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Non-Edible Oil Cakes as Organic Amendment for the Growth of *Cenchrus setigerus* and its Effect on Naturally Occurring *Azospirillum* in the Rhizosphere

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ABSTRACT

The present study was carried out to investigate the integrated effect of non-edible oil cakes with reduced dose of urea on yield and fodder value of *Cenchrus setigerus* along with gene expression of rhizospheric *Azospirillum*. Maximum yield of 23.75 t/ha was obtained when 75 % of nitrogen required by the grass was provided by Neem cake and rest by urea. Fodder value in terms of acid detergent fibre, neutral detergent fibre and crude proteins were also significantly ($P<0.05$) affected by the treatments. The population of free-living *Azospirillum* in the rhizopheric soil was directly proportional to the amount of cakes applied in soil. With Urea alone, the *nifH* and *ntrC* gene activity was found to downregulated by 1.5 and 4.0 folds respectively but integration of cakes (both *Jatropha* and neem cake) upregulated the nifH and ntrC gene expression.

Key Words: Non-edible Oil Cakes; Urea; PCR; ; Nif Genes; Upregulation; Downregulation

INTRODUCTION

Livestock is the main source of livelihood for a majority of the rural population in India. Although, India has a huge livestock population, the productivity per animal is still about the lowest in the world (Pathak and Kumar 2004). The deficiency of feed and fodder is identified as one of the major constraints in achieving desired level of livestock productivity. Chemical fertilizers although serve to improve fodder yields, their application directly or indirectly causes negative impact on chemical, physical and biological properties of the soil. Bremner (1995) reported the adverse effects of urea on the growth of seedlings, owing to production of ammonia through its hydrolysis. Therefore, sustainable productivity of livestock and fodder shortage problem could be alleviated through integrated use of organic fertilizers with minimum dose of chemicals.

Non-edible oil cakes (NEOCs) such as Jatropha, Karanja, Castor, Neem etc. are the waste products of oil extraction with high nitrogen content and therefore can be used as organic nitrogenous fertilizers (Sharma et al. 2013). These cakes, apart from providing nutrition to plants, also finds application as effective biopesticides covering a broad range of approx 413 insect pest (Schmutterer et al. 1995).

Nitrogen fixation by free-living microorganisms occurs in most soils and is the major source of fixed nitrogen for fodder crops (Widmer et al. 1999, Lovell et al. 2000). It is a well-known fact that *Azospirillum* commonly present in the rhozsphere of wide variety of tropical and sub-tropical fodder grasses. *Azospirillum*, firmly attached with the roots of grasses and mediates the process of nitrogen fixation at low level of nitrogen and oxygen by the action of nitrogenase complex (Hubbell et al. 1980). Intracellular concentrations of nitrogen and oxygen regulate nitrogenase at the transcriptional and post-translational levels (Zhang et al.

1997). Functionally important gene, *nifH* has been extensively studied to examine the variety and heterogeneity of nitrogen-fixing bacteria present in the rhizosphere of rice roots (Ueda et al. 1995) and nitrogen fixation ability in marine plants (Zehr et al. 1998). NtrC (nitrogen regulatory protein C) is basically a positively acting bacterial transcription factor that is involved in regulating the metabolism of nitrogen (Carroll et al. 2001).

In one of our previous work, we showed the importance of non-edible oil cakes in enhancing the growth and yield of brinjal (unpublished data). In current study, we focus on the role of same in increasing the yield and quality of a multicut fodder crop, *Cenchrus setigerus*. In addition to it, we also investigated the imapct of cakes on the natural population of nitrogen fixing *Azospirillum* available in the rhizosphere of *C.setigerus* and expression of their *ntrC* and *nifH* genes.

MATERIALS AND METHODS

Experimental Site

The field experiments were conducted at Micromodel complex, IIT Delhi campus. The site was located at 77.09 °E longitude and 20.45 °N latitude and elevations ranged from 228 m altitude above sea level. The field soil was sandy loam with C 0.72%, N 272 kg ha^{-1}, P 9.0 kg ha^{-1}, K 200.7 kg ha^{-1} and pH 7.5.

