International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Special Issue 1- ICMTBT | 2018 | 136-143
International Conference on Microbial Technology for Better Tomorrow ICMTBT
Held at D.Y. Patil College of Arts, Commerce and Science, Pimpri, Pune, 17-19 February
| Conference Proceedings | Research Article | Biological Sciences |Open Access |MCI Approved|
|UGC Approved Journal|

ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC BACTERIA FROM SUGARCANE (SACCHARUM spp.) GENOTYPES DEVELOPED IN INDIA AGAINST PATHOGENS

Elham Jafarzadeh[1*], Tulshiram Yeole[2] and Rutuja More[2]

[1]Ahvaz Jundishapur University of Medical Sciences, Khuzestan, Islamic Republic of Iran

[2]Department of Microbiology, Vasantdada Sugar Institute, Pune, Maharashtra, India

*Corresponding Author Email: elham_jafarzade@yahoo.com

ABSTRACT

This research work aims to isolate and characterize endophytic bacterial population from five sugarcane genotypes (Co 86032, CoC 671, VSI 434, CoVSI 9805 and CoM 0265) using cultivation-based approach. The diazotrophic endophytes were highest (51.24 X10^{6}cfu.mL^{-1}) in shoot of genotype Co86032 and were lowest (1. 63X106 cfu. mL^{-1}) in root of genotype CoVSI 9805. In this study, 83 putative endophytic bacteria were isolated, and six representatives were identified by 16S rRNA gene sequencing. The isolates belonged to six Genera namely Acetobacter, Azospirillum, Burkholderia, Herbaspirillum, Azoarcus and Agrobacterium. They were tested for antimicrobial activity against potent sugarcane pathogens such as Colletotrichum falcatum, Fusarium moniliforme, Fusarium sacchari, Helminthosporium sacchari and Ceratocystis paradoxa. The highest antimicrobial activity (clear zone of 18.5 mm) was observed in Acetobacter against Helminthosporium sacchari, regarding Azoarcus and Herbaspirillum highest antimicrobial activity (clear zone of 17.5 mm) were exhibited against Helminthosporium sacchari. However, Agrobacterium has shown highest antimicrobial activity (clear zone of 17.0 mm) against Helminthosporium sacchari, Burkholderia has shown highest antimicrobial activity (clear zone of 15.0 mm) against Helminthosporium sacchari. However, Azospirillum has shown highest antimicrobial activity (clear zone of 12.5 mm) against Ceratocystis paradoxa. It is concluded that all our endophytes except Azospirillum isolated in the present studies have shown maximum antimicrobial activity against Helminthosporium sacchari. The present study thus catalogues the cultivable diazotrophic endophytes which are associated with Indian sugarcane genotypes and displays the ability of these endophytes as a potent biocontrol agent. Thus, it can be concluded that all these endophytes have significant potential in sustainable growth of the Indian sugarcane genotypes.

KEY WORDS

Sugarcane, diazotrophic, endophytes, Acetobacter, Helminthosporium sacchari

Abbreviations:

Sh-shoot, Lf- leaf, Rt – root, BNF- Biological Nitrogen Fixation, Ag – Agrobacterium, Bu – Burkholderia, H-Herbaspirillium, Ac – Acetobacter, Az – Azospirillium, Ar – Azoarcus.

INTRODUCTION:

Microorganisms livings within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence are defined as endophytes (Hardoim *et al*., 2008; Ryan *et al*., 2008). Endophytic bacteria inhabit various tissues of seeds, roots, stems and leaves. Endophytes have received considerable attention in last 20 years due to their capacity to protect plant hosts

