5-2005

Collection, isolation and evaluation of nitrogen fixing bacteria in the Rio Grande Valley of Texas

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COLLECTION, ISOLATION AND EVALUATION OF NITROGEN FIXING BACTERIA IN THE RIO GRANDE VALLEY OF TEXAS

A Thesis

by

THOMAS M. EUBANKS

Submitted to the Graduate School of the
University Of Texas-Pan American
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Biology

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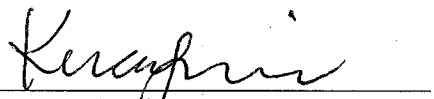

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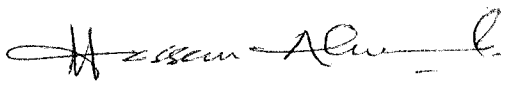

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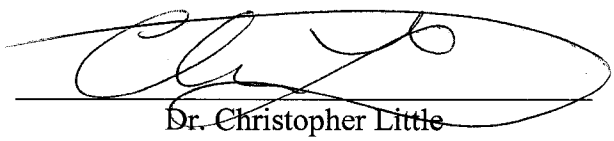

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ABSTRACT

Eubanks, Thomas M., Collection, Isolation and Evaluation of Nitrogen-Fixing Bacteria in the Rio Grande Valley of South Texas Master of Science (MS), May 2005, 52pp., 11 Figures , 3 tables, 3 Appendixes, references 19 titles.

Isolation from local soil samples with *Phaseolus vulgaris* (L.) produced 20 samples from which pure cultures were obtained. A commercial strain of *Rhizobium* #132 was obtained from Becker Underwood and included as a standard in the experiment. The results demonstrated that a variety of infective and effective of strains of *Rhizobium* nodulating *Phaseolus vulgaris* (L.) exist in the native soils of the Rio Grande Valley of Texas. A cluster of effective bacteria was identified and can serve as a basis for recommendations to inoculant industry in the Valley. An isolate collection has been established to serve as a source of material for further research.

Journal: International Journal of Plant Sciences

ACKNOWLEDGMENTS

I would like to thank my graduate committee: Dr. Luis Materon, Dr. Anxiu Kuang, Dr. Hassan Ahmad, and Dr. Christopher Little for their help in this research. This would not have been possible without the dedicated support and friendship of my advisor, Dr. Luis Materon and my co-chair Dr. Anxiu Kuang. I would also like to thank Dr. Zilbilski for use of the facilities at the Kika de la Garza Subtropical Agricultural Research Center (USDA/ARS) in Weslaco, Texas for the initial phase of the experiments.

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INTRODUCTION

Statement of the problem

Plants need nitrogen to grow, which they derive from fixed or chemically available nitrogen. Annual global production of synthetic nitrogen fertilizers by the Haber process exceeds 150 million tons per year and is projected to increase at a rate of over 5% per year (Bumb and Baanante 1996). Biological fixation is estimated at twice the industrial production but plant acquisition exceeds both by a factor of ten (Taiz and Zeiger 1998). The average time that a nitrogen molecule remains in the organic form is about 370 years (Taiz and Zeigler 1998).

Global use of fossil fuel as a feedstock for industrial synthesis of nitrogen fertilizer is not sustainable (Miller 2001). By the year 2020, fertilizer demand is expected to reach 208 million tons per year (Bumb and Baanante 1996). World population projections at current rates will exceed 11 billion people by 2050 (Miller 2001). Clearly the use of exhaustible resources to support modern agriculture is not sustainable. Advances in agricultural sustainability require an increase in the utilization of biological nitrogen fixation.

Conceptual framework

How well a legume develops at a particular site is the result of many factors among them an effective symbiosis between host plant and strain of

Rhizobium. Experience has shown that introduced legumes may fail to nodulate effectively in a new environment (Date 1982). The goals of this research are to collect, isolate and evaluate soil bacteria of the genera *Rhizobium* which are genetically and geographically affiliated within the soils of the Rio Grande Valley of Texas. Soil samples were collected from Starr, Hidalgo, Willacy and Cameron county Texas. *Phaseolus vulgaris* (L.) was grown in the different soil types to directly isolate the rhizobia. The nitrogen fixing potential or effectiveness of the isolates was evaluated with the goal of establishing an isolate collection for further research.

REVIEW OF THE LITERATURE

Isolate collection

Isolate collection, preservation and characterization of *Rhizobium phaseoli* has been successful in many areas in the world (Mott 1978; Beck, Materon and Afendi 1993; Date 1982). Common to all these protocols are records of soil type, elevation, location and vegetation present at the collection site. Standard microbiological techniques were employed to prevent contamination during collection as well as for the protocols for isolation and evaluation (Beck, Materon and Afendi 1993). Various methods are reviewed for identifying and isolating pure cultures of rhizobia (Somasegaran and Hoben 1994; Gibson 1980). Congo Red media is used to help in identification as well as the gram stain (Hahn 1966). *Rhizobium* stain gram negative and only pick up small amounts of Congo red from the media (Beck, Materon and Afendi 1993). Inoculation under sterile conditions can be used to verify inoculants (Beck, Materon and Afendi 1993). Statistical parametric evaluation is based on the experimental design and sample size (Somasegaran and Hoben 1994; Beck, Materon and Afendi 1993).

Biological Nitrogen Fixation

Krascheninikov appears to be the first to document nitrogen fixation in excised nodules in 1916 (Allison, Hoover and Minor 1939). There are at least

1,350 herbaceous and woody plants that have the ability to fix atmospheric nitrogen in the soil (Tilth 1982). In this list there are only 25 used in agriculture with beans, peas, clover and alfalfa the best known. Symbiotic relationships can occur between the members of the plant family Leguminosae and soil bacteria of the genera Rhizobium, *Bradyrhizobium, Azorhizobium, Sinorhizibium*, and *Photorhizobium*. Only certain prokaryotes can fix atmospheric nitrogen. Included are the cyanobacteria of soils and fresh and salt waters and other free living soil bacteria. The nitrogen fixing bacteria that live as symbionts in the roots of leguminous plants are the focus of this research.

Biological nitrogen fixation accounts for most of the atmospheric conversion of molecular nitrogen into ammonium. This is a key point of entry of molecular nitrogen into the biogeochemical cycle of nitrogen. The triple bond of atmospheric N_2 is very stable with a bond energy of 942 KJ/mole (Nelson and Cox 2000). Free living bacteria lack the energy to fix significant amounts of nitrogen. The symbiotic relationship between higher plants and nitrogen-fixing bacteria is a complex genetic and signaling interaction that provides this energy in the form of photosynthate. Nitrogen-fixing legumes can be divided into two groups, either amide exporters or ureide exporters based on the content of the xylem. Legumes from cooler climates tend to export amides like asparagines or glutamine while legumes of tropical origins export nitrogen in the form of ureides (Taiz and Zeigler 1998). Allantoin, allantoic acid, and citruline are the three major forms of ureides. Once formed in the peroxisomes or endoplasmic reticulum of the nodule

they are released into the xylem for transport (Taiz and Zeigler 1998). In the shoot they are they are catabolized into ammonium and combined with glutamine synthetase and glutamate to form glutamine and then into other essential amino acids via transamination (Nelson and Cox 2000).

The Infection Process

The infection process begins with the migration of *Rhizobia* toward the roots of the host legume (Taiz and Zeigler 1998). This is a chemotaxic response to the chemicals produced in the root under nitrogen limited conditions. The elaborate exchange of signaling factors and the infection process has been the focus of extensive research. Lectins may serve as molecular glue to hold the bacteria on the surface of the root hairs or they may have a role in signaling as well (Kline et al.1997 and Hirsch 1999). Isoflavonoids and betaines, secreted by the roots activate the rhizobial NodD protein which then induces the transcription of the other *nod* genes (Phillips and Kapulnik 1995). Plant genes specific to nodules are called nodulin (Nod) genes and rhizobial genes that are involved are referred to as nodulation (*nod*) genes (Heidstra and Bisseling 1996). The *nod* genes are classified as either host specific or common *nod* genes. The host specific *nod* genes like *nod*P, *nod*Q, *nod*H, or *nod*F, *nod*E, and *nod*L differ in rhizobial species and control host range. The common *nod* genes, *nod*A, *nod*B, and *nod*C are found in all rhizobial strains (Taiz and Zeigler 1998).

The Chemistry of Nitrogenase

Dinitrogenase reductase and dinitrogenase are a highly conserved complex of proteins that are called the nitrogenase complex (Nelson and Cox 2000). These enzymes are capable of reducing the very stable nitrogen triple bond to ammonia. The nitrogenase complex and the binding and hydrolysis of ATP overcome the high energy of activation. The ammonia produced is toxic and is rapidly converted into other organic forms in the root nodules before being transported up the plant via the xylem (Taiz and Zeigler 1998). The nitrogenase enzyme complex is irreversibly inactivated by oxygen so nitrogen must be fixed anaerobically (Taiz and Zeigler 1998).

Legumes regulate gas permeability in their nodules with an oxygen binding heme protein called leghemoglobin (Taiz and Zeigler 1998). The host plant produces the globin portion as a response to infection and the bacterial symbionts make the heme portion (Marschner 1995). Leghemoglobin gives nodules a pink color (Taiz and Zeigler 1998).

MATERIALS AND METHODS

Isolation Procedure

Soil samples were collected from 83 sites representing the different soil types that are found without grade (see Appendix Table 3). These sites were identified and located using 4 county soil survey maps from the Rio Grande Valley of Texas. Areas were identified on the maps and collected over a thirty-day period. Samples of ¼ pound were taken at a depth of two inches in undisturbed locations and placed in a plastic zip lock bag. A small hand shovel was then thoroughly sprayed with a wash bottle containing 50% clorox solution and rinsed with water three times. It was then transported to the next site in a 5 gal bucket containing 50% clorox and 20 grams detergent per gallon of water. The soil sample was taken, stored, and the elevation and time recorded. Locations were recorded using a GPS receiver.

Plant cultivation was performed at the USDA Kika Dela Garza Center for Subtropical research in Weslaco Texas. *Phaseolus vulgaris* (L.) was grown in four inch pots to isolate the native populations of *Rhizobium phaseoli* bacteria. Sterile Sunshine soil mix #1 was used to fill 70 % of the container. Using plastic spoons and gloves, 2 teaspoons of native soil was placed in each pot with two pinto bean seeds that had been surfaced sterilized with alcohol, clorox and water.

The pot was then filled with more potting soil. Sixteen pots were filled and planted with pinto been seeds without any native soil to serve as controls.

The plants were watered once a day by an automatic sprinkler system using reverse osmosis water. The plants were harvested after 30 days. Isolation of the rhizobial populations was done at the UTPA microbiology laboratories. The procedure consisted of removing three nodules, small, medium and large from each of the plants and surface sterilizing them. The process involves washing with water to remove all soil and debris. The nodules were then placed in 95% ethanol for 10 seconds to break the surface tension and then into 5% clorox for thirty seconds. The nodules were then rinsed 5 times in sterile reverse osmosis water. The nodule contents were then crushed with the tip of forceps. The crushed nodule was applied onto the surface of TSA media plates and placed into an incubator overnight @ 37° C. Isolates were designated from 1 to 83 as they were processed. Isolates were obtained and streaked out to single colonies. Samples of each isolate were then viewed microscopically using Gram's stain. Rhizobia specific to *Phaseolus* are small pink (Gram negative) rod shaped bacillus. Presumptive isolates were then placed on TSA slants and stored at 4°C

Evaluation of Isolates

One thousand new 4-inch pots were disinfected and filled with course grade vermiculite that had been rinsed and drained with reverse osmosis water and then autoclaved at 121°C for 45 minutes. Two *Phaseolus vulgaris* (L.) cv. Pinto Bean seeds that had been surface sterilized were planted in each pot. Pots

were then covered in saran wrap to prevent contamination. After 5 days in the greenhouse, the surfaces of the containers were sprayed with 75% ethanol and a razor was used to cut the plastic open to stand up the sprout. The other seed or sprout was removed. Another cover of saran wrap was used to reseal around the stem of each plant. After two more days, 690 plants, 2 inches tall were selected and placed into 23 groups of thirty plants per isolate. Each group was split into three groups and randomly assigned position in the greenhouse.

Inoculants were prepared from the isolate collection established in the beginning of this study. Ten ml of yeast mannitol broth (YMB) was inoculated with a loopful of each isolate of *Rhizobium* and placed in a shaker with a 37°C water bath for 2 days. Ten fold serial dilutions were used to calculate populations. Forty ml of each inoculant were prepared adjusting to 1×10^{-7} bacteria per ml for each of the isolates. One ml of each prepared was used for inoculation of 30 pinto bean sprouts in each group in a random block design. The plants were then weighed and adjusted to 480 grams with a nitrogen free Hoagland solution. The positive nitrogen controls (plants not inoculated) were watered with 300ppm of nitrogen (from ammonium nitrate) added to this mixture. The negative nitrogen controls (plants not inoculated) only contained reverse osmosis water. A commercial inoculant (#132) was obtained from Becker Underwood in Ames Iowa to compare and contrast with the local strains as well. They were harvested at 64 days when the plants were at the flowering stage. The vermiculite was carefully removed as not to detach nodules under a gentle stream of water. In the laboratory the number of flowers, and bean pods were recorded for each plant and the tops then dried for

four days at 70°C and their dry weight recorded. The numbers of lateral and crown nodules as well as total weight of the nodules were recorded for each plant.

Electron Microscopy

Preparation of the nodules for imaging was done to preserve the morphology of the nodules while under the electron beam at 20 KV. Proteins were cross-linked with FAA (10% formalin, 85% ethanol, 5% glacial acetic acid) and then the nodules dehydrated in an ethanol series (Bozzola and Russell 1999). Any moisture will prevent successful imaging. Critical point drying is successful because the sample is slowly dried totally immersed in a dense vapor phase avoiding the crushing forces of the air/liquid interface passage. Sputter coating provides a conductive coating, minimizing damage due to charge accumulation. The coating also serves to provide a source of secondary electrons for improved imaging.

Protocol for sample preparation

Nodules from each of the isolates were removed from the roots and carefully cleaned. They were washed in 70% ethanol and rinsed in distilled water three times. They were then fixed in FAA overnight and dehydrated in 70%, 80%, 90% and 100% ethanol for 30 min at each dilution. The nodules were then placed in a Samdri critical point dryer (Bozzola and Russell 1999). The unit was turned on and the electrically heated valves allowed to warm for ten minutes. The temperature was reduced to -10°C and then the sample was immersed in liquid

carbon dioxide. The first bath was allowed to bleed off slowly while being replaced with more liquid carbon dioxide to wash out the ethanol. The heater was turned on and set to 31.1°C, which caused the pressure to reach the critical point of 1,073 psi. Once the critical point was reached, the heater was left on and the sample was allowed to slowly vent over ten minutes. After drying the nodules were then mounted on SEM stubs and sputter coated for 60 seconds (Denton Desktop II). Images were obtained on a Leo 435 scanning electron microscope and kept in digital files for further printing.