Non-Edible Oil Seed Cakes (NEOC)

Non edible oil seed cakes of Jatropha and Neem were procured from Center for Rural Development and Technology, Indian Institute of Technology Delhi, India. The cakes were obtained from cold press. The cakes were dried in a hot air oven at 40 °C for 6 h to remove moisture.

Experimental Design

The experimental design was a randomized split plot of 4 m^2 quadrats and consisted of organic and inorganic treatments as given in Table 1. NEOC were applied 10 days before transplantation and the mineral N (urea) was applied in two splits i.e. half dose just before transplantation and remaining half dose at the time of peak requirements of *C. setigerus*. Phosphorous (P) and Potassium (K) were applied at recommended rate in the form of single super phosphate and murate of potash respectively as basal dose to crop and adjusted on the basis of phosphorous and potassium available in the oil seed cakes. Three replicates were maintained for each treatment.

Table 1. Treatment details of experiments using non-edible oil cakes and chemical fertilizers on *Cenchrus setigerus*.

Symbol	Treatment details
T1	Control (No cake & No fertilizers)
T2	Chemical alone (Urea)
T3	25 % Neem cake + 75% Urea
T4	25 % Jatropha cake + 75% Urea
T5	50 % Neem cake + 50% Urea
T6	50 % Jatropha cake + 50% Urea
T7	75% Neem cake + 25% Urea
T8	75% Jatropha cake + 25% Urea

Growth Parameters of *Cenchrus setigerus*

Grass yield was estimated by harvesting random 4-m^2 quadrants. Fresh biomass was further oven dried at 65 ^{0}C and qualitatively analyzed for acid detergent fibre (ADF) and neutral detergent fibre (NDF) (Van Soest et al. 1991). Crude protein content was determined by multiplying 6.25 to the plant N content that was estimated by micro- Kjeldahl method (Nelson and Sommers 1973).

Azospirillum Isolation and Enumeration

Azospirillum was isolated from the rhizospheric soil treated with different combinations of fertilizers. Further it was routinely cultivated in N-free *Azospirillum* specific media (Sigma-Aldrich), supplemented with of Congo red and autoclaved separately (Caceres 1982). The number of colony forming units (CFUs) for each sample was determined by MPN method (Doberneir 1995).

RNA Isolation and cDNA Synthesis

Pure *Azospirillum* colonies from each plate were inoculated into the corresponding suspension media for the purpose of RNA isolation. Total RNA of *Azospirillum* cells was extracted from each treatment by

using Bio Basic Rapid Bacteria RNA isolation kit after 12 h and 48 h of incubation, which were subsequently visualized on 1.5 % (w/v) agarose gel to assess their integrity.

Reverse Transcriptase reactions comprising of 1 µg of total RNA, 5 µL of M-MLV RT 5X reaction buffer (Promega), 1.25 µL of 10mM of each deoxynucleotide triphosphate, 1 µg of random hexamers (Promega) and 200 units of M-MLV RT (H-) point mutant (Promega) were carried out in a final volume of 25 µL. RT reaction vials using random hexamers were incubated at 40°C for 1 h.

Amplification of Genes Encoding nifH, ntrC and 16S rRNA Primers

Amplification of the genes of interest (*nif*H and *ntr*C) was carried out in DNA thermal cycler (BioRad Model CT1000) using Promega PCR amplification kit (Table 2). For each gene, PCR reaction mixture consisted of 2.5 µL reaction buffer, 2.5 µL of dNTP, 3 µL of each primer (Table 2), 1 µL of *Taq* polymerase, 2 µL of cDNA synthesized and addition of nuclease free water to make up the final volume to 25 µL. The reagents were purchased from Promega. The amplification conditions for each reaction mixture were: denaturation at 98 °C for 5 s, followed by 30 cycles with heat denaturation at 95 °C for 30 seconds, annealing (at different temperatures for each set of primers, Table 2) for 30s, and DNA extension at 72 °C for 1 min. The samples were subsequently maintained at a temperature of 72 °C for 2 min. The PCR products were analysed by 1.5 % (w/v) agarose gel.

