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Effect of Fungicide Seed Treatments on N_2-Fixation and Nodulation in Pea, *Pisum sativum* L.

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Sweet pea (*Pisum sativum* L.), a cold season crop, is vulnerable to a number of diseases caused by fungi, bacteria, viruses and nematodes (Begum et al. 2004). The fungal diseases of pea are mostly seed-born (Hagedorn 1984) and the percentage of seed infection and extent of fungal infection ranges from 20-70 and 1-6 percent, respectively (Anwar et al. 1994). The infected plants cannot express their inherited yield potential qualitatively and quantitatively (Hagedorn 1984).

Fungicide seed treatment is useful to reduce the loss from seed born pathogens and seedling damping-off agents (Sinclair and Backmann 1989). However, their possible side effects to non-target microorganisms such as *Rhizobium* spp. (Ruiz-Sainz et al. 1984) and legume nodulation (Alexander 1961) are of a common concern. As legumes grow in symbiosis with soil bacteria, *Rhizobium* spp., the compatibility of fungicide seed treatments with the bacteria, with the establishment of symbiosis and growth of the plant must be assessed. Considering the drastic effects of fungicides on *Rhizobium*-pea symbiosis, we conducted this study primarily to assess the effect of some selected fungicides on nodulation and nitrogen fixation in pea.

MATERIALS AND METHODS

Three strains of *Rhizobium leguminosarum* bv. viciae (PS-1, PS-3 and PS-4) were used to inoculate plants. PS-1 was obtained from the BIRCEN culture collection of Bio-fertilizer Division, NIBGE, whereas PS-3 and PS-4 were isolated and purified from pea plants. Root nodules were surface sterilized with 0.1% $HgCl_2$ and crushed in 0.98% saline solution. The extract was streaked on yeast extract mannitol agar (YEMA) plates containing congo-red (Vincent 1970a) and incubated at 30°C for 2-3 days. Off-white, wet and gelatinous with round margins bacterial colonies were selected. A single colony was picked and sub-cultured on freshly prepared YEMA plates until pure colonies had been obtained. Isolated bacteria were tested for viable numbers of cells per ml of culture by plate dilution method, gram staining reactions (Vincent 1970b), nodulation ability and nitrogenase activity (Hardy et al. 1968). The selected rhizobial strains were grown in YEM-broth media at a pH of 6.8-7.0 (Vincent 1970a) 30±2°C for 2 days in an orbital shaker for inoculum. All the experimental procedure was performed under

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controlled aseptic conditions. Chemicals of the highest purity (Sigma and Merck) were used during the isolation and verification process.

Seeds of three sweet pea cultivars (Meteor, Olympia 2000 and AM-1) were obtained from Ayub Agricultural Research Institute (AARI) Faisalabad. Commercial grades of there fungicides Vitavax (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide), Derosal (Methyl-benzimidazol-2-ylcarbamate), and Captan [N(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide] were purchased from the local market. Surface sterilized seeds were dressed with the fungicides @ 2 g kg^{-1} of dry seeds by soaking in fungicide solutions. The fungicide treated seeds along with untreated ones were sown at a depth of 1.25 cm in plastic pots (10 cm diameter) containing sterilized sand. Plants were inoculated with 2 ml of the rhizobial culture (mix culture of PS-1, PS-2 and PS-3) at the time of germination and a week after germination. Untreated and uninoculated plants were considered as control and each treatment replicated thrice. The pots were transferred to a growth chamber at day/night temperature 20/15±2°C, photoperiod 12 h and RH 65%. The plants were irrigated regularly with N-free ¼ strength Hoagland's solution (Arnon and Hoagland 1940).

