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The Nitrogen Fixation Capability of Cyanobacteria-A Natural Source as a Bio-Fertilizer For Arachis *Hypogaea*

KEYWORDS: Arachis hypogeal, cyanobacteria, nitrogen fixation, nif genes

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ABSTRACT *The cyanobacteria are widely distributed over land and water, often an environment where no other vegetation can exist. They have a considerable and increasing economic importance. The fixation of atmospheric nitrogen (N2) is a very energy intensive endeavor. The nif genes are responsible for the coding of proteins related and associated with the fixation of atmospheric nitrogen into simpler form of nitrogen which is easily utilized by plants. These genes responsible for nitrogen fixing are found in certain species of cyanobacteria. Nitrogen fixation depends on interaction between cyanobacteria nif genes, host plant and the environment. The experiments were conducted during this project to examine the presence of nif genes at molecular level in isolated cyanobacteria. The improvement in the plant/crop growth is observed, if cyanobacteria are supplemented with soil in comparison with control. The crop was selected on the basis of availability of sterile seeds of Arachis hypogeal (groundnut). Different parameters were observed in intervals to evaluate the capability and effectiveness of nitrogen fixing by cyanobacteria. Root nodules, thickness and health of root, shoot length, and yield parameter were found effective in test experiment when compared with control. The observation was obtained from 25 plants at interval of 20, 37, 45 and 65 days. The average data of treated after 20 days showed the shoot length, root length and number of nodules are 24.9, 9.4 and 0 whereas control showed 21.4, 6 and 0 respectively. Data obtained after 45 days were 32.7, 18.7 and 16.6 in treated with comparison of control which is 28, 13.1 and 3 for shoot length, root length and number of nodules. The yield parameter were obtained after 65 days and found better in treated, which suggest increase of 6 times on an average. The data obtained indicate that the nitrogen fixation increased by supplementation of cyanobacteria in soil.*

Introduction

Cyanobacteria are a gram-negative eubacteria. They are structurally diverse and widely distributed throughout the world and are also known as blue green algae (Vijaya S *et al* 2013). Cyanobacteria are characterized by their capacity to perform biological nitrogen fixation and oxygenic photosynthesis. As cyanobacteria are very resistant to extreme environmental conditions, they are assuming increasing importance in frontier areas of biotechnology (Ritika C *et al* 2011).

Cyanobacteria, formerly called blue-green algae, are a fascinating group of photosynthetic bacteria many of which also fix atmospheric di-nitrogen. Both academic and applied aspects of the biology of these organisms are studied extensively. They come in various shapes and sizes, some with and others without the specialized cells called heterocysts (Apte S *et al* 1994). Cyanobacteria originated three billion years ago and are supposed to have contributed significantly to the oxygenation of the primitive Earth's atmosphere. Chloroplasts of higher plants are believed to have evolved through endosymbiosis of cyanobacteria and plant cells (Walsby *et al* 1986). In sharp contrast to their "primitive antecedents" heterocystous cyanobacteria display remarkable evolutionary advances such as differentiation, pattern formation, intercellular communication, physiological division of labour among cell types, developmentally regulated gene rearrangements and gene expression and a range of adaptive responses of survival value resulting in their ubiquitous distribution. Among the nitrogen-fixing organisms, they occupy a unique position in being the only truly photoautotrophic aerobic nitrogen fixers and are of great consequence as nitrogen biofertilizers in nature, especially in the oceans (Capone *et al* 1982) and in tropical rice cultivation. Only the heterocystous forms can fix free nitrogen in air because they differentiate heterocysts—cells designed to exclude oxygen and therefore conducive to the activities of the oxygen-sensitive nitrogenase enzyme (Wolk *et al* 1982).

Cyanobacteria are one of the major components which provide potential source of nitrogen fixation at no cost. It plays an important role to build up soil fertility increasing the yield. The blue-green algae are capable of fixing the atmospheric nitrogen and convert it into an available form of nitrate required for plant growth. They have the abilities of photosynthesis as well as biological nitrogen fixation. The isolated cynobacterial DNA was high in quantity and quality and was suitable for molecular manipulation such as PCR amplification of *nif* genes. Data obtained will indicate usefulness of cyanobacteria species containing *nif* genes in nitrogen fixation as biofertilizers. Biofertilizers are ecofriendly and have been proved to be effective and economical alternate of chemical fertilizers with lesser input of capital and energy.