Statistical Analysis

Significance of the 5 % level was considered to demonstrate differences by using one way ANOVA by new SPSS statistical software. Upregulation and down-regulation pattern recognition was performed using Vision cap software.

RESULTS AND DISCUSSION

Effects of Non-edible Oil Cakes and Chemical Fertilizers on *C. setigerus*

C. setigerus fodder production under various treatments (T1 to T7) ranges from 18.46.7 Mg ha^{-1} to 23.75 Mg ha^{-1} (Table 3). Best yield was obtained from the pots treated with 75 % Neem cake supplemented with 25 % urea and 75 % Jatropha cake + 25% urea that is 23.75 and 22.6 Mg ha^{-1} respectively. Urea alone enhanced the yield of *C.setigerus* by only 11 %. It is evident from Table 3 that integrated use of non edible oil cakes with chemical fertilizer (urea) not only reduced the use of chemicals but significantly improved the yield also. Oil seed cakes as manure has been shown to increase total nitrogen, carbon, cation exchange capacity, soil respiration and decrease particle size (Wennberg and Nyman 2004). Ogbonna et al. (2012) observed that the use of deoiled palm kernel cake based fertilizer enhanced the maize grain yield by 74.76% over control while Cassava tuber yields by 200 %.

The data in Table 3 also revealed that with increasing proportions of Neem and Jatropha oil cakes, digestibility of grass (*C. setigerus*) increased and hence, ADF and NDF values got decreased. The ADF and NDF for treatment T7 i.e. Neem cake 75 % + Urea 25 % were 46.54 and 53.31 % respectively while for T8 (Cake + urea = 3:1) had 46.6 and 53.43% respectively. It might be due to improvement of soil quality by addition of NEOC thereby lowering the rhizosphere temperature and thereby increasing soluble carbohydrate uptake and decrease in fibre contents (Wennberg and Nyman 2004). The highest content of crude protein was also observed in T7 (8.24%) and T8 (8.37%). It might be due to the

Table 2. Primer detail of *NifH* and *NtrC* genes used for the study

Gene and Function	Base pairs	Primer sequence
NifH Nitrogenase functional gene	205	F=5'GACCCGCCTGATCCTGCACG R=5'GTTCTCTTCCAGGAAGTTGATCGA [1]
NtrC Required for full nitrogenase activity	183	F= 5'CTACGCAAGTAATGCTGC R= 5'CTTGTCACCGGCAGTTCCACCAG [2]

[1] Fani et al. (1989); [2] Holguin et al. (1999)

Table 3. Effect of non-edible oil cakes on fresh biomass yield, and qualitative composition

Treatments and details		Yield (Mg ha^{-1})	ADF (%)	NDF (%)	Crude Protein (%)	*Azospirillum* CFU g^{-1} soil
T1	Control (No cake & No fertilizers)	18.46 ± 0.53^{a}	48.60 ± 0.3^{c}	57.33 ± 0.36^{c}	6.32 ± 0.22^{a}	7.00E +06^{c}
T2	Chemical alone (Urea)	20.5 ± 1.5^{c}	48.81 ± 0.23^{c}	57.55 ± 0.25^{c}	6.42 ± 0.28^{a}	5.00E +04^{a}
T3	25 % N (75% Urea)	19.9 ± 0.9^{b}	48.99 ± 0.28^{c}	57.53 ± 0.22^{c}	6.40 ± 0.20^{a}	5.00E +06^{b}
T4	25 % J (75 % Urea)	20.0 ± 0.10^{b}	48.91 ± 0.18^{c}	57.68 ± 0.30^{c}	6.43 ± 0.20^{a}	5.00E +06^{b}
T5	50 % N(50 % Urea)	18.91 ± 0.05^{a}	47.34 ± 0.30^{b}	55.17 ± 0.5^{b}	7.27 ± 0.34^{b}	7.00E +06^{c}
T6	50 % J (50 % Urea)	19.02 ± 0.10^{b}	47.56 ± 0.4^{b}	55.52 ± 0.40^{b}	7.50 ± 0.34^{b}	6.00E +06^{c}
T7	75% N (25 % Urea)	23.75 ± 1.0^{c}	46.54 ± 0.23^{a}	53.31 ± 0.36^{a}	8.24 ± 0.16^{c}	7.00E +07^{d}
T8	75% J (25 % Urea)	22.6 ± 1.7^{c}	46.60 ± 0.23^{a}	53.43 ± 0.36^{a}	8.37 ± 0.32^{c}	9.00E +07^{d}

