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The *in vitro* Compatibility of some *Rhizobium* and *Bradyrhizobium* Strains with Fungicides

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Abstract: Laboratory experiments were conducted to study the effects of different concentrations (0,10,20,50,100,200,500 and 1000 µg/l) of the fungicides Captan, Thiram, Luxan, Fernasan-D and Milcurb on inhibition of growth and colony sizes of seven *Rhizobium* strains (4 introduced and 3 locally isolated) and ten *Bradyrhizobium* strains (5 introduced and 5 locally isolated). The effects were determined by measuring the colony size and the diameter of the zone of inhibited growth.

Fungicides differed in their effects on *Rhizobium* and *Bradyrhizobium* strains. Captan at the concentrations of 100 and1000µg/l was the most toxic, followed by Thiram, Milcurb, Luxan then Frernasan-D.Increasing the concentration of each fungicide from 0 to 10, 20, 50, 100, 200, 500 and 1000 µg/l reduced the colony sizes of rhizobial and bradyrhizobial strains with different degrees of sensitivity. All strains tolerated low fungicide concentrations (≤ 100 µg/l) but they were sensitive to high concentrations (≥500 µg/l) with varying degrees of sensitivity. *Rhizobium* strains were more tolerant than *Bradyrhizobium* strains, and no clear differences were observed between the introduced and the locally isolated strains.

Introduction

Legume seeds are often treated with insecticides and/or fungicides to protect the seeds and seedlings from harmful insects and soil microorganisms during germination and early seedling development. Chemically treated seeds are inoculated with rhizobia which may be adversely affected by these chemicals (FAO, 1984). Since large numbers of viable rhizobia are required to bring about effective nodulation, compatibility or survival of rhizobia on treated seeds is a major of concern (Kutcher *et al*,. 2002).

Fungicides markedly affect the saprophytic soil microbial population as equally as the pathogens they are designed to control. The degree of inhibition and the duration vary with the chemical, the soil and the environmental conditions (Elsheikh, 1993). Fungicides differ in their effects on the growth and survival of *Rhizobium* and *Bradyrhizobium* strains depending on the strain and the concentration of the fungicide (Hashem *et al*., 1997).

Measuring the inhibited zones of growth on solidified agar media is one of the methods used to evaluate the effects of pesticides on soil microflora, especially *Rhizobium* and *Bradyrhizobium*. The size of the inhibition zone in solid culture media may not give a true measure of toxicity to rhizobia since the test depends not only on the sensitivity of the organism but also depends on the concentration of the chemical and its ease of diffusion through the agar (Johnen and Drew, 1977). Results from this method can also be misleading. Zones of inhibited growth occur if the chemical is toxic and diffusible (Curley and Burton, 1975). Not all growth inhibition zones with fungicides represent bactericidal reaction. The zone is independent of the chemical concentration and differs with the fungicide under test (Johnen and Drew, 1977). With insecticides, the diffusion of toxic material depends on whether it is present in an emulsion or in crystal form. Diatloff (1970) found that the emulsifying agent used in the formulation was partly the toxic principle. On the other, hand crystalline forms of active ingredients have low water solubility and consequently form only small inhibition zones.

The objective of this study was to investigate the effects of some fungicides on the growth and survival of locally – isolated and introduced *Rhizobium* and *Bradyrhizobium* strains, as reflected in the size of inhibition zone and colony size.

Materials and Methods

Rhizobium and *Bradyrhizobium* strains used throughout this study were obtained from The Biofertilization Department, Environment and Natural Resources Research Institute (ENRR1) of the National Center for Research, Khartoum, Sudan. The strain affiliations and origins are shown in Table 1.

The fungicides used were obtained from the Crop Protection Department, Ministry of Agriculture, Khartoum North, Sudan. These included: Captan, Fernasan-D, Luxan, Thiram and Dimethirimol (Milcurb).

The growth medium used was yeast extract mannitol (YEM) agar described by Cleyet-Marel (1993) with 10ml Congo red per liter.