RESULTS

Only twenty five percent of the soil sample sites had native population densities of *R. phaseolus* that were recovered to pure culture. The experimental design with thirty plants per group allowed for parametric analysis of the data. Twenty native site isolates were evaluated alongside a commercial inoculant (132) and negative (0) and positive (300) controls. The results of these evaluations are presented in Figures 1-6. The results indicate that there are native isolates of *R. phaseolus* that compare favorably with the use of industrial fertilizers to produce a viable legume crop. It was also found all of the strains tested performed better than having no nitrogen at all on parameters recorded. The high dry shoot weight and low flower score in the group with 300 ppm of nitrogen indicates that high levels of nitrogen can force vegetative growth over reproductive expression. The negative nitrogen control group developed chlorosis and did not grow much after the third week. Several of the native strains merit further evaluation based on their successful performance on many of the characteristics measured.

Fig.1

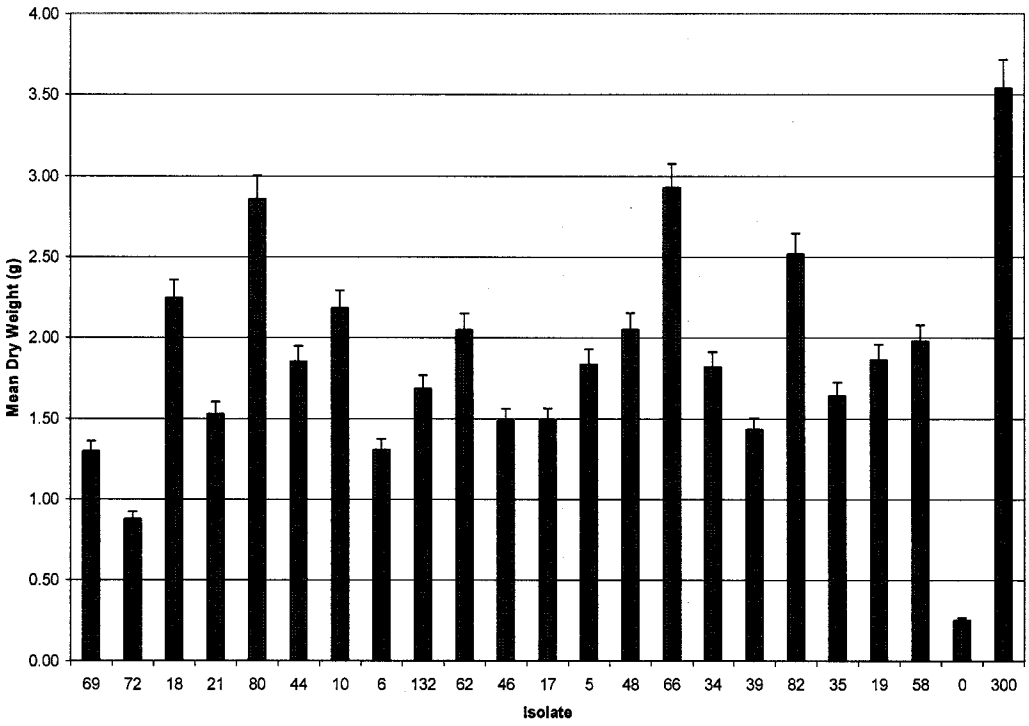


Fig.1 illustrates that the nitrogen treatment #300 produces the heaviest plant. The native isolates performed well against the commercial inoculant 132. Even the poorest of the isolates did better than no nitrogen at all in this experiment. The positive and negative nitrogen treatments are shown as 300 and 0.

Fig. 2

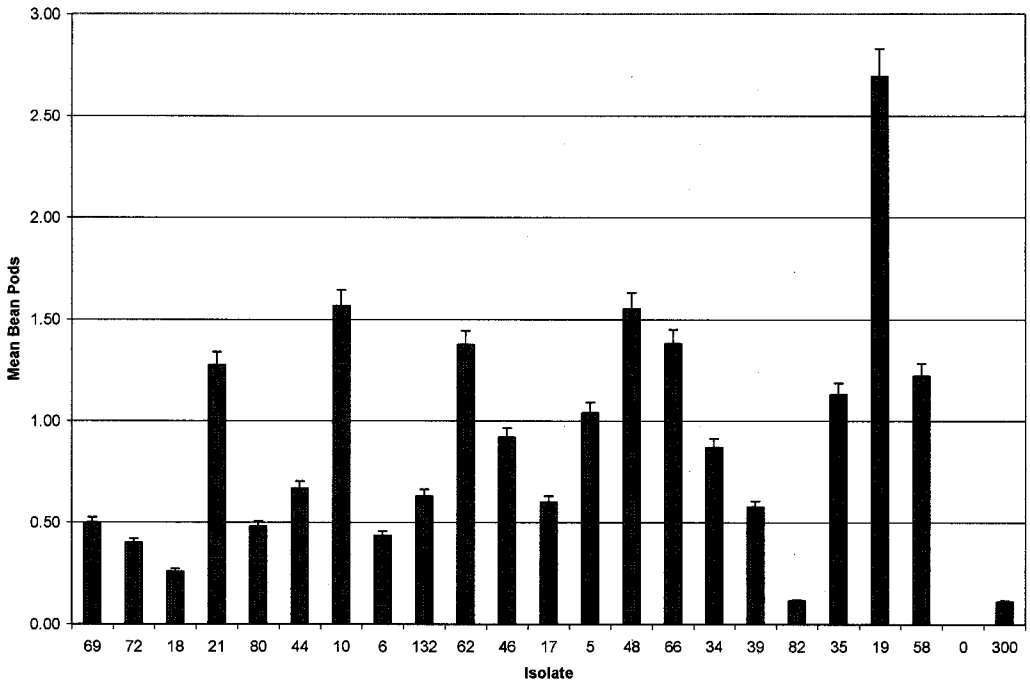


Fig. 2 illustrates that in terms of the number of bean pods on a plant, isolate #19 outperformed the commercial *Rhizobium* strain #132 and most of the others by a factor of two or more. More than half of the local isolates also did better than #132. The positive and negative nitrogen treatments are shown as 300 and 0. The high of level of nitrogen in group 300 may have either delayed blooming or inhibited it at sixty four days. Isolate # 19 merits further evaluation for the inoculant industry.

Fig. 3

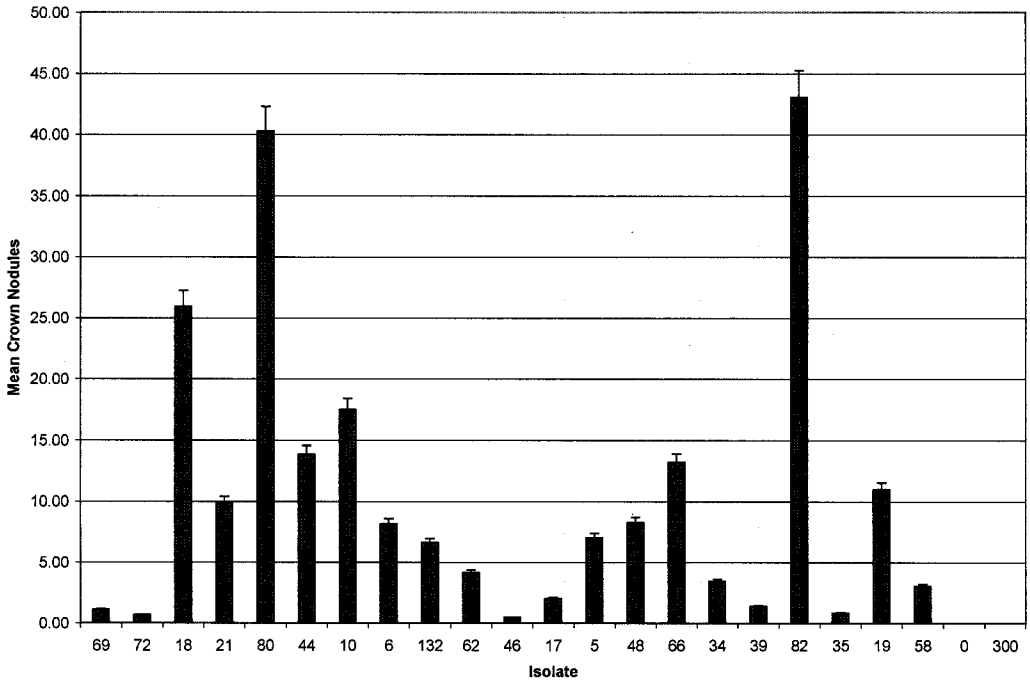


Fig. 3 shows that comparing the mean of the crown nodule number of the 21 isolates, # 82 and # 80 had the most nodules within the first inch of the root. Nine of the native isolates had less than 5 crown nodules. The zero levels recorded for the positive and negative nitrogen treatments shown as 300 and 0 demonstrate they were not contaminated.

Fig. 4

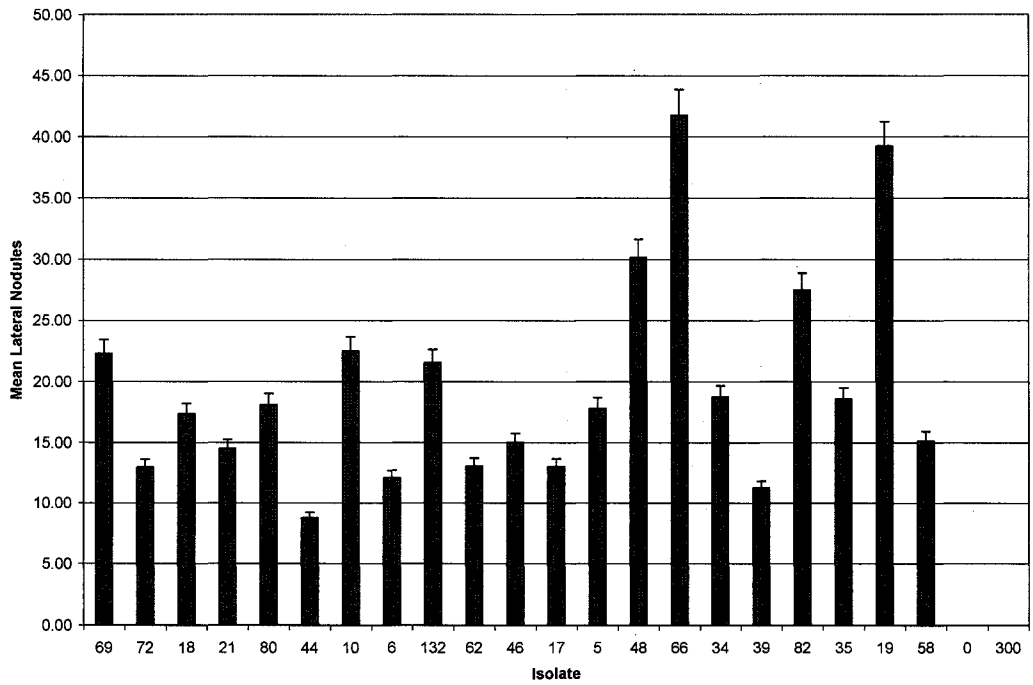


Fig. 4 illustrates that there is a four-fold variation in lateral nodule populations. The positive and negative nitrogen treatments are shown as 300 and 0. Twenty-five percent of the native site isolates had higher lateral nodule counts than the commercial strain 132. The zero scores for the nitrogen controls indicate bacterial containment strategies were successful.

Fig. 5

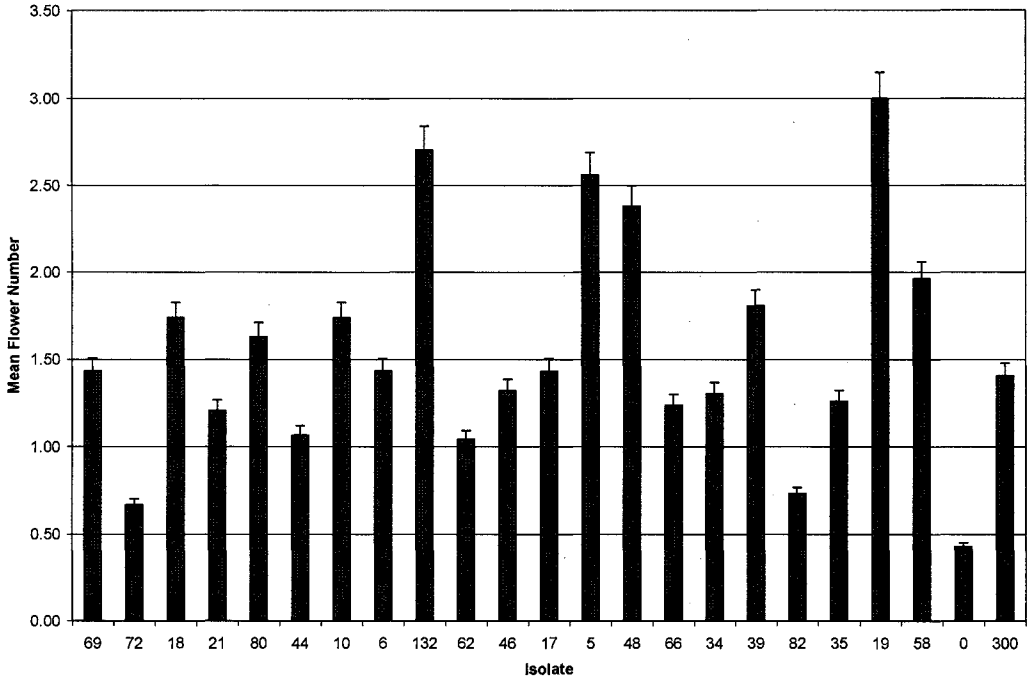


Fig. 5 shows that in terms of numbers of flowers, Becker Underwood stain #132 compares favorably with local isolates. The positive and negative nitrogen treatments are shown as 300 and 0.