increased availability of nitrogen by N- rich oil cakes and urea which consequently enhance the crude protein content by increasing nitrogen uptake.

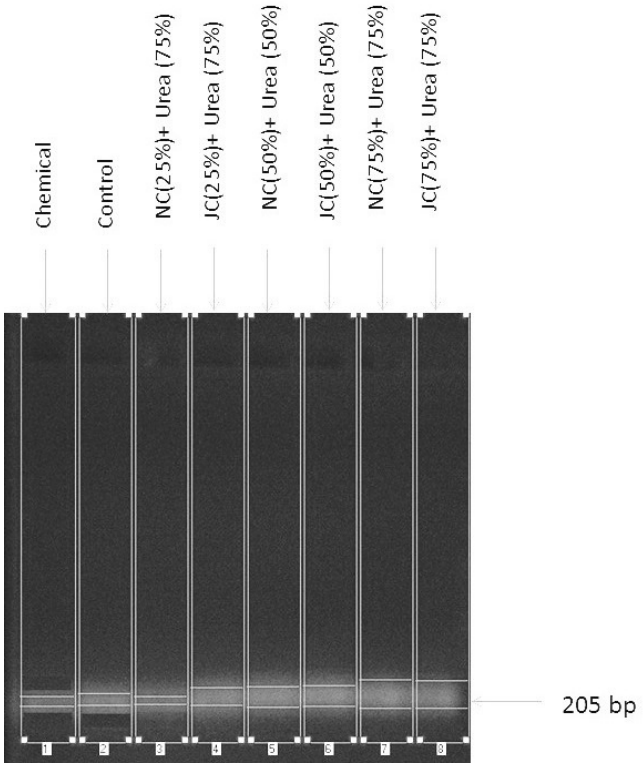
Effect of cake amendment on *Azospirillum* population

Besides other microorganisms , *Azospirillum* biomass is a living part of soil organic matter and it act as a repository of plant nutrients mainly nitrogen (N) and can therefore, have important implications for nutrient bio-availability. Table 3 shows CFU count of *Azospirillum sp.* grown in different soil treatments at the micromodel of IIT Delhi (CFU per g of soil). The count was lowest in case of chemical treatment for *Azospirillum sp.* grown in field conditions (Table 3). However, the CFU count increased with increasing percentage of Neem and Jatropha cake and this might be due to utilization of nutrients from these cakes as these cakes are good source of nutrients (Nakhro et al. 2010).

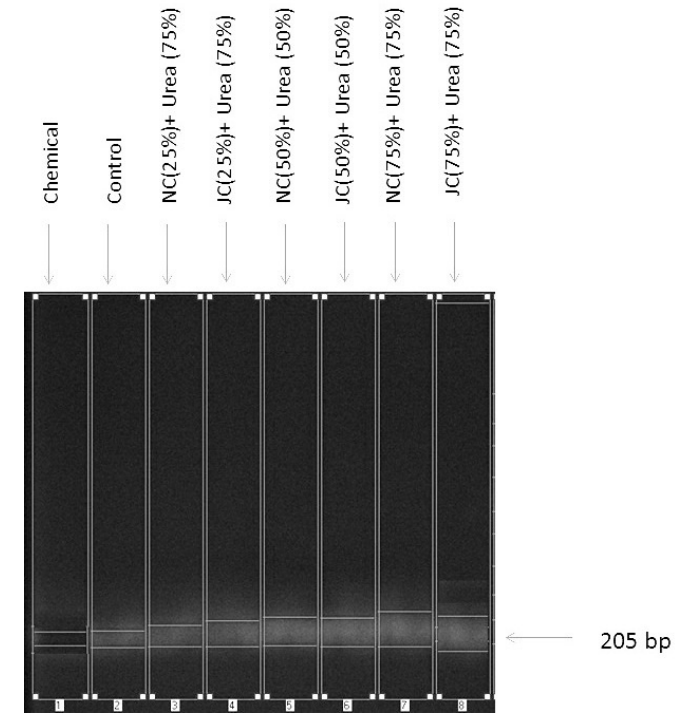
Gene Expression Analysis and Quantification of Nitrogen Fixing Genes

