A resourceful methodology to profile indolic auxins produced by rhizo-fungi using spectrophotometry and HPTLC

Dhavalkumar Patel¹ · Anoshi Patel² · Disha Vora² · Sudeshna Menon² · Sebastian Vadakan² · Dhaval Acharya¹ · Dweipayan Goswami²

Received: 8 May 2018 / Accepted: 10 September 2018 / Published online: 15 September 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Plant growth-promoting fungi play an important role in development of sustainable agriculture. In the current study, 13 fungal strains were isolated from the rhizosphere of healthy *Triticum aestivum* (wheat) plant and screened for their indolic auxin production potential. *Aspergillus flavus* strain PGFW, *Aspergillus niger* strain BFW and *Aspergillus caespitosus* strain DGFW were amongst the most efficient indolic auxin-producing strains. Indolic auxins such as indole 3 acetate (IAA), indole 3 butyrate (IBA) and indole 3 propionate (IPA) are produced by fungi. The conventional method to assess the IAA production is through a spectrophotometric assay using Salkowski's reagent, which quantifies all indolic auxins and not individual auxins. Moreover, it was also observed that the absorption maxima ($\lambda_{\max}$) of the samples, when compared to that of standard indole-3-acetic acid, showed deviation from the latter, indicative of production of a mixture of indolic derivatives by the fungi. Hence, for further profiling of these indolic compounds, high-performance thin layer chromatography (HPTLC) based protocol was standardized to precisely detect and quantify individual indolic auxins like IAA, IBA and IPA in the range of 100–1000 ng per spot. HPTLC analysis also showed that the fungal strains produce different indolic auxins in media with and without fortification of tryptophan, with the production of indolic auxins being enhanced in presence of tryptophan. Thus, this standardized HPTLC protocol is an efficient and sensitive methodology to separate and quantify the indolic derivatives.

Keywords *Aspergillus* spp. · Indolic auxins · HPTLC protocol · Assay optimization

Introduction

Microbes residing in the rhizosphere of the plants, interact with them by physical contact and/or by special small molecules secreted by microbes that act as signaling molecules for plant (Arora and Verma 2017; Hansda and Kumar 2017; Goswami et al. 2017). Most of these small molecules belong to the category of phytohormones, of which, indole-3-acetic acid (IAA) is the most understood molecule (Khatai et al. 2018; Wani et al. 2016; Pérez Montaña et al. 2014; Jasim et al. 2014). In the list of microbes that interact with plant, fungi hold a major stake as they have evolved to thrive in

the rhizosphere. Some of these fungi are pathogenic while others tend to be beneficial for plant growth. The beneficial ones are known as plant growth-promoting fungi (PGPF). Several studies have shown that PGPFs utilize tryptophan secreted by plant as root exudates, metabolize and secrete it back as IAA which in turn interacts with plant to enhance its growth. IAA is the most abundantly produced but it is not the only indole derivative that is being produced by fungi. Other indolic derivatives that may be produced by microbes include indole 3-butyric acid (IBA), indole-3-propionic acid (IPA), indole 3-pyruvic acid (IPyA), indole 3-acetamide (IAM), indole 3 acetonitrile (IAN), indole 3-lactic acid (ILA), and indole 3-acetaldoxime (IAAld) (De Palma et al. 2016; Szkop and Bielawski 2013). IBA and IPA are also known to enhance plant growth and are categorized as potent auxins along with IAA. Intensive research in deducing pathways have shown that microbes can metabolize tryptophan in several ways (Goswami et al. 2016). Thus, depending on the metabolic capabilities of fungi, it tends to produce several other indolic compounds along with producing IAA

✉ Dweipayan Goswami
dweipayan.goswami@sxca.edu.in

¹ Department of Biotechnology and Microbiology, Parul Institute of Applied Science and Research, Parul University, Bopal Guma Road, Ahmedabad, Gujarat 380058, India

² Department of Biochemistry and Biotechnology, St. Xavier's College (Autonomous), Ahmedabad, Gujarat 380009, India

(Manici et al. 2015). These indolic auxins accelerate plant growth by enhancing apical dominance and root differentiation. Detection and quantification of these indolic derivatives is an important criterion to screen potential biofertilizers for sustainable agriculture.

Spectrophotometric technique is routinely used for analyzing indolic derivatives produced by fungi. In this method, outmoded Salkowski reagent, which is prepared by mixing FeCl_3 in perchloric acid (or sulfuric acid) is used. The limitation of this method is that all the indolic derivatives have the potential to react with Salkowski reagent and form colour complexes ranging from orange to pink (Karnwal 2009; Glickmann and Dessaux 1995). Thus, the drawback of using Salkowski reagent is that it gives a non-specific color reaction with the entire spectrum of the indolic derivatives produced by the fungi and, so it detects total indolic auxins rather than precisely and specifically detecting IAA, IBA, and/or IPA individually (Szkop and Bielawski 2013). The λ_{max} of the samples obtained by Salkowski's reaction must be primarily examined before proceeding further for individual characterization of each indolic derivative, as each is reported to have specific absorption maxima (Glickmann and Dessaux 1995) and, so if there exists a mixture of indolic derivatives in culture extract then it will not show λ_{max} characteristic to any reference standard indolic auxin.

Apart from the spectrophotometric method, thin-layer chromatography (TLC) is also routinely used, which provides precise detection of indolic compounds but fails to quantify them (Dhandhukia and Thakker 2008). On the other hand, high-performance liquid chromatography (HPLC) can precisely detect and quantify all the indolic compounds in the mixture (Beni et al. 2014; Szkop and Bielawski 2013). However, its analysis requires intense calibration and longer duration (Dhandhukia and Thakker 2011).

High-performance thin layer chromatography (HPTLC) is an advanced form of TLC with high separation efficiency. The technique consists of precise sample application, standardized reproducible chromatogram development and software-controlled evaluation. HPTLC is an entire concept that incorporates a broadly standardized methodology founded on scientific facts as well as the use of validated methods for qualitative and quantitative analysis (Shawky 2013).