from several pathogens (Krishnamurthy and Gnanamanickam, 1997; Sturz and Matheson, 1996), insects (Azevedo *et al.,* 2000) and nematodes (Hallmann *et al*., 1998). It has recently been discovered that these bacteria can have beneficial effects on host plants, such as growth promotion and increased resistance against pathogens and parasiess (Hallman *et al*,1998; Elham *et al*., 2015). Though synthetic chemicals also have long been used as active agents in reducing the incidence of plants, and animals diseases, these are costly and may have potentially harmful effect on the environment and induce pathogen resistance. Therefore, biological control or the use of endophytic microorganisms or their secretions to prevent diseases offer an attractive alternative to disease management without the negative impact of chemical control. Endophytic microbes' antagonism has been demonstrated for a variety of plant pathogens such as *Alternaria, Rhizoctonia, Fusarium, Colletotrichum* and *Sclerotium*spp (Yuan and Crawford, 1995; Aghighi *et al.,* 2004, Elham *et al*., 2015). In the present study 83 bacterial endophytic strains were isolated, all of them showed antagonistic activity against *Helminthosporium sachhari.*

It has recently been recognized that endophytic bacteria play an important role in resistance to disease and that signals exist to mediate cross talk between the endophyte and its host. Now endophytic bacteria have garnered a good attention because of their intimate and no detrimental association with plants that results in production of variety of antimicrobial compounds. They have unique genetic and biological systems that may have applications outside the host plant in which they normally reside.

Sugarcane (*Saccharum officinarum*) is the main source of sugar in India and holds a prominent position as cash crop which occupies more than 5.06 million hectares of area in tropical and subtropical belt of nation (Ref). In Maharashtra state of India in 2008 to 2009 area under sugarcane cultivation has risen to 0.937 million hectares (Cooperative Sugar 2014). Even though, area under sugarcane has been increased, however, the crop productivity did not show increasing trend mainly because of decline in soil fertility status and use of chemical fertilizers and pesticides. In 1980 to 81 sugarcane yield was 92.3 t ha^{-1} but in 2012 to 13 it has come down to 66.4 t ha^{-1} (Cooperative sugar 2014). In spite of increasing efforts of the scientists towards the improved sugarcane production technology, in the last few years, sugarcane production and yield figures are showing the declining trends. Therefore, in order to improve soil fertility and sugarcane crop productivity integrated nutrient management is the best remedy.

In sugarcane and other monocotyledonous crops endophtyic bacteria like *Acetobacter, Herbaspirillum, Agrobacterium, Burkholderia, Azoarcus* and *Azospirillum* play a major role in symbiotic Biological nitrogen fixation process as well as in the production of plant growth promoting and regulating (PGPR) hormones and antimicrobial substances (Bandara *et al*., 2006; Elham *et al*., 2015). The aim of the present study was to isolate "Endophytic Microflora" of sugarcane (Saccharum sp.) and to study their potential role in enhancing Productivity and Resistance to Phytopathogens which would contribute to plant growth, disease resistance, and crop productivity.

Therefore, in the present investigation, an attempt has been made to review and accumulate the data on endophytic bacteria isolated from roots, shoots and leaves from five different Indian sugarcane genotypes and to test the metabolite they produce with antimicrobial activity against sugarcane pathogens.

MATERIAL AND METHODS:

1. Sampling

Five different sugarcane genotypes (Co 86032, Co 671, VSI 434, CoVSI 9805 and Co 0265) cultivated in the agricultural research fields of Vasantdada Sugar Institute, Pune, India (Latitude:18°31' N, 73°55' E) were selected for the present study. A total of 30 samples (root, leaf and shoot) from 8 to 10 months old plants of different sugarcane genotypes were collected in month of October. Healthy, disease free plants were selected for sampling. The samples were brought to the laboratory and stored in sterile plastic bags and kept on ice until further analysis.

