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RESEARCH ARTICLE

INDOLE ACETIC ACID PRODUCTION BY THE ACTINOMYCETES OF COFFEE PLANTATION SOILS OF WESTERN GHATS

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Glossary of Abbreviations

IAA- Indole acetic acid
SEM- Scanning electron Microscope
µg/ml- microgram per millilitre
TLC- Thin layer chromatography
HPLC-High performance liquid chromatography
rpm- rotation per minute
ISP2- International Streptomyces Project type-2
g/l- gram per litre
mg/ml- milligram per millilitre
M- molar
v/v- volume by volume
nm- nanometer
ANOVA- Analysis of variance

ABSTRACT

Background: The isolation of actinomycetes from coffee plantation soil increases the chance for the production of high auxins—IAA producers, helping in maximum increase in growth of the plants. **Objectives:** In the present study, actinomycetes were isolated from the less explored coffee plantation soils and the strains were screened for IAA producing activity which can be exploited for the production of agroactive compounds like auxins. **Methods:** The actinomycete strains were isolated from coffee plantation soils of Chikkamagalur district Karnataka, India. They were identified by molecular characterization and used for studying the IAA-producing ability. The identification of IAA was confirmed by spectrophotometric and chromatographic analyses. **Results:** A total of thirty two distinct actinomycete strains were isolated from coffee plantation soils of Chikkamagalur district Karnataka, India. Morphological and molecular studies (16S rRNA) revealed that 50% of the isolates belonged to the genus *Streptomyces*, and 50% were non-streptomycetes comprising *Actinomadura*, *Arthrobacter*, *Spirillospora*, *Sachharopolyspora*, *Nonomuraea*, *Gordonia*, *Micromonospora*, *Nocardia*, *Rhodococcus*, and *Pseudonocardia*. Of the thirty two actinomycete strains, twenty seven strains showed the IAA-producing ability which was confirmed by spectrophotometric analysis. The strain *Streptomyces violaceolatus* CMCS 016 produced maximum amount of IAA (109.24±0.2 µg/ml) in the ISP2 medium supplemented with 0.5% L-tryptophan. The chromatographic studies (TLC and HPLC) revealed that the extracted IAA had similarity to standard IAA with the same R_f value and retention time respectively. This study indicates the potential of *S. violaceolatus* CMCS 016 as a significant IAA producer. **Conclusion:** The present study is the first report on the IAA-producing potential of actinomycetes from coffee plantation soil enhancing the significance of non-rhizospheric soil which are equal proficient auxin producers compared to rhizospheric soil. The nutrient-rich soils of coffee plantations containing litter from both the tree species and the coffee plants are indeed rich sources of soil actinomycetes with biotechnological applications.

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INTRODUCTION

Auxins are the first of five significant plant growth promoters regularizing the plant's growth by stimulating cell elongation, cambial cell division, phloem and xylem differentiation, root initiation and lateral root development. They also help in the inhibition or promotion of flowering and fruiting (Sreevidya et al., 2016). Although there is the advancement in the enzymes involved in the biosynthesis of IAA, however, so far no pathway is designed to the extent that all the appropriate genes and enzymes are predicted (Simm et al., 2016). Microorganisms produce phytohormones (including IAA) influencing the growth and development of plants.

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Microbial IAA helps plants to produce the greatest number of root hairs resulting in increased nutrient absorption. The efficiency of these microbes for auxin production is used as a tool for the preference of potential plant growth promoters. Researchers have concentrated towards the efficient plant growth promoters for sustainable agricultural practices (Gupta et al., 2015). Evidently, IAA biosynthesis by microorganisms differs with the strains and external parameters such as soil type, soil physicochemical parameters, vegetation, etc (Spaepen and Vanderleyden, 2011). The actinomycetes are Gram-positive bacteria that are most widely distributed in nature. *Streptomyces* is the predominant actinomycete in soil and 60% of this genus provides potential secondary metabolites such as antimicrobial or plant growth promoting compounds of pharmaceutical and agricultural interest. Reports to date indicate that 80% of microorganisms of