The process of nitrogen fixation and the occurrence of gas vesicles are especially important to add to the success of the blue-green algae or cyanobacteria. They help in reclamation of alkaline soils. They have heterocysts, which are helpful in the fixation of atmospheric nitrogen (Teresa T *et al* 2001). They have symbiotic relationship with protozoa, fungi, and nitrogen fixing species form associations with angiosperms. Their ability to photosynthesize and also to fix atmospheric nitrogen means that their nutritional requirements are minimal. The *nif* genes are found in both free living nitrogen fixing bacteria and in symbiotic bacteria in various plants.

The *nif* genes are genes encoding enzymes involved in the fixation of atmospheric nitrogen. The primary enzyme encoded by the *nif* genes is the nitrogenase complex which is in charge of converting atmospheric nitrogen to other nitrogen forms such as ammonia, which plants can use for various purposes. Besides the nitrogenase enzyme, the *nif* genes also encode a number of regulatory proteins involved in nitrogen fixation. This study indicates the use of cyanobacteria as a biofertilizer for *Arachis hypogeal.* The optimum growth temperature found in the month of September to December

Volume : 4 | Issue : 5 | May 2014 | ISSN - 2249-555X

in Indian climatic condition, used in this study. The experiments were conducted during this project to examine the presence of *nif* genes at molecular level in isolated cyanobacteria. The improvement in the crop growth is observed, if cyanobacteria are supplemented with soil in comparison with control. Parameters like root nodules, thickness and health of root, shoot length, and yield were considered for observation in frequent intervals.

Materials and Methodology

Primer designing

The bioinformatics approach to find the conserved sequence of gene capable of nitrogen fixation in isolated cyanobacteria from lake water samples collected from different lakes of Maharashtra, India (Table 1).

The process of primer designing includes choosing the multiple sequences from NCBI database (Table 2). Multiple sequence alignment of all the obtained sequences was performed using T-Coffee software (Figure 1) and some repeated conserved stretches of nucleotides were obtained which were common to all the sequences. The resulting nucleotide stretches were cross-checked with the Primer-3 software.

Sub-culturing of isolated cyanobacteria

The Cyanobacteria was cultured in BG 11 media (Cetinkaya G *et al* 1999). Which is further screened for the single isolate by serial dilution with continues streaking. The isolated colonies from the plates were inoculated in the BG11 liquid media to obtain a pure culture of Cyanobacteria.

DNA isolation from isolated cyanobacteria

Cyanobacterial sample is dried and crushed with the help of liquid nitrogen and suspended in TE buffer. Then the cells are centrifuged at room temperature. Supernatant is removed and 500 µl TE buffer is added. Adding 1 % SDS, the sample is kept at 70°C. Before centrifuging, chloroform: isoamyl alcohol in the ratio 24: 1 is added. Finally, taking the supernatant, 5M NaCl and 100 % ethanol is added and precipitated at -20°C for 2 hours. The pellet is centrifuged and washed with 70 % ethanol. Finally, the pellet is dried and resuspended in TE buffer.

Amplification of Nif genes

Amplification of cyanobacteria DNA was performed with the help of our designed primers using Polymerised Chain Reaction (Table 3).

Primers designed from selected sequence, details are furnished below:

Primer 1 Sequence ID**:** >gi|450374|gb|L15551.1|NOSNIFH Nostoc sp. 'J. Gallon' nitrogen fixation protein (*nif* H) gene, partial cds (231 base pairs)

Primer 2 Sequence ID**:** >gi|9081940|gb|AF167547.1|Pseudanabaena PCC7403 Nif EN (*nif* EN) gene, partial cds (167 base pairs)

Preparation of soil beds

Two soil beds, Control and Treated were prepared for a comparative study of nitrogen fixation in groundnuts.

Control

A simple soil bed is prepared (natural environment) without any external additions to it (Figure 2-A) and the bacterium flora are checked by plating soil sample (Figure 2-B). Groundnuts seeds are planted by keeping a distance of 4-5 inches.

Treated

A semi poly-house (Figure 3-A) is prepared in which soil bed is made by treating autoclaved soil with Cyanobacteria culture (Figure 3-B). The cyanobacteria grown in plastic sheet covered with semi poly house. The growth was observed and mixed with autoclaved soil. Soil bed 5 inch thick was developed to plant the groundnut seeds. This treated soil is checked for contamination, again with the help of plating techniques (Figure 3-C). After this, groundnuts seeds are sowed with sowing criteria similar to that of Control soil.