2. Isolation and enumeration of endophytes

The isolation of endophytes were carried out as per procedure given by Collins and Lyne (1984) with minor modifications. In brief, the roots, leaves and stems were washed several times with sterile distilled water and the surface was disinfected with 1% (w/v) mercuric chloride followed by treatment of 70 % (v/v) ethanol. The samples were dried with sterile paper towel and cut *ca*. 3-5 cm

and 10 gm of samples were macerated in 10 ml of sterile distilled water. From the sap obtained one ml was serially diluted to 10^{-6} and 0.1 ml of these dilutions were plated in triplicate on potato dextrose agar (Döbereiner *et al.*, 1995) and several other selective media such as LGI media, Azoarcus media, Agrobacterium media, Burkholderia media, Herbaspirillum media and Azospirillum media. The plates were incubated at 30°C for 5 days and enumeration of colony forming units was performed using colony counter (Medica Instrument MFG. Co, Mumbai) for each media and dilution. The cultures were purified on respective semisolid media and preserved at 4°C for further identification. Total 83 isolates from roots, stems and leaves of sugarcane representing different types of colonies developed on agar were selected for further characterization.

3. Identification of endophytes

Identification of the putative endophytic bacteria was based on Bergey's Manual of Systematic Bacteriology (Bernner, *et.al*., 2005) and biochemical and physiological characterization. Molecular identification employing 16S rRNA gene sequencing was carried out for some representative isolates. The biochemical test performed included hydrolysis of starch, nitrate reduction, catalase test, production of hydrogen sulphide (H_2S), digestion of casein, liquefaction of gelatin, sugar fermentation and utilization of different carbon sources as per the protocols previously described (Konde and Moniz, 1967; S.A.B. 1957). The physiological characterization included observation of colony characters, cell morphology, gram character, motility, pigment production optimum temperature (20 -50 °c), pH (2-8), NaCl concentration (0.1-20%w/v) and sucrose concentration (10- 40%w/v) for growth.

Antimicrobial activity of endophytes against pathogens by well method for inhibition zone:

Pathogens causing sugarcane diseases

Following pathogens causing sugarcane diseases were obtained from Agriculture Pathology Section of VSI.

Name of diseases	Causative pathogens
Red rot disease	*Colletotrichum falcatum*
Pokkah boeng disease	*Fusarium moniliforme*
Wilt disease	***Fusarium sacchari***
Eye spot disease	*Helminthosporium sacchari*
Pineapple disease	*Ceratocystis paradoxa*

Above pathogens were spread on their respective nutrient agar and potato dextrose agar media. Wells of constant diameter (3mm) were made in each of these plates using sterile cork borer in the plate containing pathogen. 0.1 ml of different exotoxin of endophytes (Intact endophytes) was inoculated with sterile syringe into the wells (number of cells $1x10^8$/ml). Plates were incubated for 24-48 hr at 30°C and were observed for zone of inhibition. Results were recorded and interpreted according to Murray *et al.,* 1995; and Dubey and Maheshwari 2012.

RESULTS AND DISCUSSION:

In the present study, five sugarcane genotypes (Co 86032, CoC 671, VSI 434, CoVSI 9805 and CoM 0265) developed at Vasantdada Sugar Institute (VSI), Pune, India were explored for plant growth promoting endophytic bacterial population. Maximum total viable count ($51.24x10^6$) was obtained from the extracted juice of shoots of Co86032 and minimum bacterial count ($1.65x10^6$) was obtained from roots of CoVSI 9805 (Fig.1). It was also seen that the bacterial count was maximum in shoot followed by leaf and root of these varieties (Fig. 1).