various crop rhizospheres have the ability to produce auxins (Ahemad and Kibert, 2014). The global human population is growing and demanding for higher world agricultural production, resulting in the high usage of agrochemicals. However, the use of agrochemicals procures adequate crop yield, but their application causes definite drawbacks, including acidity and infertility of the soil, leading to the elimination of microorganisms. However, in the recent past, significant observations of plant growth promoting rhizosphere (PGPR) for the plant growth promotion were made to replace chemical fertilizers and pesticides (Batool and Althaf, 2017). Accordingly, coffee plantation soil is considered as the source of bioactive potential actinomycetes, therefore this soil was given the most important consideration for isolating IAA producing strains. Generally, for microbial IAA synthesis, root exudates, litter and crop residue are the organic source of tryptophan in the soil (Khamna *et al.*, 2010).

Therefore, the main reason for microbial specificity towards the plantation soil could be due to the abundant organic matter of soil extracted from high litter incorporation influencing the actinomycete populations (Bagyaraj *et al.*, 2015). The isolation of actinomycetes from plantation soils increases the chance for the discovery of the high auxins—IAA producers, helping in the maximum increase in the growth of plants. Actinomycetes produce indole-3-acetic acid stimulating plant growth by helping the growth of roots. They also produce siderophores which improve nutrient uptake. The actinomycete strains were isolated from the coffee plantation soils of Chikkamagalur district, India and their correlations with physicochemical parameters of soil was studied along with the determination of enzymatic potentials as described previously (Sameera *et al.*, 2018). In the present study, these strains were screened for the IAA producing activity by spectrophotometric method. The extract from strain exhibiting maximum IAA production was subjected to HPLC characterization.

MATERIALS AND METHODS

Isolation of Actinomycetes: Actinomycetes were isolated from coffee plantation soil collected from the tropical region of south India as described previously (Sameera *et al.*, 2018). Briefly, one gram of dry soil was taken in a 250 ml flask and 100 ml of sterile distilled water was added to it. Further, it was incubated at 28 °C on a rotary shaker at 120 rpm for one hour. The sample was allowed to settle, and then one ml sample was serially diluted upto 10⁻⁶. 0.1 ml of each of the diluted sample was plated on sterile starch casein agar (SCA) medium (composition: soluble starch: 10 g, K₂HPO₄: 2 g, KNO₃: 2 g, casein: 0.3 g, MgSO₄.7H₂O: 0.05 g, CaCO₃: 0.02 g, FeSO₄.7H₂O: 0.01 g, agar: 15 g, distilled water: 1000 ml and pH: 7.0±0.1) supplemented with cycloheximide (100 µg/ml), nystatin (100 µg/ml) and nalidixic acid (50 µg/ml) (Himedia[®], Mumbai, India) and incubated for 14 days at 28 °C. The discrete colonies were selected and streaked on sterile ISP2 (Himedia[®], India, 41 g/l) medium to obtain pure cultures. The individual colonies were observed for morphological characterization and identified by 16S rRNA gene sequence analysis.

Molecular characterization of actinomycete strains: The genomic DNA was extracted and the 16S rRNA amplification was performed using Genomic bacterial DNA isolation kit RKN 15. The concentration of DNA was measured by using

nanospectrophotometer (Thermofisher 2000C). Further, the DNA was amplified by PCR technique using PCR kit PCR 18 (Chromous Biotech[®] Pvt. Ltd., Bangalore, India) using a set of universal primers 27F and 1492R. The amplification conditions were set for an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min (denaturation), 58 °C for 2 min (annealing), 72 °C for 2 min (extension) and final extension at 72 °C for 8 min. The PCR samples were sent to the Chromous Biotech[®] Pvt. Ltd., Bangalore, India for sequencing. Accordingly, the alignment of sequences was done for the similarity and homology against the reference sequences using the BLAST[®] algorithm submitted to the NCBI GenBank submission portal to obtain accession numbers (Akshatha *et al.*, 2014).

