International Journal of Plant & Soil Science
3(8): 959-968, 2014; Article no. IJPSS.2014.8.003

SCIENCEDOMAIN *international*
www.sciencedomain.org

Effect of *Rhizobium* Based Biofertilizer Combined with *Saccharomyces cerevisiae* on the Growth of Hyacinth Bean

Chaitanya Velhal[1*], Mrudula Sant[1], Tanvi Godbole[1], Samadhan Waghmode[1] and Chandrashekhar Kulkarni[1]

[1]MITCON Biotechnology and Pharmaceutical Centre, Agriculture College Campus, Shivajinagar, Pune 411005, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author CV designed the study, wrote the protocol did the statistical analyses, and wrote the first draft of the manuscript. Authors TG and MS managed the literature searches, analyses of the study, performed the pot experiment, managed the experimental process and identified the Rhizobium species. Author CK proof read the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 1st February 2014
Accepted 31st January 2014
Published 17th May 2014

ABSTRACT

Aim: To study the combined effect of *Rhizobium* isolate *and Saccharomyces cerevisiae* on the growth of *Lablab purpureus* (hyacinth bean plant) as a potential bioinoculum for preparation of a novel biofertilizer.
Place of Study: The study was done in the laboratory of MITCON Biotechnology and Pharmaceutical Center, Pune, India; between February 2013 to December 2013.
Method: A pot experiment was arranged in two treatments (control and biofertilizer application) and provided with six replications each with 20 pots. Biofertilizer was prepared by mixing the *Rhizobial* inoculant with activated charcoal that acted as the carrier. Biochemical as well as genetic (16S rRNA sequencing) characterization proved that the isolate was from *Rhizobium* spp. Seeds were treated by placing in a solution containing *S. cerevisiae*. The seeds were then sown in pots and allowed to germinate in controlled conditions.
Results: The application of biofertilizer had a significant impact on the growth of hyacinth

**Corresponding author: E-mail: chaitanyavelhal@yahoo.com;*

bean. The treated plants showed 21.7% increase in the shoot length (*P value < 0.01)*, 6% in root length (*P value <0.001)* and 13.7% increase in the wet weight (*P value < 0.01)* as compared to control plants grown without the biofertilizer.
Conclusion: The newly prepared biofertilizer shows a significant potential for farmers looking to increase their productivity.

Keywords: Rhizobium; biofertilizer; Saccharomyces cerevisiae; growth promoter.

1. INTRODUCTION

An increase in the human population has been witnessed during the past four decades concurrently increasing the demand of food. This increase in the demand for food has led to the need for improvements better plant nutrition to get higher yields. One solution lies in the use of commercial man-made fertilizers. The use of these fertilizers has facilitated plant production at relatively low costs by using highly productive and intensive agricultural systems. The intensive use of organic or chemical fertilizers not only increases agricultural yield but also causes degradation of soil health and environment quality. The use of these inorganic fertilizers has increased exponentially since a few decades in a bid to counter the ever increasing consumer demand. The use of nitrogen (N) fertilizer has increased by almost nine folds and phosphorus (P) fertilizer by more than four folds till recently [1]. However, the abundant use of fertilizers and highly productive systems has led to environmental problems like deterioration of soil quality, surface water, groundwater, air pollution, reduced biodiversity and suppressed ecosystem function [2].

Biofertilizers, also known as microbial inoculants, are the fertilizers that help plants meet their nutritional requirements through biological fixation of essential elements as well as by enhancing rate of decomposition. This is done by supplementation with nitrogen, phosphorus and plant growth promoting rhizobacteria (PGPR) [3]. These are carrier based microbial preparations that contain beneficial microorganisms in a viable state intended for seed or soil application, which enhance plant growth through nutrient uptake and/or growth hormone production.

Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria that elicit nodule formation on legumes. The most commonly studied genera are *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Sinorhizobium* [4]. They are ubiquitous part of the soil micro-flora and in a free-living state in the rhizosphere of legumes until the point of nodulation. The nature and properties of soils allows billions of organisms to co-exist. The ability to form symbiotic relationships with members of the plant family *Fabaceae* is a unique feature associated with bacteria belonging to the family *Rhizobiaceae* [5]. Rhizobia elicit on their host the formation of nodules in which they fix nitrogen and thus, providing nutrition to the plant [5].

