International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 3 | JUL-SEPT | 2018 | 611-618
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

IN VITRO ANTAGONISM OF RESIDENT RHIZOBACTERIA, *BACILLUS AMYLOLIQUEFACIENS* SUBSP. *AMYLOLIQUEFACIENS* AGAINST THE BACTERIAL BLIGHT PATHOGEN OF BT COTTON

Raut LS[1] and Hamde VS[2]

[1]Department of Microiology, Sant Tukaram College of Arts and Science, Parbhani 431401 (M.S.) India
[2]Department of Microiology, Yogeshwari Mahavidyalaya, Ambajogai-431517 (M.S.) India

*Corresponding Author Email: lasraut76@gmail.com

ABSTRACT

Bacterial blight is one of the most important yield-reducing diseases of Bt cotton caused by Xanthomonas axonopodis pv. malvacearum. The present work was undertaken to search for efficient biocontrol agents from resident microflora of soil as an alternative for chemical management. 114 resident rhizobacterial isolates were screened in vitro antagonistic activity against bacterial blight pathogen using cross plate technique. Among the tested rhizobacterial isolates, six isolates showed antagonistic activity. The highest antagonism was exhibited by rhizobacterial isolate RLS19. Based on the inhibitory activity, the rhizobacterial isolate RLS19 was selected for further investigation. The rhizobacterial isolate RLS19 was later identified as Bacillus amyloliquefaciens subsp. amyloliquefaciens by 16S rRNA sequence analysis. To study antagonistic mechanism, the rhizobacterial isolate was evaluated for production of non-volatile metabolites and volatile metabolites. B. amyloliquefaciens subsp. amyloliquefaciens was able to produce non-volatile diffusible metabolites and showed 32 mm zone of inhibition around the well. Also, the GC-MS analysis of cell free culture filtrate reveals the presence of three volatile metabolites i.e. Pyrrolo[1,2-a] pyrimidine-2,6-dione, hexahydro- , 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- and Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-. Based on these results Bacillus amyloliquefaciens subsp. amyloliquefaciens can be served as good microbial control candidate for control of bacterial blight pathogen of Bt cotton.

KEY WORDS

Bacterial blight, Bt cotton, Xanthomonas axonopodis pv. malvacearum, Bacillus amyloliquefaciens subsp. amyloliquefaciens, non – volatile and volatile.

INTRODUCTION

Bacterial blight of cotton is one of the most important yield-reducing diseases caused by *Xanthomonas axonopodis* pv. *malvacearum* [1]. The bacterial blight disease occurs to all cotton-growing areas throughout the world [2, 3] almost every year and was shown to be a serious limiting factor of fiber production. In India, the disease is known to occur all the cotton growing areas with annual losses up 32.2 % [4], 45 % [5] 53.67 % [6] depending on the cultivar, stage of crop infection and environmental condition. In Maharashtra state, the entire cotton growing districts are affected by the bacterial blight of cotton.

For the management of bacterial blight disease, the different methods are in practice which includes the destruction of infected crop residue, crop rotation, use of resistant verities and use of different synthetic agrochemicals. In India, the farmers are attracted towards chemical treatment due to easy availability of synthetic chemicals in the market and not the severity

of the disease. The arbitrary use of synthetic chemical leads to the killing of non-target insects, killing of the beneficial microbial flora of soil, increasing farming risk, increases production costs, lowering the yield and ultimately decreases in fertility of the soil and creates environmental hazardous issues.

The microbiological control using resident rhizospheric antagonistic microorganisms will be a potential alternative approach for the management of plant diseases. There are some evidences, that *Trichoderma harzianum* [7,8,9] *Pseudomonas fluorescens* [8,9,10,11,12] and *Bacillus subtilis* [9,11,13,14] were isolated from cotton rhizosphere soil and tested individually for their effectiveness in controlling cotton diseases, including bacterial blight of cotton. Seed treatment with these bioagents enhanced seed germination, limited growth of *Xanthomonas axonopodis* pv. *malvacearum*, and induced systemic resistance in plants [9]. The present investigation has been made to focus on interactions of rhizobacterial isolates with bacterial bight pathogen for searching efficient microbial biocontrol agents, following objectives were set i) screening of resident rhizospheric bacteria against bacterial blight pathogen of cotton ii) finding the biocontrol mechanism of efficient antagonistic rhizobacteria.