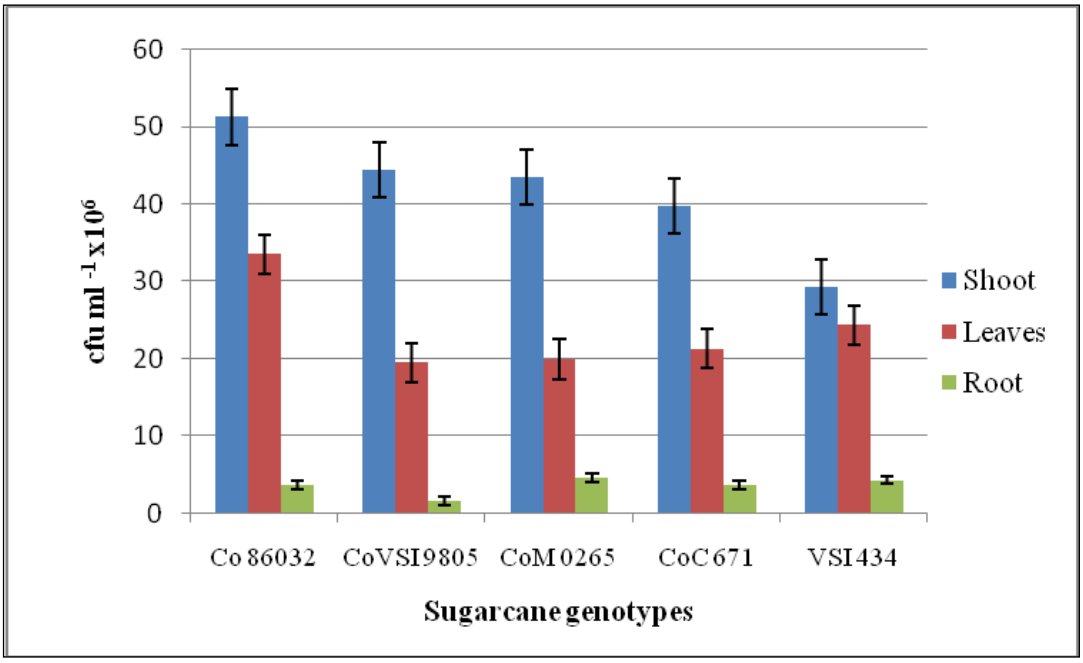

Fig. 1: Average of total cultivable endophytic bacterial population from shoots, leaves and roots of sugarcane genotypes.

A total of 83 isolates from shoots, leaves and roots of different sugarcane genotypes representing different types of colonies developed on agar were randomly chosen, analyzed by Gram staining and oxidase reaction. The potato dextrose agar medium which is reported to support diverse bacteria (Dobereiner *et al*., 1995) was used to recover any bacterial endophytes present in plant along with other selective media. All the isolates those were gram negative rods were plated on MacConkey medium (Miller, 1959) to identify candidates belonging to *Enterobacteriaceae*. The isolates were further characterized based on physiological and biochemical analysis (Table 1) up to genus level as per Bergey's Manual of Systematic Bacteriology (Bernner *et al*., 2005). In the present study it was revealed that 15 isolates each from *Acetobacter, Azospirillum, Herbaspirillum, Azoarcus, Agrobacterium* and eight isolates from *Burkholderia* were associated with the parts of sugarcane genotypes. On the basis of their morphological and biochemical characterisation these isolates were grouped within the same genus as given in Table1. The 15 isolates of *Agrobacterium* were divided into 4 groups, 8 isolates of *Burkholderia* in 3 groups, 15 isolates of *Herbaspirillum* into 2 groups, 15 isolates of Acetobacter into 4 groups, 15 isolates of *Azospirillum* into 2 groups and 15 isolates of *Azoarcus* into 3 groups. Out of 83 isolates, 30 isolates were obtained from shoots, 28 from leaves and 25 from roots. Enumeration of cultivable bacterial endophytes from five genotypes of the sugarcane revealed that endophytic bacterial communities from extracted juice were highest in shoot of genotype Co 86032 followed by genotype CoVSI 9805, CoM0265, CoC 671 and VSI 434 (Fig.1). Enumeration studies demonstrated variation in the loads of endophytes into five genotypes. The results of Biochemical and physiological characteristics of the isolates are given in Table 1.

Table1: Biochemical and physiological characteristics of the isolates.