However, under some conditions, nodulation may not occur due to the lack of suitable rhizobial strains as some soils remain void of it. For instance, acidic soils generally contain no or low population densities of the alfalfa rhizobial symbiont *Sinorhizobium meliloti*, whereas basic soils contain a low inoculum potential of *Bradyrhizobium* sp., a rhizobial symbiont of *Lupinus* spp [6]. Under such conditions, inoculation with compatible rhizobia is likely to prove highly advantageous.

Extensive work is being carried out to identify new species of endophytic yeasts as bio-control agent or as a biofertilizer. Tulhadar et al. [7] reported that combined inoculation of *Rhizobium trifolii* with *Saccharomyces cerevisiae* and other yeasts enhanced the number of nodules, length of plants and dry weight of Egyptian clover *(Trifolium alexandrinum)* seedlings grown on agar slants. Similar effects were observed when seedlings were inoculated with *R. trifolii* in the presence of dialyzed culture filtrate of *S. cerevisiae.* Singh et al. [3] found out that the nodule number, dry weight of shoot and root biomass of legumes *(Leucaen aleucocephala, Glycine max, Cajanus cajan, Phaseolus mungo, Phaseolus aureus, Vigna unguiculata)* were enhanced by inoculation with live yeast cells *(Saccharomyces cerevisiae).* Root infection (native vesicular-arbuscular mycorrhiza (VAM)) and the formation of vesicles, arbuscules and spores were also increased with yeast inoculation [8]. Differences in the effectiveness of yeast culture on plant growth were also observed.

The objective of our study was to test for the first time the effects of a newly prepared biofertilizer on the growth of *Lablab purpureus* (hyacinth bean plant). The growth was checked by comparing root length, shoot length and wet weight of the treated plants as compared to non treated controls. The main hypothesis for this study was that the yeast along with the nitrogen fixing *Rhizobium* when added as inoculants in soil would increase the efficiency of nitrogen uptake thus leading to a faster and better growth in the plant of study.

2. MATERIALS AND METHODS

2.1 Isolation and Characterization of Rhizobium from Root Nodules

The root nodules of *Glycine max* (soyabean) were taken from Agriculture College, Pune by uprooting the plant, which were then thoroughly washed. The pink colored nodules were selected for the isolation of *Rhizobium* [9]. The nodules were then surface sterilized by washing with sterile distilled water twice, followed by a 4-5 mins wash with 70% ethanol and then they were immersed in 0.1% mercuric chloride for 1-2 mins and later washed repeatedly with sterile distilled water 8-9 times to remove minute traces of mercuric chloride. The washed nodules were then crushed in 1 ml of sterile distilled water with a sterile glass rod and used for serial dilution to isolate the organisms [9]. The organisms were selected based on their morphological and biochemical characteristics. The culture was tested for the following biochemical tests; Catalase, Citrate, Methyl Red test, Vogues Proskuer, Indole as described by Lowe [10], Urease, Nitrate reduction, Starch hydrolysis, Gelatin hydrolysis and Motility by Arora [11], Oxidase, Hydrogen sulphide production as shown by Sadowsky [12] and Nitrogenase test by Wilcockson [13]. The culture was also subjected to sugar fermentation tests with glucose, mannose, xylose, galactose, raffinose, mannitol and trehalose [14,15].

Further environmental parameter testing was also performed. Salt tolerance test to check the growth of Rhizobium on 2% salt containing nutrient broth. pH tolerance test was carried out in nutrient broth by adjusting the pH range from 4 to 9. Growth at range of temperatures that were also investigated was at 23ºC, 28ºC, 37ºC and 50ºC [16]. The isolated organism was then sent for sequencing (partial 16S rRNA) to confirm that the organism was a *Rhizobial* spp.

Mass production of Rhizobium was done in yeast extract mannitol broth and incubated at 28ºC for 4 days on a shaker at 125 rpm. The isolate was sent to National Center for Cell Science, Pune for standard 16S rRNA sequencing.