MATERIAL AND METHODS

Bacterial blight pathogen

The bacterial blight pathogen of cotton used here was taken from the earlier research work conducted in the department of microbiology, Sant Tukaram College of Arts and Science, Parbhani.

Isolation of resident rhizospheric bacteria

114 resident rhizobacterial isolates were isolated from healthy Bt cotton rhizospheric soil by dilution plate technique. These isolates were labeled as RLS1 to RLS114.

Screening for the antagonistic activity against bacterial blight pathogen:

Screening for the antagonistic potential, 114 resident rhizobacterial isolates were screened against bacterial blight pathogen by cross plate technique [15]. The actively growing antagonistic rhizobacterial isolates were streaked in a narrow band across the centre of the plates containing Nutrient agar and incubated to allow the growth. After 24 h incubation, *Xanthomonas axonopodis* pv. *malvacearum* was then streaked from the edges of the plates without touching the central growth and then incubated for 48 h. The distance over which the growth of *Xanthomonas axonopodis* pv. *malvacearum* has been inhibited by antagonistic rhizobacterial isolates were noted.

Identification of antagonistic resident rhizobacterial isolate:

An effective antagonistic resident rhizospheric bacterial isolate obtained through screening against *Xanthomonas axonopodis* pv. *malvacearum*, was identified with the help of 16S rRNA sequencing at Agharkar Research Institute (ARI) Pune, Maharashtra.

In vitro antagonistic mechanism of microbial control agent:

To characterize the mechanism of microbial control agent, the efficient rhizospheric isolate was tested to produce non – volatile diffusible metabolites and volatile metabolites.

Detection of non – volatile antibacterial diffusible metabolites:

Antibacterial diffusible metabolites produced by efficient rhizobacterial isolate, *B. amyloliquefaciens* subsp. *amyloliquefaciens* against bacterial blight pathogen, *X. axonopodis* pv. *malvacearum* was studied by slightly modified Agar well Diffusion Method [16] on Muller Hinton agar plates. Antimicrobial activity (mm) was expressed as the difference between the diameter of inhibition zone and diameter of agar well [17].

Detection of volatile metabolites by GC-MS

Detection of volatile metabolites was carried out by using Gas Chromatography-Mass Spectrometry (GC-MS) analysis of Cell Free Cultural filtrate performed commercially in IIT Bombay Powai, Mumbai. The volatile metabolites were identified by comparison of their mass spectral data with those from the library of National Institute of Standards and Technology (NIST) 2007.

RESULTS AND DISCUSSION:

Screening for the antagonistic activity against bacterial blight pathogen

All the 114 resident rhizobacterial isolates were screened against the bacterial blight pathogen of Bt cotton by cross plate technique. Among these, six isolates (RLS19, RLS58, RLS76, RLS96, RLS102 and RLS114) showed considerable antibacterial activity (Table 1). The pathogen was unable to grow around a narrow band of antagonistic bacterial isolate at the

centre. The growth of pathogen was inhibited which was clearly seen in the Nutrient agar plate (Fig.1). The highest antibacterial activity was shown by rhizospheric isolate RLS19. Based on the inhibitory activity the efficient rhizobacterial isolate RLS19 was selected for further investigation.

