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Detection of salicylic acid and jasmonic acid from extracts using HPLC and HPTLC

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Abstract
Plant immunity mainly depends on two plant antagonistic hormones, jasmonic acid JA and salicyic acid SA. Both these hormones control defense responses to different types of microbes. When ever a plant is infected by a pathogen naturally, it triggers SAR by elevating the levels of SA. Plant growth promoting rhizobacteria (PGPR) can suppress the disease in plant by triggering induced systemic resistance (ISR).ISR requires Jasmonic acid (JA) and Ethylene (ET) for accumulation of defense related enzymes and defense related substances against pathogen. Interestingly, simultaneous activation of both the JA/ethylene-dependent ISR pathway and the SA-dependent SAR pathway results in an enhanced level of disease protection. In this present context emphasis on production of salicylic acid, and induction of jasmonate by *Bacillus* that promotes plant growth is carried out on groundnut plants. Easy detection of these compounds is made possible by HPLC, and HPTLC techniques is been proved in the present experimental analysis. Detection of salicylic acid extracted from the bacterial culture and Jasmonic acid from bacterially treated leaves, using effortless, cost effective, HPLC and HPTLC respectively confers great advantage over traditional techniques.

Keywords: salicylic acid, jasmonate, systemic acquired resistance, induced systemic resistance

Introduction
Plant pathogens whenever infect the plant, triggers systemic acquired resistance (SAR) by producing salicylic acid. While most of the PGPR induce resistance, by triggering induced systemic resistance (ISR) in plants. It is noteworthy to understand that there exists a antagonistic crosstalk between both the metabolites of SAR and ISR. At this juncture, it is very interesting to know that, microbes are able to induce the predominant biomolecules like, salicylic acid, jasmonate in plants, if associated with them, whenever they are infected. Also, one of the study insists the exogenous application of salicylic acid for reducing biotic stress. However, it is significant that both SAR and ISR get initiated to procure good results in the field. Farmers are exogenously applying SA, and JA to the crop field for increasing yield these days, unaware of PGPR formulations that can trigger SA and JA induction in plants.

Method
Isolation Soil from the rhizospheric regions of the groundnut plant were collected and is serially diluted from 10^{-1} to 10^{-9}. These serially diluted soil samples were transferred on to plates containing sterile nutrient agar media, later is allowed to incubation for 24-48 hrs.

Screening & Identification
All the microbes obtained were screened for their plant growth promoting abilities. These include nitrogen fixation test, phosphate solubilization assay, siderophore production assay, ammonia production assay and so on. The screened potent isolates that are capable to promote plant growth were identified on the basis of 16 s RNA sequencing, biochemical characterization, and molecular characterization.

Efficacy of the biocontrol agents
The efficacy of the potent PGP Plant growth promoters, against various pathogens that cause damage to crop was tested *in vitro* and also using pot trails.

Determining the impact of biocontrol agent using scanning electron microscope
To understand the accurate effect of the bio-control agent over the pathogen, the physical structure of the pathogen, when exposed to antagonists, can be noticed using scanning electron microscope (JOEL -JSM 5600) according to the method described by John J. Bozolla 1998. Briefly, both the sub-cultured fungal pathogen (control) that was incubated at 30^{o}C for 1~3 days, and test fungal specimens, was fixed in 2.5 percent glutaraldehyde in 0.1M phosphate buffer with pH 7.2 for 24 hrs at 4^{o} C and post fixed 2 percent in aqueous osmium tetroxide for 4 hrs. Dehydrated in series of graded alcohols and dried to critical point drying with CPD (EMS 850) unit. The processed samples were mounted over the stubs with double sided carbon conductivity tape, and a thin layer of gold coat over the samples were done by using an automated sputter coater (Model - JEOL JFC - 1600) for 3 minutes as per the standard procedures.

Detection of salicylic acid production
The efficiency of isolates to produce SA was tested in casamino acids medium with different concentrations of casamino acids (0.1 - 0.8 %) at different pH (3 - 10), temperature (20 - 55 ° C) and in static and shaken conditions. The broth cultures were incubated for 4 days and cells were separated by centrifugation at 8000 rpm for 10 min. All the harvesting procedures were carried out in dim light with samples maintained in covered ice baths (Visca 1993) [14].