Based on molecular characterization, of 32 actinomycete strains, 24 were reported in the previous study (Sameera *et al.*, 2018). The remaining eight isolates were characterized in the present study. The morphology of selected strain was studied by using scanning electron microscopy. For SEM analysis, the strain was allowed for fixation followed by dehydration by passing through alcohol in ascending order series upto 100%. The sample was mounted on stubs, splutter-coated with gold and scanned at 5.00 kV in scanning electron microscope (Carl Zeiss, Germany) and SEM image was obtained.

Screening of actinomycete strains for IAA production: The actinomycete strains were investigated for the production of IAA by following the method of Gordon and Weber (1951). The strains were streaked on ISP2 agar medium and incubated at 28 °C. After 5 days, the agar discs containing actinomycete mycelia were transferred to ISP2 broth (L-tryptophan 2 mg/ml, 0.2%), pH 7.0 and incubated (28 °C with shaking at 125 rpm, 7 days).

The mycelial broth was centrifuged (11,000 g rpm, 15 minutes) and the supernatant collected was used for the determination of the amount of IAA production by each strain. One ml of supernatant was added to two ml of Salkowski reagent [0.5 M of FeCl₃ in 35% HClO₄ in a proportion of 1:50 (v/v)] and kept in the dark for 30 minutes. The absorbance was measured spectrophotometrically at 530 nm (Beckman Coulter, DU 730 “Lifesciences”). The amount of IAA produced per milliliter culture broth was calculated on the basis of the the calibration curve of IAA obtained from standard IAA (Himedia[®], Mumbai, India) dissolved in acetone at different concentrations (50-250 µg/ml). The results were expressed as µg of IAA per ml of the extract.

Detection of IAA by Thin Layer Chromatography (TLC) High Performance Liquid Chromatography (HPLC): The crude IAA was extracted according to the method of Harikrishnan *et al.* (2014). The strains with maximum production of IAA were inoculated in ISP2 broth medium containing L-tryptophan, pH 7.0 and incubated (30 °C, 7 days) on a rotary shaker (125 rpm). The cultures were centrifuged (11,000 g rpm, 15 min, Thermofisher, Ligand XTR) and the supernatant was collected. The IAA was extracted three times from the supernatant using ethyl acetate AR-grade (1:1). IAA positive strains were further studied for the presence of indole acetic acid by a TLC-based method described by Harikrishnan *et al.* (2014). The ethyl acetate extract was spotted on a TLC plate (Silica gel coated with fluorescent indicator F254, size 20x20 cm, Merck, Mumbai, India) and developed using the mobile phase propanol and distilled water in the ratio of 8:2

(v/v). After development, the TLC plate was dried and sprayed with Salkowski reagent for the detection of IAA or indole compounds. The crude ethyl acetate extract of *S. violaceolatus* CMCS 016 was sent to Azyne Biosciences PVT LTD, Bangalore, India. The reverse phase analytical HPLC analysis of IAA was conducted using Shimadzu LC-8A (Shimadzu Corporation, Tokyo, Japan) HPLC fitted with C₁₈ column (5µm; 25 x 0.46cm, Kromasil, India). The mobile phase containing HPLC grade propanol and distilled water (8:2 v/v) was administered at a flow rate of 0.5 ml/min with UV detector (254 nm). The analysis was compared with the elution profiles of standard IAA introduced separately.

Statistical analysis: Data from this study is reported as the mean ± standard error of three independent replicate samples. Comparison of means for significance was analyzed with one-way ANOVA using Duncan's Multiple Range Test (P < 0.05).

RESULTS

Isolation of Actinomycetes: A total of 32 actinomycete strains were recovered from coffee plantation soils. The GenBank accession numbers with percent similarity of eight strains along with characteristics of colonies and spore morphology on ISP2 media and are represented in Table 1. Morphological and molecular studies revealed that 50% of the isolates belonged to the genus *Streptomyces* and 50% were non-streptomycetes namely *Actinomadura*, *Arthrobacter*, *Spirillospora*, *Actinocorallia*, *Sachharopolyspora*, *Nonomuraea*, *Gordonia*, *Micromonospora*, *Nocardia*, *Rhodococcus*, and *Pseudonocardia*.