In the current study, fungal isolates obtained from the rhizosphere of healthy *Triticum aestivum* (wheat) were screened for their potential to produce indolic auxins with the objective of preparing bioformulations for sustainable agriculture. Strains belonging to the genus of *Aspergillus* were capable of producing indolic auxins, as determined using Salkowski's spectrophotometric method. The *Aspergillus* strains have gained a negative reputation of being a plant pathogen (Mondal et al. 2000). On the contrary, there are several reports that have pointed out that *Aspergillus* may act as pathogens for harvested grains but rarely

act as pathogens for healthy growing plants; moreover, as per recent reports they are proven to be beneficial for plant growth as well (Vassilev et al. 2017; Angel et al. 2011). In our study, we observed that the λ_{max} of the samples containing indolic derivatives investigated using Salkowski's method were different from that of the λ_{max} of the standard IAA and IBA. This was the first indication that the fungal strains possibly produce mixtures of indolic compounds, which needed to be further detected and quantified. HPTLC was chosen to separate, detect and quantify the indole derivatives. Thus, this study reports a rapid, precise and sensitive methodology involving spectrophotometry and HPTLC to characterize indolic auxins produced by microbes interacting with plants.

Experimental

Materials and reagents

Standards (tryptophan, i.e., TRP, IAA, IBA and IPA) used for the assay were procured from HiMedia (Mumbai, India). Solvents used for the assay (iso-propanol, *n* butanol, methanol and 25% v/v ammonia) were procured from Merck Ltd. TLC foils (60F254) were purchased from Merck (Darmstadt, Germany).

Instrumentation

The HPTLC instrumentation assembly from CAMAG (Muttenz, Germany) has been used for the assay. The instrument consists of an (1) automatic applicator (Linomate 5) fitted with a (2) Hamilton syringe having volume capacity of 100 μl , and (3) Scanner for the scanning of developed HPTLC foils.

Standard solutions

Tryptophan, IPA, IAA and IBA procured from HiMedia were individually dissolved in absolute methanol in the concentration of 100 $\mu\text{g/ml}$, and these individual solutions were used as standards for the assay.

Isolation and identification of fungal strains

A total of 13 strains were isolated from the rhizosphere of *Triticum aestivum* (wheat) plant, all these strains were assessed for their IAA producing capabilities using traditional spectrophotometric assay that involved the use of Salkowski reagent (Brick et al. 1991; Goswami et al. 2013). Out of all, three of the most efficient IAA producing strains were used for further experiments. Three strains of fungi were identified using 18S rRNA gene sequence

analysis. Universal primer ITS2 was used for polymeric chain reaction (PCR) with a forward primer 5'-GGAAGT AAAAGTCGTAACAAGG-3' and reverse primer 5'-TCC TCCGCTTATTGATATGC-3'. PCR and 18S rRNA gene sequencing was performed at GSBTM, Gandhinagar, India. The sequence gene products were tested for sequence homology using BLASTn search program (<http://www.ncbi.nlm.nih.gov>). The gene sequence was submitted to GenBank and accession number was assigned. For phylogenetic analysis, gene sequence obtained was aligned by ClustalW using MEGA 4.0 software (Tamura et al. 2017) and a neighbor-joining (NJ) tree with bootstrap value 1000 was constructed.

Quantifying IAA produced by fungal isolates using spectrophotometric method

Conventional spectrophotometric method, which involved the use of Salkowski reagent (Brick et al. 1991; Goswami et al. 2013) was used for primary quantification of IAA from the strains under study. For the quantification, fungal isolates were cultured in the potato dextrose broth supplemented with TRP (1 mg/ml) for 168 h at 25 ± 2 °C. After the incubation period, Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl₃ solution) is mixed with culture supernatants of each of these isolates in 1:1 ratio. IAA or similar compounds if present in the supernatant will develop a pink color on reacting with Salkowski reagent which is quantified at 530 nm spectrophotometrically. Linear curve of IAA (HiMedia) in the concentrations ranging from 10 to 100 µg/ml was prepared against which IAA produced by fungal isolates were quantified.

Determination of absorption maxima ($\lambda_{\max}$)

The $\lambda_{\max}$ for each fungal extract was determined and compared with the $\lambda_{\max}$ of standard IAA and IBA. If the $\lambda_{\max}$ of sample was same as that of standard IAA or IBA, it can be said that the fungi produce pure IAA or IBA, respectively, without any other indolic derivative. However, if $\lambda_{\max}$ of fungal extract does not match with any of the standards, it indicates that fungi produce other indolic derivatives. For this, the standard IAA, standard IBA and culture supernatant were mixed with Salkowski reagent in the ratio of 1:1 (as mentioned earlier) and the spectral scans were taken from 400 to 800 nm and superimposed. The wavelength (nm) at which the O.D. was maximum, that nm is considered as $\lambda_{\max}$ of the sample standard under study. Here, for spectrophotometric study, IPA was not used as it did not produce a significant color with Salkowski reagent.

Mining of indolic derivatives from culture broth

For the extraction of indolic derivatives, a method described by Goswami et al. (2015) with modifications was employed. Fungal isolates were cultured for 168 h at 25 ± 2 °C in two batches in the potato dextrose broth supplemented with TRP (1 mg/ml of broth) (batch 1) and without TRP supplementation (batch 2). Later, using 1N HCl, culture supernatants were acidified to pH 2.5. This acidified culture supernatant was extracted using equal volume of ethyl acetate using the liquid–liquid extraction method. All the indolic derivatives favor better solubility in ethyl acetate than in aqueous culture broth under acidified conditions. Ethyl acetate being insoluble can easily be separated using separating funnel. Pooled ethyl acetate extract is air dried and after that re-suspended in one-tenth volume of methanol, which is then utilized for HPTLC measures (Goswami et al. 2015).

Standardization of HPTLC assay

Application of sample

A component of HPTLC instrument (CAMAG) Linomat 5 applicator containing 100 µl Hamilton syringe was used to load samples on TLC sized 10×20 cm. Samples were loaded which formed bands of 6 mm in width. Linomat 5 applicator was programed to load samples at the rate of 100 nl/s and with table speed adjusted to 10 mm/s. The origin was set to 15 mm and 12 mm from the edge of the plate on X-axis and Y-axis, respectively, on the TLC foil.

Calibration curves

Limit of detection (LOD) and limit of quantitation (LOQ) for TRP, IAA, IBA and IPA were determined by preparing its standard curve. Each standard was individually prepared in the concentration of 100 µg/ml and were loaded ranging from 4 to 20 µl in the form of bands on TLC (sized 10×20 cm). Volumes of standard loaded on tracks were as follows: 1 µl, 2 µl, 3 µl, 4 µl, 5 µl, 6 µl, 8 µl, 10 µl, 12 µl, 14 µl, 16 µl, 18 µl, 21 µl, 24 µl, 27 µl, 33 µl, 39 µl and 45 µl per band making total 18 bands for each standard. Thus, calibration curve of individual standard in the range of 100–4500 ng per band was developed. After the run of TLC in twin trough chamber containing mobile phase iso-propanol:*n* butanol:ammonia:water (9:3:3:1), TLC plate was air dried and scanned using Scanner 3 (a component of CAMAG HPTLC instrument) at 254 nm and its 3D densitogram was obtained, which was used to prepare standard curve.