Sr No	Genus	Total Isola--tes	Group--ed in same Genus	Colony Morpholo--gy	Gram character	Growth in sucrose concentr--ation (w/v)%	Optim um pH	Special character
1.	*Azospirillum*	15	2	Metallic sheen, Silver bright	Gram - ve rods, Circular/ Regular	5-10	6-6.5	Turns BTB indicator into dark blue colr
2.	*Azoarcus*	15	3	Creamy white/Whitish brown	Gram-ve rods, Circular/Irregular	5-15	6-6.5	Brown pigmentation
3.	*Glucanoacetobacter*	15	4	Round reddish orange	Gram -ve short rods, Circular	15	4	Growth on PDA with brownish colonies
4.	*Herbaspirillum*	15	2	Whitish	Gram-ve vibroid shape, circular	5-10%	6-6.5	Chalky white colony
5.	*Burkholderia*	8	3	Pinpoint brown	Gram-ve rods, Circular/Irregular	5-10%	6-7	Tiny brownish colony
6.	*Agrobacterium*	15	4	Watery/ white	Gram-ve rods, Circular/Irregular	5-15%	6.5	Bulky watery colonies

The representative isolates from each genus those showed maximum plant growth traits were selected for molecular identification by partial 16S rRNA gene sequencing and on analysis of the sequences by nBLAST. It was confirmed that they belong to these classified genera. The phylogenetic analysis as represented in Figure 2 depicts the closest match for each selected isolate. The strain Ag6274 was closest to *Agrobacterium* spp. 2382 (D14500); strain Bu6558 showed closest match with *Burkholderia cenocepacia* J2315 (AM74772); strain Az6274 was closest to *Azospirillum* spp. (AF112477) and strain Ar6274 found its closest match as *Azoarcus indigens* strain HZ5 (AF011345) by 99 percent homology, whereas, strain Ac6274 was similar to *Acetobacter pasteurianus,* strain M100 (BACG01000075) and strain H6558 was found to be similar to *Herbaspirillum* spp. 6415T-45 *(*DQ490985) with 100 percent homology.

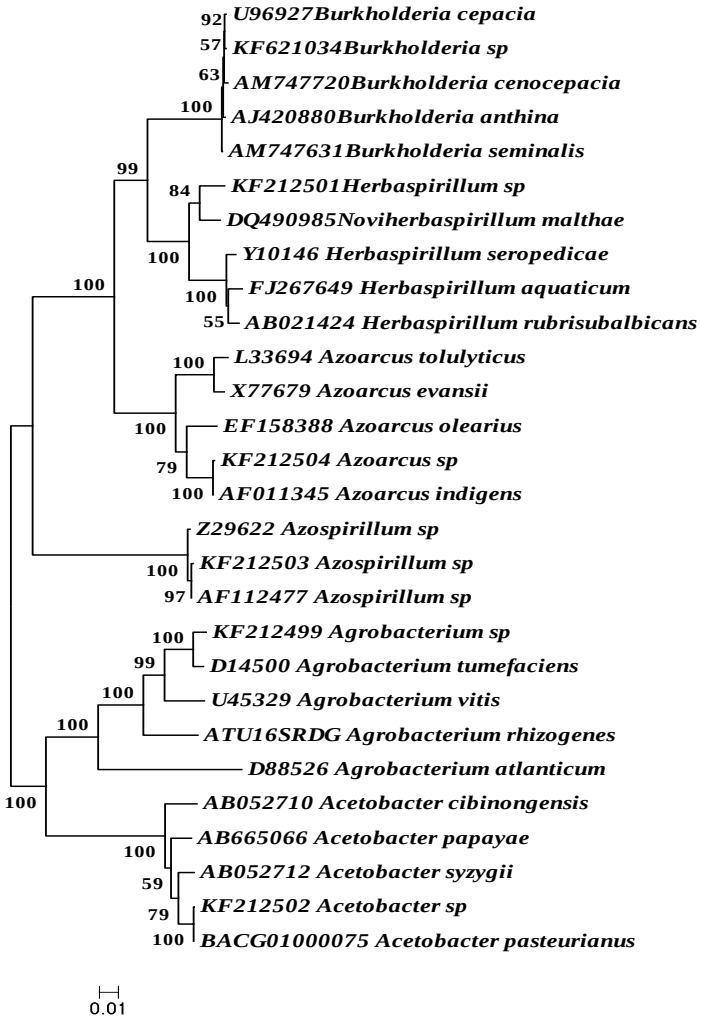

Fig.2: Phylogenetic analysis using 16S rRNA gene sequences of the representative endophytes from Indian sugarcane along with the closest respective matches obtained from NCBI GenBank databases conducted by Neighbor-Joining method. The optimal tree with the sum of branch length = 0.77368659 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. There were a total of 7347 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 software