Fig. 6

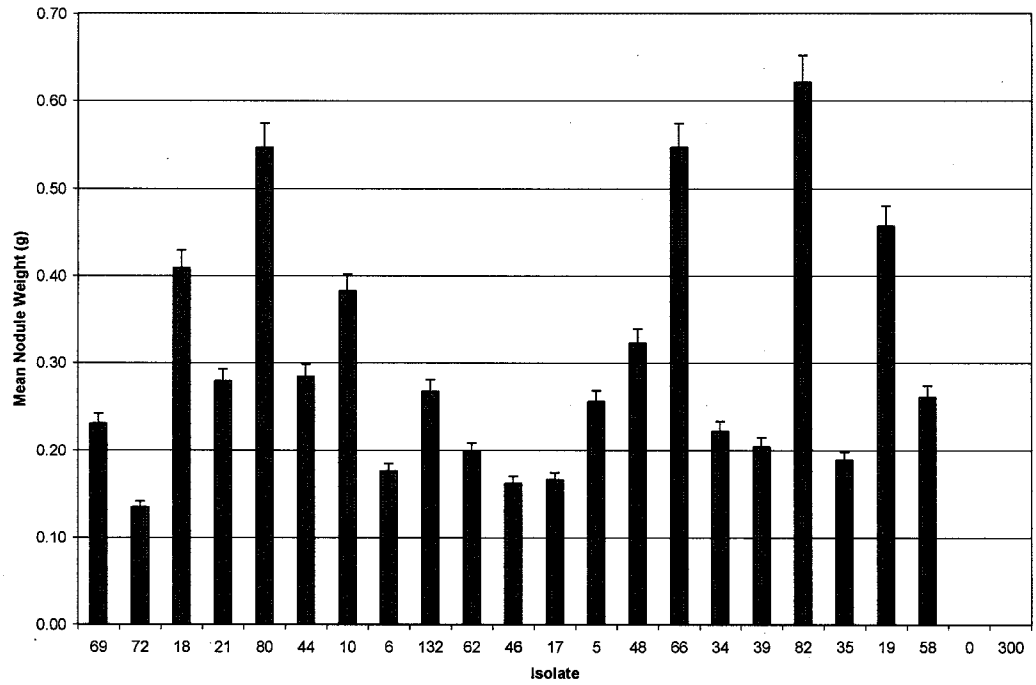


Fig. 6 illustrates that some of the isolate numbers here on the mean nodule weight figure also scored higher in the previous figures as well. The positive and negative nitrogen treatments are shown as 300 and 0. Many of the isolates that show higher mean nodule weights also scored high on many of the other characteristics observed.

Table 1. Contrast commercial *Rhizobium* #132 vs. local Isolates.

Contrast	R	DF	F value	Pr>F
Number of crown nodules	.376	1	0.12	.730
Total weight of nodules	.377	1	4.81	.028
Number of flowers	.126	1	2.22	.137
Dry shoot weight	.305	1	4.79	.029
Number of lateral nodules	.261	1	0.25	.618
Number of total nodules	.392	1	0.05	.823
Number of bean pods	.198	1	7.23	.007

Table 1 records the contrasts between the local isolates vs. the commercial *R. phaseoli* #132. This data shows that the dry shoot weight, total weight of the nodules and number of bean pods were significant at the 5% level of probability. The number of crown nodules, number of flowers, number of lateral nodules and number of total nodules were not significant at the 5% level of probability. The low R square value indicates this is not a simple or linear relationship.

Table 2. Contrast N (300 ppm) vs. *Rhizobium* Isolates

Contrast	R square	DF	F value	Pr>F
Number of flowers	.1220	1	2.24	.135
Dry shoot weight	.3452	1	4.11	.043
Number of bean pods	.2110	1	7.54	.006

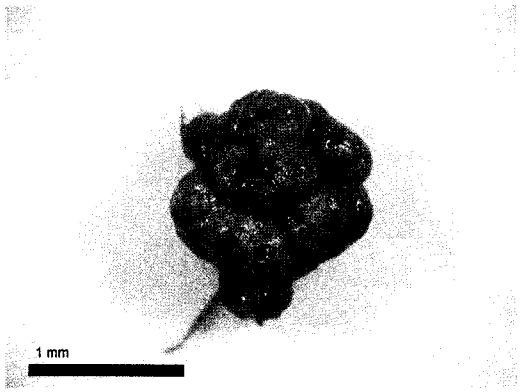
Table 2 contrasts the nitrogen treatment vs. the *Rhizobium* isolates. The dry shoot weight and the number of bean pods were significant at the 5% level of probability. The numbers of flowers were not significant at the 5% level of probability.

Table # 3

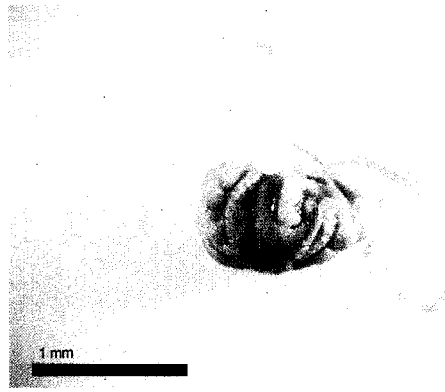
Shoot weight	Crown nodule	Lateral nodule	Nodule total	Nodule weight	Flower #	Bean pod #
N+ A	66 A	48 A	66 A	82 A	19 A	19 A
66 AB	19 AB	44 A	19 AB	80 AB	48 AB	10 B
80 AB	48 BC	66 A	48 BC	66 AB	132 AB	48 B
82 BC	82 BCD	18 A	82 BCD	19 BC	5 AB	62 BC
18 BCD	10 ECD	132 A	10 CDE	18 BCD	58 ABC	66 BCD
10 BCD	69 ECD	10 AB	132 CDEF	10 ECD	18 ABCD	21 BCD
48 ECD	132 EFCD	21 ABC	69 EFGD	48 EFCD	10 ABCD	58 BCD
62 ECD	34 EFGD	34 ABCD	34 DEFGH	44 EFGD	80 ABCD	35 BCDE
58 ECD	35 EFGD	82 ABCD	18 DEFGH	21 EFGD	6 ABCD	5 BCDE
19 ECD	80 EFGD	80 ABCDE	80 DEFGH	132EFGD	17 ABCD	46 BCDE
44 ECD	5 EFGD	19 ABCDE	5 DEFGH	58 EFGD	69 ABCD	34 BCDE
5 ECD	18 EFG	5 ABCDEF	35 EFGH	5 EFGD	39ABCD	44 BCDE
34 ECD	58 EFG	62 ABCDEF	21 EFGH	69 EFG	300 ABCD	132 BCDE
132 EFD	46 EFG	6 BCDEFG	58 EFGH	34 EFG	46 ABCD	17 BCDE
35 EFD	21 EFG	17 BCDEFG	46 FGH	39 FG	34 ABCD	39 BCDE
21 EFD	62 EFG	58 CDEFG	62 FGH	62 FG	35 ABCD	69 BCDE
17 EFD	17 EFG	39 DEFG	17 GH	35 FG	66 ABCD	80 BCDE
46 EFD	72 EFG	69 EFG	6 GH	6 FG	21 BCD	6 BCDE
39 EFD	6 FG	46 FG	44 GH	17 FG	44 BCD	72 BCDE
6 EF	39 G	35 FG	72 GH	46 FG	62 BCD	18 CDE
69 EF	44 HG	72 G	39 H	72 HG	82 DC	82 ED
72 GF	N- H	N- G	N- I	N- H	72 DC	N+ED
N- G	N+ H	N+ G	N+ I	N+ H	N- D	N- E

Table #3 is a summary table from a Ryan-Einot-Gabriel-Welsch Multiple Range rank test. Means with the same letter are not significantly different ($p < .05$). This table demonstrates the bell shaped or normal distribution of the isolates performance data for the parameters measured. N+ and N- indicate the nitrogen controls.

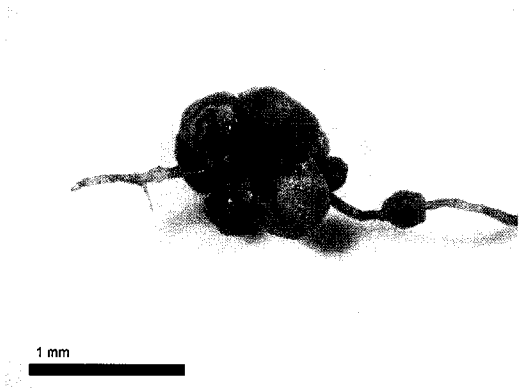
Fig.7 Photographs of the nodules in order of their shoot dry weight performance. Rank follows from left to right.