Fig.1: Screening of antimicrobial activity of rhizobacterial isolates against *X. axonopodis* pv. *malvacearum*

Table 1: Screening of antagonistic activity of rhizospheric isolates against *Xanthomonas axonopodis* pv. *malavacerum*

Rhizospheric isolate	Antagonistic activity
RLS19	+++
RLS58	++
RLS76	++
RLS96	+
RLS102	+
RLS114	+

+++ = Efficient; ++ = Satisfactory; + = Average; - =Negative

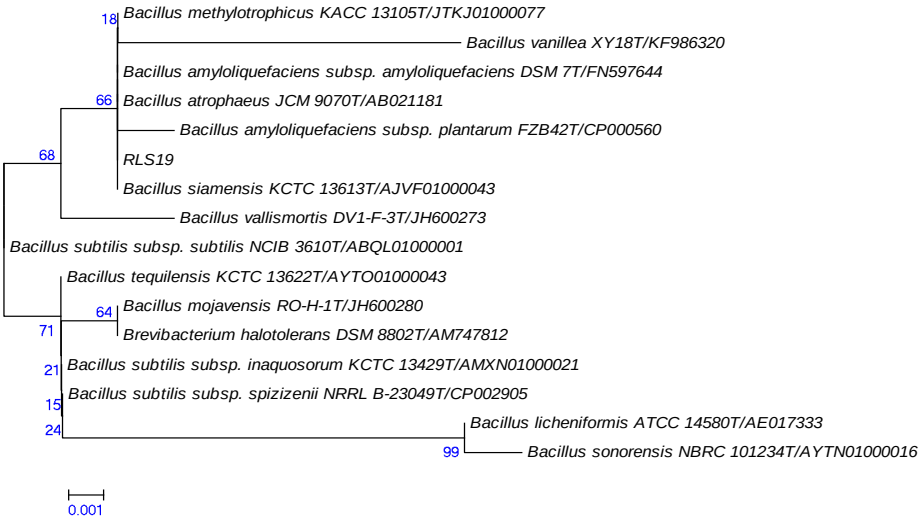
Table 2: Biochemical compounds identified in cell free culture filtrate of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*

Retention time	Compound	Formula	Mol. Wt. g/mol	Biological activity	Reference
16.73	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	Anti-microbial Algicidal	[22]
25.07	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	$C_{12}H_{22}N_2O_2$	226	Anti-fungal	[23]
25.42	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl) -2-butynyl]-	$C_{11}H_{18}N_2O_2$	210	Anti-microbial Anti-inflammatory	[24]

Identification of antagonistic resident rhizobacterial isolate:

The resident Rhizobacterial isolate RLS19 showing excellent antibacterial potential in opposition to *X. axonopodis* pv. *malvacearum* was identified as *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* by 16S rRNA sequencing with GenBank accession number DSM7T/FN597644 (Fig.2).

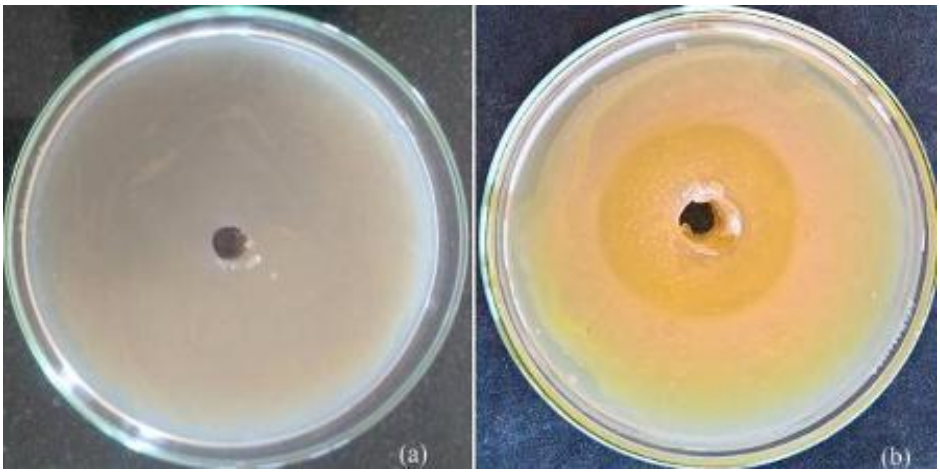
Fig.2: Phylogenetic tree of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* DSM7T/FN597644



Detection of non – volatile antibacterial diffusible metabolites

The antibacterial diffusible metabolite production was tested using Agar well diffusion method. The inoculated plates after 48 h showed a clear zone of inhibition around the well compared to control. The zone of inhibition produced by *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* by secreting non – volatile antibacterial diffusible around well was measured 32 mm in diameter (Fig.3).