Results of Antimicrobial activity of the isolates:

All the isolates were tested for plant growth promoting traits such as antimicrobial activity. In plate assay all 83 isolates were found capable of forming a clear zone on solid media against sugarcane pathogens such as *Colletotrichum falcatum, Fusarium moniliforme, Fusarium sacchari, Helminthosporium sacchari* and *Ceratocystis paradoxa.* These results showed that all the isolates have antimicrobial ability against above pathogens. The results of the antimicrobial activity of the strains of *Azospirillum, Azoarcus, Acetobacter, Herbaspirillum, Burkholderia* and *Agrobacterium* are shown in following Figures from 3a to 3f. The results of 15 isolates of *Azospirillum* against sugarcane pathogens are shown in Fig 3a, which indicated that all the isolates exhibited the antagonistic activity against all the pathogens tested. However, isolate Az2b showed maximum antimicrobial activity i. e. clear zone of 13.0 mm towards *Ceratocystis paradoxa*. In case of *Azoarcus* the results of 15 isolates of *Azoarcus against* sugarcane pathogens are shown in Fig 3b. The results showed that all the isolates exhibited the antagonistic activity against all the pathogens tested. Isolate Ar5b showed maximum antimicrobial activity i. e. clear zone of 17.5 mm towards *Helminthosporium sacchari*. The results of 15 isolates of *Acetobacter* against sugarcane pathogens are shown in

Fig 3c. The results showed that all the isolates exhibited the antagonistic activity against all the pathogens tested. Out of 15 isolates of *Acetobacter* isolate Ac1b showed maximum antimicrobial activity i. e. clear zone of 18.5 mm towards *Helminthosporium sacchari*. The results of 15 isolates of *Herbaspirillum* against five sugarcane pathogens are shown in Fig 3d. The results showed that all the isolates exhibited the antagonistic activity against all the pathogens tested. Out of 15 isolates tested isolate H1a showed maximum antimicrobial activity i. e. clear zone of 17.5 mm towards *Helminthosporium sacchari*. The results of 8 isolates of *Burkholderia against* five sugarcane pathogens are shown in Fig 3e. The results showed that all the isolates exhibited the antagonistic activity against all pathogens tested. Out of 8 isolates of *Burkholderia* isolate B5a showed maximum antimicrobial activity clear zone of 15mm towards *Helminthosporium sacchari.* The results of 15 isolates of Agrobacterium against five pathogens are shown in Fig 3f. The results showed that all the isolates exhibited the antagonistic activity against the above target pathogens. Out of 15 isolates of *Agrobacterium*, isolates Ag6a and Ag1c showed maximum antimicrobial activity i. e. clear zone of 17.0 mm towards *Helminthosporium sacchari*.

The results of antimicrobial activity of all the isolates tested, showed that most of the endophytes have potent antimicrobial activity against the major sugarcane pathogens. The results have shown highest antimicrobial activity (clear zone 18.5 mm) in *Acetobacter* against *Helminthosporium sacchari*, regarding *Azoarcus* and *Herbaspirillum* highest antimicrobial activity (clear zone 17.5 mm.) were produced against *Helminthosporium sacchari* However, *Agrobacterium* has showed highest antimicrobial activity (clear zone 17.0 mm) against *Helminthosporium sacchari*, *Burkholderia* has showed highest antimicrobial activity (clear zone) 15.0 mm against *Helminthosporium sacchari. Azospirillum* has showed highest antimicrobial activity (clear zone 12.5 mm) against *Ceratocystis paradoxa.* Therefore, it could be said that all endophytes except *Azospirillum* isolated in the present studies have shown maximum antimicrobial activity against *Helminthosporium sacchari* (Fig 3).

