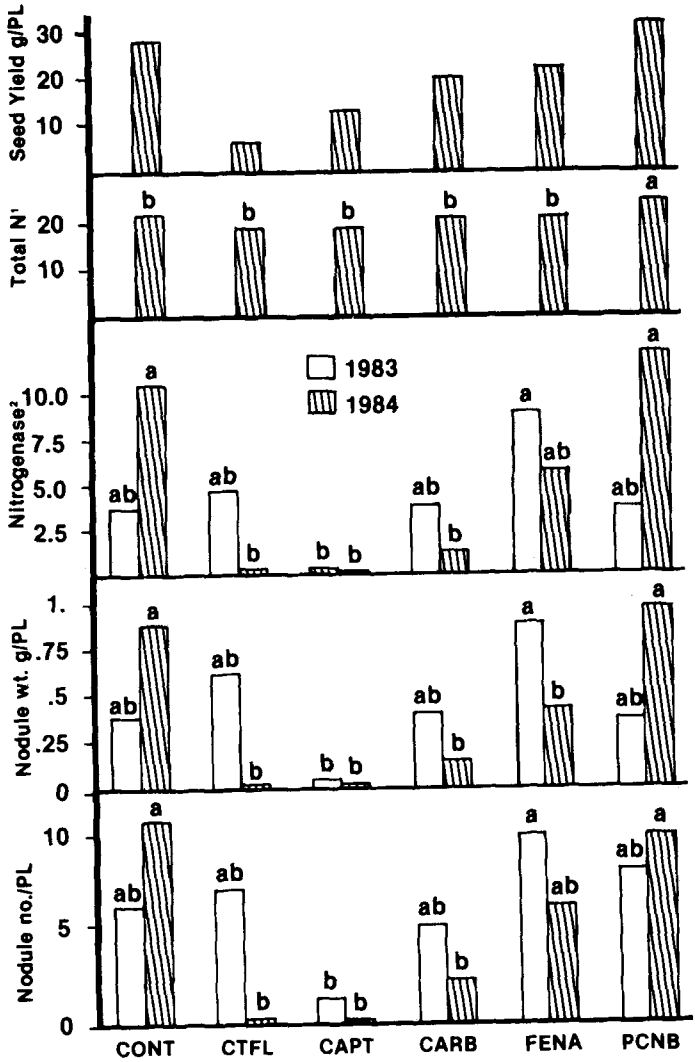

Figure 1. Effect of fungicide treatment in 1983 and 1984 growing seasons on nodule number, nodule weight, nitrogenase activity, total N content of shoot and seed yield of soybean. Within treatments values for the histogram of the same year with the same letter are not significantly different at 5% level by Duncan's New Multiple Range Test. (Statistical analysis of seed yield was not done.)
CONT = Control, CTFL = Captafol, CAPT = Captan, CARB = Carboxin, FENA = Fenaminsulf
1 = mg/g dry shoot, 2 = C_2H_2 production μmol/pl./min

captan harmful to nodulation of soybean in field experiments. Reduction in seed yield due to captan treatment was also noted by them. Similar results on these two fungicides were also obtained with R. phaseoli on Phaseolus vulgaris (Graham et al. 1980). However, Curley and Burton (1975) found that PCNB treatment along with peat inoculant was detrimental to nodulation and survival of R. japonicum on soybean seeds. Such disagreements between results arise principally because of considerable variation among species and strain of rhizobia in response to different pesticides, as reported by several workers (Faizah et al. 1980; Mallik and Tesfai 1983). Therefore, we recommend that evaluation of compatibility of fungicides with rhizobia should be done using monoculture inoculant instead of commercial inoculant which usually contain multiple strains of a species of Rhizobium.

Because captan and, to some extent, carboxin were less compatible than other fungicides we attempted to isolate strains resistant to these two fungicides. None of the three strains (3Ilb110, 3Ilb136, ATCC 10324) showed any growth in carboxin at 25 µg/ml concentration. However, all three strains grew at 100 µg/ml concentration of captan but the strain ATCC 10324 was the only one that could grow even at 250 µg/ml concentration of captan.

Table 2. Generation times of parent and captan acclimatized strains of R. japonicum ATCC 10324.

Captan µg/ml	Generation time (h)	
	Parent	Acclimatized
0	11.4	12.0
50	16.2	15.3
100	26.9	15.8
250	no growth	25.2

In order to check whether the acclimatized strain could grow at a reasonable rate in the presence of captan generation time of the parent strain ATCC 10324 was compared with that of the acclimatized strain in different concentrations of captan. Optical density was noted at 25,

50 and 75 h incubation at 28-29 C as shake culture. It is inferred from the results that the resistant strain could grow, albeight slowly, at much higher concentrations of captan than the parent strain (Table 2).

Viable cell counts of the captan-acclimatized strain by dilution plate and MPN methods were $113x10^7$ and $16x16^6$ respectively. Although no contaminant was apparent on agar plate (Congo Red as indicator) the two counts differed a great deal. Possibility of slight reduction in infectivity can not be ruled out. The acclimatized strain retained its effectivity as noted by the presence of leg-haemoglobin in the nodules.

Our results indicate that not all strains of a species can be acclimatized to a given fungicide. The strains having intrinsic tolerance to a fungicide can only be acclimatized to that fungicide but not to others. In our study the effectivity virtually remained unimpaired in the fungicide tolerance strain of R. japonicum. Similar results were obtained by Odeyemi and Alexander (1977). In contrast, Ruiz-Sanz et al. (1984) noted the complete loss of effectivity in a strain of R. trifolii acclimatized to captafol.

PCNB and fenaminsulf were more compatible than captan and captafol. The incompatibility of fungicides with peat inoculant can be overcome by using peat inoculant containing fungicide-resistant strain of rhizobia.

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