Thin layer chromatographic for salicylic acid

The extracted SA from supernatant of above centrifugation process was dried in a flash evaporator, solubilized in a small amount of methanol, and spotted on silica gel plates that had been pre-coated. The plates were then developed in a solvent system containing 9.5:5:2.5 (v/v) ratios of chloroform, acetic acid, and ethanol. The plates were immediately examined under UV light (256 nm) for blue fluorescence emission. Rf value is calculated and is compared with Rf value corresponding to that of the standard SA.

Salicylic acid determination by HPLC

The salicylic acid SA was extracted from the acidified culture supernatant of both the cultures. The filtrate was adjusted to pH 2 with 1M HCl and extracted twice with double volume of ethyl acetate. Sample is now ready for HPLC. For further quantification, to 1 ml of the extraction, 2 ml of 2M $FeCl_3$ and 1 ml of distilled water were added. (Meyer etal., 1992). The salicylic acid reacts with 2 M $FeCl_3$ to form a purple iron + SA complex in the aqueous phase with a maximum absorbance at 527 nm. The absorbance was recorded to determine SA concentration using spectrophotometer.

High performance liquid chromatography

The HPLC system consisted of following chromatographic conditions

HPLC: Azilent 1200 with EZ chrome software and PDA detector.

Mobile Phase: Acetonitrile: water (80:20v/v)

Column: C18 (waters)

Injection volume: 20 µl

Flow rate: 1ml/min

Detection wavelength: 234 nm

Retention times were determined using the detector. and chromatograms of standard and samples were compared and further calculated to quantify the concentration of salicylic acid in the samples provided.

Pot assay for bio-control activity against *Sclerotium rolfsii*

A pot assay was set up using groundnut seeds. Mass multiplication of *S. rolfsii* was carried out in potato dextrose broth at room temperature for 3 weeks (Ordentlich *et al.*, 1988) and then mycelia produced were used for the preparation of sick pots. This experiment was carried out in three sets of pots each i.e triplicates. All pots were first disinfected with 5 % $CuSO_4$ solution. First set (Positive Control) of three pots, was filled with 150 gm sterilized soil, sand (1:1) mixture with sclerotia of *S. rolfsii* artificially inoculated at 1 sclerotia/gm soil (Yaqub and Shahzad, 2005). Second set (Negative Control) of three pots, contains 150 gm of sterilized soil sand mixture. Third set (Test) of three pots, was filled with 150 gm of sterilized soil, sand mixture artificially inoculated with sclerotia of *S. rolfsii* at rate of 1 sclerotia/gm of soil. All the pots containing inoculum were incubated for 15 days at room temperature, frequently stirred and watered for colonization of fungus in the soil and then seeds treated with biocontrol agent's for 24 hrs was sown in third set pots, and untreated seeds in second set of pots. All these pots were kept at room temperature and watered regularly. Symptoms of the disease caused by the pathogens were noted. Leaves of the treated plants and untreated plant leaves of negative control (taken as control) were collected at different stages of plant growth and were prepared for analysis using HPTLC for detection of jasmonate.

Detection of jasmonic acid in treated leaves using HPTLC

The wound response of plants has been intensively investigated in many plant species by many researchers. Plant leaves were wounded with saw-toothed forceps over the midrib of the leaf and harvested 30 minutes or 120 minutes post-wounding, respectively in such cases. According to Homayon Ahmad Panahi* *et al*., (2010) [6] the method is as follows

Plant material Leaves was collected from treated plants and untreated plants from pot trial experiment. It was dried at room temperature or dried in an oven below 40ºC. The dried leaves were pulverized and sifted through a sieve of mesh size 500 lm.

Extraction

1. 5 g powder (dried leaves) was weighed and added to 50 percent methanol (75 ml).
2. Ultrasonication is carried out to the above solution for 30 min,
3. Solution was filtered and evaporated to dryness using evaporator.
4. This dry powdered methanol extract (1 g) was dissolved in 70 percent acetone–water (7 ml) and heated below 30-40ºC along with ultrasonication for 30 min.
5. Followed by step 3 again and kept aside.