Screening of strains for IAA production: Thirty two actinomycetes were isolated from the coffee plantation soil and screened for IAA production. 27 strains were able to produce IAA at a ranges of 17.36±0.6 to 109.24±0.2 µg/ml. 87.5% of *Streptomyces* spp. showed IAA production in the range of 24.29±1.3 to 109.24±0.2 µg/ml. Exceptionally, one strain, *Streptomyces violaceolatus* CMCS 016 (Fig. 1), produced the highest amount of IAA (109.24 µg/ml) among the tested strains. Next to streptomycetes, *Actinomadura* spp. was found to have a considerable amount of IAA (32.21±2.6 to 64.75±0.5 µg/ml) followed by *Arthrobacter* (73.54±6.0 µg/ml), *Nonomuraea* (53.46±1.3 µg/ml), *Spirillospora* (35.54±2.6 µg/ml), *Micromonospora* (32.26±0.7 µg/ml), *Nocardia* (22.31±0.7 µg/ml) and *Rhodococcus* (17.36±0.6 µg/ml). The results are depicted as mean ± standard error of three independent replicate samples (Fig. 2).

Detection of IAA by TLC HPLC: The IAA production was further confirmed by TLC as the R_f of the sample strains was found to be 0.9 corresponding against the standard IAA (Fig. 3). HPLC analysis revealed that the ethyl acetate extract of *S. violaceolatus* CMCS 016 contained one prominent peak of IAA with the similar retention time (2.4 minutes) with that of standard IAA. The area under the standard IAA peak was 100 % of the total peak area, while the IAA peak obtained from the culture extract of *S. violaceolatus* CMCS 016 was 32% of the total peak area. Based on contrast with the known standard IAA concentration, it was found that the strain *S. violaceolatus* CMCS 016 produced approximately 128.3 µg/ml of IAA. The sensitivity of IAA amount detected by HPLC analysis was greater than the amount detected with spectrophotometric assay. Fig. 4 shows the chromatogram of both the extract from *S. violaceolatus* CMCS 016 and standard IAA.

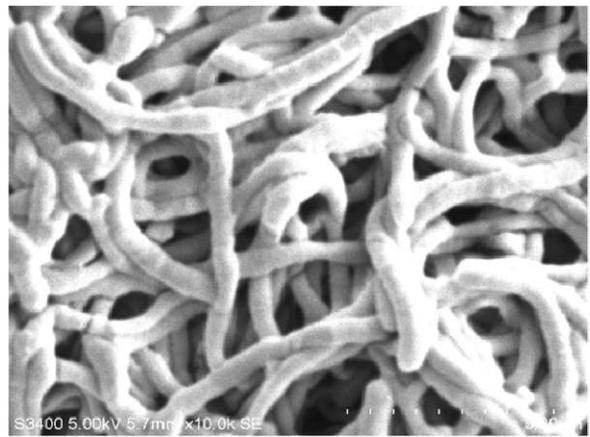


Fig. 1. Scanning electron microscopy image of *Streptomyces violaceolatus* CMCS 016 Screening of strains for IAA production

DISCUSSION

Actinomycetes constitute a significant microbial population in the soils and inhabit exorbitant proportion of soil microbial biomass. Mostly, they are found in all type of soils, rhizospheric and non-rhizospheric, in different habitats of the world. Generally, they have the ability to produce antibiotics, extracellular enzymes, bioactive compounds, plant growth hormones, and other secondary metabolites. The potentials of endophytic and rhizospheric actinomycetes to produce plant growth promoting substances (IAA) have long been known. Ahemad and Kibert (2014) recently reviewed the literature on the auxin production by rhizospheric actinomycetes. However, reports on the actinomycetes from bulk soil, producing IAA are widely dispersed.