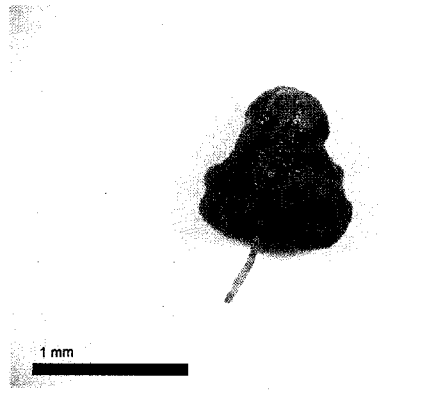
Nodule from isolate #66



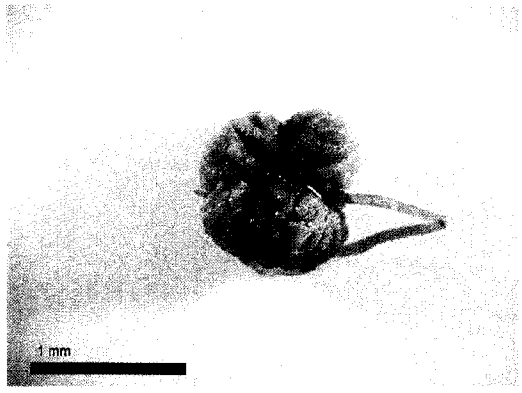
Nodule from isolate #80



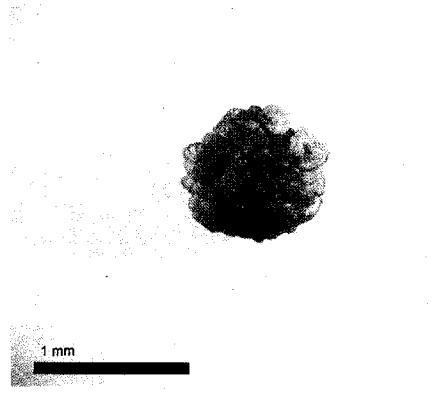
Nodule from isolate #82



Nodule from isolate #18

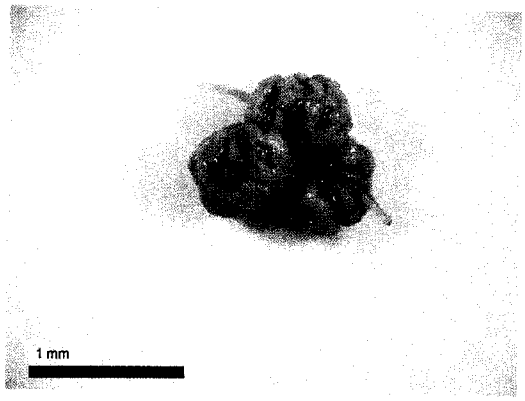


Nodule from isolate #10

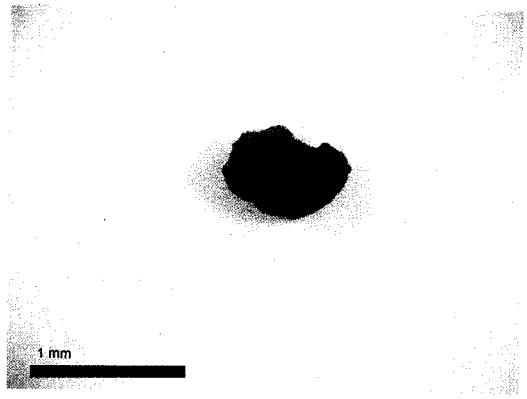


Nodule from isolate #48

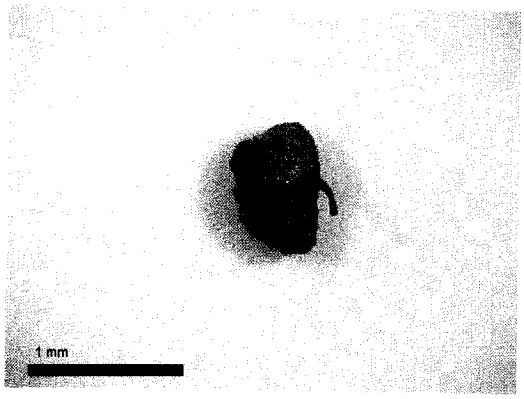
Fig.8



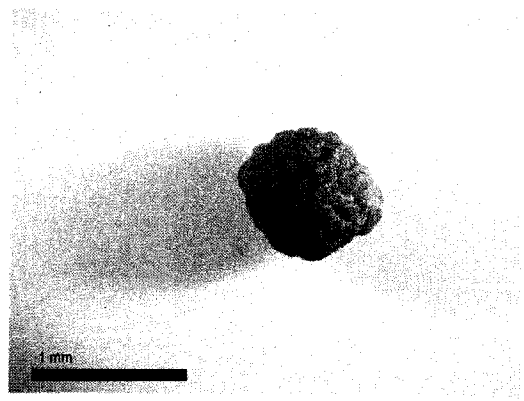
Nodule from isolate #62



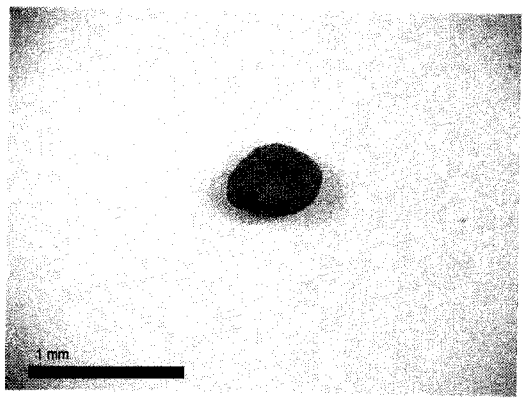
Nodule from isolate #58



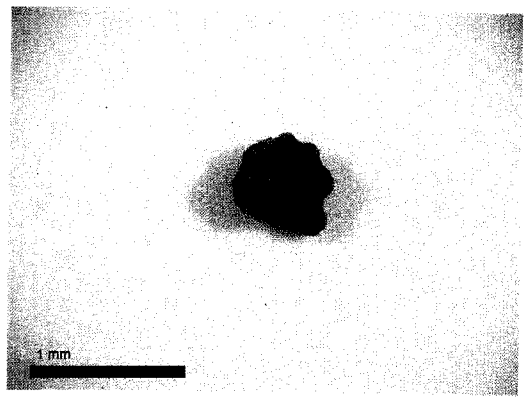
Nodule from isolate #19



Nodule from isolate #44

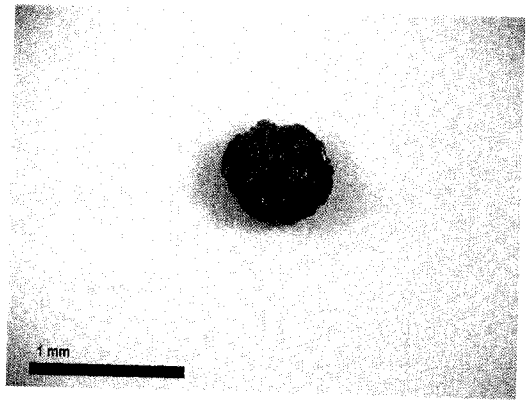


Nodule from isolate #5

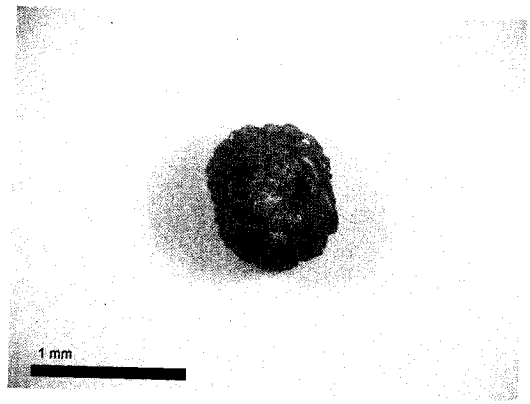


Nodule from isolate #34

Fig. 9



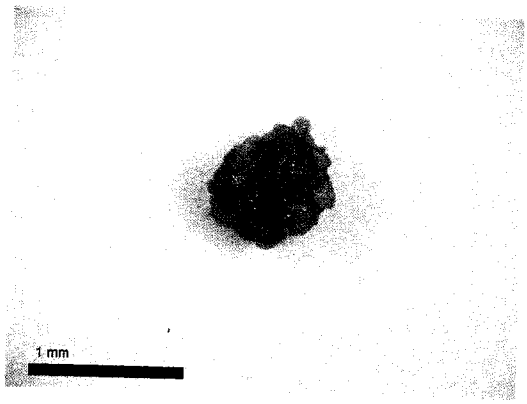
Nodule from isolate #72



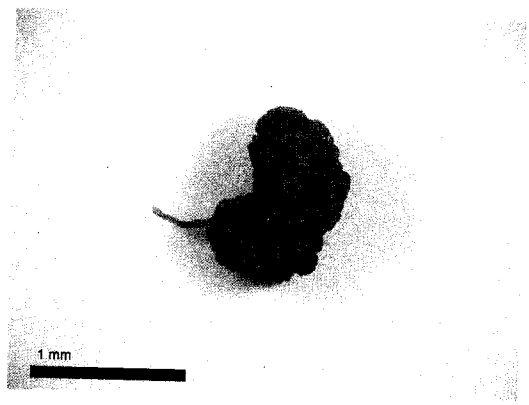
Nodule from isolate #35

Nodule from isolate #21

Nodule from isolate #17



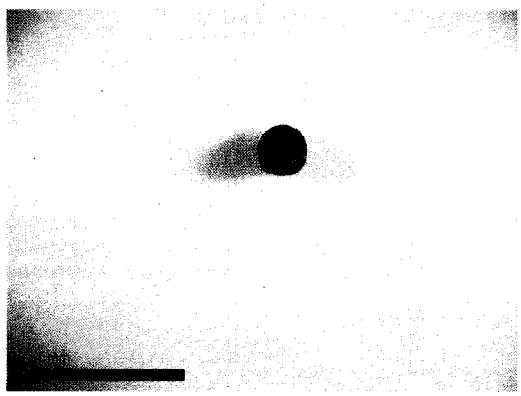
Nodule from isolate #46



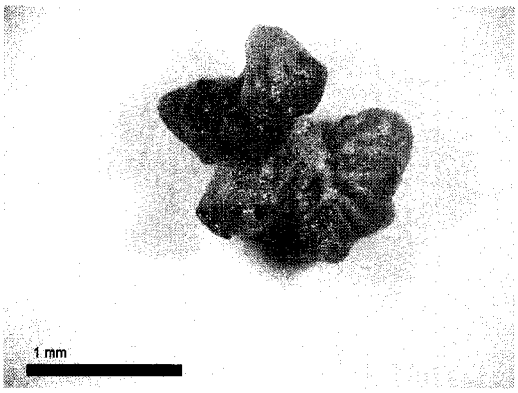
Nodule from isolate #39

Fig. 10

Nodule from isolate #6



Nodule from isolate #69



Nodule from Becker Underwood #132

HiStick® N/T

FOR USE ON DRY & SNAP BEANS

ACTIVE INGREDIENT: *Bacillus subtilis*, MBI 600 (not less than 2 x 10^8 viable spores per gram)	0.001%
OTHER INGREDIENTS:	99.999%
TOTAL:	100.000%

Contains a minimum of 2 x 10^9 viable rhizobial cells / gram acting as a rhizobial inoculant, and 2 x 10^8 cfu / gram *Bacillus subtilis* acting as a biofungicide.

KEEP OUT OF REACH OF CHILDREN

CAUTION

See additional precautionary statements inside this booklet.

30VBHSNTDB (rev. 10-02)

NET CONTENTS: 14 oz (400g)

EPA Reg. No.: 71840-2
EPA Est. No.: 71840-CAN-001

Another quality biological product from:
Becker Underwood, Inc.
801 Dayton Avenue, P.O. Box 667
Ames, IA 50010
Tel. (515) 232-5907 www.beckerunderwood.com

Fig.11 Soil Sample Location Map

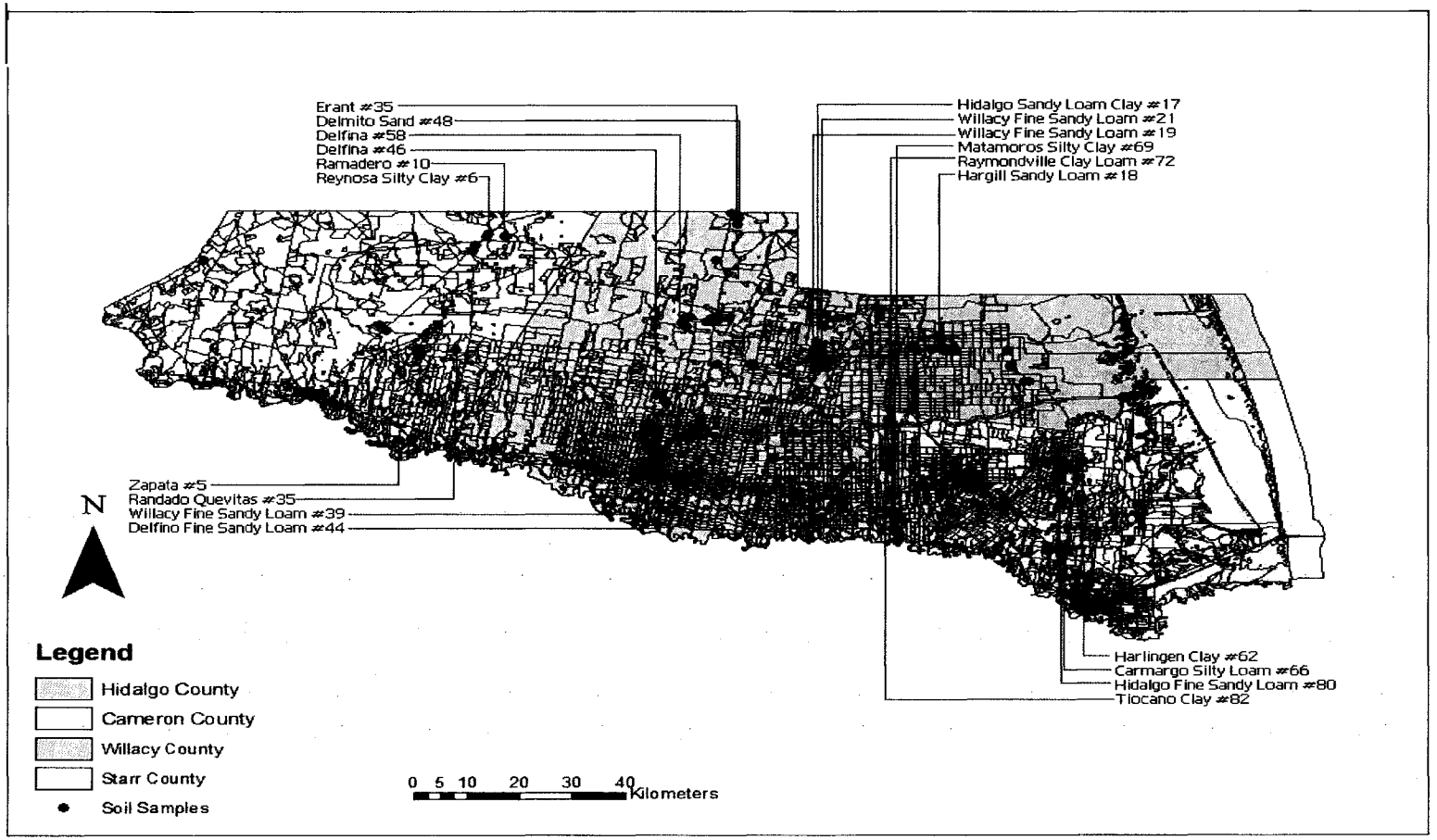


Fig. 11 shows all the soil sample locations in the Rio Grande Valley of Texas that were taken and the labeled sites that produced isolates of *R. phaseolus.*

DISCUSSION

The results indicate a variety of naturally occurring *Rhizobia phaseolus* exist in the soils of the Rio Grande Valley of Texas. Although this was the first of this kind of survey in the Rio Grande Valley of Texas, the results are similar to the research on rhizobia in other parts of the world (Mott 1978; Beck Materon and Afendi 1993). Considerable variation in the population densities and nitrogen fixing potentials were found in the soils in five countries in west Asia including Turkey (Materon 1993). This survey of biodiversity in *R. phaseolus* has revealed substantial variability in the nitrogen fixing efficiencies of the isolates. Only 25% of the sites sampled produced nodules on the host plants used in the initial stage. This is lower than the 80% of 105 sites sampled in Turkey by Keatinge (1994) but in line with the results reported by Materon (1991) between 10% and 25% found in the Turkish lowlands. No overall geographic pattern was found (see Fig. 11) was found in either the presence or efficiencies of nitrogen fixing potential in this study.

Some of the isolates clearly out performed others in terms of the dry shoot weight but the 300 parts per million of nitrogen treatment had the highest mean in this category. The high levels of nitrogen inhibited flowering and left this group on the lowest rank in terms of bean pods. Strain #66 and #80 scored the highest on the dry shoot weight, with no significant difference ($p>.05$) between them but fell

back in the rank somewhat on the number of flowers and bean pods produced which may be the most important aspect to the farmer. The controls did not become nodulated as seen on the rank position of both nitrogen treatments for nodule data. The negative nitrogen treatment grew poorly after the initial two weeks producing the lowest weight, flower and bean pod count.

In the contrast of the commercial inoculant vs. the local isolates, the number of bean pods produced, the dry shoot weight and total weight of the nodules were statistically different ($p<.05$) from the local isolates. The number of flowers, crown nodules, lateral nodules and total nodules were not significantly different ($p>.05$) from the local isolates. This product was prepared with a co-inoculant and was difficult to separate from the *Rhizobia* in the product as packaged. A pure culture was obtained from Dr. P. Somasegaren (label #132) and used in this experiment.

With the contrast of nitrogen vs. *Rhizobium* Isolates, the number of bean pods and dry shoot weight were significantly different ($p<.05$) when contrasting with 300 ppm of nitrogen. No significant differences were found ($p.>.05$) for numbers of flowers.

CONCLUSION

The low values for the R squared or correlation coefficients between most of the variables indicate that this is not a simple relationship. It appears that many of the low scoring isolates fail to fill the nodules after formation and may lack the number of functioning bacteroids that the higher scoring isolates have. The symbiosis seems to be blocked somewhere between formation of the nodule and effective functioning. The commercial strain had a very different morphology and much larger bacteroid content than those of local strains. Although the vermiculite was washed before use, the high (8.0) soil /media ph at the end of the experiment may have given the local isolates an advantage in terms of their salt tolerance over the strain from Iowa. In any case, this collection contains isolates that did better across many variables than the commercially available inoculant, Becker Underwood #132. This research provides a set of isolates containing a wide range of nitrogen-fixing characteristics as reflected in the shoot and nodule biomass of Pinto beans. It constitutes a basis to establish and expand a future collection of rhizobia adapted to the local agricultural soils. It is expected that the collection will be expanded to other cross-inoculation groups and be the source for strains to be used in inoculants for the Rio Grande Valley of Texas.

The electron micrographs may shed some light on the low R squared figures in the contrast analysis. The nodules from the higher ranks appear to be

demonstrating a higher bacteroid occupancy than those from the lower ranks. Further research is necessary to determine the nature of many of these relationships. The nodule photographs and electron micrographs illustrate the variability of nodule morphology that exists as well as a look at the interface between prokaryotic and eukaryotic organisms.