Result

Primer designing

Two primers (forward and reverse) sets of *nif* H and *nif* EN genes of cyanobacteria were synthesized.

PRIMER SET 1 (Nif H):

Left- GCAGAACGTGGTGCAGTAGA

Right- GGATAGGCATAGCGAAACCA

PRIMER SET 2 (Nif EN):

Left- CTCCTTGGGAAGATCCAACA

Right- TCGCTCCTTTTAATCCCAGA

Sub-culturing of isolated cyanobacteria

Cyanobacteria single isolated colonies where obtained by continuous streaking. Further pure culture obtained by inoculation in BG11 broth (Figure 4-A, B).

DNA isolation from isolated cyanobacteria

From the obtained cyanobacteria culture, DNA was isolated. A quality check with the help of purification techniques was done and seen in Electrophoresis unit (Figure 5). Spectrophotometer was used to check the quantity of the DNA.

Amplification of Nif genes to confirm the presence

Amplification bands were observed by doing Polymerized Chain Reaction. And the length of gene was compared with the DNA ladder. The result of amplification of Nif H (primer 1) and Nif EN (primer 2) is seen in (Figure 6-A) and (Figure 6-B), respectively.

Results of Control and Treated Plants

The effects of cyanobacteria treatment were seen on the growth and development of plants during 20 days (Figure 7-A, B), 37 days (Figure 7-C, D), 45 days (Figure 7-E, F) and 65 days (Figure 7-G, H) interval. Treated soil gave rise to healthier plants with more root nodules, greater height and thicker roots as compared to that of Control. The tabular form giving the standard deviation of growth and development readings is shown in Table 4. The data mean value with their standard deviation was plotted, which indicate the improvement in treated for all parameters taken into consideration (Graph 1 and 2).

Discussion

Cyanobacteria is known for the presence of nif genes and globally reported for their nitrogen fixation capability. The experiments were conducted during this project to examine the presence of *nif* genes at molecular level in isolated cyanobacteria. The improvement in the plant/crop growth is observed, if cyanobacteria are supplemented with soil in comparison with control. The crop was selected on the basis of availability of sterile seeds of groundnut. Different parameters were observed in intervals to evaluate the capability and effective nitrogen fixing by cyanobacteria. Root nodules, thickness and health of root, shoot length was found effected in test experiment when compared with control. The observation was obtained from 25 plants at interval of 20, 37 and 45 days. The average data of treated after 20 days showed the shoot length, root length and number of nodules are 24.9, 9.4 and 0 whereas control showed 21.4, 6 and 0 respectively. After 37 days, average data obtained for shoot length, root length and number of nodules was 28.8, 15.9 and 2 in treated in comparison to control which is 25.8, 11.3 and 0. Data obtained after 45 days were 32.7, 18.7 and 16.6 in treated with comparison of control which is 28, 13.1

Volume : 4 | Issue : 5 | May 2014 | ISSN - 2249-555X

and 3 for shoot length, root length and number of nodules. The yield parameter were obtained after 65 days and found better in treated, which suggest increase of 6 times on an average. The data obtained indicate that the nitrogen fixation increased by supplementation of cyanobacteria in soil.

Conclusion

It was concluded that the cyanobacteria found from different lake of Maharashtra, India, has the capability of nitrogen fixation. Therefore, cyanobacteria are one of the major components which provides potential source of nitrogen fixation at no cost and can be used as a bio-fertilizer. It plays an important role to build up the symbiotic association. The blue-green algae are capable of fixing the atmospheric nitrogen and convert it into an available form of nitrate required for plant growth. The number of nodules formation increases with increase in the simplest form of nitrogen available due to the cyanobacteria supplemented with soil. They can be efficiently used as bio-fertilizers which are eco-friendly and can be utilized as economical alternate of chemical fertilizers with much lesser input of capital and energy.

Table 1: Samples collected from different lakes of Maharashtra, India

Sr. No.	Name	Location
1.	Dr.Salim Ali lake	19°53'57.26"N 75°20'32.23"E
2.	Lonar lake	19°58'36"N 76°30'30"E
3.	Harsool lake	19°55′45″N 75°20′10″E
4.	Nehru udyan lake	19° 53′ 47″N 75° 23′ 54″E
5.	Sawangi lake	19°56′18″N 75°21′30″E
6.	Khultabad lake	20.009524°N 75.188799°E

Table 2: Set of *nif* gene sequences used for multiple sequence alignment.

