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Growth stimulatory effect of AHL producing *Serratia* spp. from potato on homologous and non-homologous host plants

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ABSTRACT

Plant growth promoting rhizobacteria are known to improve plant performance by developing healthy and productive interactions with the host plants. These associations may be symbiotic or asymbiotic depending upon the genetic potential of the resident microbe and promiscuity of the host. Present study describes the potential of two *Serratia* spp. strains for promotion of plant growth in homologous as well as non-homologous hosts. The strains KPS-10 and KPS-14; native to potato rhizosphere belong to genus *Serratia* based on 16S rRNA gene sequences (accession no. LN831934 and LN831937 respectively) and contain multiple plant growth promoting properties along-with the production of quorum sensing acyl homoserine lactone (AHL) molecules. Both *Serratia* spp. strains showed solubilization of inorganic tri-calcium phosphate while KPS-14 also exhibited phytase activity (1.98 10^{-10} kcat). KPS-10 showed higher P-solubilization activity (128.5 µg/mL), IAA production (8.84 µg/mL), antifungal activity and also showed the production of two organic acids *i.e.*, gluconic acid and lactic acid. Both strains produced three common AHLs: C6-HSL, 3oxo-C10-HSL, 3oxo-C12-HSL while some strain-specific AHLs (3OH-C5-HSL, 3OH-C6-HSL, C10-HSL specific to KPS-10 and 3OH-C6-HSL, C8-HSL, 3oxo-C9-HSL, 3OH-C9-HSL specific to KPS-14). Strains showed roots and rhizosphere colonization of potato and other non-homologous hosts up to one month. *In planta* AHLs-detection confirmed a likely role of AHLs during seedling growth and development where both extracted AHLs or bacteria inoculated roots showed extensive root hair. A significant increase in root/shoot lengths, root/ shoot fresh weights, root/shoot dry weights was observed by inoculation in different hosts. PGP-characteristics along with the AHLs-production signify the potential of both strains as candidate for the development of bio-inoculum for potato crop in specific and other crops in general. This inoculum will not only reduce the input of chemical fertilizer to the environment but also improve soil quality and plant growth.

1. Introduction

Plant rhizosphere is a highly dynamic niche for different interactions and plays important role in the establishment of harmful and beneficial plant-bacteria associations. Bacteria are the main colonizers of rhizosphere which rapidly colonize plant roots at different growth stages in the presence of competing microflora (Antoun and Kloepper, 2001). They affect plant growth and health due to their innate genetic potential and rhizosphere competence and develop a core microbiome in the rhizosphere of each plant. The bacteria within these core rhizosphere microbiome(s) positively influence plant productivity and health and are generally known as 'Plant Growth Promoting Rhizobacteria' (PGPR) (Sindhu et al., 2014; Martínez-Viveros et al., 2010). Various direct and indirect processes that influence plant growth include N_2-fixation, phosphorus solubilization, siderophores and phytohormone (gibberellins, auxins and cytokinins) production (Wani et al., 2007;

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Khan et al., 2009), and biocontrol of pathogens either by induction of systemic resistance, or production of antifungal metabolites and antibiotics (Ahmad, 2014). Most of the PGPR which promote plant growth live in direct association with the host plant. In certain cases, the associations are loose and can be manipulated while in other cases, the associations are highly specific and complicated.

All interactions whether symbiotic or asymbotic, are conceded by specific signal molecules which are the key determinants of the specificity at different levels of host-microbe interactions. *N*-acyl homoserine lactone (AHL) are signal molecules produced by Gram negative bacteria to monitor and detect their own population densities in a process known as quorum sensing (QS). This AHL-based QS system controls various important pathogenic and beneficial mechanisms (Deep et al., 2011) including the resistance of bacteria to oxidative, osmotic, thermal and heavy metal stress (García-Contreras et al., 2015), rhizosphere competence and adaptation to plant roots (Boyer et al., 2008; Wisniewski-Dyé et al., 2015), biocontrol and colonization ability on plant roots (Wei and Zhang, 2006), beneficial interactions (Zúñiga et al., 2013), regulation of PGP traits (Steindler and Venturi, 2007), biofilm formation (Cerqueira, 2015), root nodule formation and induction of systemic resistance (Schuhegger et al., 2006) in various plants. Up-regulation or down-regulation of QS-dependent genes in bacteria can modulate the plant response and affect directly or indirectly its overall growth or development.