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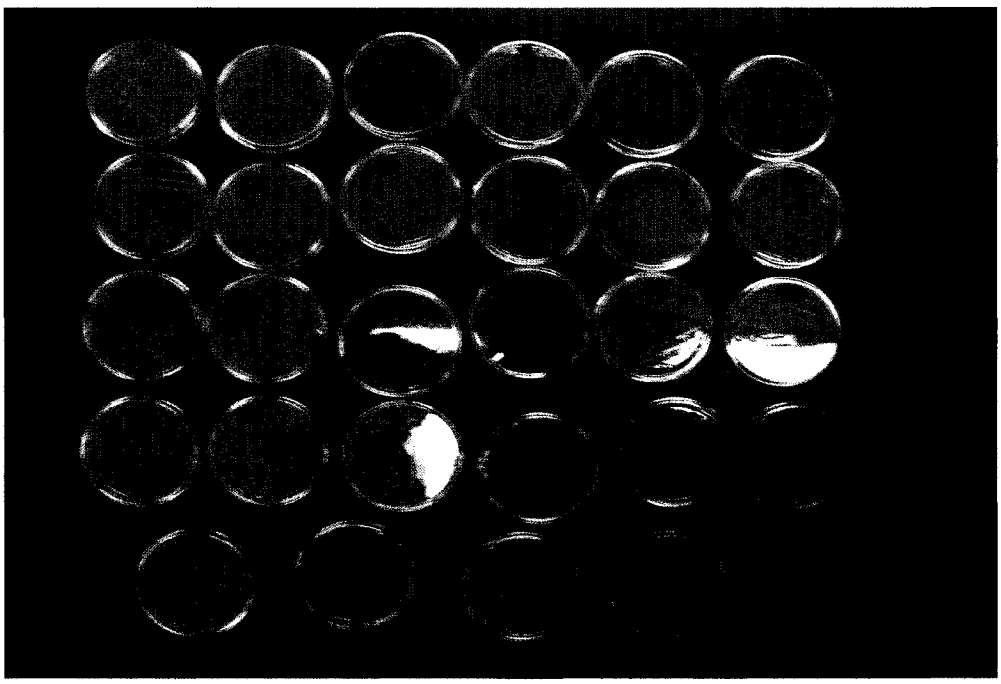
APPENDIX A

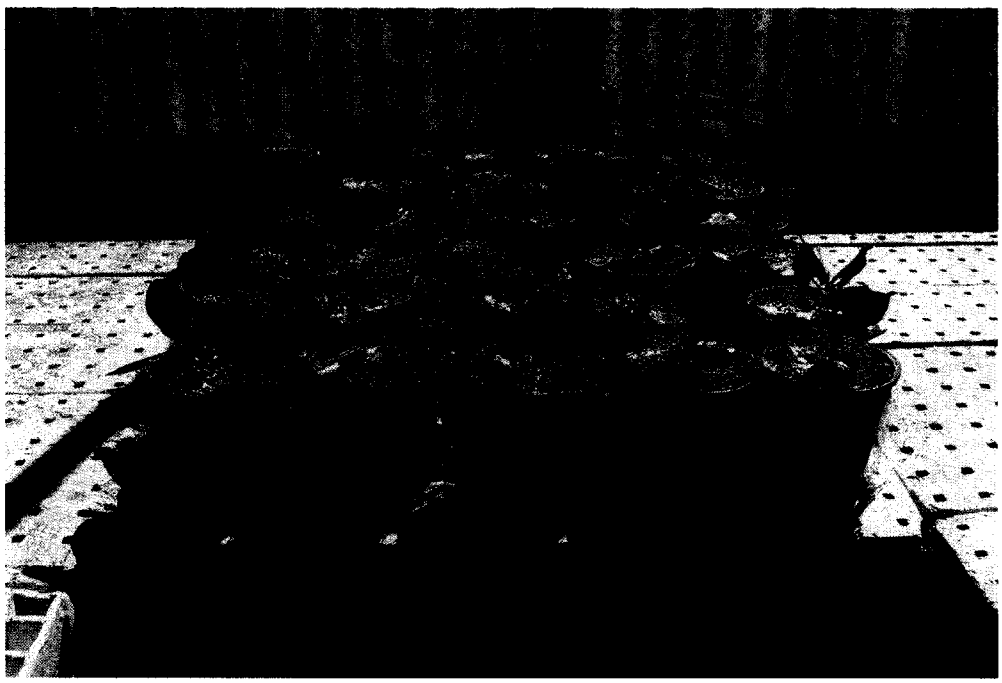
PHOTOGRAPHS OF THE EXPERIMENT

Initial isolation of *R. phaseolus* by nodulation of the host plants using the 83 soil samples collected in the Rio Grande Valley of Texas.

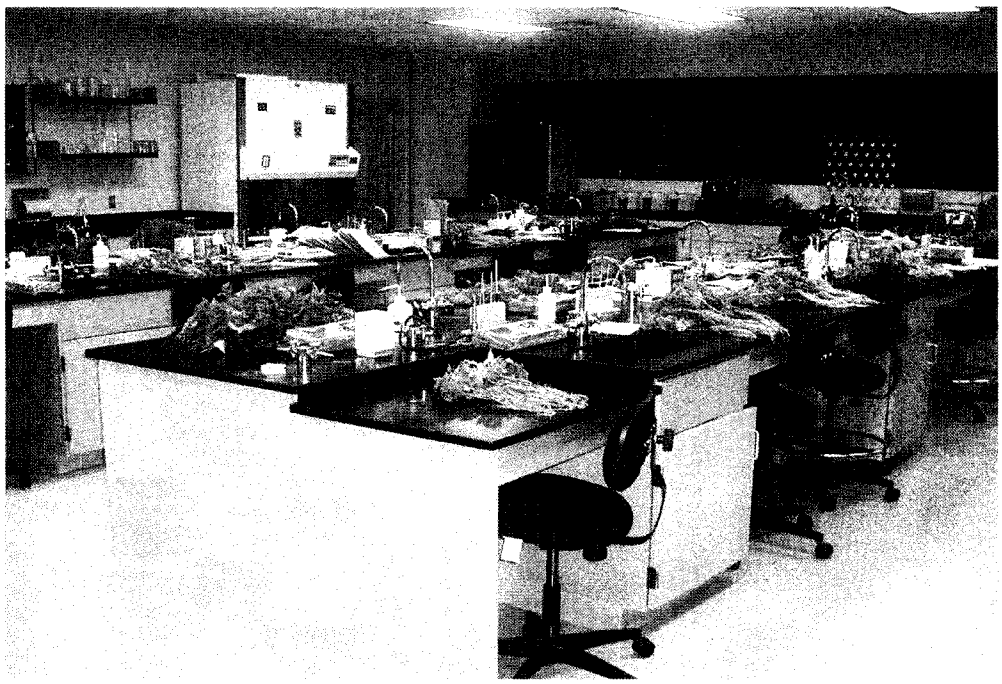


R. phaseolus isolates on YMA plates.

Plants in greenhouse after inoculation at ten days. 690 total plants were evaluated for shoot dry weights, flower numbers, nodule weights, numbers of beans, lateral and crown nodule numbers.



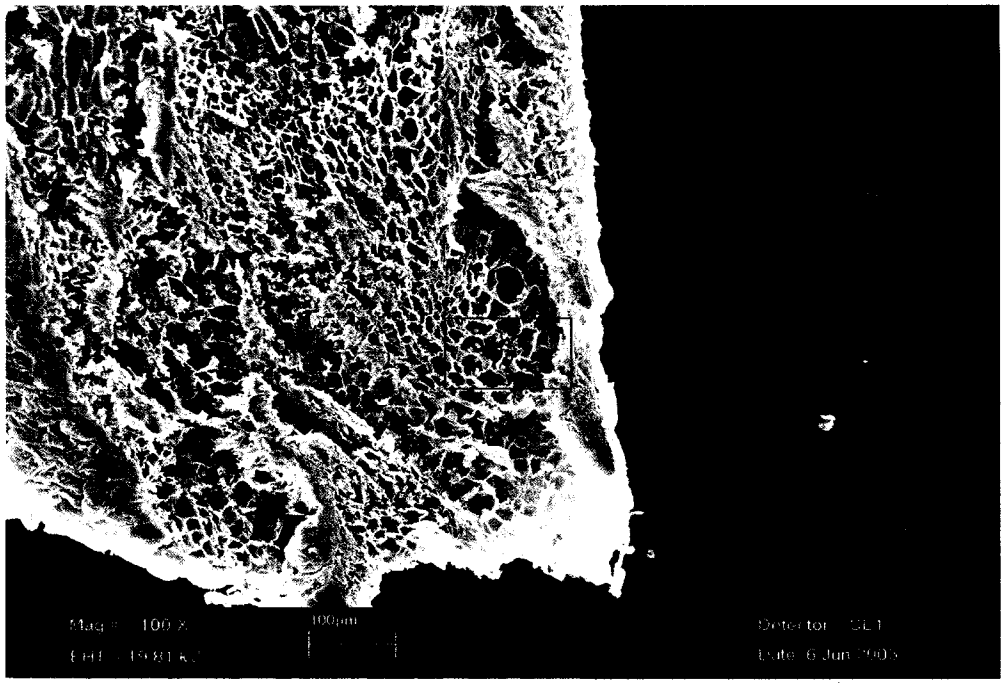
Thirty *Phaseolus vulgaris* sprouts in each group.



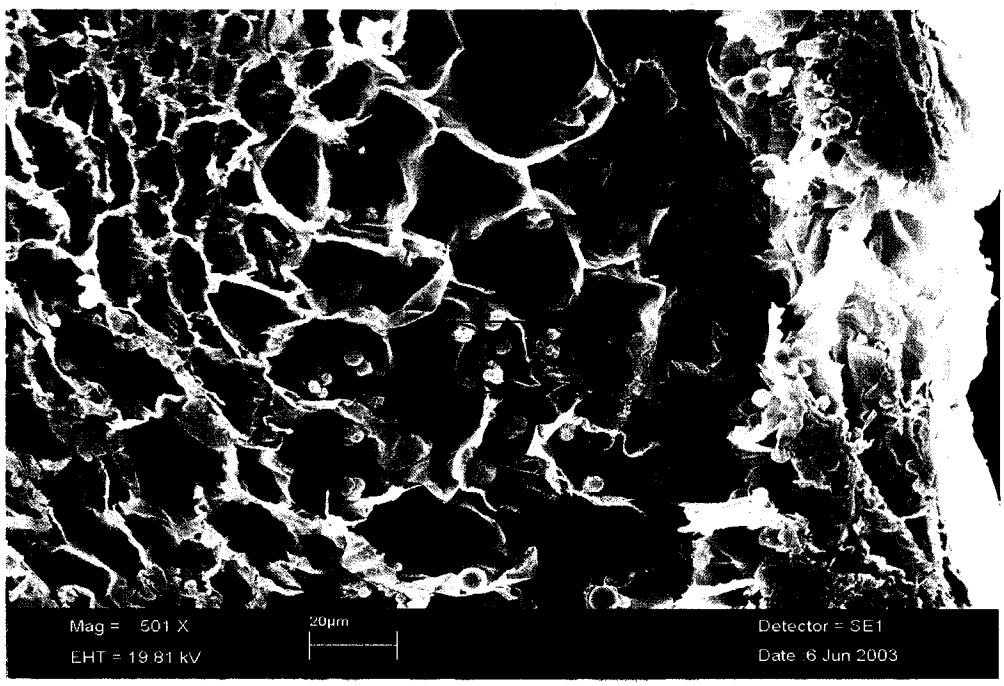
Harvested plants at 64 days. The negative nitrogen control group right front and positive nitrogen treatment opposite.

APPENDIX B

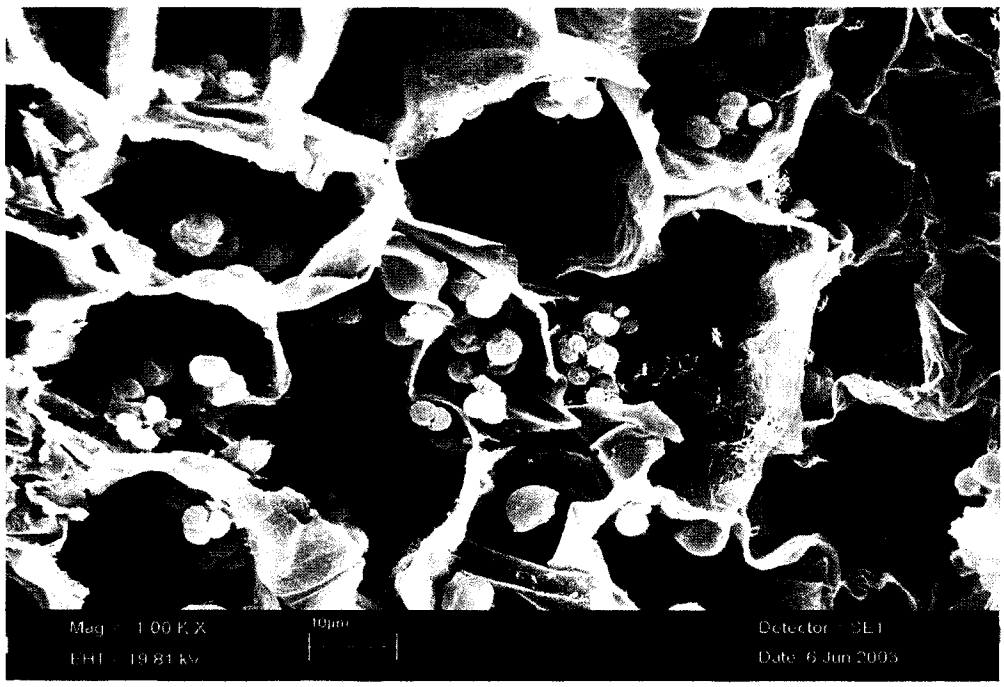
ELECTRON MICROSCOPE PICTURES



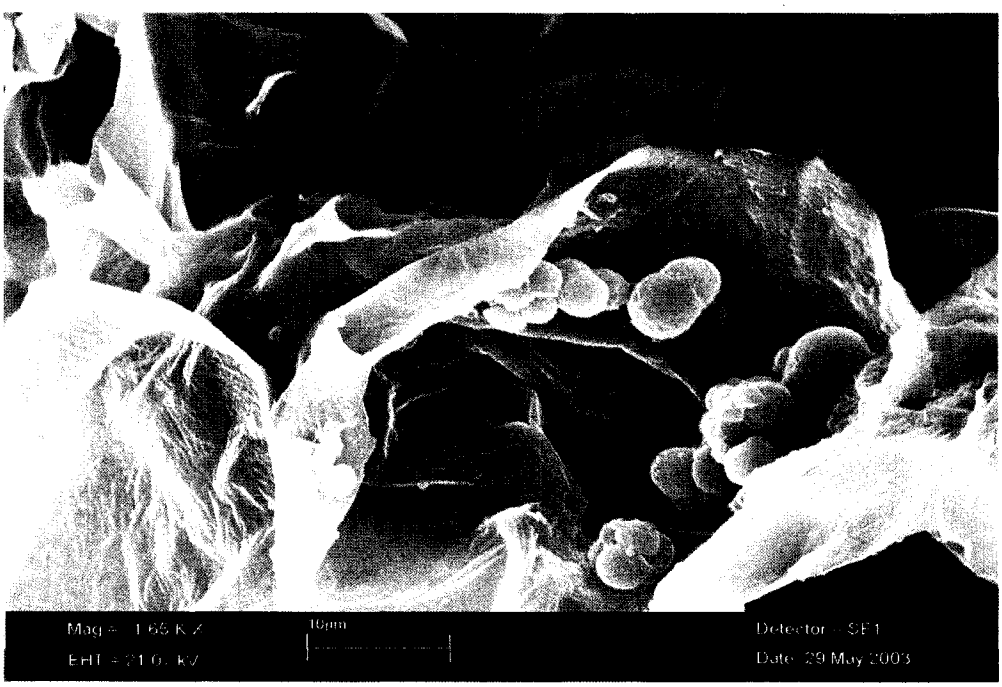
Electron micrograph of nodule cross section from isolate #21.