Effects of Captan concentration on growth of *Rhizobium and Bradyrhizobium* strains:

The effects of Captan on the growth of *Rhizobium* and *Bradyrhizobium* strains on YEM agar were tested as described by Bollich *et. al.* (1985) using measurement of diameters of the growth inhibition zones. The medium was autoclaved, cooled to 55-60°C and then poured into sterilized Petri dishes.

Discs of 0.5 cm diameter of filter paper (Whattman No.1) were soaked in medical bottles containing the required concentration of Captan then left overnight to dry. The concentrations used were 0,10,20,50,100,200,500 and 1000 µg/l active ingredient (ai)

The strains used in this exprement were: TAL 1373, TAL 1397, TAL 380, TAL 636, ENRRI 1, ENRRI 2 and ENRRI 10 for Rhizobium and TAL 1371, TAL 1113, TAL 1114, TAL 102, ENRRI 6A, ENRRI 16A and strain 2 for Bradyrhizobium.

Four discs of filter paper from different concentrations were transferred to 10-cm diameter Petri dishes containing solid YEM agar medium. Two ml of 3-5 days old suspension of the *Rhizobium / Bradyrhizobium* strain under test were mixed with 3 ml of cool YEM agar medium and then dispersed evenly over the growth medium. The Petri dishes were then incubated inverted at 37°C. Five replicates were made per treatment. The diameters of the zones of inhibition were measured after 3 and 5 days of incubation for *rhizobium* and *bradyrhizobium* strains respectively.

Effects of fungicides on growth of *Rhizobium* / *Bradyrhizobium* strains:

The above method was applied to study the effects of six fungicides at two concentrations (100 and 1000 µg/l) on the growth and survival of *Rhizobium sp* strain TAL 1397 and *Bradyrhizobium sp* strain TAL 102. The fungicides used were: Captan, Fernasan-D, Thiram, Luxan and Dimethirimol (Milcurb). The diameters of the inhibited growth zones formed were measured after 3 days of inoculation for the *Rhizobium* strain and after 5 days for the *Bradyrhizobium* strain .

Table 1: Affiliation and origins of *Rhizobium* and *Bradyrhizobium* strains used in this study.

Strain number	Strain Affiliation	Crop	Origin
TAL 1397	*R. leguminosarum* bv. *viceae*	*Vicia faba*	Morocco
TAL 636	*R. leguminosarum* bv. *Viceae*	*Lens sp*	USA
TAL 380	*R. meliloti*	*Medicago sativa*	Australia
TAL 1373	*R. mililoti*	*Medicago sativa*	Brazil
TAL 1113	*Br. spp* Cowpea group	*Sesabana grandiflora*	India
TAL 1114	*Br. spp* Cowpea group	*Sesabana gandiflora*	India
TAL 1371	*Br. Spp*	*Arachis hypogaea*	Unknown
TAL 102	*Br. Japonicum*	*Glycine max*	USA
TAL 377	*Br. Japonicum*	*Glycine max*	USA
ENNRI 1	*R. loti*	*Cajanus cajan*	Elrawakeeb *
ENNRI 2	*R. leguminosarum* bv. *phaseoli*	*Phaseolus vulgaris*	Wad Ramli *
ENNRI 10	*R. spp*	*Acacia senigal*	Khartoum *
ENNRI 20	*Br.spp* Cowpea group	*Arachis hypogaea*	Elrawakeeb *
ENNRI 6A	*Br. spp* Cowpea group	Cowpea	Eljamoeia *
ENNRI 16A	*Br. spp* Cowpea group	*Cyamopsis tetragonoloba*	Sinja *
Sheleni 12	*Br. spp* Cowpea group	*Sesabana grandiflora*	Elgazera *
Strain 2	*Br. Japonicum*	*Glycine max*	Abu Usher *

Effects of Captan concentration on colony sizes of *Rhizobium and Bradyrhizobium* strains:

Solutions of weighed amounts of Captan were added to sterilized YEMA medium contained in 500 ml conical flasks at 40 C as filter-sterilized solutions to give final concentrations of 0,10,20,50,100,200,500 and 1000 µg/l. Seventeen *Rhizobium* and *Bradyrhizobium* strains were used in this study. These strains included *Rhizobium* strains: TAL 1397, TAL 1373, TAL 636, TAL 380, ENRRI 1, ENRRI 2, ENRRI 10, and the *Bradyrhizobium* strains TAL 1113, TAL 1114, TAL 1731, TAL 377, TAL 102, ENRRI 6A, ENRRI 16A, ENRRI 20, Strain 2 and Sheleni 12.