The RNA separated at different time intervals was converted into cDNA and amplified. The amplified product was run on gel and bands were visualized to see if there is any change in the band intensity of amplified products of various treatments on different time course. Figure 1a shows the effect of various chemical and biofertilizers on the *nifH* gene at 12 hr. Of the bands quantified there was a distinctive quantitative difference in the band intensities of the treatments. Qualitatively, the expression of *nifH* gene decreases significantly in chemical treatment as compared to control. Conversely, the level of gene expression is higher in *Azospirillum spp.* in soils treated with Jatropha cake and Neem cake with the bacteria in 50% and 75% Jatropha cake treated soils showing higher gene expression than the one treated with 25% cake (Figure 1a,2a). This corresponds to the higher gene activity with biofertilizer treatment and justified out hypothesis of inactivation of N – fixing mechanism in the presence of chemical fertilizers. Similar kind of results obtained with *nifH* gene at 48 hr. However, the band intensities of chemical treatments relatively decreased as compared to 12 h treatments (Figure 1b, 2b). This concludes negative effect of chemical fertilizers on *nifH* gene activity which leads to its down regulation. In contrast, the band intensities of *nifH* gene with biofertilizer have increased showing the upregulation of *nifH*.

Figures 3 and 4 reveal the effect of chemicals and biofertilizers on the *ntrC* gene regulation. The band intensities at 12 h (Figure 3a) show the decrease in gene expression of *ntrC* with chemical. The intensity of the treatment (T1) was found to be decreased by 1.7 fold as compared to control (Figure 4a). The upturned band intensities of biofertilizer treatments (2.1 times as compared to control) showed the positive effect of biofertilizers on the *ntrC* activity. Moreover, with the time course i.e. at 48 hr, the intensity of band (~4 times decrease) with chemical treatment (T1) was likely to be disappear showing the sharp decline in the *ntrC* activity (Figure 3b, 4b). Conversely, there is more increase in the intensities of bands of biofertilizer treatments. The increase in band intensities of biofertilizer treatments followed the trend T3 =T4< T5=T6<T7=T8, which is likely same for both *nifH* and *ntrC* genes at 24 and 48 h.