Sr. no.	Name of the Organism	Gene Name	Reference (NCBI Accession ID)
1.	Rhizobium leguminosarum	Putative nif gene	X51750
2.	Clostridium pasteurianium	nifH6 gene	X07477
3.	K.pneumoniae	nifJ	X16345
4.	Azotobacter vinelandil	nif gene cluster	AF014048
5.	E.Agglomerans	nifA,nifB,nifL,nifM	X89104
6.	Heliobacterium chlorum	nifI1,nifI2,nifH,nifD, nifK,nifE,nifN,nifX,nifB,nifV	AB196525
7.	Pseudanabaena	nifEN gene	AF167547
8.	Anabaena azollae	nifE, nifN	AF167540
9.	Nostoc sp	nifH	L15551

Table 3: PCR components.

Sr. no.	PCR components	Quantity
1.	Master mix	12 µl
2.	Template DNA	8 µl
3.	Primer (right)	0.5µl
4.	Primer (left)	0.5 µl
5.	Double distilled water	4µl

Table 4: Data obtained after 20 days, 37 days and 45 days for shoot, root and number of nodules for control and treated soil.

	CONTROL			TREATED		
Days	Shoot	Root	Nodules	Shoot	Root	Nodules
20 days	21.49231± 0.820022	6.007692± 0.298501	0	24.95385± 0.547137	9.476923± 0.34194	0
37 days	25.84615± 0.706653	11.34615± 0.585399	0	28.81538± 0.494716	15.94615± 0.452061	2± 0.707107
45 days	28.14615± 0.631949	13.03846± 0.448216	3.076923± 1.037749	32.70769± 0.317442	18.76154± 0.455592	16.61538± 1.043908

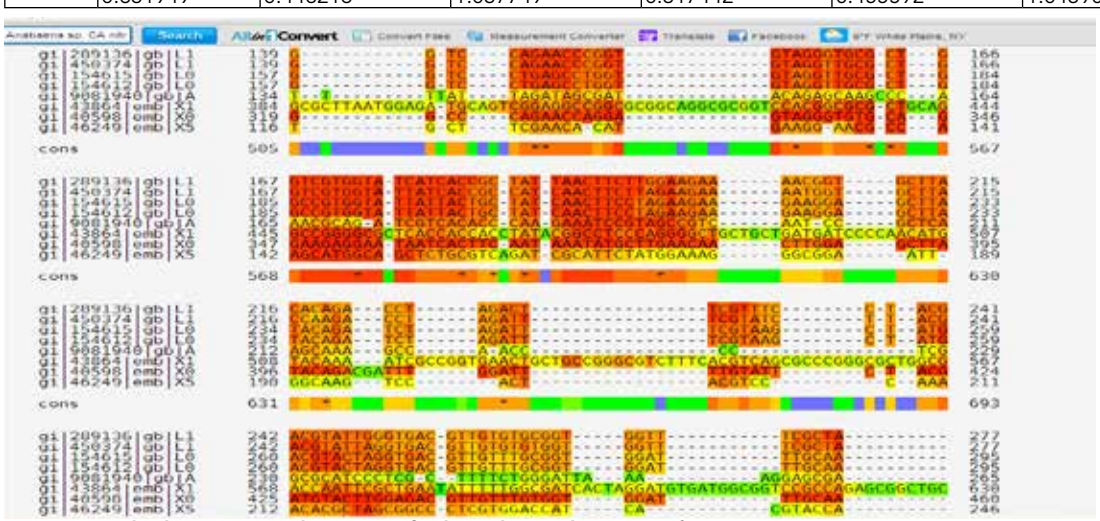


Figure 1: Multiple sequence alignment of selected cyanobacteria *nif* gene.

Volume : 4 | Issue : 5 | May 2014 | ISSN - 2249-555X

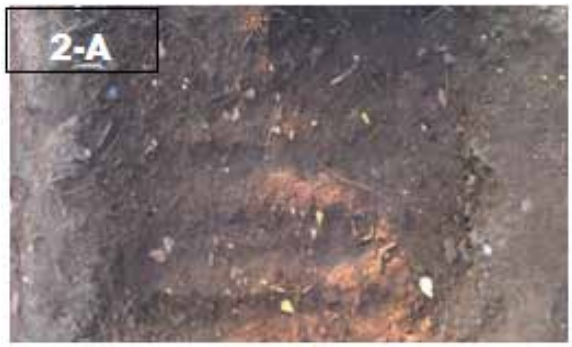


Figure 2-(A) Simple soil bed is prepared without any external additions to it.