Rhizobium and *Bradyrhizobium* strains were pinpoint inoculated in Petri dishes of each concentration under test with three replicates per treatment. The dishes were incubated at 27 C for 3 days for *Rhizobium* and 5 days for *Bradyrhizobium* strains. The diameters of colonies formed were measured after the incubation period for each treatment.

Effects of fungicides on colony sizes of *Rhizobium* and *Bradyrhizobium* strains:

The previous experiment was repeated with different fungicides, namely: Captan, Thiram, Luxan, Fernasan-D and Milcurb with concentrations of 100 or 1000 µg/l. Two strains were tested, namely; *Rhizobium sp* strain TAL 1397 and *Bradyrhizobium sp* strain TAL 102. After inoculation, the diameters of the colonies formed were measured after 3 days of incubation at 37 C for the *Rhizobium* strain and 5 days for the *Bradyrhizobium* strain.

Results

Effects of fungicides on the Sizes of inhibition zones of local and introduced *Rhizobium* and *Bradyrhizobium* strains:

With the exception of strain TAL 1397, all introduced *Rhizobium* strains (TAL) tolerated Captan concentrations $\leq$ 100 µg/l and did not form any zones of inhibited growth (Fig 1).

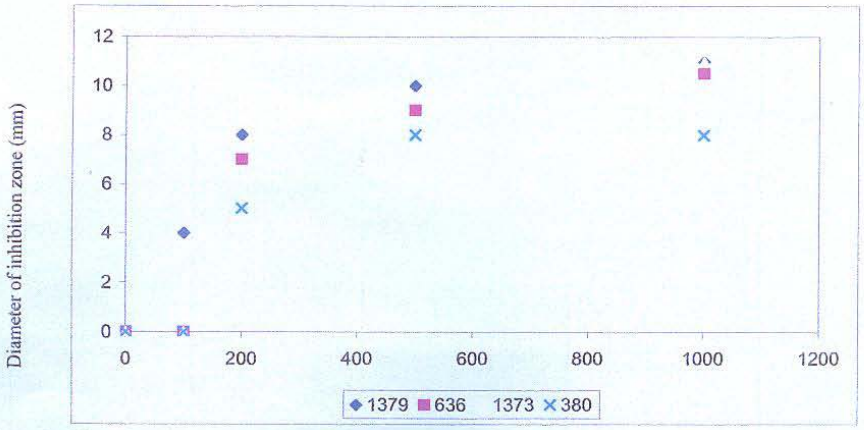

Figure1: Effect of Captan concentration on *Rhizobium* spp: Inhibition zone TAL strains

The diameter of inhibition zones formed by each of the four rhizobial strains increased with increasing Captan concentration as exemplified by strain TAL 638 (Plate1). The wide range of the diameters of the zones of inhibition (8-11 mm) at 1000 µg/l indicates the differences in strain sensitivity to Captan.

The introduced *Bradyrhizobium* strains (TAL strains) were more sensitive than the introduced *Rhizobium* strains (Fig. 2).

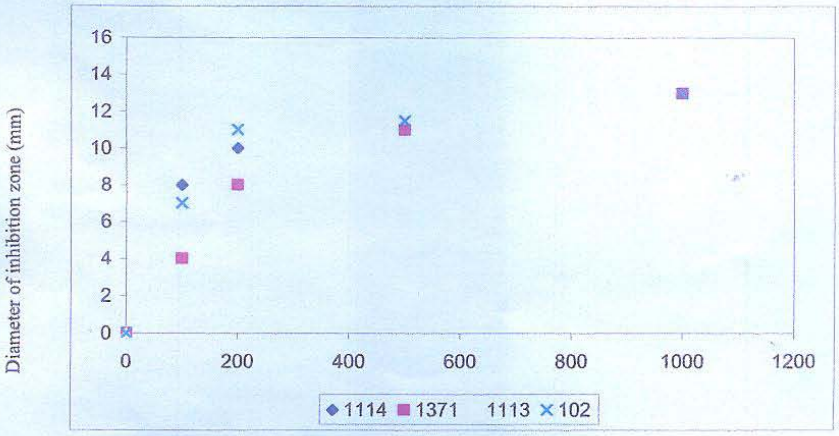

Figure2: Effect of Captan concentration on *Brdyrhizobium* spp: Inhibition zone TAL strains