Standard Vs Samples

Table 1

Track	Vial ID	Description	Volume	Type
1	Jasmonate	Standard	1.0 µl	Reference
2	Jasmonate	Standard	2.0 µl	Reference
3	Jasmonate	Standard	3.0 µl	Reference
4	Jasmonate	Standard	4.0 µl	Reference
5	Jasmonate	Standard	5.0 µl	Reference
6	Jasmonate	Standard	6.0 µl	Reference
7	Jasmonate	Standard	7.0 µl	Reference
8	SAMPLE A	Stage 1	2.0 µl	Sample
9	SAMPLE B	Stage 2	2.0 µl	Sample
10	SAMPLE C	Stage 3	2.0 µl	Sample
11	SAMPLE X	Stage 1	2.0 µl	Sample
12	SAMPLE Y	Stage 2	2.0 µl	Sample
13	SAMPLE Z	Stage 3	2.0 µl	Sample

Jasmonate (100 percent) is considered as reference for preparing standard solution. Seven jasmonate standard solution was prepared in methanol with different concentrations mentioned in the above table. Sample A, B, and C are different stages of untreated plant leaves (control). Sample X, Y, and Z are different stages of treated plant leaves using the bacterial consortium.

Chromatographic conditions

Equipment used is from Anchrom CAMAG, a semiautomatic HPTLC unit.

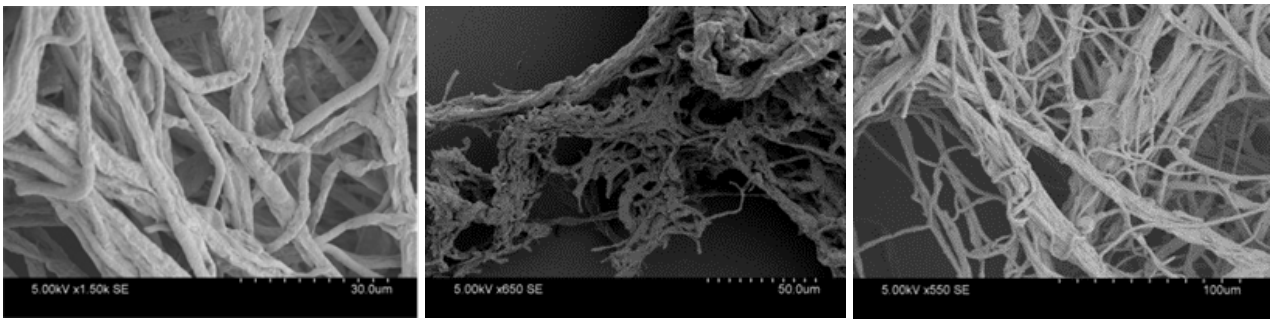
TLC chambers was washed thoroughly using methanol and are saturated for 20- 25 min with the solvent system. Applicator of the unit is automatic, and is controlled by the CPU as per the instructions given by the user. The following are the TLC plate, applicator, development chamber's and scanner conditions used in the experiment.

Table 2

Plate layout:	
Stationary phase	Merck, TLC plates silica gel 60 F 254
Plate format	200 x 100.0 mm
Application type	User
Application	Position Y: 8.0 mm, length: 8.0 mm, width: 0.0 mm
Track	First position X: 25 mm, distance: 12.4 mm
Solvent front position	70.0 mm
Application 1 - Linomat 5 (S/N: 250817):	
Sample solvent type	Methanol
Dosage speed	150 nl/s
Pre dosage volume	0.20 ul
Instrument diagnostics	Valid diagnostics
Development Chamber	
Tank	TTC 20x10
Mobile phase	Chloroform: Ethyl actate (6:4 v/v)
Saturation time	20 min
Use saturation pad	true
Volume front through	10 ml
Volume rear through	20 ml
Drying time	5 min
Drying temperature	Room temperature
Spectrum Scan developed plate 1d - Scanner 4 (S/N: 250413):	
Scanner type	Spectrum
Optimization for	Resolution
Measurement mode	Absorption
Detector mode	Automatic
Spectrum speed	100 nm/s
Data resolution	1 nm
Slit	6 x 0.45 mm, micro
Lamp	Deuterium
Wavelength range	190 nm to 450 nm
Reference spectrum	Per track, Y=10.0 mm

Results

Scanning electron micrograph of *Sclerotium* in control Vs *Sclerotium* exposed to bacterial antagonists. Mycelial body when exposed to the bacterial antagonist is found to be physically shrunk.