Potato (*Solanum tuberosum* L.) is the major non-grain staple food used in many parts of the world (Calvo et al., 2010; Hanif et al., 2015). It contains most of the vitamins and minerals that are essentially required by human body. Potato produces highest dry matter per unit area and time as compared to other field crops which is the major reason behind the increased interest of scientists to utilize potato to ensure food security in coming years. The crop requires high fertilizer application which increases the cost of crop production. Furthermore, the excessive fertilizers degrade the environment and a major source of nitrogen (N) and phosphours (P) pollution in the soil and water. We hypothesized that the AHLs-producing bacteria may positively influence plant growth in general and potato in specific. This study was, therefore, conducted to evaluate the potential of AHL-producing potato rhizosphere bacteria with other plant growth promoting abilities for the growth of potato. We identified a number of plant growth promoting bacteria having multiple traits, of which, two bacteria were able to produce AHL molecules. The involvement of AHLs produced by these bacteria for plant growth was investigated. The ultimate objective was to select potential PGPR for the development of potato biofertilizer for sustainable potato production.

2. Material and methods

2.1. Bacterial isolation and identification

Rhizosphere soil samples were collected from potato plants growing in the field located in District Sheikhupura, Pakistan (42.40 °N, 59.16 °E). Bacteria were isolated from one gram of soil using standard serial dilution plating technique (Somasegaran and Hoben, 1994) on to LB agar medium. Two different bacterial morphotypes KPS-10 and KPS-14 obtained on LB agar plates, were selected, characterized and analyzed in details.

Genomic DNA was extracted using genomic DNA purification kit (Invitrogen). The 16S rRNA gene was amplified using universal primers as previously described (Hanif et al., 2015; Naqqash et al., 2016). Amplified PCR products were sequenced by Macrogen (Seoul, South Korea) and accession numbers were obtained by submitting the sequences in NCBI GenBank.

2.2. Phenotypic microarray

The metabolic potential of bacteria was evaluated through GENIII MicroPlate phenotypic microarray (Biology, USA). Freshly grown bacterial culture were suspended in 'Inoculation Fluid' (IF), provided with MicroPlate panel. After achieving the desired cell density, suspension (100 µL) was inoculated to the GENIII MicroPlate which analyzed the bacterial ability of utilizing 71 different carbon and 23 chemical sensitivity assays using multichannel pipette. The plate was incubated for 48 h at 28 ± 2 °C to form the finger print and then analyzed qualitatively and quantitatively on VERSA max micro-plate reader (Molecular Devices, USA) with softmaxpro-software (Line et al., 2011).

2.3. P-solubilization and mineralization potential of bacteria

Pre-grown bacterial strains were spot-inoculated onto Pikovskaya's agar plates (Pikovskaya, 1948) and incubated at 28 ± 2 °C for 5–7 days. The formation of halo zone around bacterial colonies was considered as the potential of the bacterial strain to solubilize insoluble tri-calcium phosphate present in Pikovskaya's medium. For quantification, bacterial strains were inoculated in 100 mL Pikovskaya's broth in triplicate and grown in an orbital shaker for 10 days at 150 rpm at 28 ± 2 °C. From each replicate 20 mL culture was harvested at 5, 7 and 10 days post inoculation and cell-free supernatant was collected by centrifugation at 13,000 × *g* for 10 min. P-solubilization was determined through Phospho molybdate blue color method as described by Murphy and Riley (1962). For HPLC analysis, the cell-free supernatant was filtered and then analyzed at 210 nm on HPLC equipped with Turbochrom software (Perkin Elmer, USA) and C-18 column using 30:1:70 (v/v/v) methanol: acetic acid: water as mobile phase (Shahid et al., 2012) against the following organic acids standards; gluconic, malic, lactic, oxalic, tartaric and ascorbic acid (Sigma–Aldrich).

