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Infectivity and Competitive Ability of Fast and Slow Growing *Rhizobium* Strains on Soybeans

C. MAK, E.L. TAN, S.T. LIM and MOHD AZMI
Department of Genetics and Cellular Biology
University of Malaya
59100 Kuala Lumpur, Malaysia

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ABSTRAK

Tiga strain Rhizobium *termasuk dua pembiak cepat 440 dan Mar 1 dan satu pembiak lambat 110 telah digunakan secara tunggal dan secara kombinasi dua untuk menginokulasi dua varieti kacang soya iaitu Palmetto dan Kahaha. Strain-strain* Rhizobium *dapat dikenali dengan tanda-tanda antibiotik. Eksperimen dilakukan dalam Leonard Jar dan juga di ladang yang mana kacang soya telah ditanam untuk beberapa tahun.Dalam eksperimen Leonard Jar, perlakuan-perlakuan* Rhizobium *menghasilkan bintil dan berat kontang pokok yang signifikan dan lebih baik dibandingkan dengan kontrol. Bagi percubaan di ladang, kesan* Rhizobium *yang signifikan tidak diperhatikan. Pada amnya, Kahala menunjukkan inokulasi Rhizobium yang lebih berkesan dibandingkan dengan Palmetto. Ujian-ujian pengasingan menunjukkan kebarangkalian yang baik untuk mendapat kembali strain-strain asal dalam bintil. Tetapi, ada strain yang lebih kuat dalam perasingan. Mar 1 gagal didapati kembali apabila ia berasing dengan strain 440 atau strain-strain asli dalam tanah. Tetapi strain 440 dan strain 110 berjaya didapati kembali dalam semua pelakuan.*

ABSTRACT

Three Rhizobium *strains including two fast-growers 440 and Mar 1 and one slow-grower 110 were used singularly and in combination of two to inoculate two soybean varieties, namely Palmetto and Kahala. The* Rhizobium *strains were indentifiable with antibiotic markers. The experiment was conducted in both the Leonard Jar and in the field where soybeans had been cultivated for many years. In the Leonard Jar experiment, all* Rhizobium *treatments performed significantly better than the control in nodule formation and plant dry mass production. For field trial, no significant* Rhizobium *effects were observed. In general, Kahala was more responsive to* Rhizobium *inoculation than Palmetto. The isolation tests indicated that the probality of recovering the original inoculated strains in nodules was high. However, some strains were more competitive than the others. Mar 1 failed to be recovered in competition with strain 440 or with indigeneous strains in the soil. On the other hand, strains 440 and 110 could be recovered successfully in all cases.*

INTRODUCTION

Rhizobium strains in association with soybean has the unique property of fixing atmospheric nitrogen. In general, soybean performance could be affected by *Rhizobium* strains inoculated. In addition, other factors such as soybean genotypes, environmental conditions and a complex interaction involving both biotic factors (Trinick 1985 and Blackweell 1985) also affect the growth and yield of the plant.

A *Rhizobium* strain is successful if it is competitive, persistent and effective. Though *R. japonicum* is typically a slow grower and host specific, the fast-growing variety of *R. japonicum* (*R. fredii*) has also been successfully isolated

(Keyser *et al.* 1982). A successful and effective fast-growing strain could improve the host performance by producing early nodulation and fixing nitrogen because rapid multiplication means better chance of early colonization of a biosphere.

The present study was undertaken to (a) determine the infectivity of some fast and slow growing *Rhizobium* strains on soybeans and (b) to ascertain their competitive ability in both a sterile environment and non-sterile soil in the field.

MATERIALS AND METHODS

Three *Rhizobium* strains with identifiable markers were used singularly and in combination of two to inoculate two soybean varieties namely Palmetto and Kahala. The *Rhizobium* strains used were:

Strain 110 : slow-grower, resistant to both streptomycin and spectinomycin at 100 μg ml^{-1}

Strain 440 : fast-grower, streptomycin resistant but spectinomycin sensitive at 100 μg ml^{-1}

Strain Mar : fast-grower, streptomycin sensitive but spectinomycin resistant at 100 μg ml^{-1}

Strains 440 and 110 were obtained from University of California, Davis whereas strain Mar was isolated from a field in MARDI, Serdang. A total of 14 treatments, inclusive of the non-inoculated control were studied, both in Leonard Jar and in the field.