Plants were harvested 45-days after the second inoculum and used for data collection. The nodulated roots were subjected to acetylene reduction assay (ARA) by the method described by Hardy et al. (1968). The noduled roots were placed in a 13 ml vacationer tubes fitted with rubber stoppers. Then 10% (v/v) air was replaced with acetylene and incubated for 1 h at 30 °C. A sample volume of 10 µL was injected into a Carlo-Erba gas chromatograph (Model CE 8000) fitted with a micro column PORAPACK-Q and equipped with a hydrogen flame ionization detector (FID). Oxygen-free nitrogen was used as a carrier gas at a flow rate of 5 mL min^{-1}. Other conditions were as follow: 100 °C oven temperature; 120 °C injection port temperature; 180 °C detector temperature, a 2 min temperature hold before the run and 3 min after the run, and the total run time was 10 min. Peak identification was performed by a Chromcard data-handling program which did all of the evaluation and quantification (Chromcard-32 bit GC Trace, Ver 1.06, October 1998). The quantitative data were reported as nmoles h^{-1} $vial^{-1}$.

The roots were removed from vacationer tubes to count the number of nodules per plant, and for observation of size (large, small or medium) and colour (pink or white) of the nodules. The nodules were detached from the roots and their fresh weights recorded. The nodules were wrapped in an aluminum foil, dried in an oven at 70±2° C for 72 h and dry weights recorded. Soon after harvest, fresh weights of shoots and roots were recorded. The plants were dried in an oven for 72 h at 70±2° C and dry weights recorded. Estimation of shoot nitrogen was conducted according to the procedure described by Bremner (1965) using the Micro-Kjeldahl apparatus.

Analysis of variance (ANOVA) of the data was computed using a COSTAT computer package (CoHort Software, 2003, Monterey, California). The comparison of means was done by the same computer package, using Duncan's New Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

All fungicide treatments severely affected the nodulation ability of *Rhizobium* because the size of nodules was reduced significantly. The maximum size of nodules was observed in non-treated and *Rhizobium* inoculated plants, whereas fungicidal seed treatments reduced it (Table 1). No nodule was formed in un-inoculated plants (Fig.1a). Cultivar Meteor showed the best nodulation ability with Derosal treatment which decreased under both Captan and Vitavax treatments. A similar trend was shown by cvs. AM-1 and Olympia 2000. The colour of the nodules was pink in all inoculated, non-treated plants, whereas it was slightly pick in Derosal and off-white under Vitavax and Captan treatments.

In non-treated plants, the pink colour of nodules was due to the presence of leghemoglobin, a protein present in the cytosol of infected nodule cells at higher concentrations (Marschner 1995), that gives legume nodules a distinct pink colour (Salisbury and Ross 1992). Off-white nodules in all fungicide treatments might have been due to inhibitory effect of fungicides on leghemoglobin synthesis. It seemed that although, globin protein of leghemoglobin was produced in nodules, the gene for which is coded by the host plant in response to infection thread formed by bacteria (Marschner 1995), yet the heme protein of leghemoglobin was not produced, the gene for which is coded by the bacteria themselves (Apple-by 1984), Although sufficient rhizobia survived in fungicide treatments, still they were unable to produce heme protein of leghemoglobin.

The nodule size was large in non-treated and *Rhizobium* inoculated plants while it was smaller in fungicidal seed treatments. This might have been a consequence of the fact that the bacteria surviving in the nodules of the host plants were unable to multiply at normal rates and were ineffective in symbiosis with their host plant. In fungicide treatments, small size of nodules indicated that the bacteria capable of surviving on agrochemical treated seeds might lose their symbiotic effectiveness over time (Martenson and Wilter 1990).

In our studies, fungicide treatments drastically affected number of nodules plant^{-1} (Eberbach and Dougles 1991). Nodulation was adversely affected by Vitavax (Malik and Tesfai 1985; Tesfai and Malik 1986). Captan had a less detrimental effect on nodulation (Dunfield et al. 2000) as compared to Vitavax and Derosal, which proved to be the least detrimental with respect to this variable (Revellin et al. 1993). No nodule was formed in non-inoculated plants due to the absence of appropriate strain of the nodule forming bacteria (Duczek and Buchan 1981).