The four *Bradyrhizobium* strains that were tested did not tolerate concentrations ≥ 100 µg/l, whereas the growth was normal in lower concentrations with no zones of inhibited growth formed. The diameters of the zones of inhibition increased sharply with increasing Captan concentration from 0 to 200 µg/l but with a steady increase between 200 and 1000 µg/l. Plate 2 Shows inhibition zones by strain TAL 1130.

Of the local (ENRR1) *Rhizobium* strains, two tolerated concentrations up to 100 µg/l (Fig. 3) while ENRRI 1 showed an inhibition zone of ≈4.5 mm. All the three strains showed a sharp decrease in growth, forming large diameters of inhibited zones between 100 and 500 µg/l, with gradual or no decrease above that concentration.

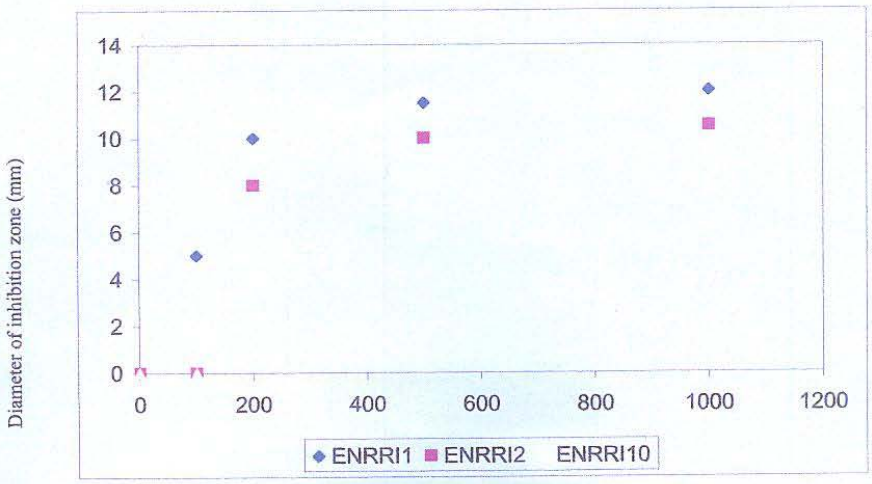

Figure3: Effect of Captan concentration on *Rhizobium* spp: Inhibition zone ENRRI strains

The strains differed in their sensitivity to Captan concentration even at the concentration of 200 µg/l. Plate (3) depicts inhibition zones formed by *Rhizobium* strain ENRRI 10. The local *Bradyrhizobium* strains grew normally in the presence of Captan concentrations of < 100 µg/l. The three strains were very sensitive to concentrations of 100-1000 µg/l with varying degrees depending on Captan concentration (Fig. 4). The zones of inhibited growth formed by local *Bradyrhizobium* strains were larger in diameter (Plate 4).