2.2 Selection and Processing of Carrier Material

The carrier material used was fine activated charcoal. It was ground to a fine powder so as to pass through 212 micron IS sieve which was then sterilized [17]. Then it was spread on a clean, dry, glass tray and the bacterial cultures were added to the sterilized carrier materials and manually mixed in the ratio of 1:1. The moisture content was maintained to about 35-40%. After proper mixing with activated charcoal, it was left for 5 days by covering the trays at 24ºC. Thereafter, *Rhizobium* inoculants could either be used directly or packed and stored [17]. The bacterial load was calculated at about $3.2x10^{10}$ cells per ml of inoculum at the time of preparation [17].The packets were stored in a cool place away from the heat or direct sunlight. The packets were kept in fridge in lots in polythene bags.

2.3 Pot Experiment

The efficiency of the biofertilizer was tested by running a pot culture experiment and monitoring the growth of hyacinth bean plants. *S. cerevisiae* was used in combination with *Rhizobium* to check if it enhances the growth of the plants.

The experiment was arranged in two treatments (control i.e. without addition of biofertilizer and test i.e. application of *Rhizobium spp* combined with *S. cerevisiae*). Each treatment consisted of 20 plants and the whole treatment was replicated six times.

0.04 gms of *S. cerevisiae* (dry granules) was revived by placing in sterile distilled water and then mixed with 5 gms of biofertilizer under aseptic conditions. Seeds of hyacinth bean were mixed in the prepared slurry and allowed to stand still for 30 mins. Test and control seeds were directly sown into their respective pots. Each pot contained about 200 gm of soil. The phenotypic parameters i.e. stem height; root length and the wet weight of the plants were measured after the 15^{th} day from sowing. Soil used for the whole experiment was taken from a single source (black farm soil provided by Agriculture College, Pune). The plants were kept in a polyhouse with humidity ranging from 70 – 80%. The temperature of the polyhouse was not controlled and varied with local conditions (max: 32ºC, min: 15ºC).

2.4 Statistical Analyses

The experiment data was taken from a total of 6 trials each having 20 treated plants and 20 control plants.

All the data obtained from the pot experiment was analyzed using Minitab (version 15). Paired T test was carried out between the control values and treated values of root length, shoot length and wet weight respectively. The graphs were plotted in MS Excel with the error bars representing standard error as calculated from Minitab.

3. RESULTS AND DISCUSSION

3.1 Biochemical and Morphological Tests

The isolated *Rhizobium* strain was observed to be producing white colored, circular, raised, convex and semi-translucent colonies. Under the microscope the cells were observed to be rod shaped, non spore forming and stained gram negative. Live cells were found to be

motile. Certain biochemical tests were also performed on the strain, which are detailed in Table 1 below.

Table 1. Biochemical Characterization of *Rhizobium* isolate

Sr. no	Tests	Results
1	Urease	Positive
2	Nitrate reduction	Positive
3	Starch hydrolysis	Positive
4	Gelatin hydrolysis	Negative
5	Catalase	Positive
6	Oxidase	Positive
7	Methyl Red test	Negative
8	Vogues Proskuer	Negative
9	Indole	Negative
10	Citrate	Negative
11	Hydrogen sulphide production	Negative
12	Nitrogenase	Positive

The culture was also subjected to various sugar fermentation tests and was found to be able to reduce glucose, mannose, xylose, galactose, raffinose, mannitol and trehalose. These results are in consensus with all the previous reported tests done during the isolation of *Rhizobium* [18]. The sequence obtained from NCCS was found to of *Rhizobium* spp after it was run through the NCBI database using BLAST. Maximum similarity was obtained at 99% with 95% query coverage (e value = 0).

3.2 Temperature, pH and Salt Tolerance

An optimum growth was observed at room temperature (28ºC to 30ºC). The organisms were found to be temperature sensitive as at higher and lower temperatures, a low growth was observed that might be due to a hindrance in the metabolic activity.

pH is an important parameter for the growth of the organism. Best growth of *Rhizobium* was reported at neutral pH i.e. 7 and very less growth was observed in the medium with pH 4.0 and 9.0.This may be explained with the help of the study carried out by Kucuk et al. [17] in 2006 reporting that salt stress significantly reduces nitrogen fixation and nodulation in legumes. In addition, Hashem et al. [19] in 1998 had proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemo taxis and root hair colonization, thus directly interfering with root nodule function.