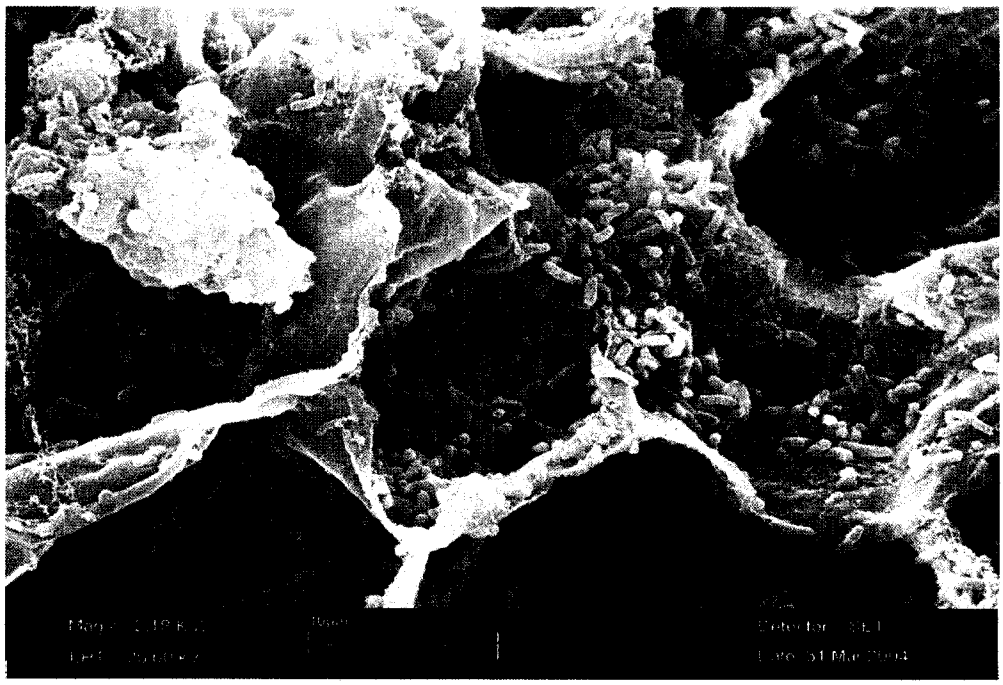
Electron micrograph of nodule cross section from isolate #21.



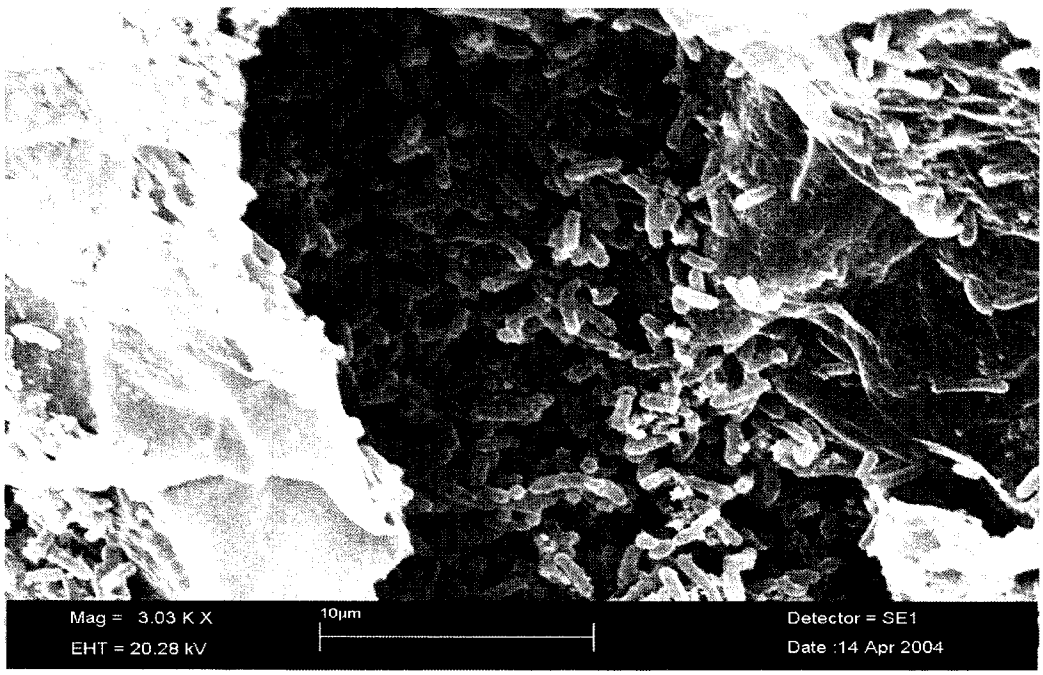
Electron micrograph of nodule cross section from isolate #21.



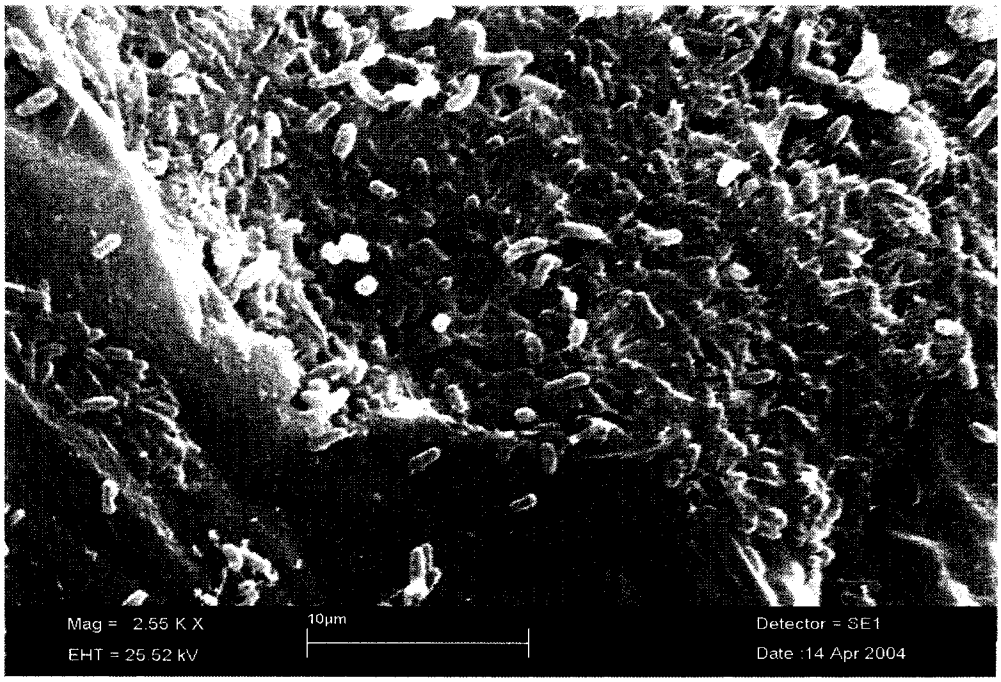
Electron micrograph of nodule cross section from isolate #21.



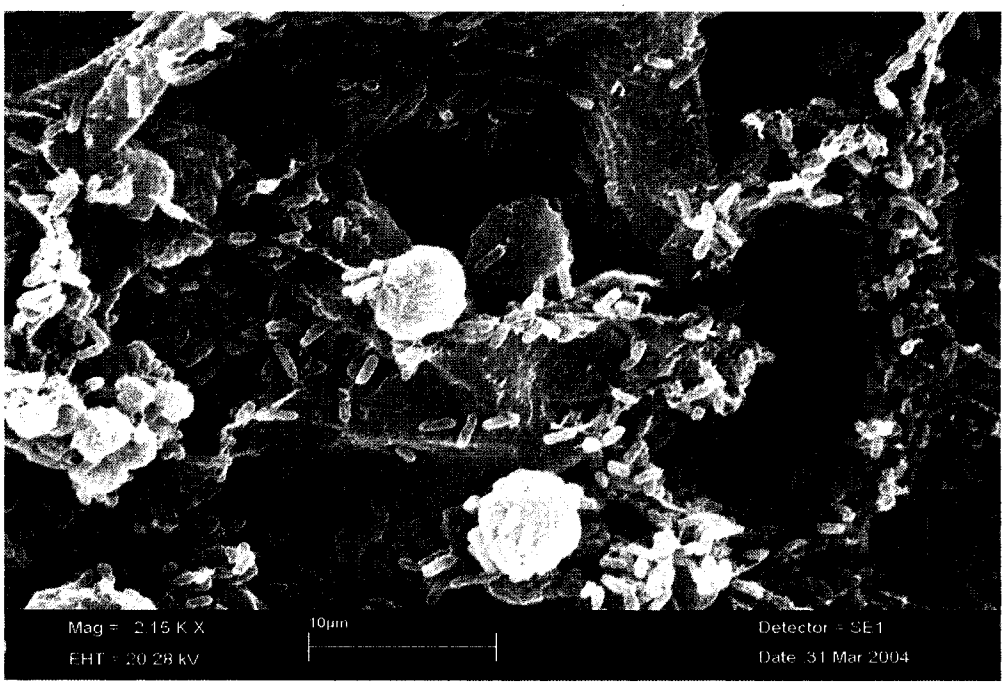
Electron micrograph of nodule cross section from isolate #66.



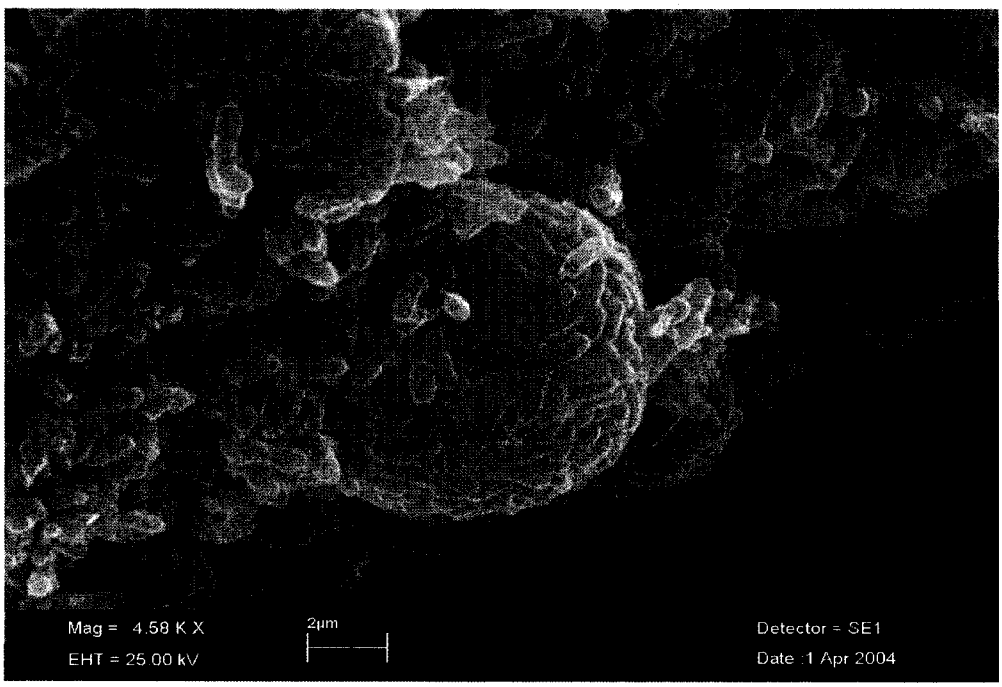
Electron micrograph of nodule cross section from isolate #66.



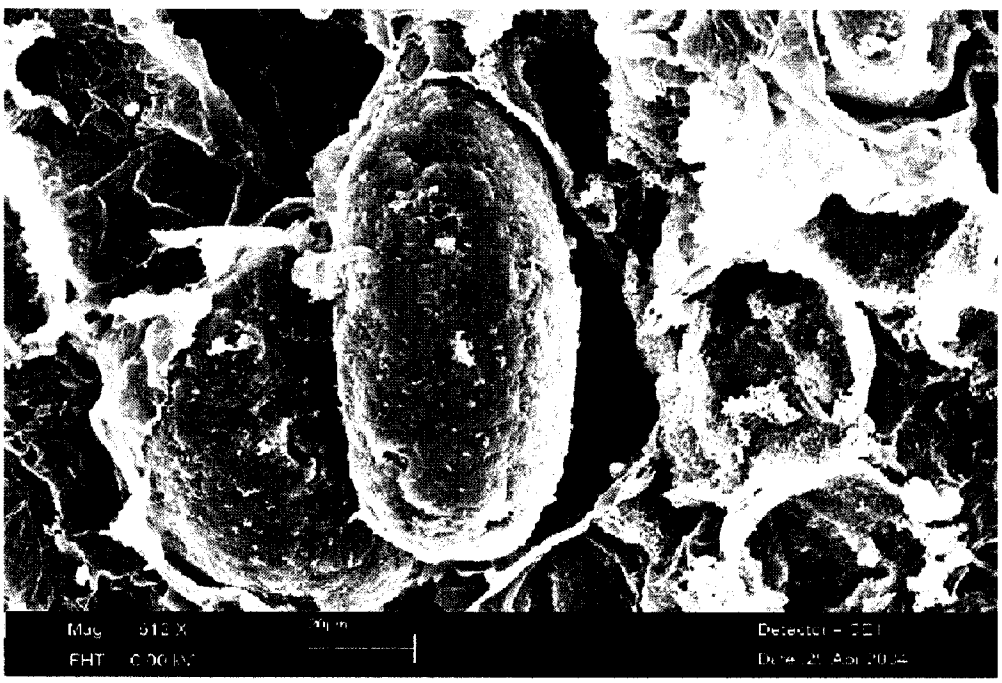
Electron micrograph of nodule cross section from isolate #66.