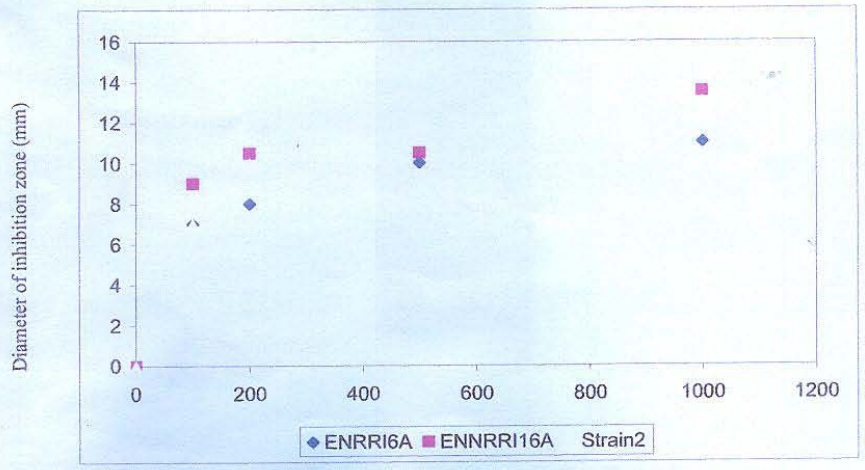

Figure4: Effect of Captan concentration on *Bradyrhizobium* spp: Inhibition zone ENRRI strains

The result obtained on the effects of Captan on the size of zones of inhibited growth prompted widening the investigation to include four other fungicides,viz. Thiram, luxan, Fernasan - D and Milcurb, on the introduced R*hizobium* strain TAL 1397 and *Bradyrhizobium* strain TAL 102. The 100 µg/l concentration of all fungicides had no effect on either of the two strains under test, indicating the tolerance of these strains to this fungicide concentration (Table 2).However the two strains responded differently to the different fungicides at the concentration of 1000 µg/l. Captan had the most deleterious effect on both strains. *Rhizobium* strain TAL 1397 was more tolerant to the fungicides than *Bradyrhizobium* strain TAL 102 except for Milcurb to which the two strains responded similarly. This difference in strain sensitivity was clearer with Captan and Thiram.

Table 2: Inhibition zones (mm) of *Rhizobium* and *Bradyrhizobium* strains due to five fungicides.

Fungicide	*Rhizobium* Strain TAL 1397		*Bradyrhizobium* Strain TAL 102	
	Fungicide concentration (µg/l)			
	100	1000	100	1000
Thiram	0	9	0	10.5
Captan	0	9	0	11.5
Luxan	0	8	0	9
Fernasan-D	0	6	0	7
Milcurb	0	8	0	8

Effects of fungicides on Colony size:

Investigations of the effects of five fungicides on colony size of *Rhizobium* strain TAL 1397 and *Bradyrhizobium* strain TAL 102 showed that the two strains failed to grow or to form colonies of ≥2 mm at the concentration of 1000 µg/l with all fungicides tested. At the low fungicides concentration (100 µg/l), the strains responded differently, depending on the fungicide. Fernasan-D and Milcurb showed low toxicity at 100 µg/l as the two strains formed colonies with diameters ≥ 5 mm. The *Rhizobium* strain was more tolerant to the fungicides than the *Bradyrhizobium* strain, which failed to grow at 100 µg/l when Captan was used. The former strain formed larger colonies than the latter strain with Luxan at this concentration (Table 3).

All Seventeen strains tested grew normally forming colonies with diameters ≥ 5 mm in the low concentrations (0-20 µg/l) of Captan (Table 4).However, all strains failed to tolerate Captan concentrations of 500 and 1000 µg/l. The tested strains differed greatly in their sensitivity to concentrations of 100 and 200 µg/l. All *Rhizobium* strains tolerated 50 µg/l, whereas many *Bradyrhizobium* strains formed colonies of 2 - 5 mm, at this concentration, and showed much more reduction in colony size in higher concentrations.

Table 3: Effect of fungicides on the colony sizes of *Rhizobium* and *Bradyrhizobium* strains.

Fungicide	Fungicide concentration (µg/l)			
	Strain TAL1397		Strain TAL 102	
	100	1000	100	1000
Captan	+	-	-	-
Thiram	+	-	+	-
Luxan	+	-	++	-
Fernasan-D	++	-	++	-
Milcurb	++	-	++	-

Where:
- = No growth or colonies with diameter ≤ 2 mm.
+ = Colonies with diameter 2 – 5 mm.
++ = Colonies with diameter ≥ 5 mm.

Table 4: The effect of Captan concentration on the colony size of *Rhizobium* and *Bradyrhizobium* strains.