Fig.3: Production of antibacterial diffusible metabolite by *B. amyloliquefaciens* subsp. *amyloliquefaciens* against bacterial blight pathogen (a) Control (b) Test



The different researchers have been reported that rhizobacterial isolates able to produce non-volatile low-molecular-weight diffusible antibiotics that suppress the growth of plant pathogens [18]. It has been reported that *Bacillus subtilis* and *Pseudomonas aeruginosa* inhibited the growth of bacterial blight pathogen by 22 mm and 15 mm respectively [19]. *In vitro* antibacterial activity of seven strains of *Pseudomonas fluorescence* against bacterial blight pathogen of cotton was tested. Among these, *Pseudomonas fluorescens* strain MMP and Pf1 were most effective showing 10 mm zone of inhibition [20]. The antagonistic potential of twenty-one rhizobacterial isolates belonging to *Pseudomonas* and *Bacillus* sp. were tested against *Xanthomonas axonopodis* pv. *malvacearum.* Among these, *Pseudomonas fluorescens* strain Pf32, Pf93 and *Bacillus subtilis* B49 were found effective in inhibiting the bacterial blight pathogen in 26.7, 25.4 and 6.1 mm respectively [11]. Similarly, fourteen strains of *Paenibacillus* were evaluated for antagonistic activity against *Xanthomonas axonopodis* pv. *malvacearum*. Among 14 isolates, *Paenibacillus* strain 4 and strain 8

had inhibitory effects and was measured 11 mm and 8.5 mm respectively against the phytopathogen [21].

In Present study *B. amyloliquefaciens* subsp. *amyloliquefaciens* showed 32 mm zone of inhibition which was greater than the isolates tested by previous rsearchers [11, 19, 20, 21].

Detection of volatile metabolites by GC-MS

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of cell free cultural filtrate of *B. amyloliquefaciens* subsp. *amyloliquefaciens* was performed to detect the novel compounds and secondary metabolites responsible for the antibacterial activity. The cell free cultural filtrate showed 6 peaks in the GC-MS chromatogram (Fig.4). Based on the abundance of peaks, three peaks of chromatogram were compared to mass spectral data from NIST (2007) library. The GC-MS spectral data compared with library search successfully enabled the identification of three compounds namely Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-, 2, 5-Piperazinedione, 3,6-bis(2-methylpropyl)- and Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]- (Fig.5, Fig.6, Fig.7). The active principles with their retention time (RT), molecular formula, molecular weight (MW), and biological activity are presented in Table 2.

Fig.4: GC-MS Chromatogram of cell free culture filtrate of *B. amyloliquefaciens* subsp. *amyloliquefaciens*