The experiments also showed that the cells were able to grow in 1.2% NaCl containing medium but unable to grow on higher concentration, showing that the isolate was sensitive to the salt concentration.

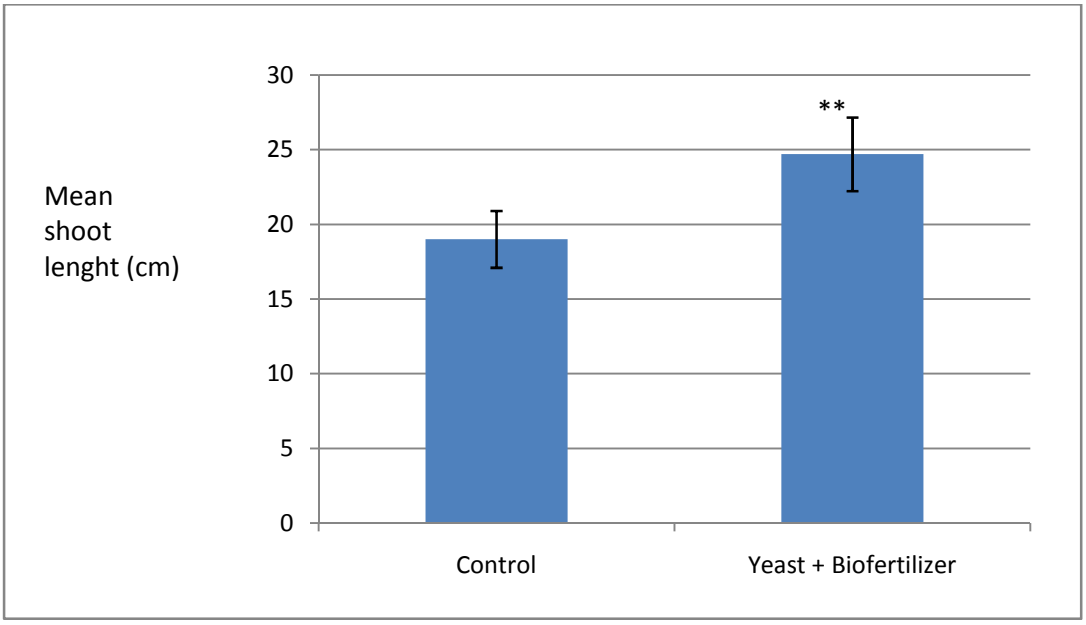
3.3 Hyacinth Growth

It was observed that the shoot length, root length and the wet weight of the hyacinth increased significantly when treated with biofertilizer formulation (Graph 1, Graph 2 and

Graph 3). The increment was about 21.6% in shoot length, 6% in root length and 13.7% in wet weight.

Thus this formulation has showed a positive effect on the growth of the treated plants.

Graph 1 exhibits the difference between the shoot length of the test and control hyacinth plant. It was seen that there was an increase of 21.67% in the mean shoot length of the plant treated with the formulation as compared to the control. The *P value* for this experiment was found to be 0.0036 (*P < 0.01)* and hence the increase in growth as observed in treated plants can be considered statistically significant as compared to that of the control.