1 2 3

1. *Sclerotium* in control
2. *Sclerotium* Vs *Bacillus Zhangzouensis*
3. *Sclerotium* Vs *Bacillus velexensis*

4 5 6 7

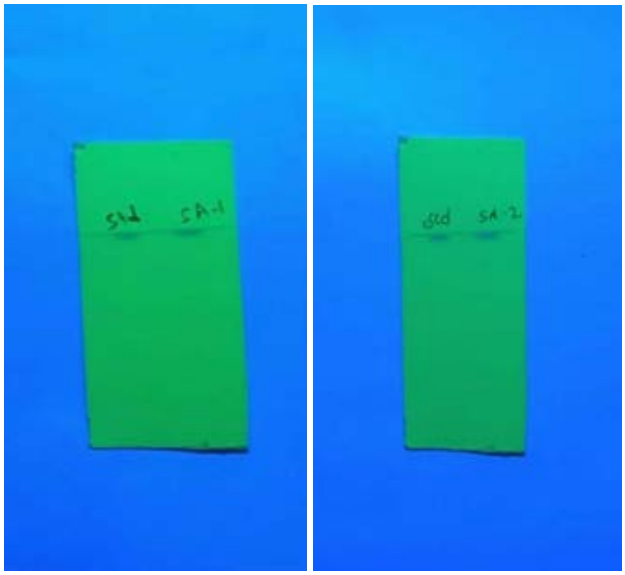
Pot trails: Symptoms exhibited by the plants in the control (Pathogen alone)

4. Sick pot showing sclerotial bodies
5. Chlorosis on leaves
6. Partially withered leaves
7. Withered leaves
8. Control Vs *Bacillus zhangzouensis and Bacillus velezensis*
9. Healthy, mature, flowering treated plants grown using bacterial consortium.