We performed the modifications in the solvent system that we reported in our previous publication where we used iso-propanol:*n* butanol:ammonia:water (10:6:3:1) (Goswami

et al. 2015) as in this solvent, the R_f of IPA and IBA identical and unresolved, this is because we have introduced IPA as one more standard which was not included in our previous report. To overcome this limitation, we optimized solvent system as iso-propanol:*n* butanol:ammonia:water (9:3:3:1), and we achieved distinct R_f for IBA and IPA which we have used in this procedure.

All the four standards (IAA, IBA and IPA) in five replicates were loaded on single TLC plate in varying concentrations making 20 tracks in total. Briefly, each standard was loaded on five tracks where the concentration loaded was 4 μ l (4000 ng), 8 μ l (8000 ng), 12 μ l (12,000 ng), 16 μ l (16,000 ng) and 20 μ l (20,000 ng), respectively. The TLC plate was subjected to run in presaturated twin trough chamber containing mobile phase iso-propanol:*n* butanol:ammonia:water (9:3:3:1). TLC was permitted to run till the mobile phase created solvent front at 8 cm on TLC plate (sized 10×20 cm). On development, TLC plate was air dried and scanned using Scanner 3 using absorbance–reflectance mode at 254 nm.

Densitometric analysis of chromatogram

TLC foils after the chromatographic run were analyzed CAMAG TLC Scanner 3 for quantitative densitometric analysis. Scanner 3 was programmed to scan at the rate of 20 mm/s at 254 nm using deuterium source and baseline correction was adjusted to the lowest slope. Slit dimension was adjusted to 6 mm length×0.45 mm width, and 100 μ m per step of resolution was achieved using filter factor Savitsky Golay 7.

Precision of method

The sensitivity and accuracy of the method was determined by loading 4000 ng and 8000 ng of each standard (i.e., IPA, TRP, IAA and IBA) individually on separate tracks on single TLC plate (sized 10×10 cm). On performing the run and densitometric analysis, the concentration on each spot of the TLC plate was determined by comparing the peak area of the spot with previously obtained standard curve. The rate recuperation of every standard was the deliberated amount, considering the stacked measure of every standard as 100%.

Assessment of IAA, IBA and IPA from extricated indolic derivatives from fungal cell-free supernatant

Standards (TRP, IPA, IAA and IBA) and the methanolic extracts of the selected fungal species that contain indolic derivatives were loaded on the same TLC plate. Two bands spiked with each standard (TRP, IPA, IAA and IBA) separately and two bands loaded with each fungal methanolic extract in different volumes were loaded on TLC plate (sized

10 cm×20 cm). For *Aspergillus flavus* strain PGFW, *Aspergillus niger* strain BFW and *Aspergillus caespitosus* strain DGFW, the methanolic extricates stacked per band were 20 μ l and 30 μ l separately. The TLC plate was subjected to run in presaturated twin trough chamber containing mobile phase iso-propanol:*n* butanol:ammonia:water (9:3:3:1). On completion of chromatographic run, TLC plates were air dried and separated spots were checked utilizing Scanner 3 (CAMAG) in absorbance–reflectance mode at 254 nm. Densitometric examination was performed to measure IAA, IBA and IPA in the fungal methanolic separates; evaluated values were contrasted with quantitative values obtained using the spectrophotometric technique.

Pearson correlation coefficient (PCC or Pearson's *R*) was computed for determining the analogy between HPTLC inferred values with spectrophotometric estimations for derived values of IAA. Pearson's *R* is a measure of the direct connection (reliance) between two factors (1) HPTLC determined estimations of IAA and (2) spectrophotometric inferred estimations of IAA giving a '*R*' value. Coefficient of determination (R^2) was likewise ascertained, which is implied coefficient of assurance peaks to the percent (%) of the information that is the nearest to the line of best fit.

Results

Strain identification

Three strains designated as PGFW, BFW and DGFW with a potential of high production of IAA was isolated from *Triticum aestivum*. 18 s rRNA gene sequence analysis using BLASTn and phylogenetic analysis was performed to identify these strains. The analysis showed that fungal isolate PGFW showed maximum similarity with *Aspergillus flavus*, isolate BFW showed maximum similarity with *Aspergillus niger* and isolate DGFW showed maximum similarity with *Aspergillus caespitosus* (Fig. 1). Accession number KY964055 was assigned to *A. flavus* strain PGFW, KY964054 was assigned to *A. niger* strain BFW and KY964055 was assigned to *A. caespitosus* strain DGFW. Accession numbers to these fungal cultures were assigned by GenBank.

IAA production by PGPF strains analyzed using the spectrophotometric method

Spectrophotometric analysis confirmed the production of indolic auxin derivatives by all the three *Aspergillus* strains in media without tryptophan as well as in media fortified with tryptophan. Spectrophotometric investigation evaluated that in presence of TRP and in absence of TRP, *A. flavus* strain PGFW produced 112.29 ± 8.34 μ g/ml and

Fig. 1 18S rRNA gene sequences of fungal strains BFW, PGFW and DGFW along with similar sequences retrieved from GenBank using BLASTn were used for phylogenetic analysis. Multiple alignments of data were performed using ClustalW. Neighbor joining tree constructed using MEGA 4.0, distances and clustering in the neighbor joining tree contained bootstrap values based on 1000 replications listed as per percentages at the branching points

85.67 ± 4.33 $\mu\text{g/ml}$ of IAA, respectively, *A. niger* strain BFW produced 139.72 ± 7.44 $\mu\text{g/ml}$ and 82.67 ± 3.22 $\mu\text{g/ml}$ of IAA, respectively, and *A. caespitosus* strain DGFW produced 156.08 ± 5.44 $\mu\text{g/ml}$ and 106.62 ± 6.44 $\mu\text{g/ml}$ of IAA, respectively (Table 2), although these quantities connote the aggregate indolic subordinates produced rather than just IAA. In this manner, the qualities determined utilizing spectrometric assay is the measurement of aggregate indolic subordinates, which are viewed as identical to the measure of IAA. The fundamental conclusion from the spectrophotometric examination is that strains can beneficially utilize TRP and synthesize indolic compounds.