Rhizobium/Bradyrhizobium strains		Captan Concentration (µg/l)							
		0	10	20	50	100	200	500	1000
Rhizobium strains									
TAL	1397	++	++	++	++	+	+	-	-
TAL	1373	++	++	++	++	+	-	-	-
TAL	636	++	++	++	++	++	+	-	-
TAL	380	++	++	++	++	+	+	-	-
ENRRI	1	++	++	++	++	+	-	-	-
ENRRI	2	++	++	++	++	++	+	-	-
ENRRI	10	++	++	++	++	++	+	-	-
Bradyrhizobium strains									
TAL	1114	++	++	++	++	-	-	-	-
TAL	1371	++	++	++	++	+	-	-	-
TAL	1113	++	++	++	+	-	-	-	-
TAL	102	++	++	++	++	+	-	-	-
TAL	377	++	++	++	++	-	-	-	-
ENRRI	6A	++	++	++	+	+	-	-	-
ENRRI	16A	++	++	++	+	-	-	-	-
ENRRI	20	++	++	++	++	-	-	-	-
Shel.	12	++	++	++	+	-	-	-	-
St.	2	++	++	++	++	+	+	-	-

Where:
- = No growth or colonies with diameter ≤ 2 mm.
+ = Colonies with diameter 2 – 5 mm.
++ = Colonies with diameter ≥ 5 mm.

Table 2: Inhibition zones (mm) of *Rhizobium* and *Bradyrhizobium* strains due to five fungicides.

Fungicide	*Rhizobium* Strain TAL 1397		*Bradyrhizobium* Strain TAL 102	
	Fungicide concentration (µg/l)			
	100	1000	100	1000
Thiram	0	9	0	10.5
Captan	0	9	0	11.5
Luxan	0	8	0	9
Fernasan-D	0	6	0	7
Milcurb	0	8	0	8

Effects of fungicides on Colony size:

Investigations of the effects of five fungicides on colony size of *Rhizobium* strain TAL 1397 and *Bradyrhizobium* strain TAL 102 showed that the two strains failed to grow or to form colonies of ≥2 mm at the concentration of 1000 µg/l with all fungicides tested. At the low fungicides concentration (100 µg/l), the strains responded differently, depending on the fungicide. Fernasan-D and Milcurb showed low toxicity at 100 µg/l as the two strains formed colonies with diameters ≥ 5 mm. The *Rhizobium* strain was more tolerant to the fungicides than the *Bradyrhizobium* strain, which failed to grow at 100 µg/l when Captan was used. The former strain formed larger colonies than the latter strain with Luxan at this concentration (Table 3).

All Seventeen strains tested grew normally forming colonies with diameters ≥ 5 mm in the low concentrations (0-20 µg/l) of Captan (Table 4).However, all strains failed to tolerate Captan concentrations of 500 and 1000 µg/l. The tested strains differed greatly in their sensitivity to concentrations of 100 and 200 µg/l. All *Rhizobium* strains tolerated 50 µg/l, whereas many *Bradyrhizobium* strains formed colonies of 2 - 5 mm, at this concentration, and showed much more reduction in colony size in higher concentrations.

Table 3: Effect of fungicides on the colony sizes of *Rhizobium* and *Bradyrhizobium* strains.

Fungicide	Fungicide concentration (µg/l)			
	Strain TAL1397		Strain TAL 102	
	100	1000	100	1000
Captan	+	-	-	-
Thiram	+	-	+	-
Luxan	+	-	++	-
Fernasan-D	++	-	++	-
Milcurb	++	-	++	-

Where:
- = No growth or colonies with diameter ≤ 2 mm.
+ = Colonies with diameter 2 – 5 mm.
++ = Colonies with diameter ≥ 5 mm.

Table 4: The effect of Captan concentration on the colony size of *Rhizobium* and *Bradyrhizobium* strains.