Leonard Jar Experiment

Seeds of Palmetto and Kahala varieties were surfaced sterilized with 3% chlorox and washed thoroughly with sterile distilled water before inoculating them with a suspension (10^9/ml) of an appropriate strain or combination of strains of *Rhizobium.* Two germinated seeds were grown in the Leonard Jar assembly. A N-free nutrient solution was applied daily to ensure sufficient nutrients in the sand and to overcome the slow rate of absorption by the wick. The jars were placed in a completely randomized block design with 2 replications. Seedlings of 5 weeks old were harvested from each jar for the determination of (a) number of nodules, (b) dry mass of nodules, (c) plant dry mass and (d) strain identifications test.

Field Experiment

Seeds of both varieties were similarly surface-sterilized and washed thoroughly before they were inoculated with appropriate inoculants which were mixed with Christmas Rock Phosphate (1g/5ml culture solution). The inoculated seeds were kept overnight before planting in the field. A randomized complete block design with 3 replications was used. Each replicate consisted of 14 plots, each representing one treatment combination. Each plot was a raised bed of 1m long, 0.3m wide and 0.2m high.

Distance between plots was 0.4m. A total of 40 inoculated seeds per treatment were sown per plot of two rows with planting points 10 cm apart. The plants were thinned out to maintain a uniform population of 20 plants per plot after three weeks. Five plants were randomly selected from each plot five weeks after planting. The plants were uprooted carefully, cleaned and dried for the study of similar characteristics as listed in the Leonard jar experiment.

Isolation and Identification of Rhizobium *Strains from Nodules*

Nodulated plants of five weeks old from both the Leonard Jar and field experiments were collected and washed with running water to remove adherent dirt materials. Noduless from each treatment combination was detached from the roots and surfaced sterilized with 0.1% $HgCl_2$ for five minutes followed by five rinsings with sterile distilled water (Kowalski *et al.* 1974). The nodules were cut into two halves and placed in YEM agar plates incorporated with indicator bromothymol blue. The plates were then kept at 28°C for 48 hours. Appearance of yellow colonies would indicate a fast-growing strain(s) whereas a blue colony would indicate a slow-growing strain(s) of *Rhizobium japonicum.* The colonies were later transferred into a YEM broth for each individual type of colony isolated per treatment combination. After five days of growth at 28°C,

the suspension concentration was determined. A serial dilution was then done to obtain an average of 80–100 cells per ml. This diluted suspension was then used in spread plate cultures. The spread plate was of YEM agar with bromothymol blue and incorporated with the appropriate antibiotics. The different strains were differentiated according to the following anticipated results:

Rhizobium strains	Bromothymol blue incorporated with Streptomycin (100 μg ml^{-1})	Spectinomycin (100 μg ml^{-1})
110	Blue colony, resistant	Blue colony, resistant
440	Yellow colony, resistant	No growth, sensitive
Mar 1	No growth, sensitive	Yellow colony, resistant

RESULTS

Effects of Rhizobium *Treatments on Nodulation and Plant Growth*

Plants of five weeks old were used to measure nodulation response and plant growth in terms of number of nodules, dry mass of nodules and plant dry mass. Analysis of variance for these traits is presented in Table 1. In both the Leonard Jar and field experiments, varietal variation was highly significant for the three characteristics measured, with Kahala performing better than Palmetto. With respect to *Rhizobium* treatments, significant variation was observed only in the Leonard Jar experiment (Table 1), where all *Rhizobium* treatments, whether applied singularly or in combination of two, induced significantly better nodulation response (Table 2) and plant growth (Table 3) than the control. In fact, there was no formation of nodules in the control treatment. However, differences among the different strains and their combinations were non-significant (Tables 1, 2 and 3). For nodule dry mass and plant dry mass, differential response was observed because soybean varieties showed significant interaction with *Rhizobium* treatment (Table 1).

For the field experiment, inoculation with different strains of *Rhizobium* regardless of individual or mixture of two, did not show significant difference in nodulation response and plant growth when compared to the uninoculated control (Tables 1, 2 and 3). However, interaction between soybean varieties and *Rhizobium* treatments was significant only for nodule dyr mass.

Recovery of Rhizobium Strains

The fast-grower is an acid producer, changing the indicator bromothymol blue to yellow whereas the slow-grower, being an alkaline producer would change the indicator to blue. Two types of antibiotics (100 μg ml^{-1}) were used to differentiate the two fast-growing strains. Strain Mar 1 is sensitive is to streptomycin whereas Strain 440 is sensitive to spectinomycin. The slow-grower i.e. 110 is however, resistant to both antibiotics.

Results of the identification tests are presented in Table 4. In the Leonard Jar experiment, there was distinct recovery of the *Rhizobium* strain(s) introduced except for the mixture of strains 440 and Mar 1, where strain Mar 1 could not be recovered. No nodules were found in the control.