Determination of λ_{max}

The λ_{max} of standard IAA and IBA were found to be 516 nm and 460 nm, respectively (Fig. 2a). The λ_{max} of strain *A. niger* strain BFW was found to be 475 nm (Fig. 2b). Similarly, λ_{max} of strain *A. flavus* strain PGFW (Fig. 2c) and *A. caespitosus* strain DGFW (Fig. 2d) was found to be 510 nm and 470 nm, respectively. None of these three strains showed any peak corresponding to pure IAA or IBA. This is an indication that there might be other indolic derivatives that react with Salkowski reagent to give color that might cause shift in λ_{max} when compared to pure IAA or IBA. To confirm this,

we used the HPTLC technique, which will be represented further in this manuscript.

HPTLC

Calibration curves

Calibration curves for individual standards (TRP, IAA, IBA and IPA) were developed for comparative visualization and the assessment of retention factor (R_f) on single TLC plate (Fig. 3). The calibration curve also provides a basis to prepare standard curve of each working standard (TRP, IAA, IBA and IPA) individually in the form of absorbance unit (AU) versus concentration of standard loaded per spot. For preparing calibration curve, each standard was loaded individually ranging from 100 to 4500 ng per band. Densitometric investigation found that TRP had a straight connection with concentration of standard stacked and peak area of the created spot in the range of 100–1000 ng per spot. IAA showed linearity in the range of 100–1000 ng per spot, IBA linearity in the range of 50–500 ng per spot and IPA showed linearity in the range of 100–500 ng per spot. Here, densitometric assessment of TLC plate on development portrayed the R_f values of working standards as follows: TRP 0.48, IAA 0.58, IBA 0.62 and IPA 0.59. LOD and LOQ for TRP was determined to be 200.1 ng and 640.4 ng per spot, respectively; for IAA these values were found to be 96.1 and 291.2 ng per spot, respectively; for IBA these values were found to be 83.4 and 252.7 ng per spot, respectively; and for IPA these values were found to be 103.4 and 311.3 ng per spot, respectively (data not shown).

Precision of method

To decide the precision of the strategy, 4000 ng and 8000 ng of every standard per band were stacked on TLC plate, and permitted to run in the twin trough chamber containing mobile phase iso-propanol:*n* butanol:ammonia:water (9:3:3:1) for 90 min. On development, the TLC plate was checked at 254 nm to determine peak area for each created spot. From this information, concentration of standard in the particular spot was estimated by looking at its individual peak area from standard calibration curve. Considering the stacked sum as 100%, the deliberated amount of every standard so acquired has been referred to as rate recuperation value. Rate recuperation obtained in each standard is shown in Table 1.

Determination of IAA, IBA and IPA from fungal cell-free supernatant containing indolic derivatives

After standardization to precision, this technique was utilized to identify and measure IAA, IBA and IPA from

Table 1 Rate recuperation of standards by HPTLC method

Standards	Track	Expected value (in ng)	ng obtained from HPTLC assay	Rate recuperation (%)
TRP	Run 1	4000	4006.22	100.16
	Run 2	8000	8007.04	100.09
IAA	Run 1	4000	4008.87	100.22
	Run 2	8000	8005.30	100.07
IBA	Run 1	4000	4008.93	100.22
	Run 2	8000	8005.95	100.07
IPA	Run 1	4000	4001.55	100.04
	Run 2	8000	8002.39	100.03

fungal culture extracts. Track 1 to track 8 were stacked with standards (every standard, i.e., TRP, IPA, IAA and IBA were stacked on two tracks making eight tracks altogether) as shown in Fig. 4. The rest of the tracks (9th to 20th track) on same TLC foil contain extracted indolic compounds from fungal culture medium. With reference to Fig. 4, the 2D densitogram of the spots obtained for the tracks loaded with samples is represented in Fig. 5, where the Absorption Units (AU) on the Y-axis of graph for each spot obtained is used for calculating the concentration of respective indolic compound.

For the experimental set of culture, *A. flavus* strain PGFW grown in absence of TRP, its 20 μ l and 30 μ l methanolic extract were loaded on two different bands. On development, an aggregate of five resolved bands developed, of which three bands demonstrated a R_f similar to IAA, IPA and IBA (Fig. 4). On loading 20 μ l sample, the IAA, IPA and IBA estimated were 3681.30 ng, 3856.56 ng and 3519.32 ng, respectively, and in 30 μ l sample, IAA, IPA and IBA estimated were 5024.03 ng, 6003.33 ng and 5053.13 ng, respectively. Thus, IAA, IPA and IBA in the fungal isolate extract were computed to be 17.58 ± 1.17 μ g/ml, 19.64 ± 0.51 μ g/ml and 17.22 ± 0.53 μ g/ml, respectively, in absence of TRP. While in the other set where culture was grown in presence of supplemented TRP, from the 20 μ l of stacked concentrate, the IAA, IPA and IBA distinguished were 6271.95 ng, 4972.86 ng and 7170.15 ng separately and for 30 μ l of stacked concentrate, the identified IAA, IPA and IBA esteems were 8654.61 ng, 8011.15 ng and 10466.06 ng, respectively. Thus, IAA, IPA and IBA in the methanolic fungal extract were ascertained to be 30.10 ± 1.77 μ g/ml, 25.78 ± 1.30 μ g/ml and 35.37 ± 0.68 μ g/ml, respectively, in presence of TRP. The spectrophotometric analysis computed the amount of IAA produced to be 85.67 ± 4.33 μ g/ml without TRP and 132.29 ± 8.34 μ g/ml with TRP, which is significantly different from the concentrations calculated through HPTLC.