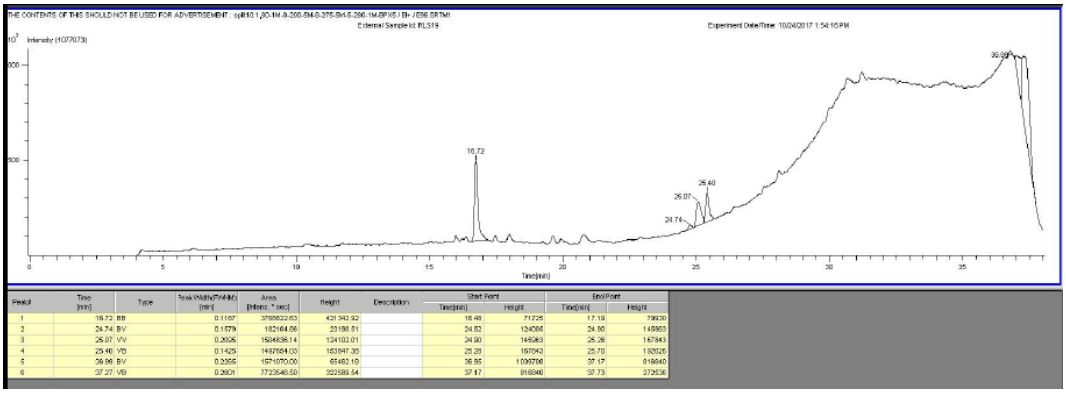


Fig.5 : Mass spectrum of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- with Retention Time (RT) = 16.73

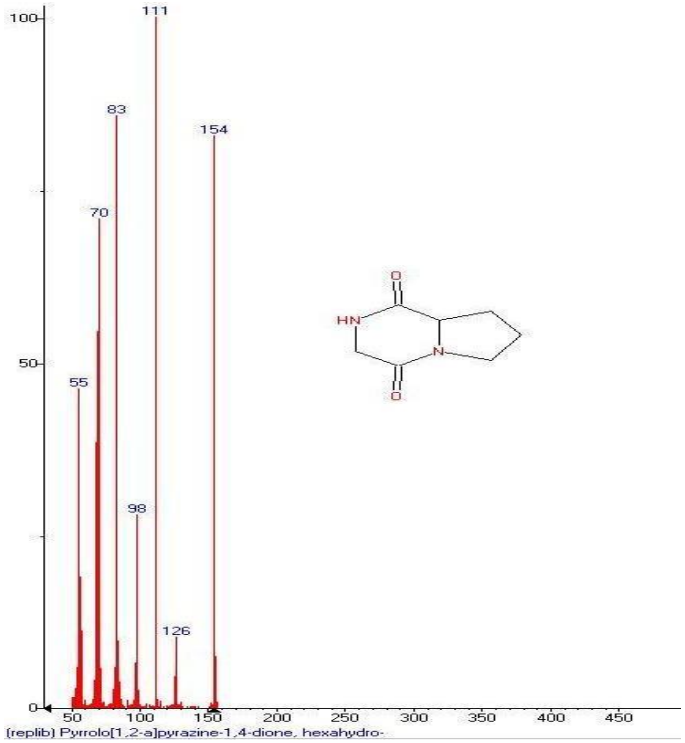


Fig.6 Mass spectrum of 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- with Retention Time (RT)= 25.07

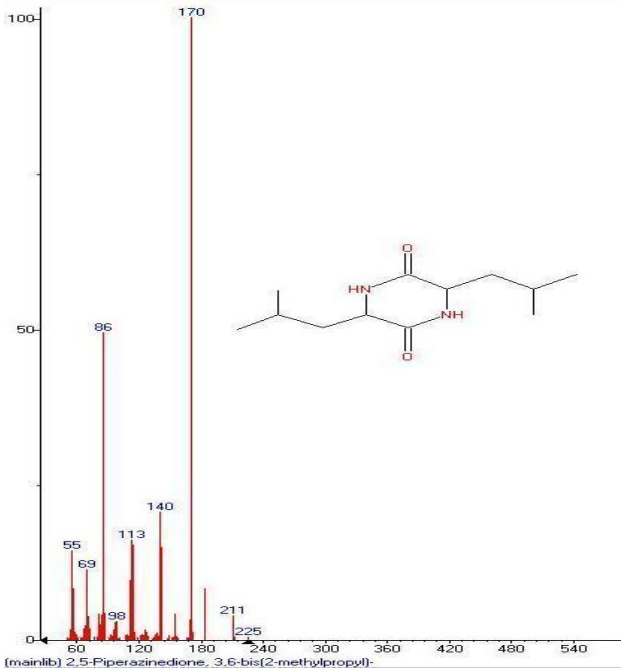
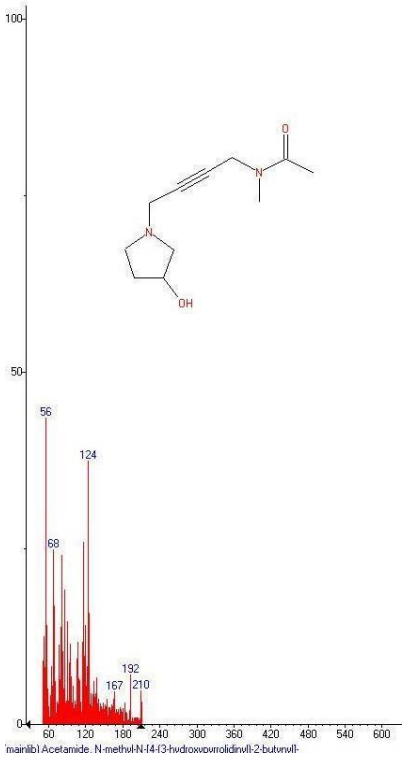