8

9

TLC

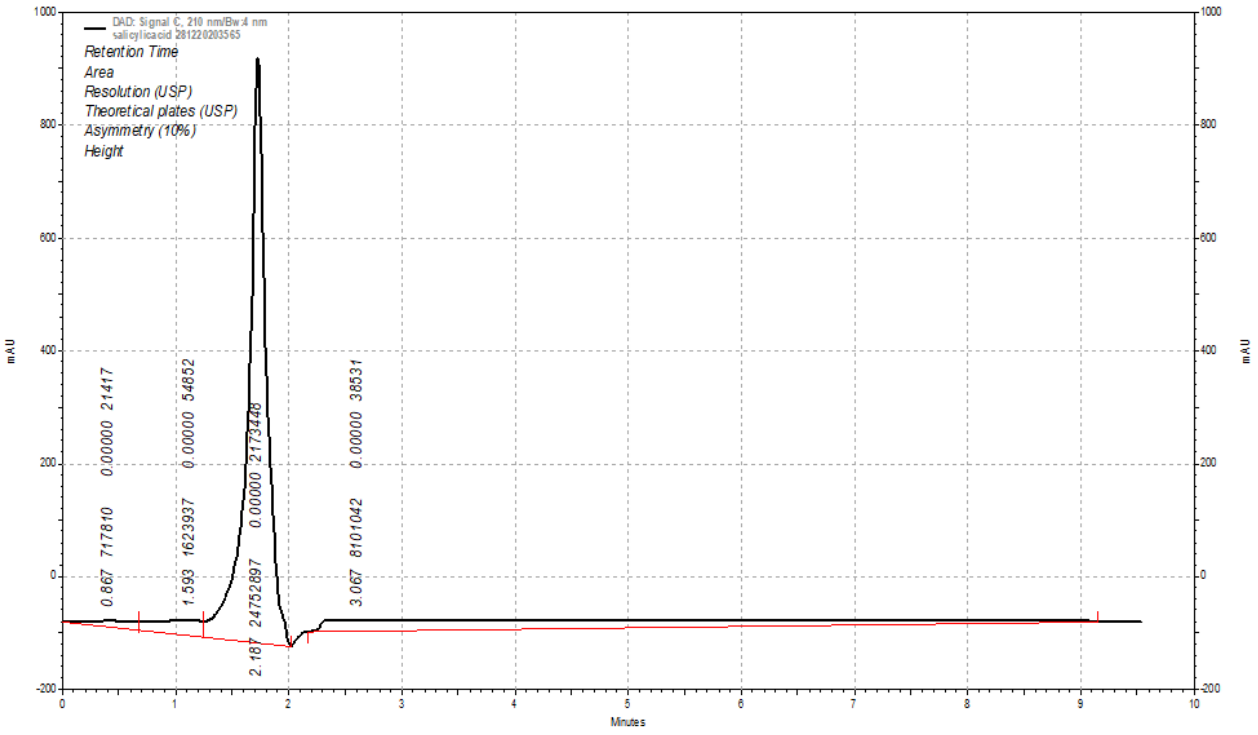


A B

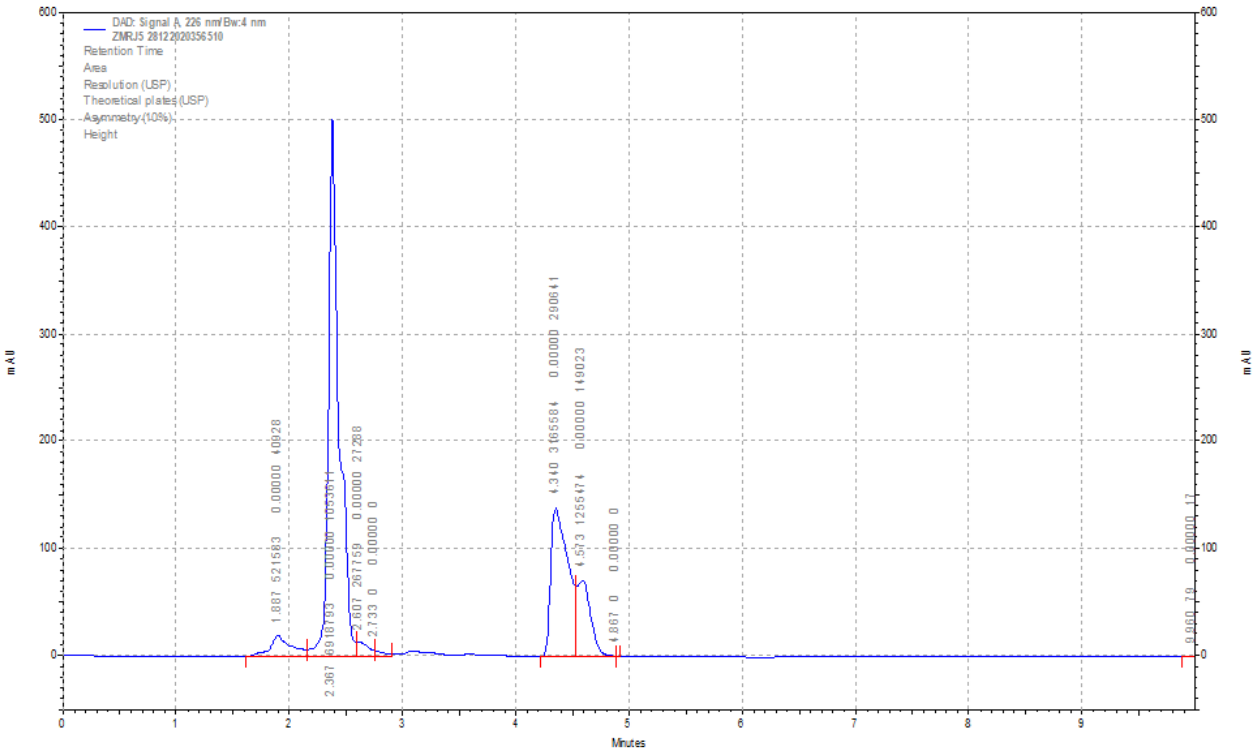
$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

