The Bioscan 8(2): 477-480, 2013
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

ISOLATION AND CHARACTERIZATION OF NITRIFYING BACTERIA BACILLUS ANTHRACIS MTCC NO. 2104 FROM LONGSTANDING CULTIVATION OF BT COTTON RIZHOSPHERE SOIL

P. SIVA RAAGINI*, K. VIJAYA LAKSHMI AND K. USHASRI
Department of Applied Microbiology, S.P.M.V.V. Tirupati - 517 502 A. P. INDIA
e-mail: sivaraagini@gmail.com

KEY WORDS
Bt cotton
Rhizosphere soil
Screening
Nitrifying activity
MPN method

Received on :
03.10.2012

Accepted on :
17.03.2013

***Corresponding author**

ABSTRACT
In the present study, an attempt has been made to isolate the Nitrifying bacteria from the total of eleven bacteria of rhizosphere soil of Bt cotton. Among the eleven isolates, six isolates were positive in primary screening for nitrifying activity. The cultures of these six isolates were secondary screened, by a micro titer system based on the most probable number (MPN) method with an increased accuracy. Finally, one isolated bacteria which shows maximum activity labeled as Bt rhizosphere soil (Btrs7) was selected and characterized. This is further conformed by IMTECH, Chandigarh and identified as *Bacillus anthracis* with MTCC no 2104.

INTRODUCTION

Cotton is an important fiber crop of global significance, which is cultivated in tropical and sub-tropical regions of more than seventy countries, in the entire world. In India cotton being cultivated over an area of about 9.5million hectares representing approximately one quarter of the global area of 35 million hectares under this crop (Khadi.B,M 2007/2008). Cotton plays vital roles as a raw material for nearly all food and other agricultural products, the clothing (including the primary minerals for synthetics), chemical and building industries.

Further, it depends to some degree on the products taken from the soil. In the long term, agriculture must be sustainable. Sustainability of primary production depends on the maintenance of soil fertility, The aim of this work is therefore to examine the impact of long standing cultivation of transgenic cotton varieties modified to express the Cry1Ac insecticidal toxin Bacillus thuringiensis (Bt) cotton on the nitrifying bacterial populations in rhizosphere soil, in which cotton was grown, in order to identify any impacts on the soil nitrifying bacteria. The rhizosphere is the zone of interaction between the soil, plant roots and microbiota. This microenvironment is characterized by increased biomass microbes and other microfauna, and is strongly influenced by the interface of the plant root system and its exudates (Bowen and Rower,1999)Soil organisms play a crucial role in soil health. Therefore, it is necessary to understand how different agricultural practices affect them. Bt crops may be problematic for long-term soil health, as they express proteins that are known to be toxic to a range of non-target organisms. An unknown number of species make up the soil food web and could be affected by Bt - yet tests have been conducted on very few, in very few soil types and ecosystems. If, under field conditions, the Bt deposited in the soil by these crops has an impact on soil organisms – bacteria, there will necessarily be downstream effects. If Bt crops kill or otherwise reduce the activity of any of these soil organisms, they will disturb the web of relationships necessary for carrying out essential ecosystem functions, such as decomposition and nutrient cycling.

Nitrifying bacteria play a important role in the dynamic cycling of reduced nitrogen throughout our global ecosystem. Nitrogen is most extensively distributed element in nature with atmosphere(78%) as the chief reservoir (Singh and Kashyap, 2007) However, nitrogen has traditionally been considered one of the most important plant nutrients 95-99% of the potentially available nitrogen in the soil is in organic forms either in plant or animal residues, which is not directly available to plants and can be converted to available forms by microorganisms (Sridhar, 2000). The nitrogen is the key element of the biogeochemical cycle that describes the transformation of nitrogen and nitrogen–containing compounds in nature. Amongst different N cycle processes vis-à-vis ecosystem functioning and environmental concerns, the process of nitrification stands out to be the most important process, This process was discovered by the Russian microbiologist, Sergei Winogradsky.Nitrification is the biological oxidation of ammonia with oxygen into nitrite

followed by the oxidation of these nitrites into nitrates (Focht and Verstraete, 1977).