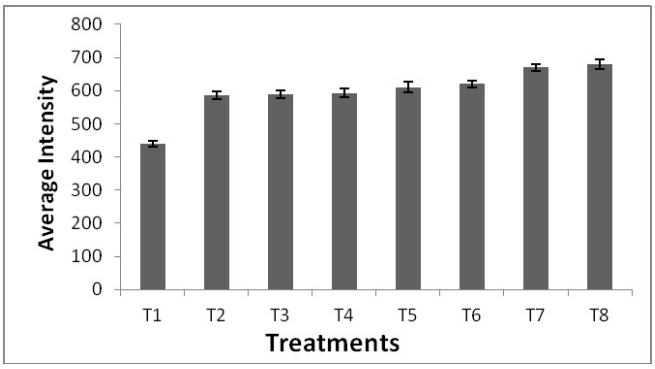


a

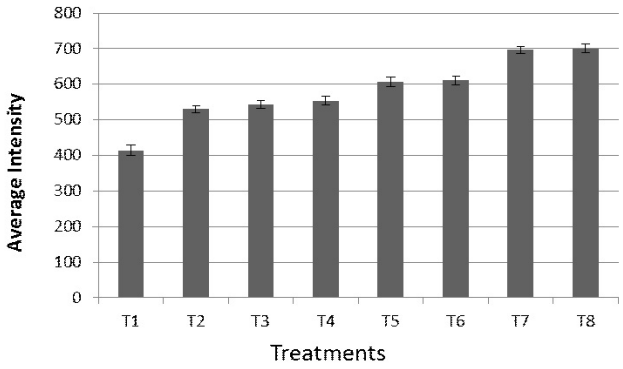


b

Figure 1. Gel analysis of amplification of *nifH* gene of *Azospirillum* treated with various biofertilizers and chemical fertilizer. a) after 12 h b) after 48 h



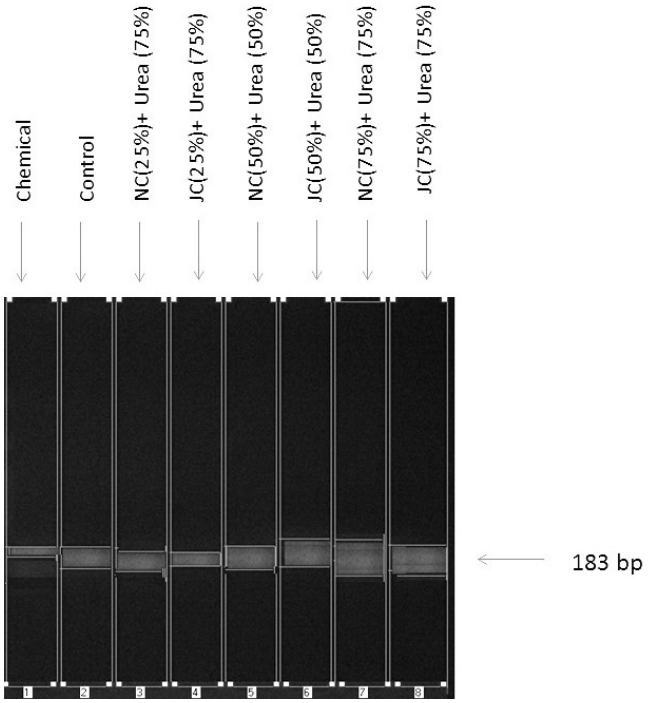
a



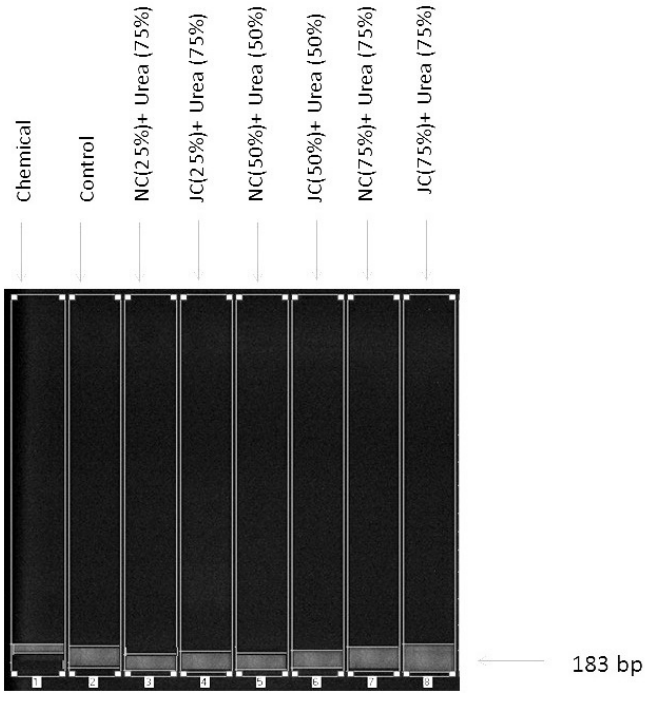
b

Figure 2 Quantification of band intensities of amplified *nifH* gene of *Azospirillum* treated with various biofertilizers and chemical fertilizer. a) after 12 h b) after 48 h