The decrease in nodulation may be attributed to the death of rhizobia by fungicides (Kishinevsky et al. 1992; Martensson 1992). Another possibility that

may explain poor nodulation is that fungicides did not cause root hair infection and deformation (curling) necessary for nodulation (Martensson 1992) or may have induced root hair deformations, reminiscent of bacterial induced root hair deformations, which have been reported to prevent the susceptibility of plants to bacterial infection. Agrochemicals affect the plant in a manner that the bacterial induced deforming factor and other related compounds (Truchet et al. 1991) was incapable to influence morphogenic activity of the plants, which is necessary for nodulation (Martensson 1992). The phenomenon can also be explained on the basis of fungicidal effect on the phenotypic characteristics of *Rhizobium* which may change the fatty acid methyl ester (FAME) and biology profiles of rhizobia (Dunfield et al. 2000). The FAME profiles were altered, probably in response to change in cellular metabolism (Rose, 1989). In addition, cellular fatty acids were involved in nodulation, but their role remains to be determined (Pueppke 1996). Thus, the alteration in fatty acid and biology profiles by agrochemicals might suggest a decreased capacity of a plant to nodulate (Dunfield et al. 2000). The differential nodulation in the three varieties of pea showed the specificity of nodulation by varieties (Udaiyan et al. 1995) and strains (Shin and Oh 1989).

Table 1. Mean squares from the analysis of variance of data for different vegetative and growth parameters of three cultivars of pea (*Pisum sativum* L.) when seeds were dressed with three different fungicides prior to sowing.

Source of variation	Degrees of freedom	Number of nodules	ARA of nodules	Fresh wt. of nodules	Dry wt. of nodules
Treatment (T)	4	378.3***	40197.54***	0.00412***	48.87***
Genotype (G)	2	392.6***	40712.44***	0.00279***	37.94***
G x T	8	43.35***	4914.49***	0.00046***	3.565***
Error	30	5.8	59.04	0.0000074	0.236
	Degrees of freedom	Fresh wt. of shoot	Dry wt. of shoot	Shoot nitrogen	
Treatment (T)	4	0.096***	4.868***	3.831***	
Genotype (G)	2	0.157***	0.001***	3.158***	
G x T	8	0.009***	4.588***	0.137***	
Error	30	2.516	0.044	0.0071	

*** = Significant at $p < 0.001$

Nitrogen fixation (nitrogenase activity) may be inhibited by the fungicides used as seed treatments against pathogens (Fisher and Hayes 1981). In our studies, the maximum nitrogenase activity was detected in the *Rhizobium* inoculated but non-treated plants, which decreased in all fungicide treatments. Derosal was proved to have the least detrimental effect on nitrogenase activity (Revellin et al. 1993; Udaiyan et al. 1995) as compared to Vitavax which proved to be the most detrimental (Fisher and Hayes 1981); Captan had less drastic effect as compared to Vitavax (Dunfield et al. 2000). In inoculated and fungicide treated plants, higher nitrogenase activity was observed in Meteor, as compared to those in AM-1, and Olympia 2000. Nitrogenase activity was not detected in control, in which no nodule was formed.

The decrease in nitrogenase activity indicated that the rhizobial population present in nodules was not so effective in nitrogen fixation due to the inhibitory effects of fungicides (Kishinevsky et al. 1992). While examining the correlation among different attributes it is evident, that the decrease in nitrigenase activity was directly related to the decrease in nodulation by fungicide treatments. A reason for this inhibitory effect could also be that these chemicals inhibit photosynthesis and the synthesis of acetolactate synthase, both processes being important for the nitrogen fixing activity (Stenersen 2004; Ware and Whitacre 2004). Photosynthesis provides nodules the energy for the reduction of nitrogen, and the acetolactate synthase regulates the synthesis of aromatic compounds (Martensson 1992). However, it could also be due to inhibition or decrease in leghemoglobin synthesis by fungicides. Leghemoglobin regulates the supply of oxygen to bacteria present in nodules. The low leghemoglobin contents were found to have accelerated the supply of oxygen to nodules, which consequently depressed the nitrogenase activity in the nodules of treated plants (Stachey et al. 1997). The difference in nitrogenase activity in the three pea cultivars was probably due to the differential response of cultivars to nodulation.