In the present study owing to importance of rhizosphere bacteria and their affective role in nitrification, bacteria were isolated from Bt cotton rhizosphere soil and screened for nitrifying activity.

MATERIALS AND METHODS

Collection of Soil Sample

Rhizosphere soil was carefully separated from the roots with a narrow spatula, and sharp pointed knife. Only soil in close association with roots was used for analysis. Plants grown for longer than five weeks had extensive root systems and the adjacent soils were so closely associated with roots. Harvested rhizosphere soils were transferred to closed containers and stored at 8ºC overnight, pending the analyses to be undertaken the next day.

Rhizosphere soil samples from Bt cotton was serially diluted and were plated on Nutrient Agar (NA) and incubated at 37ºC for 24hr for the isolation of bacteria. Replicates were maintained for each dilution Single isolated colonies were sub cultured for the isolation of pure culture (Cappucino and Sherman, 2008).

Primary screening of rhizospheric bacteria for nitrification

A total of eleven bacterial strains were isolated from Bt cotton Rhizospheric soil. Primary screening was done individually for all the eleven isolates and determined for the production of nitrites and nitrates (Cappucino and Sherman2008).

1) Determination of Nitrite production

Ammonium sulfate broth was inoculated to all eleven isolates and was incubated for three weeks. Trommsdorf's reagent and sulfuric acid were used to test the presence of Nitrites (Cappucino and Sherman, 2008).

2) Determination of Nitrate production

Nitrite broth was inoculated to eight isolates which were positive in the nitrite production and incubated for three weeks. Diphenylamine reagent and sulfuric acids were used to test the presence of Nitrates (Cappucino and Sherman, 2008).

Secondary screening of rhizospheric bacteria for nitrification

Bacterial isolates which were positive in the primary screening were screened out for the best nitrifying bacteria by a micro technique based on the Most Probable Number (MPN) method, developed for the enumeration of ammonia and nitrite oxidizing bacteria by (Rowe *et al.*,1977). A blue colour reaction indicates that these end products have been formed and the well was scored as positive. The absence of a blue colour was scored as negative. The MPN values were calculated according to the table provided by (De Man,1975) and (Parnow, 1972).

Identification of bacterial isolate

Further identification of the bacterial isolate was carried out based on the morphological, cultural and biochemical characteristics according to the guidelines of Bergey's Manual of Systemic Bacteriology. The bacterial isolate from Bt was

Table 1: Morphological and biochemical tests for identification of bacterial isolate

Identification tests	Bacterial isolate Btrs7
Colony morphology	
Configuration	Round, Concentric, Cream, Wrinkled
Margins	Smooth
Surface	Butyraceous
Elevation	Slightly raised
Pigmentation	-
Opacity	Opaque
Gram's reaction	Positive
Cell shape	Rods
Size(µm)	2-4 µm in length, width 1.0 -1.2 µm
Spores	+
Motility	+
Physiological tests	
Growth at temperature	
4^0C	-
10^0C	±
30^0C	+
37^0C	+
40^0C	+
45^0C	+
50^0C	+
55^0C	-
Growth in NaCl (%)	
2	+
4	+
6	+
8	+
10	-
Growth at pH	
5	-
6	+
7	+
8	+
9	-
Growth under aerobic condition	+
Biochemical tests	
Indole test	-
Methyl red test	+
Voges proskauer test	-
Citrate utilization test	+
H_2S production	-
Gelatin hydrolysis	-
Urea hydrolysis	-
Starch hydrolysis	+
Lecithinase	+
Lipase (Tween 80 hydrolysis)	+
Catalase test	+
Oxidase test	+
Denitrification	-
Arginine dihydrolase	-
Phosphate solubilization	+
Chitinase	+
Casein hydrolysis	+
Degradation of Tyrosine	+
Nutritional characteristics	
Starch	+
Maltose	+
Glucose	+
Mannitol	-
Sucrose	-
Fructose	+

Table 1: Cont.....