In the present investigation, IAA producing abilities were studied by actinomycete strains from the non-rhizospheric fertile soil of coffee plantations. Generally, IAA, a major phytohormone, is considered the most significant native auxin. In recent times, the plant growth promoting activities have been studied from the endophytic actinomycetes (Vurukonda *et al.*, 2018) and actinomycetes from rhizospheric soils of medicinal plants (Khamna *et al.*, 2009), rice (Harikrishnan *et al.*, 2014), tomato, wheat (Anwar *et al.*, 2016) etc. Noticeably, in the present study, 80.5% of the strains were able to produce auxin ranging from 17.36±0.6 to 109.24±0.2 µg/ml in the presence of L-tryptophan (2 mg/ml). Correspondingly, 50.9% of the mangrove actinomycete isolates produced IAA in the range of 0.21 to 165.74 µg/ml (Suksaard *et al.*, 2017). However, 81 % of the isolates from the rhizospheric soil of medicinal plants produced IAA ranging from 5.47±0.7 to 143.95±0.2 µg/ml (Khamna *et al.*, 2009).

S. violaceolatus CMCS 016 isolated from the coffee plantation soil showed high ability to produce IAA (109.24±0.2 µg/ml). This study is the first report on the IAA production by *S. violaceolatus* isolated from plantation soils. Besides, production of IAA by *S. nobilis* WA-3, *S. kunmingensis* WC-3, and *S. enissocaesilis* TA-3 was recorded by Anwar *et al.* (2016) and in *S. atrovirens* by Ameer and Ghoul (2012). Additionally, *Actinomadura*, *Nocardia*, *Nonomuraea*, were reported to produce IAA (Khamna *et al.*, 2009). Accordingly, the culture filtrate of the most active isolate *S. violaceolatus* CMCS 016 was used to extract IAA for characterization by TLC and HPLC. TLC of culture extracts and standard IAA revealed the similar R_f values.