For the experimental set of culture, *A. niger* strain BFW grown in absence of TRP, its 20 µl and 30 µl of methanolic extract were stacked on two distinct bands. On development, an aggregate of four components were distinguished out of which just two compounds corresponded to a R_f similar to IAA and IBA (Fig. 4). From 20 µl of stacked concentrate, the IAA and IBA estimated were 2629.49 ng and 2049.50 ng, respectively, and for 30 µl of stacked concentrate, the IAA and IBA estimated were 3410.67 ng and 3416.34 ng, respectively. Thus, IAA and IBA extracted in the fungal culture supernatant was computed to be 12.26 ± 1.26 µg/ml and 10.82 ± 0.81 µg/ml, respectively, in absence of TRP. Whereas, in the set of culture grown in presence of TRP, from 20 µl of extract loaded on TLC, the IAA and IBA distinguished estimated were 4696.79 ng and 4753.48 ng individually and for 30 µl of stacked concentrate, identified IAA and IBA values were 6904.41 ng and 6934.97 ng, respectively, when grown in presence of TRP. Thus, IAA and IBA produced in the fungal broth was calculated to be 23.25 ± 0.33 µg/ml and 23.44 ± 0.46 µg/ml, respectively, in presence of TRP, whereas the spectrophotometric technique proposed that the IAA produced was estimated to be 82.68 ± 3.22 µg/ml without TRP and 139.73 ± 7.44 µg/ml with TRP.

For the experimental set of culture, *Aspergillus caespitosus* strain DGFW grown in absence of TRP, its 20 µl and 30 µl of its methanolic extract were stacked on two distinct bands. On development, an aggregate of eight compounds were distinguished out of which just two compounds corresponded to a R_f similar to IAA and IBA (Fig. 4). From 20 µl of stacked concentrate, the IAA and IBA estimated were 3023.79 ng and 4545.67 ng, respectively, and for 30 µl of stacked concentrate, IAA and IBA were 4192.09 ng and 6567.31 ng, respectively. Thus, IAA and IBA extracted in the fungal culture supernatant were computed to be 14.55 ± 0.81 µg/ml and 22.31 ± 0.59 µg/ml, respectively, in absence of TRP. While in the other set where culture was grown in presence of supplemented TRP, from the 20 µl of stacked concentrate, the IAA and IBA detected were 5037.28 ng and 6279.31 ng, respectively, and for 30 µl of stacked concentrate, identified IAA and IBA values were 8369.34 ng and 9610.31 ng, respectively. Thus, IAA and IBA produced in the fungal broth was calculated to be 26.54 ± 1.98 µg/ml and 31.72 ± 0.45 µg/ml, respectively, in presence of TRP, whereas the spectrophotometric method suggested that the IAA produced was calculated to be 106.62 ± 6.44 µg/ml without TRP and 156.08 ± 5.44 µg/ml with TRP.

Pearson's R for determining the correlation between HPTLC-derived values with the spectrophotometric-derived values of IAA from fungal extracts suggests that there is a partial positive correlation, where the value of R is 0.84. This suggests that there is a tendency for an increase in the

value of HPTLC-derived values of IAA for an increase in the spectrophotometric-derived values of IAA and vice versa (Table 2). The value of R^2 , the coefficient of determination, is 0.70, which suggests that only 70% of the data fits the linear regression line of correlation.

Tables 2 and 3 represent the amount of IAA and IBA produced by these three isolates of *Aspergillus* spp., respectively, while Table 4 represents the amount of IPA produced by *Aspergillus flavus* only, the other two strains were not able to produce IPA. Figure 3 represents the total 20 tracks loaded and Fig. 4 shows the 2D densitogram image of produced IAA, IPA and IBA of these three fungal isolates in presence and absence of TRP in the media.

Discussion

Fungi produce metabolites to which the plant responds in various ways. The phytohormones that are produced by the fungi include auxins, cytokinins, gibberellins, abscisic acid, ethylene, jasmonic acid and salicylic acid (Chancelud and Morel 2016). Of these, the auxins play a vital role in rooting, apical dominance, budding, etc. Many chemically different molecules constitute the auxins, most notably IAA, IBA and IPA; however, other similar compounds, such as indole-3-acetonitrile, indole-3-acetamide and indole-3-acetaldehyde also act as intermediates for their biosynthesis and are not known to exert any hormonal effects (Maor et al. 2004). However, fungi show a capability to synthesize all these auxins, along with which they also leach out indolic intermediates thereby having a profile of indolic derivatives (Carreno-Lopez et al. 2000).

TRP is the known precursor for IAA production. The biosynthesis of IAA is broadly categorized to occur via two different pathways; TRP dependent and TRP independent (Goswami et al. 2016; Idris et al. 2007). Similar findings are also reported by Afzal et al. (2017) where the bacterial strains under their study were also able to produce a trace amount of IAA in the absence of tryptophan and comparatively more in the presence of tryptophan. Under rare instances, amount of IAA produced by any fungal isolate grown in absence of supplemented TRP is at part with to that of one growing in presence of TRP (Idris et al. 2007; Maor et al. 2004). Similar observations were obtained in the present study where, *A. flavus*, *A. niger* and *A. caespitosus* showed a better auxin profile when supplemented with TRP. Barazani and Friedman (2000) have shown that IAA production is dependent on the presence of TRP in the medium as, the organism utilizes the indole moiety from TRP to produce IAA. Similar findings were also reported by Ahmad et al. (2008) that increase in the IAA production by microbes was in corroboration with an increase in TRP supplementation in the medium.

Table 2 HPTLC analysis of IAA from three *Aspergillus* spp

Strains	Culture media	Sample loaded (µl)	Area under curve	ng of IAA per spot	ng of IAA per ml sample	µg of IAA per sample	µg of IAA per ml of broth	µg of IAA per ml of broth	Spectrophotometric results showing production (µg of IAA per ml of broth)
<i>A. flavus</i> strain PGFW	Without TRP	20	10,173.00	3681.30	184,065.01	184.07	18.41	17.58 ± 1.17	85.67 ± 4.33
		30	13,519.10	5024.04	167,467.90	167.47	16.75		
	With TRP	20	16,628.90	6271.95	313,597.51	313.60	31.36	30.10 ± 1.78	132.29 ± 8.34
		30	22,566.50	8654.61	288,487.16	288.49	28.85		
<i>A. niger</i> strain BFW	Without TRP	20	7551.90	2629.49	131,474.72	131.47	13.15	12.26 ± 1.26	82.67 ± 3.22
		30	9498.60	3410.67	113,689.14	113.69	11.37		
	With TRP	20	12,703.60	4696.79	234,839.49	234.84	23.48	23.25 ± 0.33	139.72 ± 7.44
		30	18,205.00	6904.41	230,147.14	230.15	23.01		
<i>A. caespitosus</i> strain DGFW	Without TRP	20	8534.50	3023.80	151,189.81	151.19	15.12	14.5 ± 0.81	106.62 ± 6.44
		30	11,445.90	4192.09	139,736.49	139.74	13.97		
	With TRP	20	13,552.10	5037.28	251,863.96	251.86	25.19	26.54 ± 1.92	156.08 ± 5.44
		30	21,855.60	8369.34	278,978.06	278.98	27.90		
Correlation of HPTLC values of IAA with spectrophotometric values of IAA from fungi under study									
Pearson correlation coefficient (Pearson's <i>R</i>)									0.84
Coefficient of determination (<i>R</i> ²)									0.70