Fig.7: Mass spectrum of Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]- with Retention Time (RT) = 25.42



Some microorganisms have ability to produce low molecular weight volatile metabolites which are responsible for inhibition of bacterial growth. The bioactive compound 3,6-bis(2-methylpropyl)-2,5-piperazinedione produced by *Lactobacillus plantarum* AF1 showed the antifungal activity against *Aspergillus flavus* ATCC 22546 [25]. Characterization of the crude secondary metabolites of *Streptomyces sp*. indicated

the presence of chemical compounds that were present in GC-MS chromatogram *of B. amyloliquefaciens* subsp. *amyloliquefaciens* i.e. Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro, and derivative of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- i.e. Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) but not 2, 5-Piperazinedione, Cyclohexanecarboxylic acid, property with different levels of activity against Gram positive and Gram negative pathogens [26]. In another kind of analysis where the ethyl acetate extract of *Pseudomonas plecoglossicida* by analyzed using GC- MS and reported the presence of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- ; 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- with antimicrobial activity [27]. To find the metabolite and mechanistic basis of antifungal property exhibited by endophytic *Bacillus amyloliquefaciens* BmB 1, the crude extract analyzed for the presence of volatile compounds by GC-MS analysis and confirmed the presence of, benzene acetic acid, Pyrrolo [1, 2- a]pyrazine-1, 4-dione hexahydro-, Pyrrolo[1, 2-a]pyrazine-1, 4-dione hexahydro-3-(2-methylpropyl), octadecanoic acid, 2, 5-piperazinedione, 3-benzyl-6-isopropyl, Pyrrolo[1, 2- a] pyrazine-1, 4-dione hexahydro-3-(phenylmethyl), diisooctyl phthalate [28]. Similarly, the crude extract of the *Bacillus* sp.WG4 analysed by GC-MS reveals the presence of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro - derivative like Pyrrolo [1,2- a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) as a basis of antifungal activity against *Pythium myriotylum* [29].

All these reports indicate that the volatile compounds, Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-, 2, 5-Piperazinedione, 3, 6-bis(2-methylpropyl)- and Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]- as produced by B. amyloliquefaciens subsp. amyloliquefaciens and confirmed by GC-MS, displayed importance in the inhibition of the bacterial blight pathogen of Bt cotton, *X. axonopodis* pv. *malvacearum.*

CONCLUSIONS

The uses of *Bacillus* sp for the microbiological control agent have different benefits over other bacterial species, for instance, effortless cultivation, the capacity of sporulation and long shelf life. The rhizobacterial isolate *B. amyloliquefaciens* subsp. *amyloliquefaciens* showed strong antibacterial activity against X. *axonopodis* pv. *malvacearum.* Also, the rhizobacterial isolate able to produce diffusible non-volatile and volatile metabolites. The GCMS analysis of crude extract reveals the presence of Pyrrolo pyrazine compound and their derivatives. The inhibitory activity observed is the cumulative effect of diffusible metabolites, volatile metabolites and enzymes secreted by *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens.* These results indicate that the isolate can serve as an effective microbial control agent for controlling the bacterial blight disease of cotton. Further production of siderophores, cell wall degrading enzymes and study the microbial control ability *in vivo* are needed to be investigating for preparation of commercial microbial control agent.