Rhizobium/Bradyrhizobium strains		Captan Concentration (µg/l)							
		0	10	20	50	100	200	500	1000
Rhizobium strains									
TAL	1397	++	++	++	++	+	+	-	-
TAL	1373	++	++	++	++	+	-	-	-
TAL	636	++	++	++	++	++	+	-	-
TAL	380	++	++	++	++	+	+	-	-
ENRRI	1	++	++	++	++	+	-	-	-
ENRRI	2	++	++	++	++	++	+	-	-
ENRRI	10	++	++	++	++	++	+	-	-
Bradyrhizobium strains									
TAL	1114	++	++	++	++	-	-	-	-
TAL	1371	++	++	++	++	+	-	-	-
TAL	1113	++	++	++	+	-	-	-	-
TAL	102	++	++	++	++	+	-	-	-
TAL	377	++	++	++	++	-	-	-	-
ENRRI	6A	++	++	++	+	+	-	-	-
ENRRI	16A	++	++	++	+	-	-	-	-
ENRRI	20	++	++	++	++	-	-	-	-
Shel.	12	++	++	++	+	-	-	-	-
St.	2	++	++	++	++	+	+	-	-

Where:
- = No growth or colonies with diameter ≤ 2 mm.
+ = Colonies with diameter 2 – 5 mm.
++ = Colonies with diameter ≥ 5 mm.

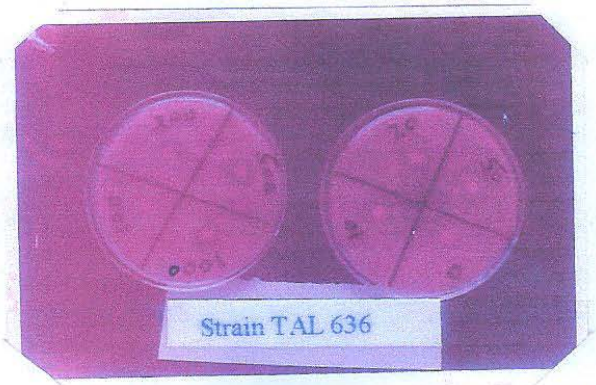

Plate 1: Effect of Captan concentration (µg/l) on *Rhizobium spp*: the diameter of the zones of inhibited growth (mm) formed by strain TAL 636.

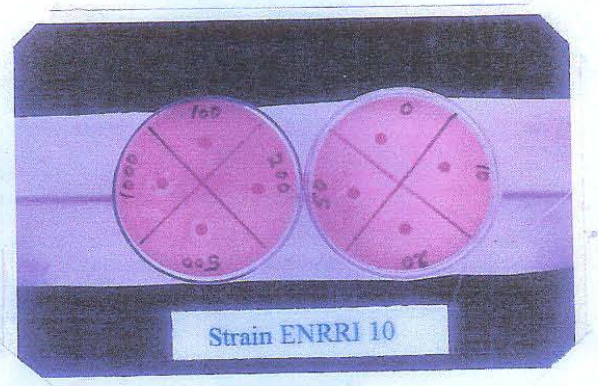

Plate 2: Effect of Captan concentration (µg/l) on *Rhizobium spp*: the diameter of the zones of inhibited growth (mm) formed by strain ENRRI 10.

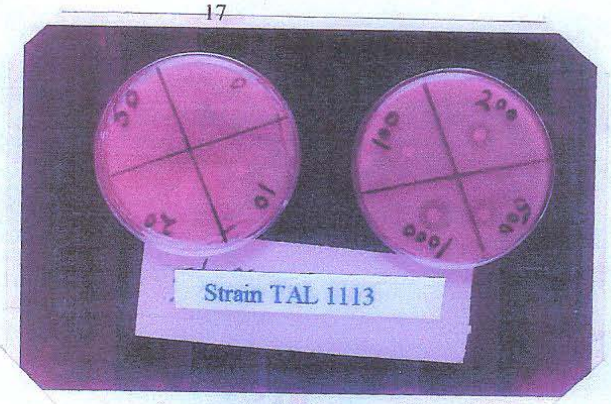

Plate 3: Effect of Captan concentration (µg/l) on *Bradyrhizobium spp*: the diameter of the zones of inhibited growth (mm) formed by strain TAL 1113.