Where *R* = 1, signifies total positive correlation; *R* = 0, signifies absolute no correlation; 0 < *R* < 1, signifies correlation; *R* = − 1, signifies negative correlation

Spectrophotometry is the most widely used procedure to detect the presence of indoles, which give a color reaction with Salkowsky's reagent. Under acidified conditions the indolic compound reacts with Fe³⁺ to give a pinkish-red color, and each of the indolic auxin has a specific $\lambda_{\max}$ that falls in the range of 510–550 nm which enables quantification (Szkop et al. 2012; Glickmann and Dessaux 1995). However, in the present study, we deduced that if two or more such indolic compounds occurs as a mixture, the $\lambda_{\max}$ of such solution will differ from characteristic $\lambda_{\max}$ values of each compound occurring independently. So, to individually detect and quantify IAA, IBA, IPA or other indolic compounds occurring as mixture using spectrophotometry becomes impossible (Goswami et al. 2016). The $\lambda_{\max}$ of the colored complexes of fungi culture extracts produced on reacting with Salkowski reagent was different from that of standard IAA and IBA. Thus, it would be completely wrong to portray our samples producing only IAA. Therefore, a more advanced approach is inevitable, and we further used HPTLC to analyze our samples. But, if the samples after reacting with Salkowski reagent would have given $\lambda_{\max}$ like that of IAA, then one can claim that the microbe under study can efficiently produce IAA without other indolic derivatives.

In our current research, the shift in the absorption maxima ($\lambda_{\max}$) was detected while using the traditional spectrometric method for the detection of IAA as represented in

Fig. 2. In both the conditions, in presence or in absence of TRP the shift in $\lambda_{\max}$ was perceived giving the aggregate of all indolic derivatives. For these unpredictable derivatives TLC technique is used in general, which gives only the number of compounds that are produced, failing to quantify their amounts. To overcome this limitation, an innovative technique of using HPTLC was carried out where the assay procedure was not only able to detect other indolic compounds, but also was able to quantify the amount in which they were produced.

On performing HPTLC of the fungal extract, it was found that quite a few other compounds were also present along with IAA, such as IBA and IPA. So, from standard curve and calibration curve IBA and IPA were quantified. It was noted that while determining IAA using the spectrophotometric technique its production was more in presence of TRP compared to the media in which TRP was absent. So, the only conclusion that can be deduced from spectrophotometric analysis is that strains can efficiently metabolize TRP and produce indolic derivatives in larger quantities. While using the HPTLC technique, other indole compounds like IAA, IBA and IPA were also detected. Moreover, using HPTLC, these indole compounds were not only detected but also were quantified. Similarly using HPTLC, it was also deduced that strains can efficiently metabolize TRP and produce indolic derivatives in larger quantities.

Fig. 2 **a** The lambda max ($\lambda_{\max}$) of standard IAA that is 516 nm and $\lambda_{\max}$ of IBA that is 460 nm is displayed, **b** the shift in lambda max ($\lambda_{\max}$) of *A. niger* strain BFW is shown, **c** the shift in lambda max

($\lambda_{\max}$) of *A. flavus* strain PGFW is shown and **d** the shift in lambda max ($\lambda_{\max}$) of *A. caespitosus* strain DGFW is shown

Thus, a systemic approach to analyze indolic derivatives has been standardized. There are reports where researchers have employed TLC to detect IAA but lacked quantification. Mohite (2013) isolated bacteria that could metabolize TRP to produce IAA and extracted it. Extracted IAA was compared to its extract with standard IAA on TLC chromatograms (TLC slide prepared with silica gel G and calcium carbonate. Propanol:water in ratio of 8:2 was used as solvent system.) and it was found that the spot with R_f value of 0.57 corresponding to standard IAA was obtained. Thus, it was confirmed that IAA was produced by rhizospheric isolates using TLC chromatograms but failed to provide its quantification. Gravel et al. (2007) isolated soil microbes and

confirmed that they produced IAA using the TLC method but quantified it using spectrophotometry which may have provided error in the quantified value. Several researches also made the use of HPLC (Szkop and Bielawski 2013; Patal and Saraf 2017). HPLC is not only time-consuming but often requires stringent conditions for accurate determination of substances, and could prove to be costlier (Dhandukia and Thakker 2011). In most of these researches, quantification by HPTLC was not given a focus.

We recently reported the novel methodology to simultaneously detect and quantify IAA as well as IBA using HPTLC (Goswami et al. 2015). This methodology allowed not only detection of several compounds

Fig. 3 The 3D densitogram of the same TLC foil where intensity of peak describes the area under the curve is displayed. It can be observed that as the concentration of standard TRP, IPA, IAA and IBA increases as the area under the curve for corresponding spot also increases

Fig. 4 The image of TLC foil under 254 nm UV light is displayed where track 1 and 2 are laden with standard TRP (20 μ l and 30 μ l, respectively), track 3 and 4 are laden with standard IPA (20 μ l and 30 μ l, respectively), track 5 and 6 are laden with standard IAA (20 μ l and 30 μ l, respectively), track 7 and 8 are laden with standard IBA (20 μ l and 30 μ l, respectively), track 9 and 10 are laden with extracted indolic derivate from *A. flavus* in absence of TRP in media (20 μ l and 30 μ l, respectively), track 11 and 12 are laden with extracted indolic derivate from *A. flavus* strain PGFW in the presence of TRP (20 μ l

and 30 μ l, respectively), track 13 and 14 are laden with extracted indolic derivate from *A. niger* strain BFW in absence of TRP in media (20 μ l and 30 μ l, respectively), track 15 and 16 are laden with extracted indolic derivate from *A. niger* strain BFW in the presence of TRP (20 μ l and 30 μ l, respectively), track 17 and 18 are laden with extracted indolic derivate from *A. caespitosus* strain DGFW in absence of TRP in media (20 μ l and 30 μ l, respectively), track 19 and 20 are laden with extracted indolic derivate from *A. caespitosus* strain DGFW in the presence of TRP (20 μ l and 30 μ l, respectively)

but rapid quantification as well. We adapted the method represented in the publication with modifications as our previous work dealt with bacterial isolates and in this

report, we used fungal strains. The solvent system has been modified to analyze one more indolic auxin, IPA. The previously reported solvent system iso-propanol:n