ACKNOWLEDGMENTS

We acknowledge the Principal, Yogeshwari Mahavidyalaya, Ambajogai Dist. Beed (M.S.) India for providing us the good laboratory facilities during this research work.

REFERENCES

[1] Vauterin L., Hoste B., Kersters K., Swings J. Reclassification of X*anthomonas*. Int. J. Syst. Evolut. Microbiol, 45: 472-489, (1995)

[2] Hillocks RJ. Fusarium wilt. CAB Int., Wallingford, UK. 1992.

[3] Hussain T., Tahir M. Chemical control of bacterial blight of cotton. Pak. J. Phytopathol, 5: 119-121, (1993)

[4] Venkateswarlu D., Srinivas T., Rao NH. Epidemiological studies on bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum*. J. Cotton Res. Dev, 20: 270-272, (2006)

[5] Ingole OV., Patil BR., Raut BT., Wandhare M.R. Epidemiological studies on bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum*. J. Cotton Res. Dev, 22: 107-109, (2008)

[6] Raut SA., Ingole OV., Kolhe AV. Influence of sowing dates on incidence and intensity of bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum*. J. Plant Dis. Sci, 5: 110-112, (2010)

[7] Karkachi NE., Gharbi S., Henni MKJE. Biological control of *Fusarium oxysporum* f. sp. *lycopersici* isolated from Algerian tomato by *Pseudomonas fluorescens*, *Bacillus cereus*, *Serratia marcescens* and *Trichoderma harzianum*. Res. J. Agron, 4: 31-34, (2010)

[8] Jagtap GP., Jangam AM., Dey U. Management of bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum*. Scientific J. Microbiol, 1: 10-18, (2012)

[9] Raghavendra VB., Siddalingaiah L., Sugunachar NK., Nayak C., Ramachandrappa NS. Induction of systemic

resistance by biocontrol agents against bacterial blight of cotton caused by *Xanthomonas campestris* pv. *malvacearum*. Int. J. Phytopathol, 2: 59-69, (2013)

[10] Chattannavar SN., Hosagoudar GN., Ashtaputre S.A. Chemical and biological management of major foliar diseases of cotton. Karnataka J. Agric. Sci, 23: 599-601, (2010)

[11] Salaheddin K., Valluvaparidasan V., Ladhalakshmi D., Velazhahan R. Management of bacterial blight of cotton using mixture of *Pseudomonas fluorescens* and *Bacillus subtilis*. Plant Prot. Sci, 46: 41-50, (2010)

[12] Singh D., Singh N., Gil, JS., Singh P. Management of bacterial blight and leaf spot diseases of upland cotton using *Pseudomonas fluorescens*. J. Cotton Res. Dev, 26: 99-100, (2012)

[13] Medeiros FH., Souza RM., Medeiros FC., Zhang H., Wheeler T., Payton P., Ferro HM., Paré PW. Transcriptional profiling in cotton associated with *Bacillus subtilis* (UFLA285) induced biotic-stress tolerance. Plant and soil, 347: 327-337, (2011)

[14] Medeiros FHVD., Souza RMD., Ferro HM., Zanotto E., Machado JDC. Screening of endospore-forming bacteria for cotton seed treatment against bacterial blight and damping-off. Adv. Plants Agric. Res, 2: 56-61, (2015)