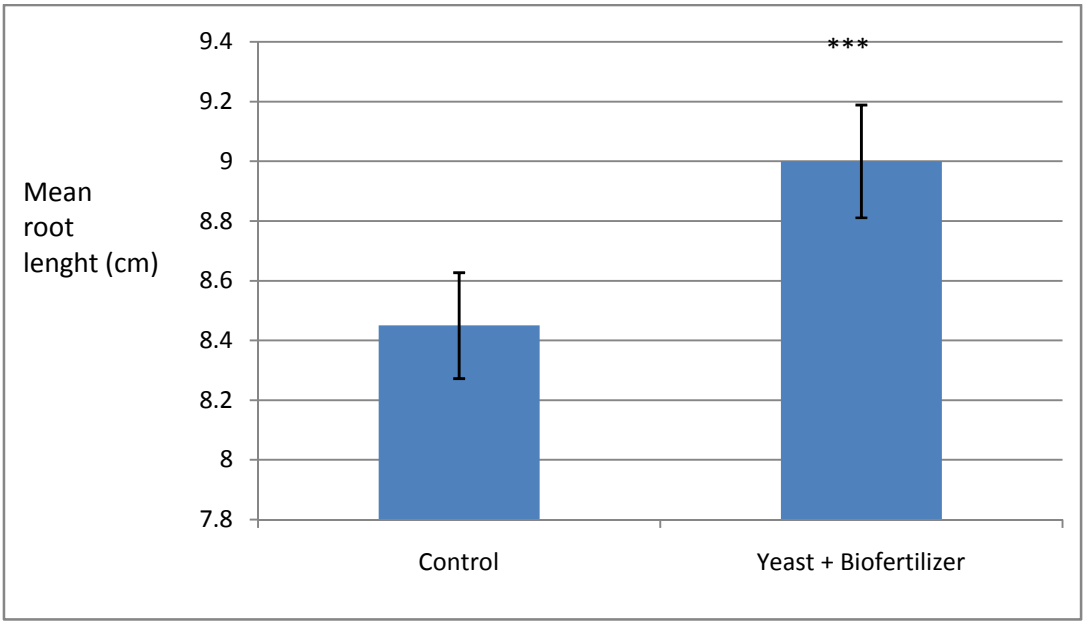


Graph 1. The effect of biofertilizer application on the shoot length of hyacinth after 15 days of sowing

** P value < 0.05, ** P value < 0.01, *** P value <0.001, Values are expressed in Mean ± S.E.M (n= 6) Error bars represent standard errors*

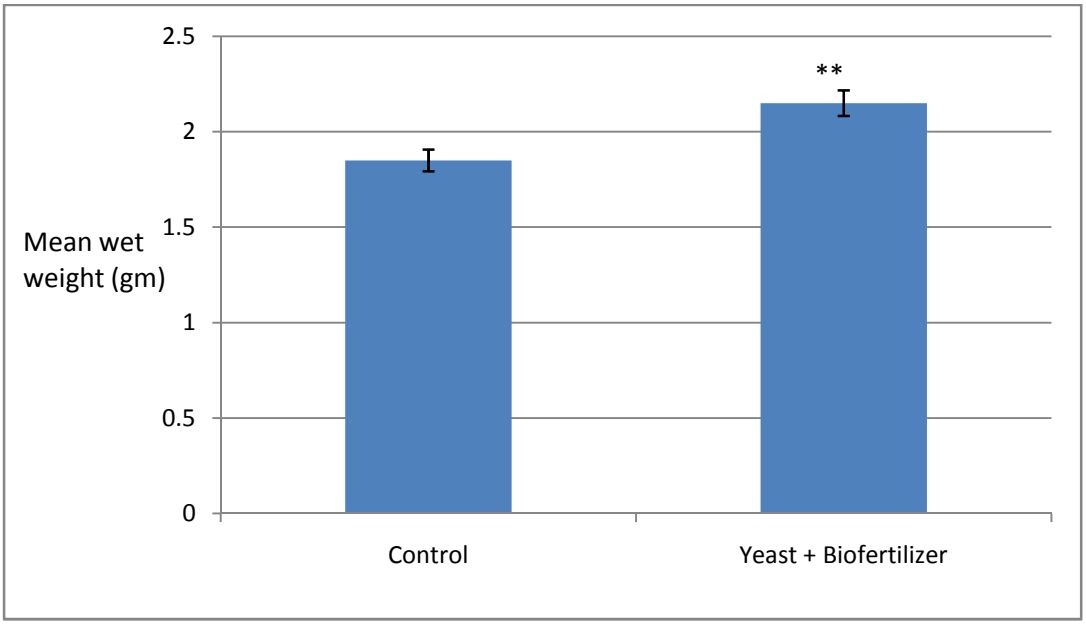
Graph 2 shows the difference between the mean root length of the control and the test hyacinth bean plant. It was observed that there was a significant increase of 6% in the root length of test plants. The *P-value* in this case was less than 0.001, which signifies a statistically significant increase in shoot length of treated samples as compared to the shoots of untreated control.

There was a 13.7% increase in the wet weight of the plant that was supplied with the yeast and biofertilizer formulation as compared to the control plant. This may be due to the improved quality of nutrition of the plant due to the formulation that was supplied to it. Graph 3 below shows this difference between the control plants and the treated plants.