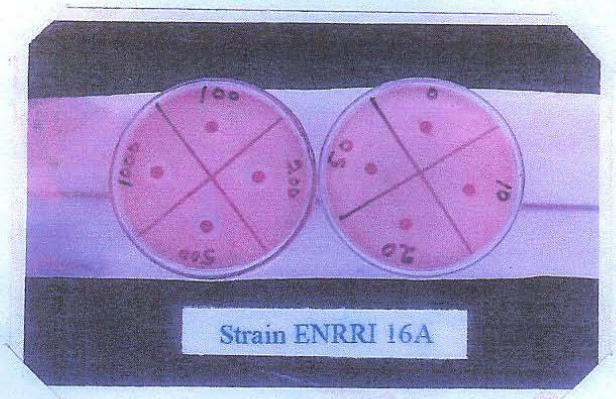

Plate 4: Effect of Captan concentration (µg/l) on *Bradyrhizobium spp*: the diameter of the zones of inhibited growth (mm) formed by strain ENRRI 16A.

Discussion

The results obtained show that high concentrations of Captan (500-1000 µg/l) formed large diameters of inhibition zones indicating the sensitivity of tested strains to these concentrations. These results revealed that the tested strains, whether *Rhizobium* or *Bradyrhizobium*, tolerate low concentrations but they varied in their response to moderate concentrations (100 and 200 µg /l) depending on the strain sensitivity and Captan concentration. Generally, it is concluded that strains of *Rhizobium or Bradyrhizobium* whether introduced or locally isolated differ greatly in their sensitivity to Captan. *Rhizobium* strains were more tolerant than *Bradyrhizobium* strains at any concentration tested, whereas, no clear differences in sensitivity were detected between the introduced and locally isolated stains.

As different rhizobial and bradyrhizobial strains differ in their sensitivity to fungicide concentration, fungicides react differently with *Rhizobium or Bradyrhizobium* strains depending on the fungicide toxicity. From the

results it was clear that *Rhizobium* and *Bradyrhizobium* strains tolerate low fungicide concentration (100 µg/l). On the other hand, different fungicides react differently on rhizobia and bradyrhizobia. Similarly, *Rhizobium* and *Bradyrhizobium* strains differ in their sensitivity to fungicides with variations of 10-11% between them. The results obtained showed that with all tested fungicides, except Milcurb, the *Rhizobium* strain TAL 1397 was more tolerant than the *Bradyrhizobium* strain TAL 102. Variations in the effects of fungicides on *Rhizobium* and *Bradyrhizobium* strains depend on fungicide toxicity. Thiophanate-methyl at 50-500 µg/l stimulated *Rhizobium* growth in YEM broth indicating that the fungicide does not have any bactericidal activity (Lakshmi and Gupta, 1997) whereas Mancozeb decreased the growth of two *Rhizobium* strains by 50% (Castro *et al.*, 1997).

The results obtained indicate that the growth of *Rhizobium and Bradyrhizobium* strains was completely inhibited at Captan concentration of $\geq$ 500 µg/l indicating the high sensitivity of these stains to these concentrations. Low Captan concentrations, on the other hand, did not affect the tested strains, which grew normally forming colonies of $\geq$8mm. At moderate Captan concentrations (100 and 200 µg/l), the strains differed greatly in their sensitivity to either concentration. The results obtained showed that all fungicides at the concentration of 1000 µg/l were toxic to the two strains tested but their toxicity varied considerably with the strain.

It is concluded that *Rhizobium* and *Bradyrhizobium* strains vary in their sensitivity to the fungicides and that *Bradyrhizobium* strains are more sensitive than *Rhizobium* strains but no clear differences were observed between the introduced and locally isolated strains. On the other hand, fungicides react differently with the strains depending on their toxicity and concentration. Many research workers reported these variations in strain sensitivity and fungicide toxicity. Strzelec and Martyniuk (1994) report a reduction in the growth of three strains of *Rhizobium leguminosarum* and four strains of *Rhizobium meliloti* with *R. meliloti* surviving well on fungicide treated seeds. Similarly, strains USDA 3384 and strain USDA 3456 varied in their sensitivity to fungicide treatment depending on fungicide toxicity and concentration (Hashem *et al.*, 1997).

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