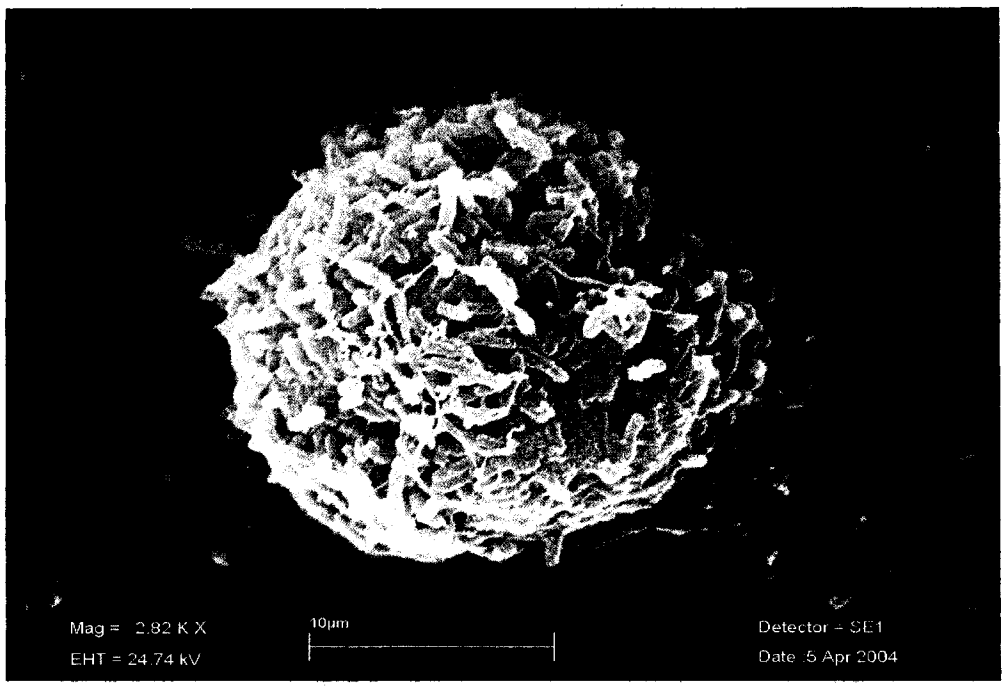
Electron micrograph of nodule cross section from isolate #66.



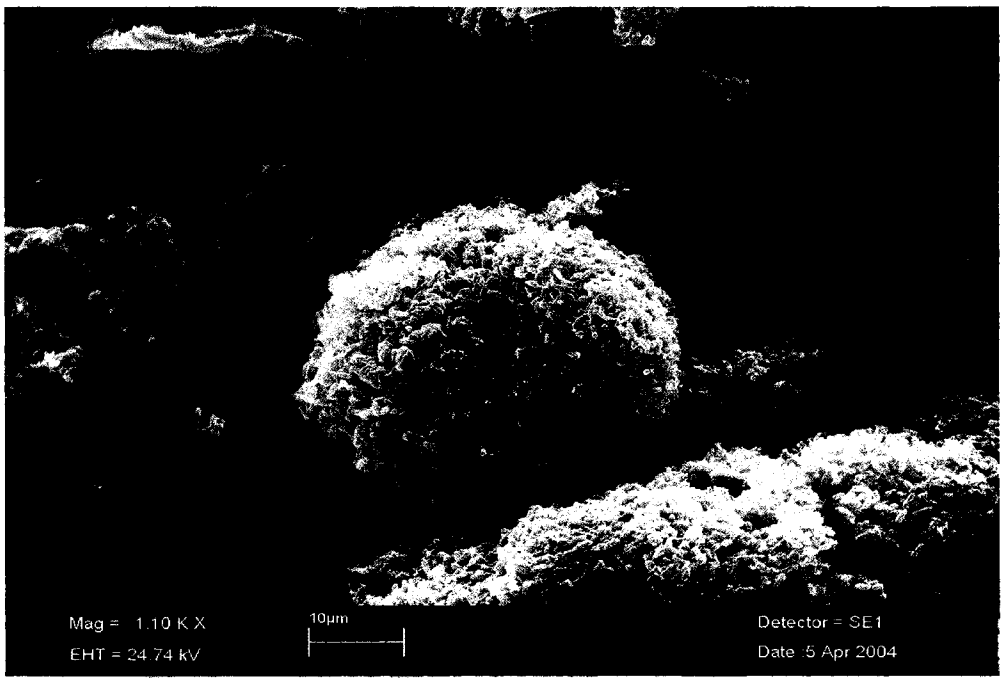
Electron micrograph of nodule cross section from isolate #66.



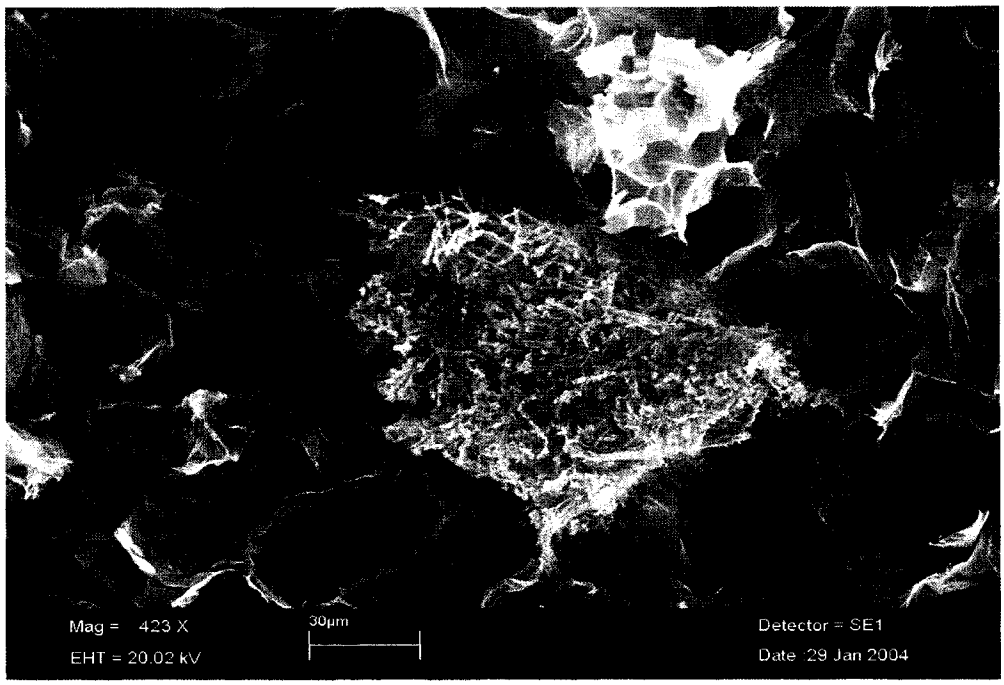
Electron micrograph from isolate #132.



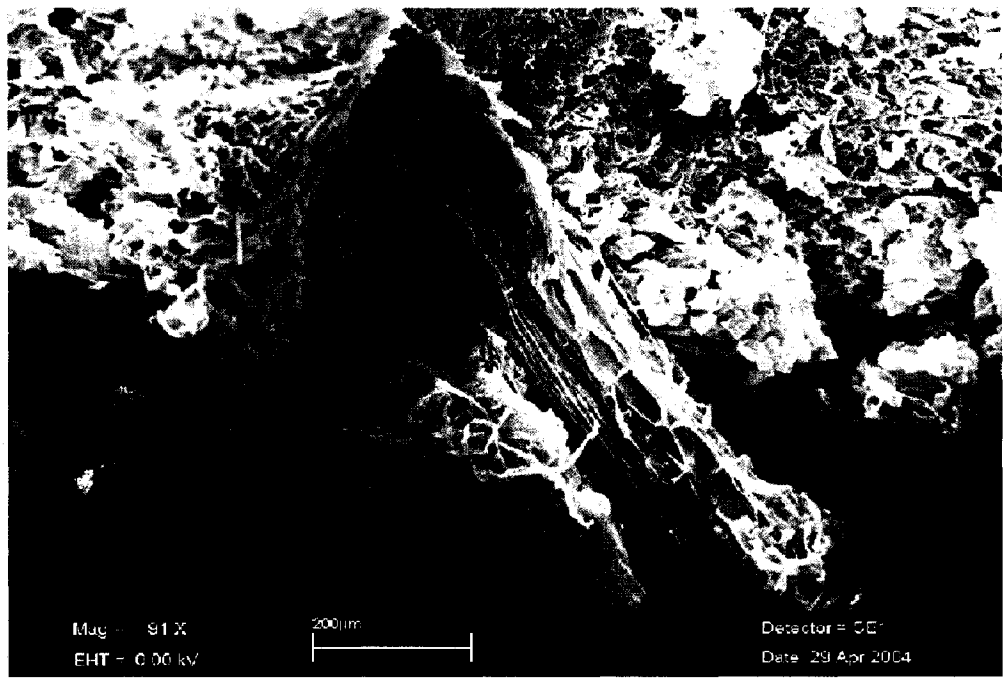
Electron micrograph of isolate #82.



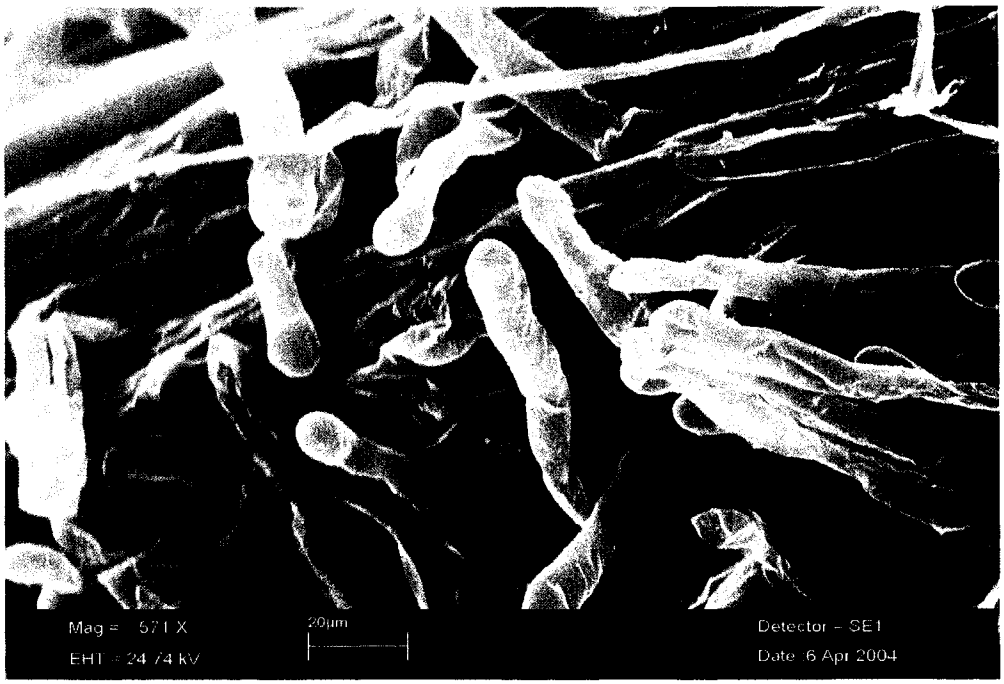
Appendix Fig 17. Electron micrograph of isolate #80.



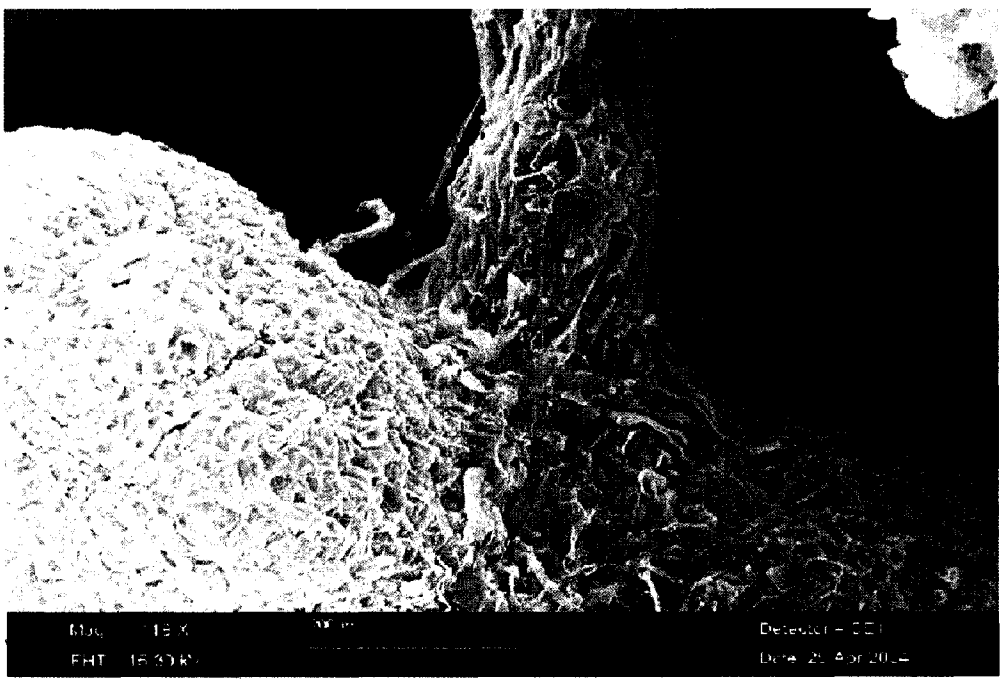
Electron micrograph of isolate #72.



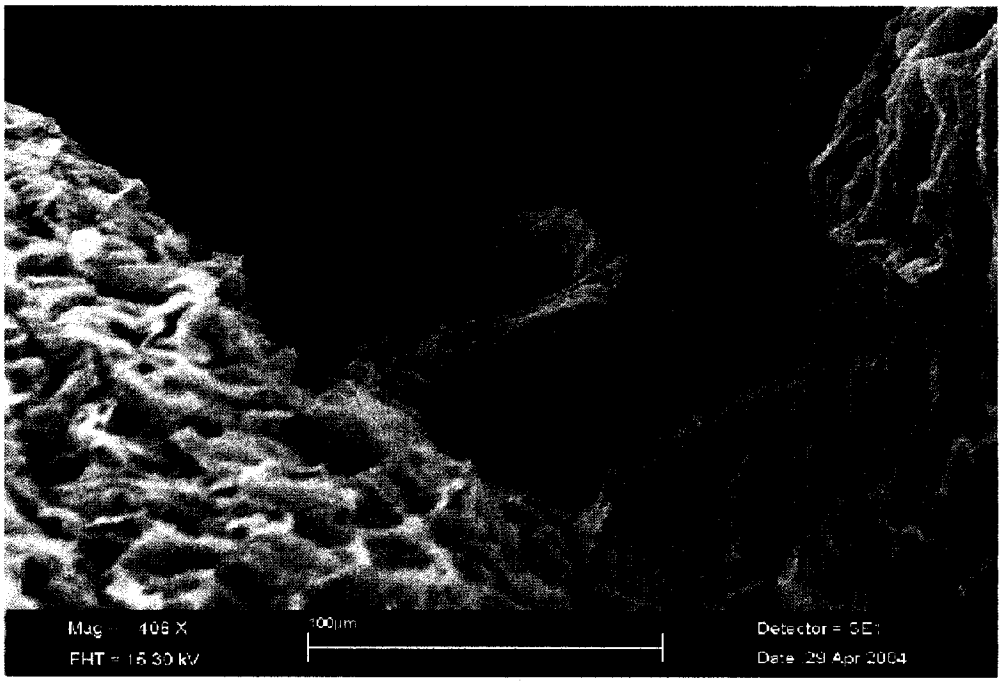
Electron micrograph of root / nodule transition.