Fig. 5 A 2D densitogram of fungal extracts containing indolic compounds is displayed **a** track laden with extract of *A. flavus* strain PGFW without TRP (track 10 of Fig. 3), **b** track loaded with extract of *A. flavus* strain PGFW with TRP (track 12 of Fig. 3), **c** track laden with extract of *A. niger* strain BFW without TRP (track 14 of Fig. 3),

d track laden with extract of *A. niger* strain BFW with TRP (track 16 of Fig. 3), **e** track laden with extract of *A. caespitosus* strain DGFW without TRP (track 18 of Fig. 3), **f** track laden with extract of *A. caespitosus* strain DGFW with TRP (track 20 of Fig. 3)

butanol:ammonia:water (10:6:3:1) when used showed R_f of IPA and IBA to be identical indicating that these were not separated. By optimizing the solvent system proposition as iso-propanol:*n* butanol:ammonia:water (9:3:3:1), distinct R_f for IBA and IPA were obtained. Moreover, in our previous report we did not use this methodology to critically

examine the $\lambda_{\max}$ of samples that were analyzed spectrophotometry. Significantly, the coefficient of determination (R^2) computed of IAA for this data was 0.70 compared to 0.41 for a previously published paper. This signifies that there is more than 40% improvement in the precision of the method with the solvent used for this experimentation.

Table 3 HPTLC analysis of IBA from three *Aspergillus* spp.

Strains	Culture media	Sample loaded (µl)	Area under curve	ng of IBA per spot	ng of IBA per ml sample	µg of IBA per sample	µg of IBA per ml of broth	µg of IBA per ml of broth
<i>A. flavus</i> strain PGFW	Without TRP	20	20,493.70	3519.33	175,966.45	175.97	17.60	17.22 ± 0.53
		30	27,944.90	5053.13	168,437.63	168.44	16.84	
	With TRP	20	38,229.40	7170.15	358,507.62	358.51	35.85	35.37 ± 0.68
		30	54,240.90	10,466.06	348,868.53	348.87	34.89	
<i>A. niger</i> strain BFW	Without TRP	20	13,353.30	2049.51	102,475.30	102.48	10.25	10.82 ± 0.81
		30	19,993.40	3416.34	113,878.14	113.88	11.39	
	With TRP	20	26,489.20	4753.48	237,673.94	237.67	23.77	23.44 ± 0.46
		30	37,086.90	6934.97	231,165.77	231.17	23.12	
<i>A. caespitosus</i> strain DGFW	Without TRP	20	25,479.70	4545.68	227,283.86	227.28	22.73	22.31 ± 0.59
		30	35,303.30	6567.83	218,927.54	218.93	21.89	
	With TRP	20	33,901.70	6279.31	313,965.62	313.97	31.40	31.72 ± 0.45
		30	50,083.70	9610.31	320,343.76	320.34	32.03	

Table 4 HPTLC analysis of IPA from *Aspergillus flavus* strain PGFW

Strains	Culture media	Sample loaded (µl)	Area under curve	ng of IPA per spot	ng of IPA per ml sample	µg of IPA per sample	µg of IPA per ml of broth	µg of IPA per ml of broth
<i>A. flavus</i> strain PGFW	Without TRP	20	22,132	3856.56	192,828.32	192.83	19.28	19.65 ± 0.51
		30	32,561	6003.33	200,111.16	200.11	20.01	
	With TRP	20	27,555	4972.86	248,643.47	248.64	24.86	25.78 ± 1.30
		30	42,315	8011.15	267,038.56	267.04	26.70	

Acknowledgements Authors are thankful to the Gujarat State Biotechnology Mission (GSBTM) for providing the funding under FAP 2016 GSBTM/MD/PROJECTS/SSA/5041/2016-17 project and St. Xavier's College Ahmedabad for providing necessary facilities.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest whatsoever.

References

- Afzal I, Iqbal I, Shinwari ZK, Yasmin A (2017) Plant growth-promoting potential of endophytic bacteria isolated from roots of wild *Dodonaea viscosa* L. J Plant Growth Regul 81(3):399–408
- Ahemad M, Kibret M (2014) Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J King Saud Univ Sci 26(1):1–20
- Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163(2):173–181
- Angel SMM, Flores MAS, Badillo MGC, Saavedra MTR, Osuna MAI, Flores SC (2011) The plant growth-promoting fungus *Aspergillus ustus* promotes growth and induces resistance against different lifestyle pathogens in *Arabidopsis thaliana*. J Microbiol Biotechnol 21(7):686–696
- Arora NK, Verma M (2017) Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. 3 Biotech 7(6):381
- Barazani OZ, Friedman J (2000) Effect of exogenously applied L-tryptophan on allelochemical activity of plant-growth-promoting rhizobacteria (PGPR). J Chem Ecol 26(2):343–349
- Beni A, Soki E, Lajtha K, Fekete I (2014) An optimized HPLC method for soil fungal biomass determination and its application to a detritus manipulation study. J Microbiol Methods 103:124–130
- Brick JM, Bostock RM, Silversone SE (1991) Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. Appl Environ Microbiol 57:535–538
- Carreno-Lopez R, Campos-Reales N, Elmerich C, Baca BE (2000) Physiological evidence for differently regulated tryptophan-dependent pathways for indole-3-acetic acid synthesis in *Azospirillum brasilense*. Mol Gen Genet 264(4):521–530
- Cassán F, Vanderleyden J, Spaepen S (2014) Physiological and agronomical aspects of phytohormone production by model plant growth promoting rhizobacteria (PGPR) belonging to the genus *Azospirillum*. J Plant Growth Regul 33(2):440–459
- Chanclud E, Morel JB (2016) Plant hormones: a fungal point of view. Mol Plant Pathol 17(8):1289–1297
- De Battista JP, Bacon CW, Severson R, Plattner RD, Bouton JH (1990) Indole acetic acid production by the fungal endophyte of tall fescue. Agron J 82(5):878–880