Our study suggests that sugarcane is a potent source of bacterial endophytes with wide biological activity against pathogenic fungi.

Even though bacteria can produce a wide range of secondary metabolites, the biological function of most of these is not fully understood. It is assumed that they are involved in a number of processes including cell-cell signalling, inter-species signalling, a possible carbon release valve and that these compounds can also promote plant growth and act as microbial inhibiting agents (Wheatley, 2002; Vesperman *et al*., 2007; Kai *et al*., 2009). Our study suggests that endophytes play an important role in enhancing yields probably through improved nutrient uptake, growth hormone production and disease control. Increased environmental awareness has prompted the development of biological alternatives to chemical crop protection agents. Unfortunately, most of these biocontrol agents have not fulfilled their initial promise, their failure usually being attributed to poor rhizosphere competence and the difficulties associated with the instability of bacterial biocontrol agents in long term culture (Schroth *et al*., 1984; Weller, 1988). However, intimate relationship between endophytic bacteria and their hosts make them natural candidates for selection as biocontrol agents (Chen *et al*., 1995; Van Buren *et al*., 1993). It was found that 61 of 192 endophytic bacteria strains recovered from potato stem tissues were effective biocontrol agents against *Clavibacter michiganensis* subsp. *Sepedonicus* (Van Buren *et al*. 1993).

Communities of endophytic bacteria within a population have been shown to act as agents of biological control. Chen et al. (1995) showed that of 170 endophytic bacterial strains isolated from the internal tissues of cotton, 40 possessed biological control activities against *Rhizoctonia solani* in cotton and 25 induced systemic resistances to *Colletotrichum orbiculare* in cucumber. The mechanism by which endophytes can act as biocontrol agents include the production of antifungal or antibacterial agents (Lambert *et al*., 1987; Leyns *et al*.,1990; Maurhofer *et al*., 1992), siderophore production (Kloepper *et al*., 1980; Duijff *et al*., 1993), nutrient competition (Lockwood, 1990) niche exclusion (Cook & Baker, 1983) and indirectly through the induction of systemic acquired host resistance or immunity (Chen *et al*., 1995; Tuzun and Kloepper, 1994). Therefore, further studies are necessary to assess the ability of the isolates to confer protection against plant pathogens and their role in enhancing growth and yield of plants under field conditions.

CONCLUSION:

1. Among all the 83 isolates, Acetobacter strain, Ac6274 has shown maximum antimicrobial activity (18.5 mm) against *Helminthosporium sacchari* compared to rest of the isolates.
2. Endophytic bacteria are manufacturer of plethora of bioactive antimicrobial metabolites.
3. Therefore, a rich pool of bacterial species is yet to be discovered and investigated over the coming years for Antimicrobial activity**.**

Expected Outcome:

1. Present Investigation based on lab and pot culture studies are foundation for application of endophytic bacteria having antimicrobial activity as bioinoculants for reducing RDN.
2. After confirmation of the efficiency at field scale on large area they could be used as bio-agro products for saving 100% RDN.
3. Thus, mass production of these endophytic bacteria as bioinoculants and their mass application to sugarcane crop will be a milestone**.**

Need for more Research:

1. Cross inoculation of isolated efficient endophytes of sugarcane to different cereal-crops and horticularal crops for BNP.
2. Studies on PGPR hormone production and antimicrobial properties of endophytic Nitrogen fixing bacteria and its applications for sugarcane and other crops (especially horticulture crops) is essential in field of agriculture with respect to crop productivity and Nitrogen economy.

REFERENCES

Note: Please send References as per journal format.

***Corresponding Author:**
Elham Jafarzadeh*
Email: **elham_jafarzade@yahoo.com**