Figure 2-(B) Simple bed soil is checked by plating techniques

Figure 3-(A) Poly house

Figure 3-(B) Soil bed is made by treating autoclaved soil with Cyanobacteria culture.

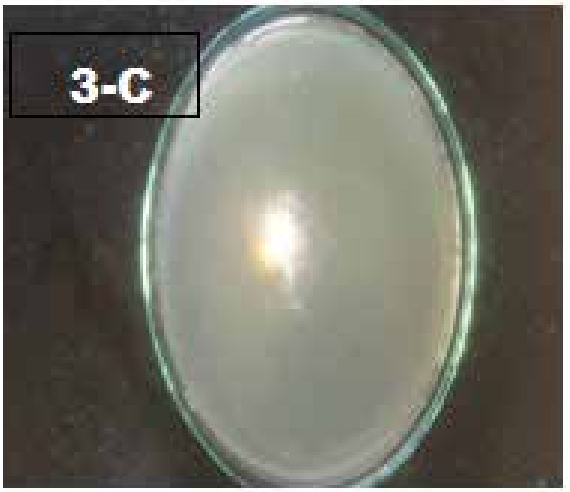


Figure 3-(C) This treated soil is checked by plating techniques.

Figure 4-(A) Cyanobacterial streaking,

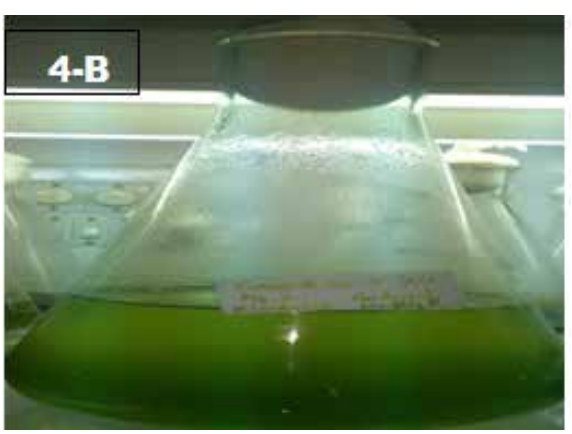


Figure 4-(B)Pure cyanobacterial culture.

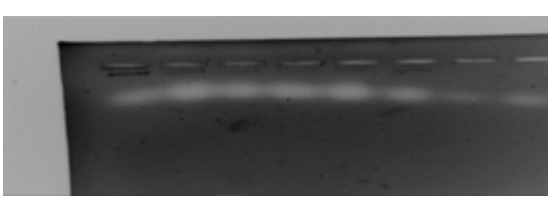


Figure 5: Purified quality DNA bands.

Volume : 4 | Issue : 5 | May 2014 | ISSN - 2249-555X

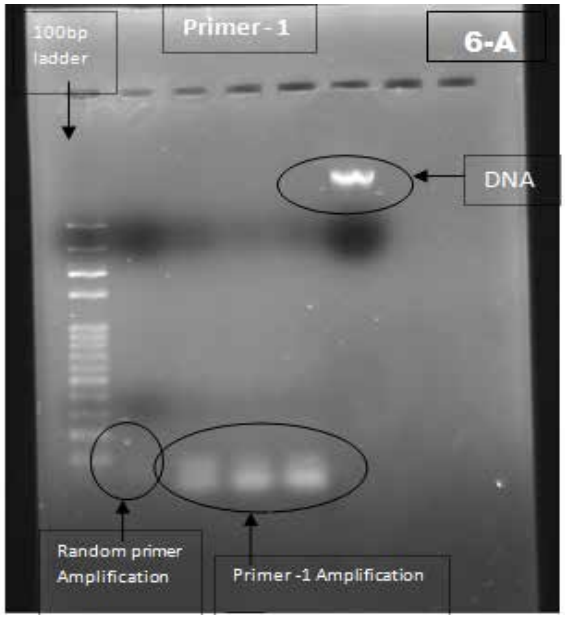


Figure 6-(A) Amplification result of NifH (primer 1).