Phytase activity was determined by inoculation of bacteria on phytase-screening medium agar plates and incubation at 28 ± 2 °C for 48 h. Phytase activity was quantified using the ferrous sulfate- molybdenum blue method (Huang et al., 2006) as described in detail previously (Hanif et al., 2015).

Genomic DNA was used to amplify *pqq*E gene (900 bp) which encodes pyrroloquinoline quinine, a co-factor for glucose dehydrogenase enzyme by using primers set pqqF (5́-GARCTGACYTAYCGCTGYCC-3́) and pqqR2 (5́-TSAGSAKRARSGCCTGRCA-3́) (Perez et al., 2007). The amplicons were purified by using Wizard® SV Gel and PCR Clean-up System (Promega) sequenced commercially from LGC Genomics, Germany. The sequence was analyzed by using "sequence scanner" software package and phylogenetic relationship was determined by MEGA6 software.

2.4. Production of indole-3-acetic acid (IAA) and antifungal activity

Single colonies of each bacterial strain were inoculated to 100 mL LB broth in flasks containing L-tryptophan (100 mg L^{-1}) as precursor of IAA biosynthesis. Cultures were grown at 28 ± 2 °C for 48 h with continuous shaking (150 rpm). Supernatant was collected by centrifugation at 13,000 *g* for 10 min and mixed with 2 drops of O-phosphoric acid and 4 mL of Salkowski's reagent. Reaction mixture was incubated at room temperature for 20 min and observed for the development of pink color. For quantification, culture supernatant was acidified (pH 2.8) and extracted with equal volume of ethyl acetate. The extract was evaporated to dryness, collected in methanol and then filtered with 0.22 µm nylon filter. Purified extract was analyzed on high performance liquid chromatography (HPLC; Varian pro-star) at λ = 260 nm and C-18 column as mobile phase using methanol: acetic acid: water (30:1:70 v/v/v) at a flow rate of 1 mL min^{-1} as described (Tien et al., 1979).

Antifungal potential was checked against broad-host range pathogenic fungus *Fusarium oxysporum* on Potato dextrose agar (PDA) as described in detail Ali et al., 2020 using a 6 mm fungal disc placed in the center of PDA plates. Bacterial strains were streaked around the four corners of the disc. Plates were incubated at room temperature for 7

days and antifungal activity was checked by measuring the growth inhibition zone between bacteria and pathogenic fungus as compared to control (fungus without any bacterial strain).

2.5. Reverse phase thin layer chromatography and tandem mass spectrometry analysis of AHL

The reporter/indicator strain *Chromobacterium violaceum* (CV026) and reference strain *Rhizobium leguminosarum* A34 containing plasmid (pRL1J1) were previously obtained from Dr. Allen J. Downie Lab at Johns Inns Centre (UK). Both strains were maintained on LB and TY media supplemented with kanamycin (25 μg/mL) and incubated at 28 ± 2 °C for 48 h with continuous shaking at 150 rpm. AHLs were detected by overlay assay (McLean et al., 2004). Briefly, 100 μL of overnight grown test strain was spread on LB agar plate. The indicator strain CV026 grown individually in TY media was mixed with molten semi solid LB-agar (0.7 %) and spread over the test strain. The agar plates were kept untouched until the agar solidified and then incubated at 28 ± 2 °C for 24 h. Production of AHL was confirmed by the development of purple colour on the plate. The reference strain *R. leguminosarum* A34 containing plasmid (pRL1J1) was used as positive control.

Bacterial strains were grown at 28 ± 2 °C for 5 days in LB at continuous shaking. Cell free supernatant was collected by centrifugation. The AHLs were extracted from spent supernatant using ethyl acetate as described in detail earlier (Ali et al., 2016) and confirmed by reverse phase thin layer chromatography (RP-TLC) using C18 reverse-phase plates (Merck) as described previously (Imran et al., 2014).