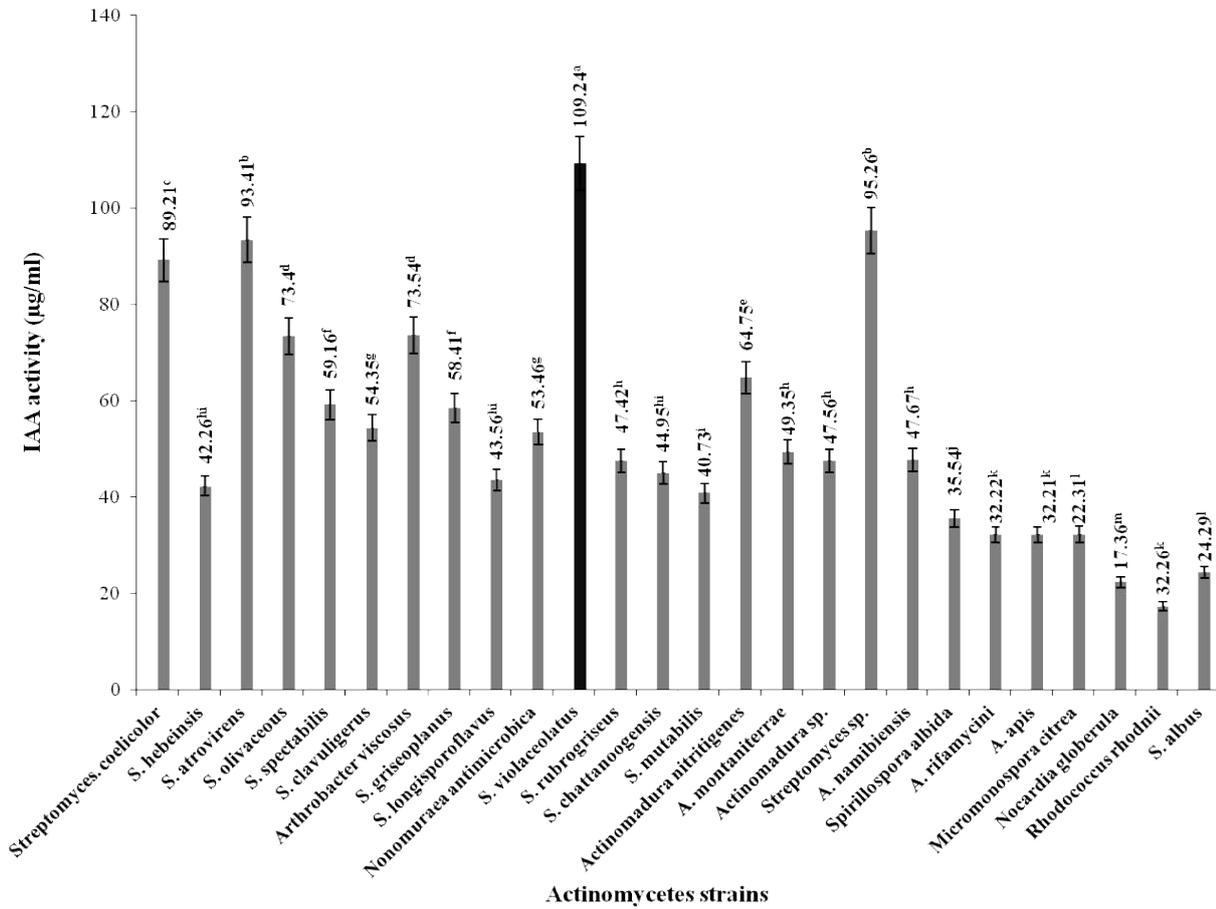


Fig. 2. Range of IAA production in the groups of Actinomycete strains

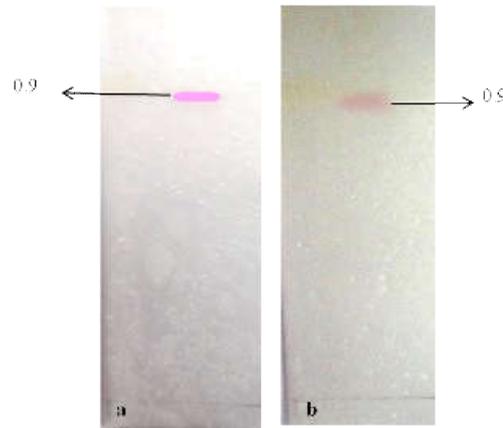


Fig. 3. Detection of IAA production by *S. violaceolatus* CMCS 016 in thin layer chromatography compared with standard IAA (a- standard IAA; b- IAA from *S. violaceolatus* CMCS 016 extract)

Table 1. GenBank accession numbers and colony characteristics of actinomycete strains from the coffee plantation soils

Isolate code	Actinomycete strain	Accession no.	Similarity (%)	Colony Characteristics*		
				Color	Pigments	Spore Characteristics
CMCS 025	<i>Gordonia terrae</i>	MK053897	100	Pink to orange	-	Rod-Coccus
CMCS 026	<i>Micromonospora citrea</i>	MK053898	100	Pastle orange	Yellow orange	Single spores
CMCS 027	<i>Nocardia globerula</i>	MK053899	100	Pink to red	-	Rod-Coccus
CMCS 028	<i>Rhodococcus rhodnii</i>	MK053900	100	Red	-	Rod-Coccus
CMCS 029	<i>Streptomyces coelicoflavus</i>	MK053901	100	Grey	-	Rod-Coccus
CMCS 030	<i>Streptomyces albus</i>	MK056266	100	White	-	Spirales
CMCS 031	<i>Streptomyces himastatinicus</i>	MK053910	100	Black	-	Oval to barrel-shaped spores in spiral chains
CMCS 032	<i>Pseudonocardia artemisiae</i>	MK053911	100	Brown	-	Rod shaped spores

* Based on the Bergey's manual of systematic bacteriology (Goodfellow et al. 2012)