Xylose	-
Rhamnose	-
Meso-inositol	-
Glycerol	+
Succinate	+
²-alanine	-
L-histidine	-
L-lucine	-
D-alanine	-
Antibiotic resistance	
Penicillin G	-
Ampicillin	-
Chloramphenicol	+
Erythromycin	+
Streptomycin	+
Tetracycline	-
Gentamycin	-
Tobramycin	+
Rifampicin	+
Polymyxin	+

identified as strains of the genus *Bacillus* based on the morphological and biochemical characteristics. Btrs7 was further submitted to IMTECH, Chandigarh for 16Sr* sequencing.

RESULTS AND DISCUSSION

The results were presented in the following Table and in Figs. 1-7.

DISCUSSION

A total of eleven bacterial isolates were isolated from the Bt cotton rhizosphere soil all the eleven isolates were subjected to primary screening to determine their ability to nitrification. The isolates were incubated in Ammonium sulphate and Nitrite broth. Out of the eleven, eight isolates had exhibited blue black colour in Ammonium sulphate broth upon the addition of Tromsdorff's reagent and six isolates had exhibited deep blue colour in Nitrite broth with the diphenylamine reagents, indicating the positive reactions. These six isolates which were positive in primary screening were further subjected to secondary screening to isolate the best nitrifier through microtitre plate method by (Rowe *et al.,* 1977). As the six isolates were inoculated in Ammonium Calcium Carbonate medium and incubated for three weeks. Blue colouration upon

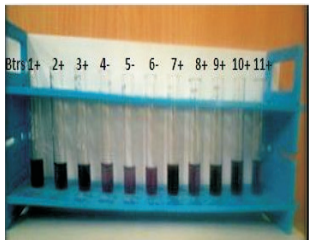

Figure 1:

Determination of nitrate production

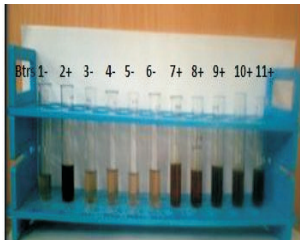

Figure 2:

Determination of nitrate production

Primary screening of nitrifyng bacteria

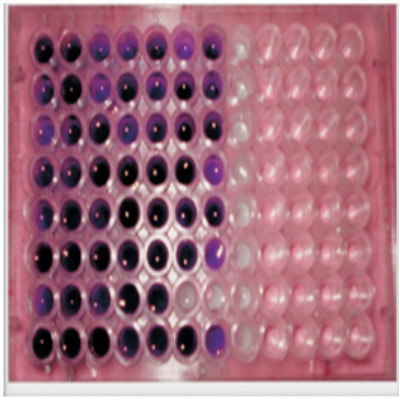

Figure 3:

Secondary screening of nitrifyng bacteria Microtiter Plate Method

the addition of diphenylamine reagent indicated that the end products were formed and the wells were scored as positive. The MPN values were calculated according to the Table provided by (De Man, 1975) and (Parnow, 1972). Among the six, one of the best nitrifier labeled as Btrs7 was selected for the morphological, physiological, Biochemical and molecular characterization. Morphological studies revealed that the isolate Btrs7 is aerobic endospore forming, non pigmented and wrinkled with concentric colonies. The growing cell was Gram positive, motile with rod shape. The isolate grew well in nutrient broth at pH range of 5.7 to 8.0 and showed salt tolerance at Nacl concentration up to 8% (w/v). Bacterial growth was observed in the temperature ranging from 10ºC - 50ºC with an optimum growth around 37ºC. The isolate is positive for the utilization of glucose, glycerol, Maltose, starch, fructose and succinate. The isolate shown positive results for Methyl