Before mass spectrometry analysis, AHL extracts were purified by solid phase extraction (Li et al., 2006) and run on LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Scientific, USA) equipped with an ESI source for the complete profiling of AHLs employing the conditions described in detail previously (Ali et al., 2016). The standards used in the analysis were 3oxoC10-HSL and C12-HSL (Sigma). The ESI-mass spectra obtained were used to monitored $[M+H]^+$, $[M + H_2O]^+$ and $[M + Na]^+$ ions for AHLs as described previously (Ali et al., 2014). AHLs were identified by finger prints of the daughter ion peaks by conducting the MS/MS of the isolated peaks. The structures of AHLs were drawn using ChemBio DrawUltra 12.0.

2.6. In vitro and in vivo inoculation assays

The homologous host potato (variety Kuruda) and four non-homologous hosts; rice (var. Super Basmati), wheat (var. Sehar-06), rapeseed (var. Abaseen-95), soybean (cv. Fs-85) and maize (var. Sahiwal-2002) were used in these experiments. Wheat, rice and maize are major agricultural crops grown in maximum area in Pakistan while soybean and rapeseed are minor crops grown in small patches.

2.7. Exp.1: potato inoculation response under P-deficient condition

As the strains were isolated from potato, analyses were carried out to see the potential of both strains for potato root colonization, rhizosphere survival and plant growth. Sand was sterilized by soaking in 0.5 N nitric acid for 24 h, washed thoroughly to remove the acid, then air-dried and autoclaved. No source of nutrients (*e.g.*, fertilizer) was added in sand except Tri-calcium phosphate (TCP) (300 mg/kg sand). The TCP was added as insoluble inorganic phosphorus (P) source to see the potential of inoculated bacteria for its solubilization *in vivo*.

Potato tubers (2 − 3 cm) were surface sterilized with sodium hypochlorite (10 % v/v) for 5 − 8 min followed by extensive washing with sterilized water. Bacteria were grown in 100 mL LB for 24 h at 28 ± 2 °C. After growth, cells were harvested by centrifugation at 4000 × *g* for 15 min and room temperature and resuspended in saline (0.89 % w/v NaCl). Single tubers, with more than two eye buds, were dipped into inoculum individually (10^9 CFU/mL) for 15 min and then placed in pots containing 600 g of sand.

There were two bacterial inoculation treatments along-with one non-inoculated control treatment; all replicated four times. The pots were placed in growth room at 16/8 h light/dark and 20/8 °C day/night temperature each pot was irrigated with autoclaved water and Hoagland's solution (Arnon and Hoagland, 1940) without P source in order to induce P-deficient conditions. 2nd inoculation was done at 15 days after seedling emergence by mixing the inocula with Hoagland solution at the rate of 2 mL per plant. Data regarding growth parameters (including root and shoot length and fresh and dry weights) was recorded after 60 days of sowing.

The rhizosphere survival of inoculated bacteria was analyzed by recovering the cells on LB plates. Roots of the potato plants were carefully removed, gently shaken in sterilized distilled water to remove the loosely adhered sand. One gram of tightly root-adhered sand was used to count bacterial population attached to the roots using standard dilution plating technique (Somasegaran and Hoben, 1994).

2.8. Ultrastructure analysis of inoculated potato roots

Root colonization at ultra-structure level was analyzed by using transmission electron microscope (TEM). Potato plants roots after 45 days of germination were carefully uprooted, washed with sterile water and cut into 1 − 3 cm pieces. Embedding was done in water agar to cut 2 − 3 mm^3 small agar cubes. Sample fixation in (5 % gluteraldehyde), dehydration and polymerization were carried out as described in detail previously (Naqqash et al., 2016). Ultrathin sections cut with ultramicrotome (RMC-700; Boeckeler Instruments, USA) were observed under transmission electron microscope (JEOL, USA) for root colonization analysis.