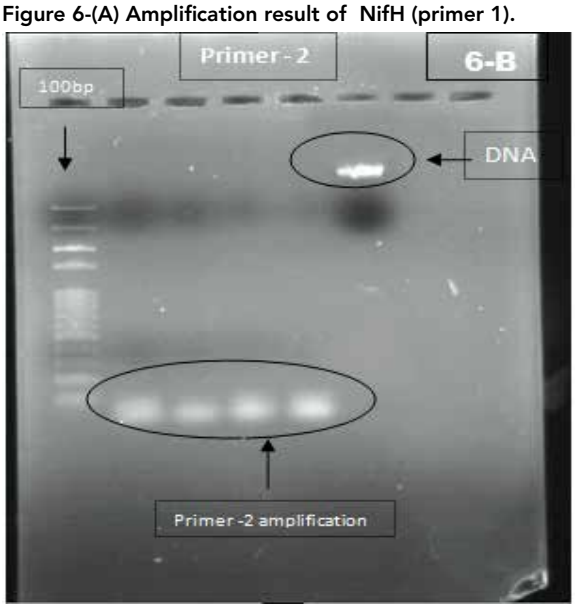


Figure 6-(B) Amplification result of NifEN (primer 2).

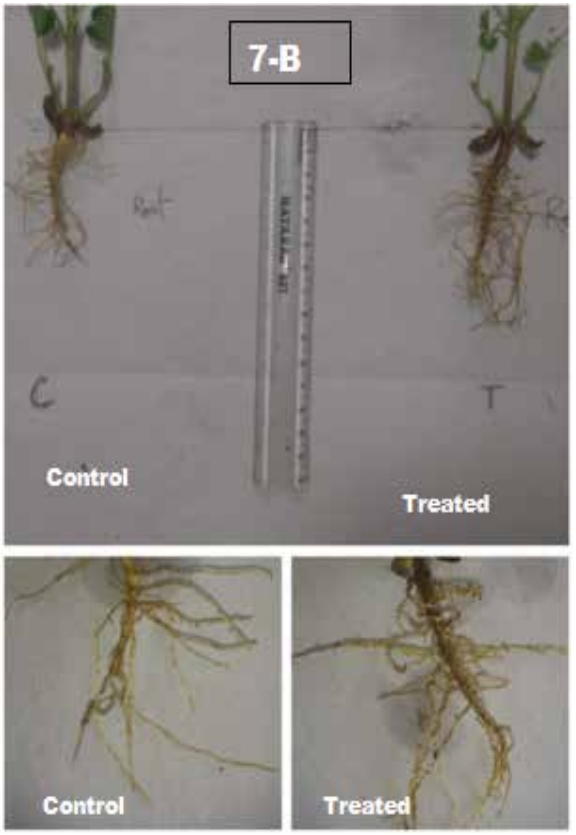


Figure 7-A, B: The growth of control and treated shoot and roots after 20 days.

Volume : 4 | Issue : 5 | May 2014 | ISSN - 2249-555X

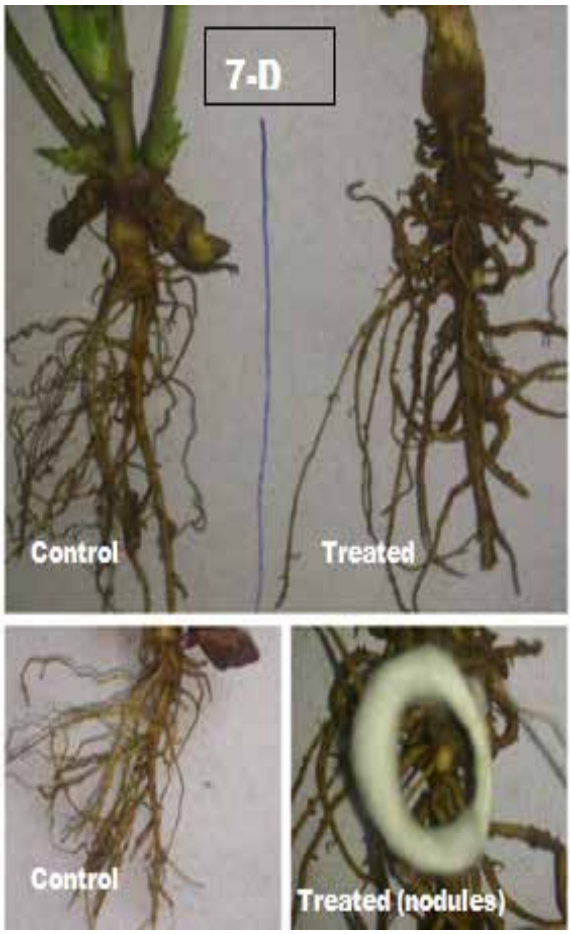


Figure 7-(C, D) The growth of control and treated shoot, roots and nodules after 37 days.