$$Rf, A = \frac{2.95}{4} = 0.7$$

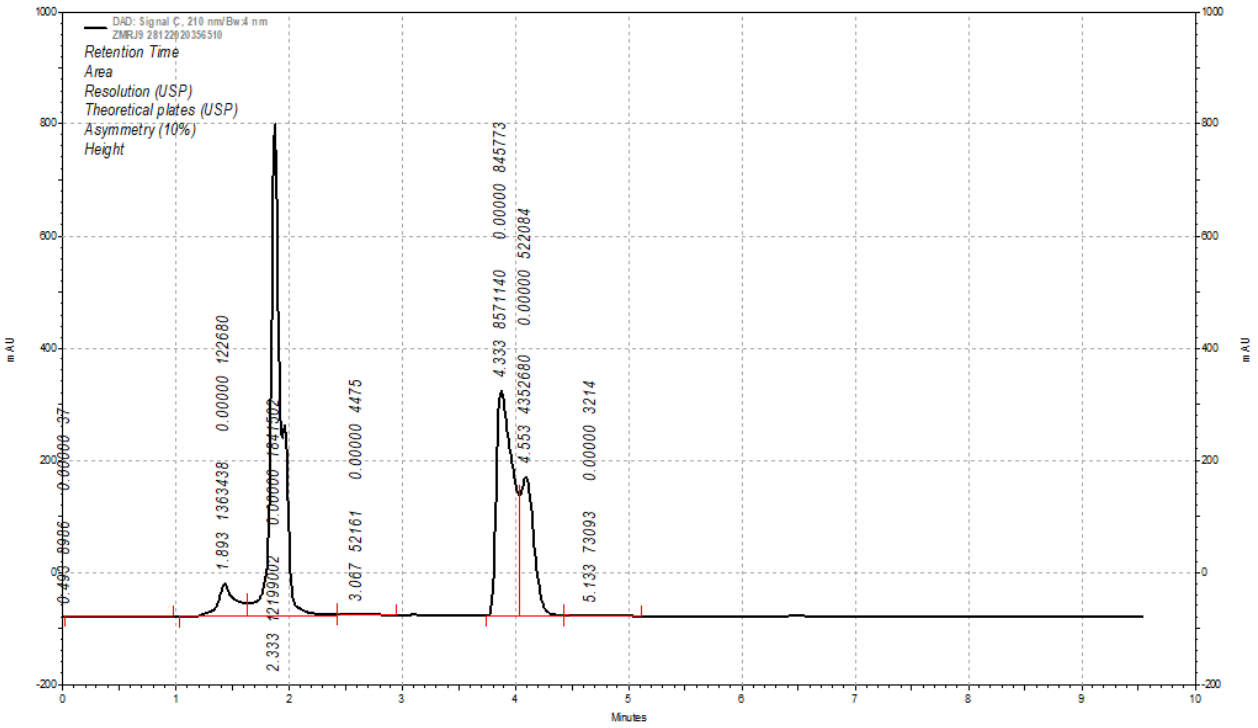
$$Rf, B = \frac{3.01}{4} = 0.75$$



HPLC at 234NM STD



Sample 1: ZMRJ5



Sample 2: ZMRJ9

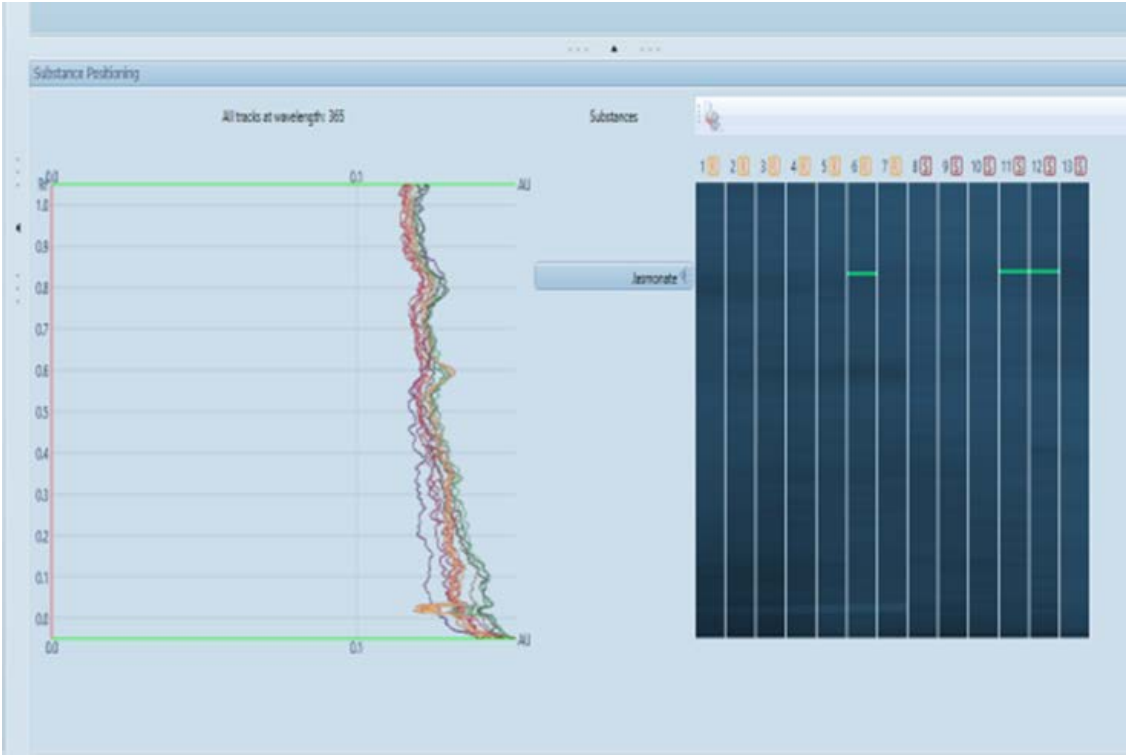
Table 3

	Retention time(min)	Peak area
Standard (20 µg/ml) SA	2.187	24752897
Sample 1 ZMRJ5	2.33	19276612
Sample 2 ZMRJ9	2.33	12199002