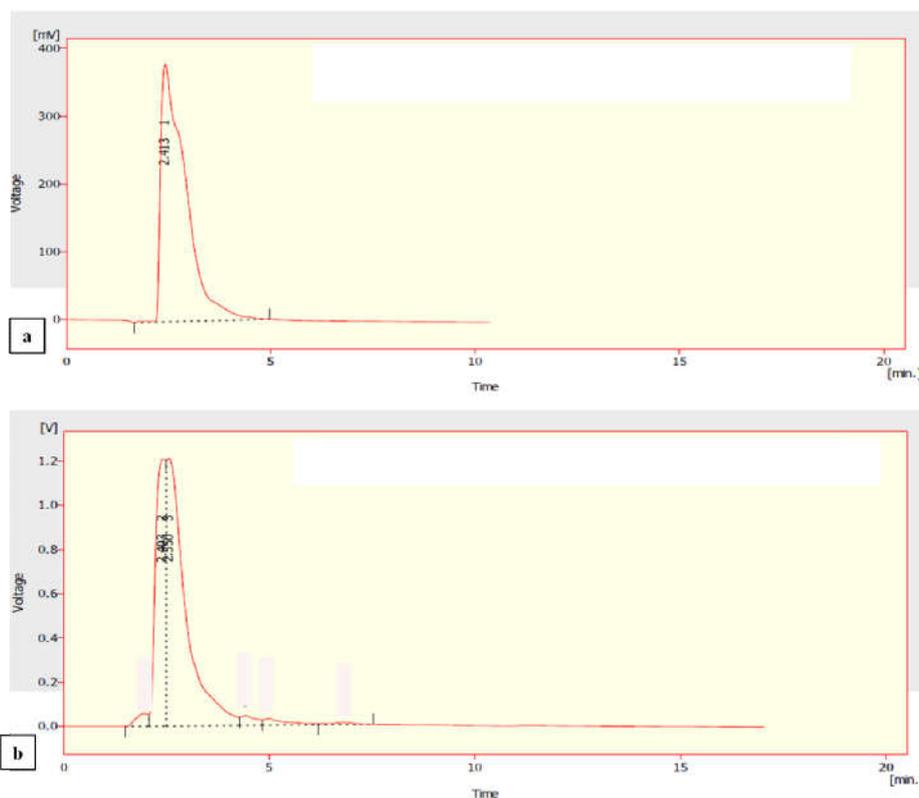


Fig. 4. HPLC profile of IAA production by *S. violaceolatus* compared with the standard IAA (a- standard IAA; b- IAA from *S. violaceolatus* CMCS 016 extract)

The TLC findings are in agreement with other reports (Abd-alla *et al.*, 2013; Harikrishnan *et al.*, 2014). Similarly, the chromatograms of the extract and standard IAA obtained from HPLC showed peaks at the same retention time (2.4 RT). Our results of HPLC analysis are consistent with the previous findings of (Ameur and Ghoul, 2012; Harikrishnan *et al.*, 2014). These studies indicated that the HPLC chromatogram of standard IAA and the fermentation broth showed major peaks at retention time 17.5 minutes and 24.9 minutes (respectively). The studies reported that the biosynthesis of IAA by plant growth promoters vary among various species in addition to the culture conditions and substrate availability (Abd-alla *et al.*, 2013). Pant and Agarwal (2014) reported that the actinomycetes isolated from rhizosphere are more effective than isolates from the bulk soil. Conversely, the present study confirms that the strains from non-rhizospheric soil are also equal efficient auxin producers compared to rhizospheric soil strains. This is facilitated by the nutrient-rich soils of coffee plantation containing litter from both the tree species and the coffee plants that favors the growth of soil actinomycetes.

Conclusion

From the present study, it has been demonstrated that, coffee plantation soils harbour rich actinomycete diversity. *Streptomyces* spp. have the ability to produce high amounts of IAA. However, investigation on seed germination assay is required to reveal the potential of these organisms in plant growth promotion which may be useful in sustainable agricultural practices.

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

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