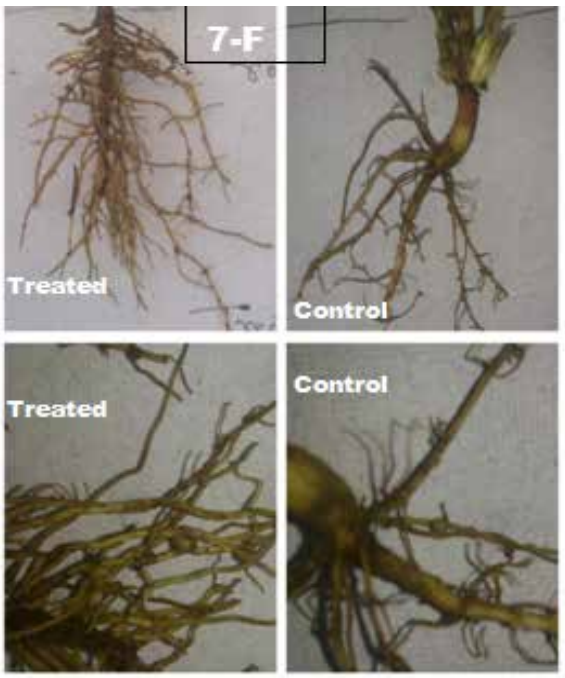
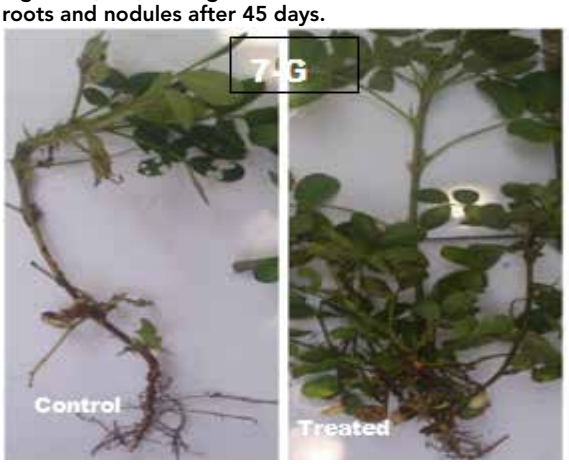


Figure 7-(E, F): The growth of control and treated shoot, roots and nodules after 45 days.



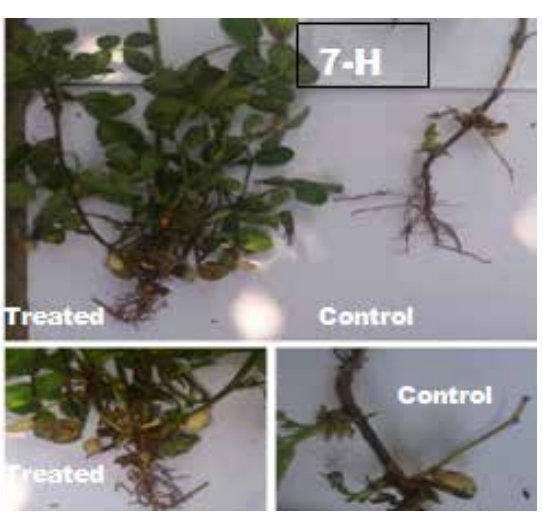
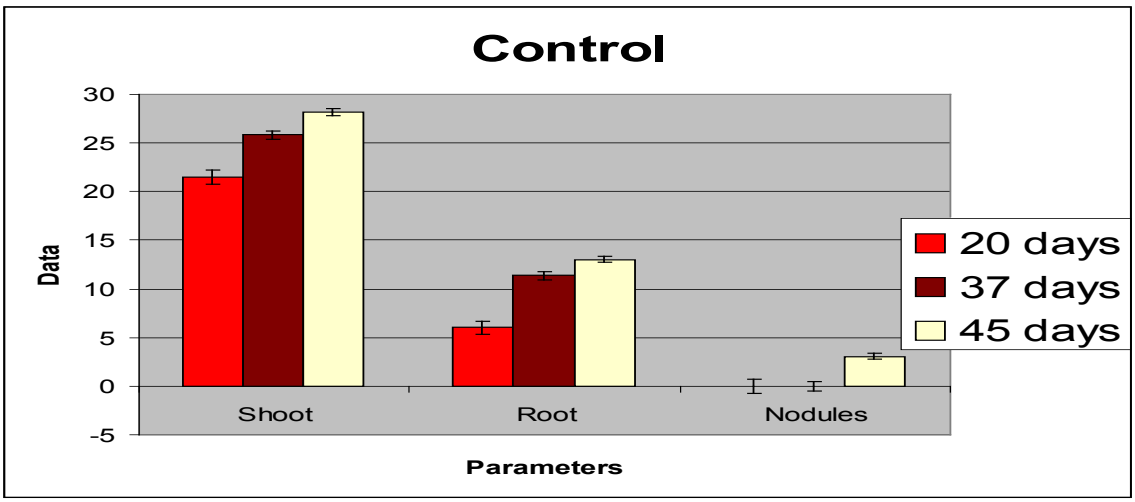