- De Palma M, D'Agostino N, Proietti S, Bertini L, Lorito M, Ruocco M, Tucci M (2016) Suppression subtractive hybridization analysis provides new insights into the tomato (*Solanum lycopersicum* L.) response to the plant probiotic microorganism *Trichoderma longibrachiatum* MK1. *J Plant Growth Regul* 190:79–94
- Dhandhukia PC, Thakkar VR (2008) Separation and quantitation of jasmonic acid using HPTLC. *J Chromatogr Sci* 46(4):320–324
- Dhandhukia PC, Thakker JN (2011) Quantitative analysis and validation of method using HPTLC. In: Srivastava M (ed) High-performance thin-layer chromatography (HPTLC). Springer, Berlin, pp 203–221
- Etesami H, Alikhani HA, Hosseini HM (2015) Indole 3 acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. *MethodsX* 2:72–78
- Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl Environ Microbiol* 61(2):793–796
- Goswami D, Vaghela H, Parmar S, Dhandhukia P, Thakker JN (2013) Plant growth promoting potentials of *Pseudomonas* spp. strain OG isolated from marine water. *J Plant Interact* 8(4):281–290
- Goswami D, Pithwa S, Dhandhukia P, Thakker JN (2014) Delineating *Kocuria turfanaensis* 2M4 as a credible PGPR: a novel IAA-producing bacterium isolated from saline desert. *J Plant Interact* 9(1):566–576
- Goswami D, Thakker JN, Dhandhukia PC (2015) Simultaneous detection and quantification of indole 3 acetic acid (IAA) and indole 3 butyric acid (IBA) produced by rhizofungi from L tryptophan (TRP) using HPTLC. *J Microbiol Methods* 110:7–14
- Goswami D, Thakker JN, Dhandhukia PC (2016) Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. *Cogent Food Agric* 2(1):1127500. <https://doi.org/10.1080/23311932.2015.1127500>
- Gravel V, Antoun H, Tweddell RJ (2007) Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). *Soil Biol Biochem* 39(8):1968–1977
- Hansda A, Kumar V (2017) Cu-resistant *Kocuria* sp. CRB15: a potential PGPR isolated from the dry tailing of Rakha copper mine. *3 Biotech* 7(2):132
- Hartmann A, Singh M, Klingmuller W (1983) Isolation and characterization of *Azospirillum* mutants excreting high amounts of indole acetic acid. *Can J Microbiol* 29:916–923
- Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann Microbiol* 60(4):579–598
- Idris EE, Iglesias DJ, Talon M, Borriss R (2007) Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens*. *Mol Plant Microbe Interact* FZB42(6):619–626
- Jasim B, Jimtha John C, Shimil V, Jyothis M, Radhakrishnan EK (2014) Studies on the factors modulating indole-3-acetic acid production in endophytic fungal isolates from *Piper nigrum* and molecular analysis of ipdc gene. *J Appl Microbiol* 117(3):786–799
- Karnwal A (2009) Production of indole acetic acid by fluorescent *Pseudomonas* in the presence of L tryptophan and rice root exudates. *J Plant Pathol Microbiol* 91:61–63
- Khati P, Bhatt P, Kumar R, Sharma A (2018) Effect of nanozeolite and plant growth promoting rhizobacteria on maize. *3 Biotech* 8(3):141
- Manici LM, Kelderer M, Caputo F, Mazzola M (2015) Auxin-mediated relationships between apple plants and root inhabiting fungi: impact on root pathogens and potentialities of growth-promoting populations. *Plant Pathol* 64(4):843–851
- Maor R, Haskin S, Levi-Kedmi H, Sharon A (2004) In planta production of indole-3-acetic acid by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. *Appl Environ Microbiol* 70(3):1852–1854
- Martínez-Morales LJ, Soto-Urzuá L, Baca BE, Sánchez-Ahédó JA (2003) Indole-3-butyric acid (IBA) production in culture medium by wild strain *Azospirillum brasilense*. *FEMS Microbiol Lett* 228(2):167–173
- Meiners SJ, Phipps KK, Pendergast TH, Canam T, Carson WP (2017) Soil microbial communities alter leaf chemistry and influence allelopathic potential among coexisting plant species. *Oecologia* 203(4):1155–1165
- Mohite B (2013) Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *J Soil Sci Plant Nutr* 13(3):638–649
- Mondal G, Dureja P (2000) Fungal metabolites from *Aspergillus niger* AN27 related to plant growth promotion. *Indian J Exp Biol* 38:84–87
- Patel T, Saraf M (2017) Biosynthesis of phytohormones from novel rhizobacterial isolates and their in vitro plant growth-promoting efficacy. *J Plant Interact* 12(1):480–487
- Pérez Montañó F, Alías Villegas C, Bellogín RA, Del Cerro P, Espuny MR, Jiménez Guerrero I, Cubo T (2014) Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. *Microbiol Res* 169(5):325–336
- Robinson M, Riov J, Sharon A (1998) Indole 3 acetic acid biosynthesis in *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. *Appl Environ Microbiol* 64(12):5030–5032
- Shameer S, Prasad TNKV (2018) Plant growth promoting rhizobacteria for sustainable agricultural practices with special reference to biotic and abiotic stresses. *J Plant Growth Regul* 84:1–13
- Shawky E (2013) Determination of synephrine and octopamine in bitter orange peel by HPTLC with densitometry. *J Chromatogr Sci* 52(8):899–904
- Sivasakthi S, Usharani G, Saranraj P (2014) Biocontrol potentiality of plant growth promoting bacteria (PGPR)-*Pseudomonas fluorescens* and *Bacillus subtilis*: a review. *Afr J Agric Res* 9(16):1265–1277
- Swain MR, Naskar SK, Ray RC (2007) Indole 3 acetic acid production and effect on sprouting of yam (*Dioscorea rotundata* L.) minisets by *Bacillus subtilis* isolated from culturable cowdung microflora. *Pol J Microbiol* 56(2):103
- Szkop M, Bielawski W (2013) A simple method for simultaneous RP-HPLC determination of indolic compounds related to fungal biosynthesis of indole 3 acetic acid. *Antonie Van Leeuwenhoek J Microbiol* 103(3):683–691
- Szkop M, Sikora P, Orzechowski S (2012) A novel, simple, and sensitive colorimetric method to determine aromatic amino acid aminotransferase activity using the Salkowski reagent. *Folia microbiol* 57(1):1–4
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24(8):1596–1599
- Vassilev N, Malusa E, Requena AR, Martos V, López A, Maksimovic I, Vassileva M (2017) Potential application of glycerol in the production of plant beneficial microorganisms. *J Ind Microbiol Biotechnol* 44:735–743
- Wani SH, Kumar V, Shriram V, Sah SK (2016) Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *Crop J* 4(3):162–176