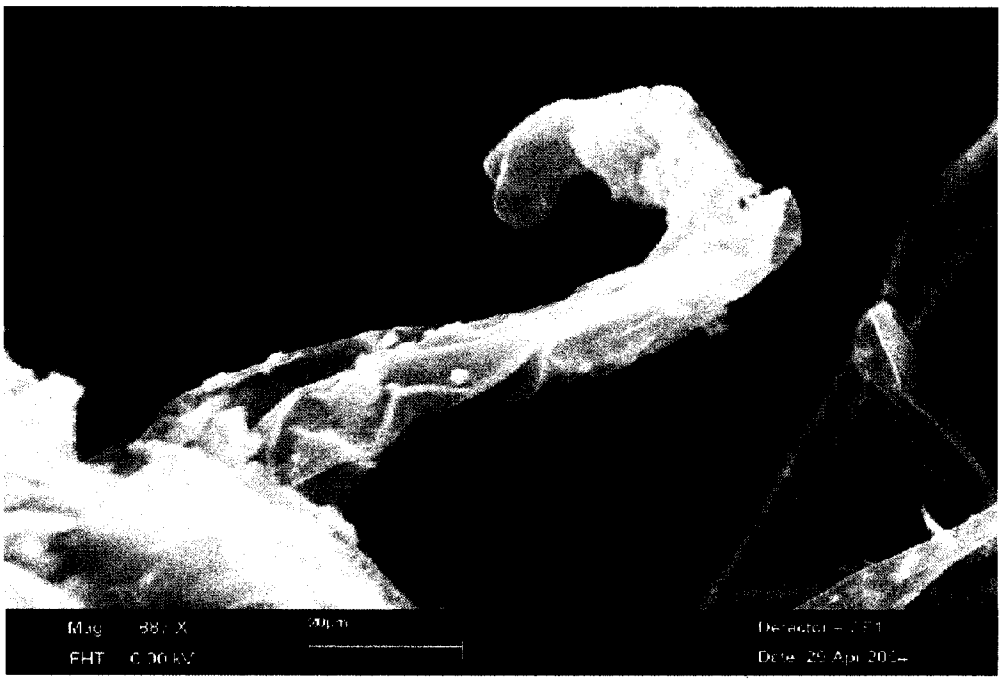
Electron micrograph of root hairs.



Electron micrograph of nodule root connection area.



Electron micrograph of root tip curling.



Electron micrograph of root tip curling at higher magnification.

APPENDIX C

DATA RECORDS

Appendix C Serial dilution data

Soil type / Strain #	Serial dilution @ 48 hrs CFU/ml
132	2×10^{-9}
18	2×10^{-10}
19	2×10^{-9}
17	2×10^{-9}
48	1×10^{-9}
72	1×10^{-8}
6	2×10^{-8}
69	2×10^{-7}
44	3×10^{-9}
62	2×10^{-9}
39	2×10^{-8}
35	1×10^{-8}
58	5×10^{-7}
10	2×10^{-8}
46	2×10^{-9}
82	3×10^{-8}
34	3×10^{-9}
66	9×10^{-8}
21	2×10^{-9}
5	5×10^{-9}
40	2×10^{-8}

Appendix C Temperature Data

Date	Max Temp.°C	Min. Temp.°C	Average Temp.°C
02-15-04	18.8	2.2	10.5
02-16-04	22.2	6.6	14.4
02-17-04	25	5.5	15
02-18-04	25	8.3	16.6
02-19-04	27.7	12.2	20
02-20-04	30	13.8	21.6
02-21-04	25	14.4	20
02-22-04	27.2	16.6	22.2
02-23-04	27.7	15.5	21.6
02-24-04	25	12.2	18.3
02-25-04	18.8	7.2	12.7
02-26-04	18.8	3.8	11.1
02-27-04	23.8	6.1	15

Date	Max Temp.°C	Min. Temp.°C	Average Temp.°C
02-28-04	23.8	12.2	18.3
02-29-04	29.4	18.8	24.4
03-01-04	27.2	17.2	22.2
03-02-04	28.8	18.3	23.8
03-03-04	29.4	21.1	25
03-04-04	28.3	22.7	25.5
03-05-04	29.4	17.7	23.8
03-06-04	29.4	13.3	21.6
03-07-04	26.1	13.3	20
03-08-04	26.6	14.4	20.5
03-09-04	27.7	12.2	20
03-10-04	27.2	11.6	20
03-11-04	24.4	16.6	20.5
03-12-04	25.5	16.5	20.5
03-13-04	24.4	18.8	21.6
03-14-04	24.4	19.4	21.6
03-15-04	22.7	17.7	20.5
03-16-04	26.6	17.2	22.2
03-17-04	28.8	16.1	22.7
03-18-04	28.3	20	24.4
03-19-04	28.3	21.6	25
03-20-04	26.6	18.8	22.7
03-21-04	28.3	17.7	22.2
03-22-04	26.6	14.4	20.5
03-23-04	26.6	15.5	21.1
03-24-04	26.1	18.3	22.2
03-25-04	27.7	20.5	24.4
03-26-04	27.7	24.4	23.3
03-27-04	28.3	20.5	24.4
03-28-04	29.4	19.4	24.4
03-29-04	30	17.2	23.8
03-30-04	27.2	14.4	21.1
03-31-04	29.4	12.7	21.1
04-01-04	28.8	14.4	21.6
04-02-04	28.8	20.5	25
03-30-04	27.2	14.4	21.1
04-03-04	30	20.5	25.5
04-04-04	28.8	21.1	25
04-05-04	26.6	17.7	22.2
04-06-04	29.4	20	24.4
04-07-04	30	20.5	25.5
04-08-04	27.2	15.4	23.3
04-09-04	29.4	17.7	23.3
04-10-04	32.7	22.7	27.7

Date	Max Temp.°C	Min. Temp.°C	Average Temp.°C
04-11-04	27.2	13.8	20.5
04-12-04	22.7	11.1	17.2
04-13-04	24.4	8.8	16.6
04-14-04	25.5	12.2	18.8
04-15-04	25.5	15	20.5
04-16-04	25.5	16.6	21.1
04-17-04	27.2	17.2	22.2
04-18-04	27.7	19.4	23.8

Soil type	GPS	Elevation (M)
1. Sarita fine sandy loam	26.42.57N 98.31.40W	101.8
2. Lomita loamy fine sand	26.41.46N 98.32.35W	89.0
3. Matamoros silty clay	26.16.35N 98.38.46W	25.9
4. Brenan fine sand	26.40.59N 99.33.15W	27.4
5. Zapata	26.31.22N 98.41.26W	143.8
6. Reynosa silty clay loam	26.22.11N 98.47.44W	43.8
7. Delmita fine sandy loam	47.43.10N 98.30.18W	86.5
8. Falfurias fine sand	26.43.49N 98.30.46W	95.0
9. Jimenez Cuemado	26.22.35N 98.47.39W	40.5
10. Ramadero loam	26.31.37N 98.41.12W	135.3
11. Delmito fine sand	26.43.08N 98.28.36W	83.2
12. La Gloria silty loam	26.22.30N 98.48.50W	43.2
13. Carmargo clay loam	26.16.25N 98.39.24W	33.5
14. Tiocano clay	26.26.53N 97.57.19W	11.5
15. Porfirio clay loam	27.24.16N 97.35.58W	0.0
16. Delfina	26.02.39N 97.31.45W	20.4
17. Hidalgo clay loam	26.28.57N 97.43.11W	3.0
18. Hidalgo sandy loam	26.29.07N 97.56.59W	8.8
19. Wilacy sandy loam	26.28.55N 97.44.47W	14.9
20. Racombes clay loam	26.26.58N 97.57.18W	12.1
21. Wilacy sandy loam	26.28.56N 97.44.24W	13.4
22. Hargill fine sandy loam	26.26.43N 97.57.21W	13.1
23. Usterthents loamy	26.27.16N 97.56.28W	6.7
24. Lyford sand clay loam	26.28.05N 97.45.17W	.61
25. Hargill fine sandy loam	26.27.29N 97.57.13W	9.1
26. Mercedes clay	26.21.26N 97.47.48W	4.2
27. Delfina	26.21.28N 97.45.20W	32
28. Rio sandy clay loam	26.29.10N 97.57.19W	4.2
29. Raymondville	26.28.57N 97.43.26W	13.1
30. Incell clay	26.24.30N 97.35.57W	.3

Soil Type	GPS	Elevation (M)
31. Racombes sandy clay	26.28.56N 97.44.51W	9.4
32. Willamar sandy loam	26.28.19N 97.37.10W	2.4
33. Jarron clay loam	26.26.57N 97.37.01W	4.8
34. Randado Cuevitas	26.32.34N 98.10.21W	22.5
35. Erant	26.28.64N 98.34.05W	9.7
36. Delfina	26.45.42N 98.05.03W	14.3
37. Hidalgo sandy loam	26.19.29N 98.08.05W	27.4
38. Brenan sandy loam	26.18.41N 98.13.50W	33.5
39. Wilacy sandy loam	26.26.56N 98.07.07W	21.9
40. Hidalgo Sandy Loam	26.18.21N 98.14.09W	31.3
41. Hidalgo sandy loam 28	26.20.20N 98.02.05W	28.3
42. Racombes clay loam	26.17.51N 98.14.13W	25.9
43. Ramondville	26.17.06N 98.13.18W	33.2
44. Del Fino sandy loam	26.26.54N 98.03.05W	21.3
45. Rio	26.17.10N 98.13.46W	26.5
46. Del Fina	26.21.46N 98.12.46W	25.9
47. Randado	26.22.14N 98.13.30W	37.4
48. Delmito sand	26.32.23N 98.09.13W	13.7
49. Sarita 42	26.46.28N 98.05.51W	14.6
50.Hebronville sandy loam	26.40.40N 98.07.01W	13.7
51. Willacy sandy loam	26.26.28N 98.03.34W	19.8
52. Tiocano clay	26.31.45N 98.10.25W	20.4
53. Sarita 43	26.46.28N 98.05.53W	19.2
54. Rio clay loam	26.32.08N 98.07.46W	16.4
55. Reynosa clay	26.32.52N 98.06.60W	15.2
56. Hidalgo 25	26.14.46N 98.01.64W	12.1
57. Rio 59	26.17.52N 98.03.42W	16.7
58. Delfina	26.32.10N 98.07.47W	17.9
59. Comitas	26.32.04N 98.10.22W	19.8
60. Delmito Randado	26.32.18N 98.08.35W	20.7
61. Benito clay	26.13.46N 97.49.31W	6.0
62. Harlingen clay	26.07.35N 97.49.24W	10.6
63. Orelia clay loam	26.13.26N 97.30.45W	6.0
64. Hidalgo sandy loam	26.15.37N 97.49.32W	5.4
65. Wilacy fine loam	26.18.49N 97.49.24W	2.4
66. Camargo silty loam	26.04.11N 97.48.54W	12.1
67. Laredo Olmito	26.02.40N 97.31.44W	21.3
68. Willamor	26.11.29N 97.30.36W	7.6
69. Matamoros silty clay	24.04.20N 97.48.55W	10.6
70. Olmito silty clay	26.06.32N 97.49.55W	8.2
71. Mercedes clay loam	26.14.14N 79.49.31W	6.7
72. Ramondville clay loam	26.20.17N 97.49.13W	27.4

Soil Type	GPS	Elevation (M)
73. Racombes clay loam	26.20.31N 97.49.12W	29.8
74. Rio Grande silty loam	26.04.11N 97.48.55W	10.6
75. Laredo silty clay loam	26.06.25N 97.51.00W	12.1
76.Laredo Reynosa	26.04.28N 97.49.05W	12.2
77. Lyford clay loam	26.13.55N 97.33.52W	17.9
78. Rio clay loam	26.18.13N 97.49.55W	2.1
79. Camargo clay loam	26.03.50N 97.48.05W	12.8
80. Hidalgo sandy loam	26.12.27N 97.30.29W	12.2
81. Lozano sandy loam	26.13.39N 97.32.25W	12.5
82. Tiocano clay	26.06.31N 97.48.36W	16.7

VITA

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U.S. Patent # 6,704, 553. March 9, 2004. System and method for providing automatic tuning of a radio receiver and for providing automatic control of a cd\ tape player.

U.S. Patent Application Serial # 09\460,131. Feb 3, 2004

ACADEMIC BACKGROUND

Master's program in Biology at the University of Texas- Pan American. Entered 2001.
Cumulative GPA 3.96 Graduation May 2005. Thesis: Collection, Isolation and Evaluation of Nitrogen Fixing Bacteria in the Rio Grande Valley of Texas.
Additional course work in chemistry (22hrs) not required in Masters program taken to prepare for research in Molecular Biology.

Double Major B.A. in Psychology and B.S. in Biology Pan American University 1984.
American Community High School. Addis Ababa, Ethiopia. 1974

JOB HISTORY

USDA \ ARS Biological research technician. June 2004-Jan 2005. Protein extraction and 2-D electrophoresis, HPLC analysis, Bio-Rad FX Molecular Imager, Protein IEF and Protein II casting.
Microbiology lab, fall 2003, spring 2004
Anatomy and Physiology lab, spring 2003. Two sections
Electron microscope lab, spring 2003, spring 2004.
Owner operator of Eubanks Nursery and in Edinburg Tx. 1984-2001
Production manager Amrock Energy, Lockhart Tx. 1981-1983
Owner/ trainer thoroughbred racing stables. Ruidoso, New Mexico 1975-1980.