The seed treatment with fungicides and inoculation of seedling with rhizobia had a significant effect on nodule fresh and dry weights (Table 1). The maximum fresh and dry weights of nodules were recorded in untreated and *Rhizobium* inoculated plants which decreased in Derosal, Vitavax and Captan treatments. No weight was recorded in un-inoculated and non-treated controls because no nodule formation was observed in these treatments. The cultivars also differed significantly in fresh and dry weights of nodules and the maximum fresh and dry weights of nodules were recorded in Meteor followed by AM-1 and Olympia 2000 (Fig 1c & 1d). In cv. Olympia 2000, no difference was found in Vitavax and Derosal treatments, whereas in Captan treatment, no nodule was formed (Fig.1c & 1d). In inoculated as well as fungicide treated plants, the maximum fresh and dry weight of nodules was observed in Meteor followed by AM-1 and Olympia 2000. Derosal had a less detrimental effect on nodule weight, which is in agreement with the earlier findings of Catroux and Arnaud (1991). In contrast, in some other studies, Vitavax was found to have a less detrimental effect (Malik and Tesfai 1985; Tesfai and Malik 1986). Captan affected the nodule weight so adversely that the lowest nodule weight was recorded in this treatment (Duczek and Buchan 1981). The nodule weight appeared to be highly correlated with number of nodules plant^{-1}.

In all cultivars, fresh weight of shoots was significantly affected by seed treatment with fungicide and rhizobial inoculum (Table 1). All the pea cultivars responded differently to different fungicide treatments and the maximum fresh weight of shoot was observed in AM-1, which was followed by Meteor and Olympia 2000 (Fig. 1e). In AM-1 and Meteor, maximum fresh weight was recorded in inoculum with *Rhizobium* alone followed by Captan, Derosal, Vitavax, and control treatments. In Olympia 2000, although the maximum shoot fresh weight was recorded in untreated and inoculated plants, no significant difference was observed among the fungicide treatments (Fig. 1e). Although the maximum