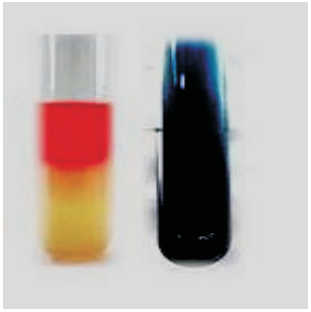

Figure 4: Methyl red, citrate

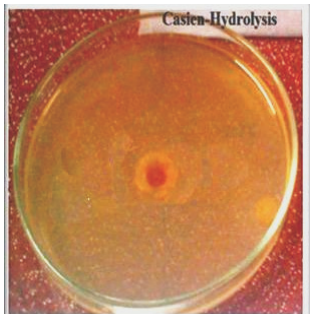

Figure 5: Casein hydrolysis

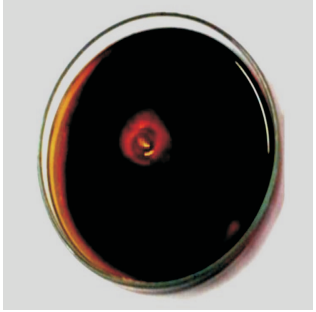

Figure 6: Starch hydrolysis

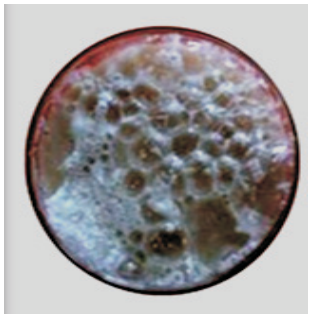

Figure 7: Catalase hydrolysis

red, Citrate utilization, Nitrate reduction, casein hydrolysis, starch hydrolysis, Degradation of, Lecithinase, oxidase, chitinase, Lipase phosphate solubilisation reactions and degradation of Tyrosine. Negative towards indole, urea production, vogose proskaer, gelatin liquification, H_2S production, utilization of arginine, xylose ,sucrose, Rhamnose, Meso-inosital, β-alanine, L-histidine, L-lucine. D-alanine and mannitol . Morphological, biochemical, physiological characteristics isolate was identified as genus *Bacillus* and Btrs7 was further molecular characterized by16S rna sequence analysis was done by IMTECH, Chandigarh identified and confirmed as *Bacillus anthracis* with MTCC NO. 2104. Based on the above characteristics *Bacillus anthracis* was able to oxidize ammonia and nitrite resulting in the nitrate formation there by showing the ability to nitrification.

REFERENCES

Bowen, G. D. and Rower, A. D. 1999. Microorganisms in Soils-Roles in Genesis and Functions - Scribd... to their participation in the soil biomass, microorganisms play a crucial role ... Conversely, soil organisms are known to ... York. pp. 641–669

Cappucino, J. G. and Sherman, N. 2008. Nitrification. In Microbiology a Laboratory Manual. International student edition. 7th edition. *Addison Wesely Publication*. pp. 343-344.

De Man, J. C. 1975. The probability of most probable numbers. *Euro J. Appl. Microbiol*. **1:** 67-78.

Focht, D. D. and Verstraete, W. 1977. Biochemical ecology of nitrification and Denitrification. *Advan. Microb. Ecol.* **1:** 135-214.

Khadi, B. M. 2007/2008. Trends in Cotton Area, Production and Productivity in India.

Parnow, R. J. 1972. Computer program estimates bacterial densities by means of the most probable numbers. *Food Technol*. **26:** 56-62.

Rowe, R., Todd, R. and Waide, J. 1977. Microtechnique for most probable number Analysis. *Appl. Environ. Microbiol.* **3:** 675-680.

Singh and Kashyap 2007. Variations in soil N-mineralization and nitrification in seasonally dry,tropical forest and savanna ecosystems in Vindhyan region, India *Tropical Ecology*. **48(1):** 27-35, 2007 ISSN 0564-3295.

Sridhar, R. 2000. Impact of pesticides on soil microorganisms. *Research periodical publishing House*. pp. 78-83.