In the field experiment, strain identification is rather difficult and often non-specific because of the presence of indigenous strain(s) in the soil. For example, when the nodules of plant inoculated with strain 440 was examined, the agar with indicator impregnated turned yellow and blue, suggesting the presence of a fast-grower, probably strain 440; and also slow-grower(s), probably indigenous strains(s). However, only the slow-growers were detected from the nodules of plants inoculated with strain 110, suggesting the recovery of strain 110 and/or other indigenous slow-growers. The nodules of plants grown in soil inoculated with Mar 1 strain only contained fast-growing strains sensitive to spectinomycin. Similarly, the originally inoculated spectinomycin resistant Mar 1 strain was not recovered in a mixture with either strain 440 or strain 110. In a mixture of strains 440 and 110, both strains were recovered. However, the relative proportion of the two strains could not be ascertained because the identification was qualitative. In the uninoculated plot, the harvested nodules showed only the presence of slow-growing strain(s).

TABLE 1
Analysis of variance for number of nodules, dry mass of nodules (mg/plant) and plant dry mass (g/plant) in the Leonard Jar and the field (parenthesis) experiments.

Item	df	Mean Squares		
		Number of Nodules	Dry Mass of Nodules	Plant Dry Mass
Replicates	1 (2)	78.89 ** (5.90)	113.00 ** (31.65)	0.02 ** (3.64**)
Varieties (V)	1 (1)	549.14** (378.23**)	763.89** (3724.30**)	0.15** (15.54**)
Rhizobium treatments (R)	6 (6)	58.19** (6.84)	78.43** (149.49)	0.01* (0.73)
V x R	6 (6)	5.56 (1.31)	67.84** (2/5.77*)	8.33×10^{-3}* (0.35)
Remainder	13 (26)	16.88 (6.37)	14.41 (66.89)	2.33×10^{-3} (0.56)

*, ** significant at $P < 0.05$ and $P < 0.01$ respectively.

TABLE 2
Mean values of nodule number and nodule dry mass in the Leonard Jar and the field (parenthesis) experiments for different *Rhizobium* treatments

(a) Number of nodules/plant

Rhizobium treatments	Soybean Varieties		Mean
	Palmetto	Kahala	
440	1.50 (3.83)	18.00 (9.92)	9.75 (6.87)
110	5.75 (3.58)	9.25 (9.17)	7.50 (8.83)
Mar 1	4.00 (6.00)	13.50 (10.08)	8.75 (8.04)
440 + 110	3.50 (1.00)	16.25 (8.92)	9.87 (4.96)
440 + MAR 1	4.50 (3.33)	11.50 (7.42)	8.00 (5.37)
110 + MAR 1	5.50 (3.00)	18.50 (10.33)	12.00 (6.66)
Control	0.00 (3.67)	0.00 (10.25)	0.00 (6.96)
Mean	3.57 (3.49)	12.42 (9.44)	7.99 (6.46)

LSD_{05} (for rhizobium treatment comparison) = 6.28 (3.00)

(b) Dry mass of nodules (mg/plant)

Rhizobium treatments	Soybean Varieties		Mean
	Palmetto	Kahala	
440	2.25 (8.40)	18.25 (32.13)	10.25 (20.26)
110	3.87 (9.20)	4.25 (8.47)	4.06 (8.83)
MAR 1	5.25 (12.83)	13.25 (17.10)	9.25 (14.96)
440 + 110	2.50 (0.93)	15.75 (25.13)	9.12 (13.03)
440 + MAR 1	2.00 (1.80)	15.00 (31.20)	8.50 (16.50)
110 + MAR 1	2.25 (10.67)	24.75 (33.40)	13.50 (22.03)
Control	0.00 (8.07)	0.00 (36.30)	0.00 (22.18)
Mean	2.59 (7.41)	13.04 (26.25)	7.81 (16.83)

LSD_{05} (for rhizobium treatment comparison) = 5.80 (11.89)

TABLE 3
Mean values of plant dry mass (g/plant) in the Leonard Jar and the field (parenthesis) experiments for different *Rhizobium* treatments

Rhizobium treatments	Soybean Varieties		Mean
	Palmetto	Kahala	
440	0.22 (1.41)	0.41 (3.40)	0.31 (2.40)
110	0.20 (1.78)	0.33 (2.41)	0.26 (2.09)
MAR 1	0.24 (1.55)	0.38 (2.93)	0.31 (2.24)
440 + 110	0.19 (0.87)	0.39 (1.99)	0.29 (1.44)
440 + MAR 1	0.22 (1.13)	0.35 (2.77)	0.28 (1.95)
110 + MAR 1	0.19 (1.75)	0.46 (2.40)	0.32 (2.08)
Control	0.18 (1.93)	0.17 (2.99)	0.17 (2.46)
Mean	0.20 (1.49)	0.36 (2.70)	0.28 (2.09)