Graph 2. The effect of biofertilizer application on the root length of hyacinth after 15 days of sowing

** P value < 0.05, ** P value < 0.01, *** P value <0.001, Values are expressed in Mean ± S.E.M (n= 6) Error bars represent standard errors*



Graph 3. The effect of biofertilizer application on the wet weight of hyacinth after 15 days of sowing

** P value < 0.05, ** P value < 0.01, *** P value <0.001, Values are expressed in Mean ± S.E.M (n= 6) Error bars represent standard errors*

Fig. 1. Difference in shoot length can be seen with the control (second from right) being the smallest while the rest show at least 20% more growth

Serial dilution and plate enumeration was carried out to check the number of viable cells in the final biofertilizer preparation which showed 2.9 x 10^{10} cfu/ml after 2 months of storage at room temperature.

This study demonstrates for the first time the combined effects of yeast (*S. cerevisiae*) and *Rhizobium* on plants as seen by the obvious differences in growth in figure 1 above. Mechanism of nitrogen fixation is quite well known and is not discussed here. The interaction amongst yeast and *Rhizobium* has definitely proved to increase plant growth. There are studies showing that yeast is capable of interaction with other symbiotic bacteria in natural conditions [20]. This interaction may cause a more efficient nitrogen fixation amongst *Rhizobial spp* [20]. Yeast such as *Saccharomyces* spp not only affects plant and microbial growth but also plays a role in soil aggregate formation and maintaining structure of soil. This could be another reason as to the increased growth seen in treated plants. Maldonade et al. [21] showed that certain species of yeast other than *Saccharomyces* synthesized carotenoids which are important constituents in plant nutrition as it is used as a precursor for vitamin A.

Nassar et al. [22] explained that this growth promoting activity was due to the production of indole-3- acetic acid (IAA) and indole-3- pyruvic acid (IPYA) by endophytic yeasts. They observed significant increase in the growth of the plant by measuring root and shoot length, dry weight and also the in-planta levels of IAA and IPYA [22].

Amprayn et al. [23] showed that using yeast alone caused a 16 – 35% increase in the dry weight of rice plant. They found out that the *Candida* spp of yeast could mobilize organic as well as inorganic phosphates which could amount to better nutrition and growth of the plant.

Certain yeast spp can solubilize insoluble inorganic phosphates and make them readily available for plants [23]. Amprayn et al also concluded that the same yeast strain could produce polyamines (spermine and spermidine) which increases cell division in roots leading to faster growth [23,24]. Apart from this a large number of yeast spp are also responsible for mineralization of organic materials, carbon and energy dissipation through soil. Major number of yeast species has also been linked to play some role in the natural nitrogen and sulphur cycle of soil [23,24].

The results further prove previous established studies that state increase in the number of yeast cells in soil causes a significant rise in the growth of the plant in that soil. Amongst all the work done in this field, only the work of Amprayn et al on rice plant shows a higher overall increase in plant growth (16 – 35%) [23] as compared to the results observed in this study (6 – 22%).

4. CONCLUSION

In this work, a novel biofertilizer was developed using a species of *Rhizobium*, which was isolated from the root nodules of soya bean plant. It was characterized with the help of morphological and biochemical tests. The isolated strain was proven to be of *Rhizobium* spp by DNA sequencing. Using this strain, a biofertilizer was successfully produced using activated charcoal as the carrier and its effect was studied on hyacinth bean in combination with *S.cerevisiae*.

An overall increase in the growth of the plant treated with the formulation was observed. Increase in biomass was observed in shoot length (21.67%), root length (6%) and wet weight (13.7%) of the bean plant. These results indicate a significant increment in the productivity of plants treated with the newly developed biofertilizer. This novel biofertilizer can be produced at a higher commercial level and its effect checked on various high yielding agricultural plants. It is necessary to continue researching in this field as it has the potential to be highly profitable for farmers as well as provide a way to a more sustainable future.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mekki BB, Amal G Ahmed. Growth, Yield and Seed Quality of Soybean (*Glycine max* L.) As Affected by Organic, Biofertilizer and Yeast Application. Research Journal of Agriculture and Biological Sciences. 2005;1(4):320-324.
2. Brockwell J, Bottomley PJ, Thies JE. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: A critical assessment. Plant Soil. 1995;174:143-180.
3. Singh CS, Kapoor A, Wange SS. The enhancement of root colonization of legumes by vesicular-arbuscular mycorrhizal (VAM) fungi through the inoculation of the legume seed with commercial yeast *(Saccharomyces cerevisiae)*. Plant and Soil. 1991;131:129-133.
4. Geetha SJ, Joshi SJ. Engineering Rhizobial Bioinoculants: A Strategy to Improve Iron Nutrition. The Scientific World Journal. 2013;2013:1-15.
5. Denarie J, Debelle F. Rosenberg C. Signaling and host range variation in nodulation. Ann. Rev. Microbiol. 1992;46:497-531.