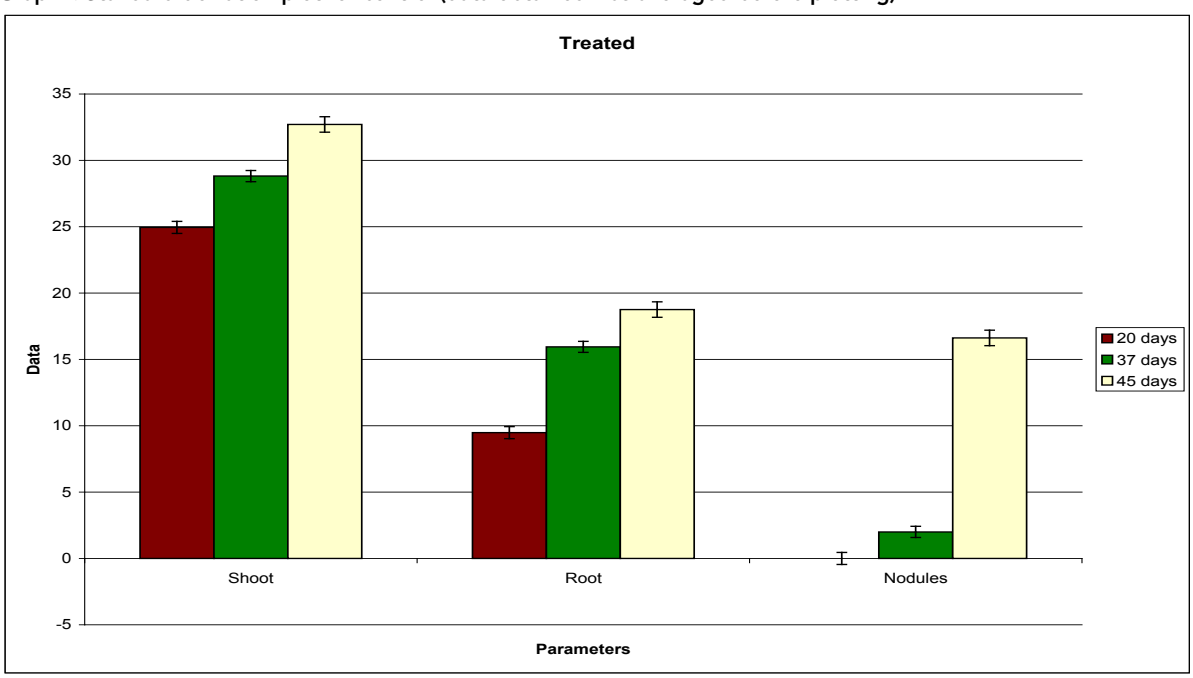
Figure 7-(G, H) The growth of control and treated shoot, roots, nodules and fruits after 65 days.

Graphs

Volume : 4 | Issue : 5 | May 2014 | ISSN - 2249-555X



Graph1: Standard deviation plot for control (data obtained was averaged before plotting)



Graph 2: Standard deviation plot for treated (data obtained was averaged before plotting)

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