LSD_{05} (for rhizobium treatments comparison) = 0.07 (0.88)

DISCUSSION

In both the field and Leonard Jar experiments, a significant varietal variation was noted in the nodulation response where the variety Kahala produced more nodule number and nodule dry mass than Palmetto. In addition, Kahala also performed better than Palmetto in terms of plant dry mass, suggesting that Kahala was more responsive to *Rhizobium* inoculation.

With regards to *Rhizobium* treatments, no parellel conclusion can be drawn from the two experiments. In the Leonard Jar experiment where sterile sand was used, all strains whether inoculated singularly or in combinations of two, induced significantly higher nodulation response in comparison to the uninoculated control where no nodules were formed. In terms of number of nodules and nodule dry mass, the combination of fast-grower (strain Mar 1) and slow-grower (strain 110) produced the best nodulation response. As strain 110 showed relatively poor nodulation when applied singularly, such a positive competitive advantage (Johnson and Means 1964) suggests the coexistence of mutually adaptive strains. In fact, both strains were recovered from nodules of plants inoculated with this mixture.

The field experiment failed to give any significant variations with regards to both nodulation response and plant growth. The indigenous strains in the soil presumably to be more effective and adaptive, could have buffered or masked the effects of the introduced strains resulting in non-significant difference between *Rhizobium* treatments and the control. Hence, the soybean is unlikely to benefit from *Rhizobium* inoculation if the soil already contained a natural population of more adaptive *Rhizobium*.

The results of the competition experiment revealed that though the probability of recovering the original inoculant strains in nodules was very high, some strains were found to be more competitive than the others. For example, strain Mar 1, a locally isolated fast-grower was found to be a poor competitor. In the Leonard Jar experiment, a mixture of Mar 1 with 440 revealed only the presence of strain 440 in the nodules harvested. In the field experiment, the vulnerability and ineffectiveness of Mar 1 was also evident because it failed to be recovered when used singularly or in combination with other strains. On the other hand, both strains 440 and 110 could compete successfully with the indigenous strains to cause nodulation. Whether they were applied singularly or in combination with other strains, they still could be recovered in the nodules.

The nodules of plants grown in soil inoculated with Mar 1 strain contained fast-growers sensitive to spectinomycin, suggesting the presence of another fast grower different from Mar 1 because the latter is spectinomycin

TABLE 4
Identification of the *Rhizobium* strains from nodules of plants grown in the Leonard Jar and the field (parenthesis) experiments.

Original inoculant	Colour indicator	Antibiotics Streptomycin	Spectinomycin	Inference
440	Y (Y & B)	Y^R (Y^R B^R)	Y^S (Y^S B^R)	440 (410 and indegenous slow-grower)
110	B (B)	B^R (B^R)	B^R (B^R)	110 (110 and other slow-grower?)
Mar 1	Y (Y)	Y^S (Y^R)	Y^R (Y^S)	Mar 1 (Fast-grower but not Mar 1)
440 + 110	Y & B (Y & B)	Y^R B^R (Y^R B^R)	Y^S B^R (Y^S B^R)	440 & 110 (440 and 110)
440 + Mar 1	Y (Y)	Y^R (Y^R)	Y^S (Y^S)	440 (440 and other fast-grower but not Mar 1)
110 + Mar 1	Y & B (Y & B)	Y^S B^R (Y^R B^R)	Y^R B^R (Y^S B^R)	110 & Mar 1 (110 and other fast-grower but not Mar 1)
Control	ns (B)	ns (B^R)	ns (B^R)	Nil (Indigenous slow-grower)

Key: B = Blue; Y = Yellow; B^R = Blue, resistant to antibiotics; B^S = Blue, sensitive to antibiotics; Y^R = Yellow, resistant to antibiotics; Y^S = Yellow, sensitive to antibiotics; ns = no sample available.

resistant. However, another indigenous fast-grower is unlikely to be found in the soil because the control treatment indicated that the soil contained only indigenous slow-grower(s). Thus, it is speculated that strain Mar 1 is rather unstable under field conditions. Spontaneous mutation might have occurred during growth of the strain in the course of nodule development such that the strain has become spectinomycin sensitive (Johnson and Beninger 1975).

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