6. Kaur H, Sharma P, Kaur N, Gill BS. Phenotypic and Biochemical Characterization of Bradyrhizobium and *Ensifer spp*. isolated from Soyabean rhizosphere, Bioscience Discovery. 2012;3(1):40-46.
7. Tuladhar KDY, Subbarao NS. Interaction of yeasts and some nitrogen fixing bacteria on nodulation of legumes. Plant and Soil. 1985;84,287-291.
8. Socolow RH. Nitrogen management and the future of food: Lessons from the management of energy and carbon. Proceedings of the National Academy of Sciences USA; 1999.
9. Vance CP. Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable sources. Plant Physiology. 2001;127:390-398.
10. Lowe GH. The rapid detection of lactose fermentatation in paracolon organism by demonstration of 6- D-galactosidase. J. Med. Lab. Technol. 1962;19:21-25.
11. Arora DR. The Text Book of Microbiology New Delhi: CBS Publisher. 2003;41-48.
12. Sadowsky MJ, Keyser HH, Bohlool BB. Biochemical characterization of fast and slow growing rhizobia that nodulate soybeans. Int. J. Syst. Bact. 1983;33(4):716-722.
13. Wilcockson J, Werner D. Nitrogenase activity of *Rhizobium japonicum* growing on agar surfaces in relation to slime production, growth and survival. J. Gen. Micro. 1978;108:151-160.
14. Mishra BK, Dadhich SK. Methodology of nitrogen biofertilizer production. J. Adv. Dev. Res. 2010;1:3-6.
15. Hugh R, Leifson E. The taxonomic significance of fermentative versus. Metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol. 1953;66:24.
16. Singh B, Kaur R, Singh K. Characterization of Rhizobium strain isolated from *Trigonella foenumgraecum* (Fenugreek). Africa. J. Biotech. 2008;7(20):3671-3676.
17. Kucuk C, Kivanc M, Kinaci E. Characterization of *Rhizobium* Sp. Isolated from Bean. Turk. J. Biol. 2006;30:127-132.
18. Michael JS, Grahm PH. Root and stem nodule bacteria of legumes. Prokaryotes. 2006;2:818- 841.
19. Hashem FM, Swelim DM, Kuykendall LD, Mohamed AI, Abdel-Wahab SM, Hegazi NI. Identification and characterization of salt and thermo-tolerant *Leucaena* nodulating *Rhizobium* strains. Biol. Fert. Soil. 1998;27:335-341.
20. Botha A. The importance and ecology of yeasts in soil. Soil Biology and Biochemistry. 2011;43(1):1-8.
21. Maldonade IR, Rodriguez-Amaya DB, Scamparini AR. Carotenoids of yeasts isolated from the Brazilian ecosystem. Food Chemistry. 2008;107(1):145-150.
22. Nassar AH, El-Tarabily KA, Sivasithamparam K. Promotion of plant growth by an auxin-producing isolate of the yeast *Williopsis saturnus* endophytic in maize (*Zea mays* L.) roots. Biol Fertil Soils. 2005;42:97–108.
23. Amprayn KO, Rose MT, Kecskés M, Pereg L, Nguyen HT, Kennedy IR. Plant growth promoting characteristics of soil yeast (*Candida tropicalis* HY) and its effectiveness for promoting rice growth. Applied Soil Ecology. 2012;61:295-299.
24. Xin G, Glawe D, Doty SL. Characterization of three endophytic, indole-3-acetic acid-producing yeasts occurring in Populus trees. Mycological Research. 2009;113(9):973-980.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=530&id=24&aid=4606