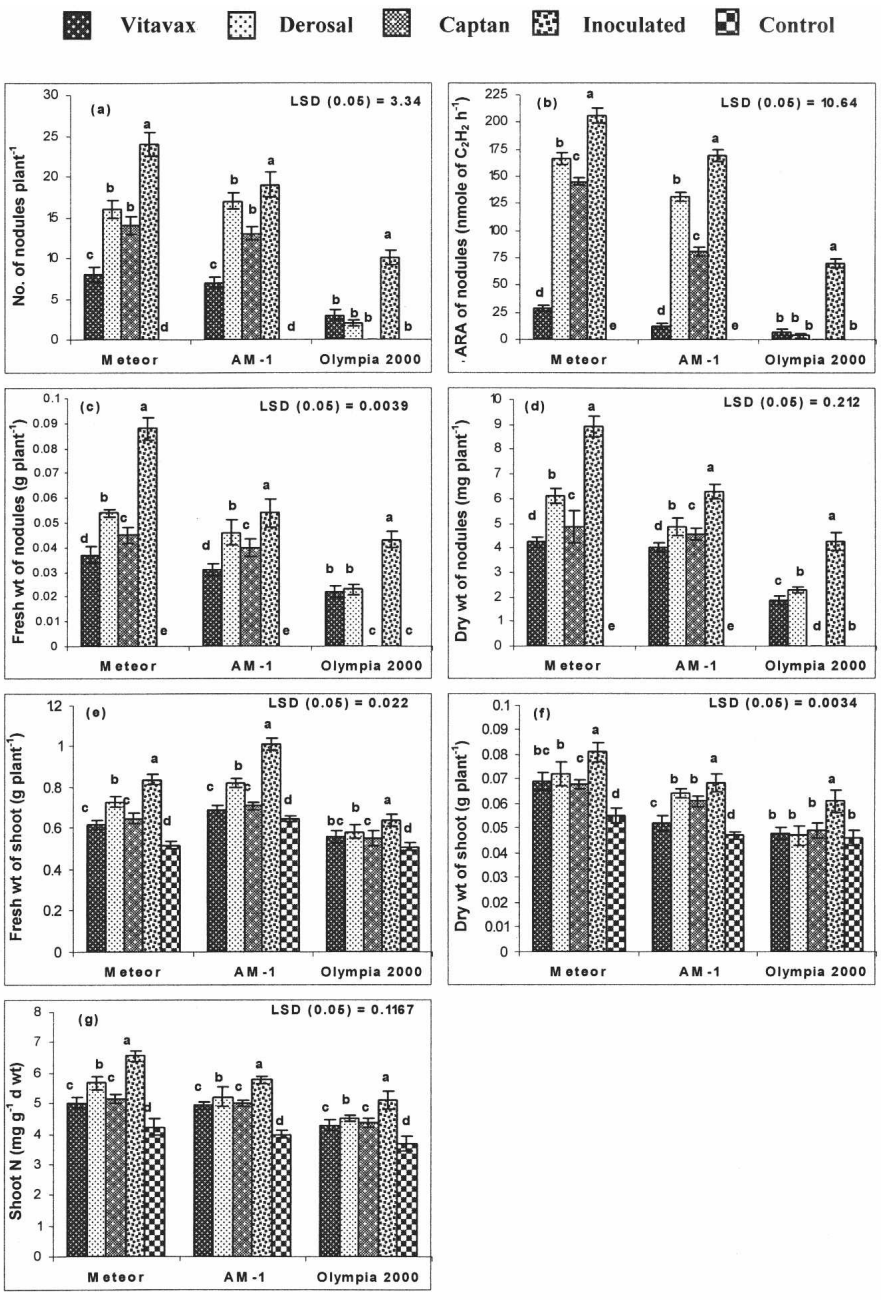

Figure 1. Effect of three different fungicides and inoculation of seedling with rhizobium on (a) Number of nodules (b) ARA of nodules (c) Fresh wt. of nodules (d) Dry wt. of nodules (e) Fresh wt. of shoot (f) Dry wt. of shoot and (g) Shoot nitrogen in different pea (*Pisum sativum*) cultivars.

number of nodules and nitrogenase activity were observed in Meteor, maximum shoot fresh weight was observed in AM-1 and the lowest in Olympia 2000. This contradiction may be explained by cultivar specific vegetative growth of plants. Although the maximum shoot fresh weight was recorded in AM-1, the maximum dry weight was recorded in Meteor. It seemed that shoot dry weight was highly correlated with the nodulation and nitrogenase activity of nodules and better nitrogen accumulation.

All the fungicide treatments had a significant effect on the dry weights of shoot and the maximum dry weights were recorded in inoculum alone followed by Captan and Vitavax, Derosal, and control treatments (Table 1). All cultivars differed significantly with respect to shoot dry weight and the maximum shoot dry weight was recorded in Meteor followed by AM-1 and Olympia 2000 (Fig. 1f). The maximum shoot fresh and dry matter production in inoculated plants may be attributed to better nodulation and the highest nitrogenase activity. The lowest shoot fresh and dry weights in control were correlated with the absence of nodulation due to this treatment. All fungicides significantly affected the shoot matter production, which might have been due to decrease in nodulation and nitrogenase activity.

Fungicidal seed treatments and Rhizobial inoculum had a significant effect on the N content of shoot (Table 1). The maximum shoot N was recorded in inoculum alone and the lowest in control. Cultivars also differed significantly with respect to this variable. The maximum shoot N was recorded in Meteor, followed by AM-1 and Olympia 2000 (Fig.1g). The maximum shoot nitrogen contents were in inoculated and non-treated plants, which indicated the effectiveness of *Rhizobium* for nitrogen fixation. The lowest shoot nitrogen contents were observed in control treatment. In well-nodulated (inoculated and non–treated) plants, higher nitrogen contents may have been due to the higher concentration of leghemoglobin in nodules, large number of nodules and hence high nitrogenase activity. In fungicide treatments, low nitrogen contents may be attributed to poor nodulation and less nitrogenase activity, which was due to inhibition in leghemoglobin synthesis. Leghemoglobin regulates the supply of oxygen to the bacteria present in nodules and hence prevents the oxygenated inactivation of nitrogenase enzyme (Stachey et al. 1997). The highest nitrogen contents in Meteor and the lowest in Olympia 2000 might have been due to the differential nodulation ability of the three cultivars.

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