[15] Deshmukh MB., Sridhar KR. Distribution and antimicrobial activity of actinomycetes of a freshwater coastal stream. Asian J. Microbiol. Biotechnol. Environ. Sci, 4: 335-340, (2002)

[16] Perez C., Pauli M., Bazerque P. An antibiotic assay by the agar well diffusion method. Acta. Biol. Med. Exp, 15: 113-115, (1990)

[17] Monteiro L., Mariano RDLR., Souto-Maior AM. Antagonism of *Bacillus* spp. against *Xanthomonas campestris* pv. *campestris*. Brazilian Arch. Biol. Technol, 48: 23-29, (2005)

[18] Beneduzi A., Ambrosini A., Passaglia LM. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. Gen. Mol. Biol, 35: 1044-1051, (2012)

[19] Rashid A., Khan MA. Evaluation of antagonistic organisms against *Xanthomonas campestris* pv. *malvacearum in vitro* and the inoculated cotton plant for the control of bacterial bight disease. Pak. J. Agric. Sci, 37: 135-139, (2000)

[20] Eddin KS., Marimuthu T., Ladhalakshmi D., Velazhahan R. Biological control of bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* with *Pseudomonas fluorescens*. Arch. Phytopathol. Plant. Prot, 40: 291-300, (2007)

[21] Osman TM., Algam, SA., Ali ME., Osman EH., Mahdi AA. *In vitro* screening of some biocontrol agents against *Xanthomonas axonopodis* pv. *malvacearum* isolated from infected cotton plants. Int. J. Agric. For. Plant, 2: 270-278, **(**2016)

[22] Shrivastava A., Gupta MK., Singhal PK., Shrivastava P. Extracellular release of non-peptide group compounds by antifungal *Bacillus* and *Brevibacillus* Strains. Curr. Bioact. Comp, 13: 259-267, (2017)

[23] Altaee N., Kadhim MJ., Hameed IH. Detection of volatile compounds produced by *Pseudomonas aeruginosa* isolated from UTI patients by gas chromatography-mass spectrometry. Int. J. Curr. Pharm. Rev. Res, 7: 462-470, (2017)

[24] Kadhim MJ., Mohammed GJ., Hussein H. Analysis of bioactive metabolites from Candida albicans using (GC-MS) and evaluation of antibacterial activity. Int. J. Pharma. Clinic. Res, 8: 655-670, (2016)

[25] Yang EJ., Chang HC. Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. Int. J. Food Microbiol, 139: 56-63, (2010)

[26] Karanja EN., Boga HI., Wamunyokoli F., Muigai AW., Nonoh JO., Kinyua J. Growth characteristics and production of secondary metabolites from selected novel *Streptomyces* species isolated from selected Kenyan national parks. In *Proceedings of the 5th JKUAT Scientific, Technological and Industrialization Conference* (Juja: Jomo Kenyatta Univ. Agric. Technol, 51–80, (2017)

[27] Anandbabu R. *Isolation, identification and screening of alkaliphilic bacteria from mangrove sediment and alkaline industry-contaminated soil for antibacterial property and chromium removal efficiency* (Doctoral dissertation). Pondicherry University, India., (2015)

[28] Jimtha JC., Jishma P., Arathy GB., Anisha C., Radhakrishnan EK. Identification of plant growth promoting Rhizosphere *Bacillus* sp. WG4 antagonistic to *Pythium myriotylum* and its enhanced antifungal effect in association with *Trichoderma*. J. soil sci. plant Nutr, 16: 578-590, (2016)

[29] Jasim B., Benny R., Sabu R., Mathew J., Radhakrishnan EK. Metabolite and mechanistic basis of antifungal property exhibited by endophytic *Bacillus amyloliquefaciens* BmB 1. Appl. Biochem. Biotechnol, 179: 830-845, (2016)

Received:04.05.18, Accepted: 07.06.18, Published:01.07.2018

***Corresponding Author:**
Raut LS*
Email: **lasraut76@gmail.com**