This correlates with the CFU count we had obtained earlier. Our results are also in corroboration with Sharma et al 2012, who observed the increase in fodder grasses yield by the use of microbial biofertilizers (*Azospirillum* and AMF). Nitrogen fixation in *A. brasilense* is regulated by a gene cluster consisting of 22 genes, spanning a region of 65 kb of DNA (Singh et al. 1989). The NtrBC system plays a pivotal role in transcription of genes of nitrogen fixation cascade. The relative degree of phosphatase and kinase activities of NtrB regulates the activation of *ntrC*. At low levels of nitrogen, α-ketoglutarate concentration increases with respect to glutamine, resulting in uridylation of PII, and phosphorylation and activation of *ntrC*. The RNA polymerase carrying σ^{54} factor (RpoN) is activated by phosphorylated NtrC, leading to transcription of n*if*HDK operon. The expression of *nifH* gene which contains an RpoN-dependent promoter occurs under conditions of nitrogen limitation and low oxygen level (Zhang et al.

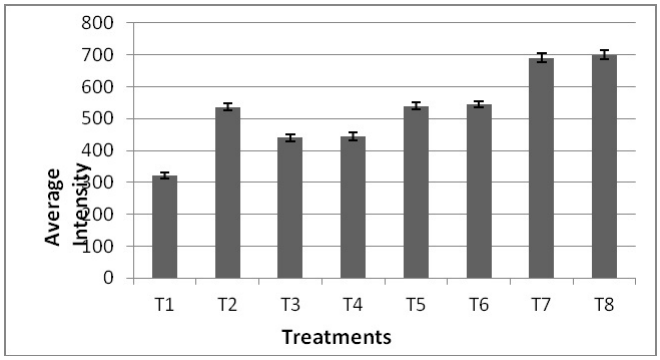


a

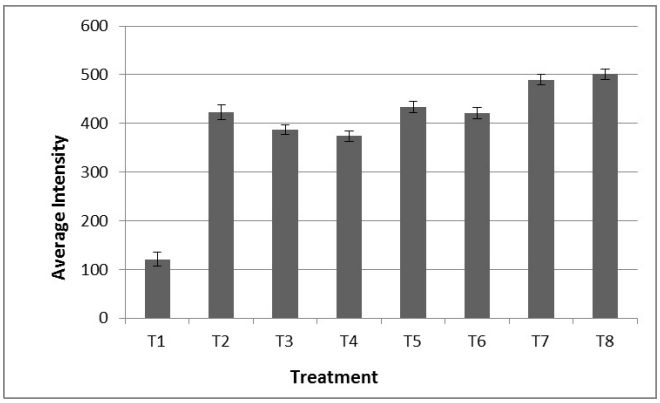


b

Figure: 3 Gel analysis of amplification of *ntrC* gene of *Azospirillum* treated with various biofertilizers and chemical fertilizer. a) after 12 h b) after 48 h



4a



4b

Figure 4 Quantification of band intensities of amplified *ntrC* gene of *Azospirillum* treated with various biofertilizers and chemical fertilizer. a) after 12 h b) after 48 h

T1: Chemical; T2: Control; T3: Neem cake (25%) + Urea (75%); T4: Jatropha cake (25%) + Urea (75%); T5: Neem cake (50%) + Urea (50%); T6: Jatropha cake (50%) + Urea (50%); T7: Neem cake (75%) + Urea (25%); T8: Neem cake (75%) + Urea (25%)

2008). Our results conclude that the use of nitrogenous chemical fertilizers not only decrease the nitrogen fixing capability of the *Azospirillum* but also have deterimental effect on the native population of the bacteria.

CONCLUSION

It may be concluded that the consumption of urea could be minimized by providing nitrogen through non-edible oil cakes *per se* and neem cake in particular for *C. setigerus*. The integrated use of non-edible oil cake not only enhanced the yield of fodder crop but also increased the natural population and nitrogen fixing ability of free-living nitrogen fixer, *Azospirillum*. Such strategy of integrating nitrogenous organic fertilizers with urea

would not only produce high quality forage but also improve the soil fertility and minimize the environmental pollution at large.

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