Concentration of unknown sample 1- ZMRJ5 is 15.575µg/ml (by comparing with standard peak area) Similarly, Concentration of unknown sample 2- ZMRJ9 is 9.856 µg/ml.

Note: ZMRJ5, ZMRJ9 are *Bacillus zhangzouensis* and *Bacillus velezensis* respectively

HPTLC



C

D

C. Reference 6 Vs X,Y stages of sample 2
D. Samples correlating spectrum over reference 6

Table 4

Substance name	Track	Rf
Jasmonate standard	6	0.829
Sample 11	11	0.835
Sample 12	12	0.835

Table 5

Results:			
Jasmonate	**(2 sample assignments) @ 366 nm**		
Sample 'SAMPLE X'	648.9 µg/ml	(CV unavailable)	(1 applications)
1.947 mg in 5.000 mg			
Volume: 2.0 µl	648.9 µg/ml	(CV unavailable)	(1 replicas)
Track 11	648.9 µg/ml	1.298 µg	
Sample 'SAMPLE Y'	737.8 µg/ml	(CV unavailable)	(1 applications)
2.213 mg in 5.000 mg			
Volume: 2.0 µl	737.8 µg/ml	(CV unavailable)	(1 replicas)
Track 12	737.8 µg/ml	1.476 µg	

Discussion

The two potent plant growth promoting rhizobacteria identified was *Bacillus zhangzouensis* and *Bacillus velezensis.* During lab trials, these bacilli inhibited the growth of sclerotium on potato dextrose agar media, though a selective medium for fungus. Mycelium of test samples as shown in scanning electron micrographs became very thin when compared to mycelia of *sclerotium* in control. *Bacillus zhangzouensis* is found to be more effective in inhibiting the growth of sclerotial bodies than *Bacillus velezensis.*

Further, to know whether the organisms are able to produce salicylic acid, a simple technique of extraction of salicylic acid from both the bacilli separately was carried out and quantified using HPLC. Nearly15.575µg/ml of salicylic acid is found to be produced by *Bacillus zhangzouensis* and 9.856 µg/ml of salicylic acid is produced by *Bacillus velezensis.* From the above observation it can be concluded that these two bacilli are able to produce salicylic acid whenever needed to induce SAR in plants. For further adequate information and assurance emphasis on study pertaining the knowledge of salicylic acid production in plants induced by bacilli at biomolecular level with respect to plants upon which they are carried out is considerable as one of the future perspective.

Apart from that during pot trails, these bacterial antagonists have shown biocontrol activity against the pathogen. the following aspects were noticed.

Plants grown in positive control only with the pathogen exhibited symptoms to large extent. Plants grown in negative control with untreated and water alone, has shown minimum symptoms. Plants grown in the test sample pots exhibited least to no symptoms. Leaves of these treated plants were collected and plant material for analysis is prepared at different stages of plant. Jasmonate is detected using HPTLC at pod stage (sample X) where, production of jasmonate was observed in treated leaves. This condition was also noticed during middle stage(sample Y) that is after 30 days, but, at mature stage that is at 60 days(sample Z) healthy plant with no symptoms were noticed but no jasmonate production in treated leaves was noticed.

Intrepretation is as follows;

Treated plant leaves was detected with jasmonate production in 11th and 12th that is X, and Y stages of sample 2 with reference to 6th standard in which standard solution contains 6 microlitres of standard. These treated leaves has shown the concentration of JA 1.947 mg in 5.000 mg and 2.213 mg in 5.000 mg of leaf extract respectively. The result indicates that more concentration of JA available during pod and middle stages of plant than at the mature stage is may

be due to jasmonate induction by bacilli, thus would have reduced the risk of disease in plants. It can be concluded that both SA and JA can be produced and can be induced by bacilli when present in the proximity of root region. It depends on the plants defense strategy mechanism to elicit SAR or ISR or both based on the bioformulation used.

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