2.9. Exp.2: Confocal microscopy analysis for root colonization

A broad host range plasmid PBBRMCS-1 encoding amp^R and harboring a yellow fluorescent protein gene (*yfp*) was used to transform both strains KPS-10 and KPS-14 (Wu et al., 2010, Shahid et al., 2012). Transformants were screened on LB agar containing ampicillin (50 μg/mL) and confirmed under confocal laser scanning microscope (CLSM, Olympus FluoView Ver.1.3) at 480 nm. The yfp-tagged strains were named as KPS-10^{yfp} and KPS-14^{yfp}.

Seeds of both homologous and non-homologous hosts were surface sterilized with sodium hypochlorite (10 % v/v) for 10 min, washed 4–5 times with sterile distilled water. Sterilized seeds were then imbibed in sterile water for 2 − 4 hours and germinated in the dark on Whatman on water agar at 25 ± 2 °C. KPS-10^{yfp} and KPS-14^{yfp} inoculum was grown ($\approx 10^8$ CFU mL^{-1}) in LB-ampicilln, cells were harvested at 8000 × *g* and re-suspended in saline (0.85 %). Three days old seedlings were inoculated with 1 mL each of KPS-10^{yfp} and KPS-14^{yfp} and grown at day/night temperature 25/20 °C, light dark periods 14/10 h. Only non-contaminated seedlings were processed for root colonization studies. Colonization of 15-days old inoculated roots was analyzed under confocal laser scanning microscope (CLSM, Olympus Fluoview Ver.1.3) as described previously (Shahid et al., 2012). The experiment was carried out with three replicate for each treatment along-with non-inoculated control seedlings as control.

2.10. Exp.3: Effect of purified AHLs on non-homologous host

Purified AHLs were checked for plant growth response on water agar medium. The AHLs were mixed in cool sterile water agar (@2 μL/mL) and poured in the plates till the medium solidified. The sterilized seeds were germinated on the plates and let them grow under controlled conditions for 7 days.

2.11. Exp.4: Inoculation response on non-homologous hosts under axenic conditions

Aseptically germinated seedlings on water agar were inoculated with wild type KPS-10 and KPS-14 strains using conditions and hosts as described above. After inoculation, seedlings were grown at day/night temperature 25/20 °C, light dark periods 14/10 h up to 7 days and observed for following traits:

2.12. Root hair development and bacteria attached to roots

After 7 days roots were observed under light microscope (Leica DMLS). Root hairs of inoculated seedlings were compared to control (un-inoculated seeds) and photographic images were recorded. The number of CFU attached to each host was calculated per gram fresh weight of root using standard serial dilution plating technique (Somasegaran and Hoben, 1994).

2.13. In planta detection of AHLs

For *in planta* analysis of AHL production, seedlings were analyzed through overlay assay described previously by (McClean et al., 1997) and modified in this study to detect AHLs production in the vicinity of growing root. In this assay, the pre-inoculated germinated seedlings were aseptically placed onto LB-plates and overlaid with the over-night grown culture of indicator strain (mixed in 0.7 % w/v LB agar). The non-inoculated roots were kept as control. The plates were kept at 28 ± 2 °C for overnight growth and the production of AHLs was confirmed by the development of blue color on the growing root.

Seedlings were observed for root length, shoot length, shoot and root fresh and dry weights after 7 days and data was recorded.

2.14. Statistical analysis

Data for pot experiment was analyzed statistically by analysis of variance (ANOVA) technique, using the Statistix (version 8.1) software, and least significant difference test (Fisher LSD) at 5 % probability was used to compare the differences among treatment means.

3. Results

3.1. Strains identification, characterization and in vitro assays for plant beneficial traits

Bacterial isolate KPS-10 formed round, dark-red colonies while KPS-14 formed white-colored colonies with irregular margins on LB agar medium. Cells of both isolates were motile and rod-shaped. Sequence analyses of 16S rRNA gene of bacterial isolate KPS-10 (accession no. LN831934) showed 99.26 % while KPS-14 (accession no LN831937) showed 99 % similarity to the same type strains of *Serratia plymuthica* strains NCTC 12961 (LS483469.1) and NBRC 102599 (NR-114158.1). Hence both isolates were designated at *Serratia* spp. Phylogenetic analysis of both bacterial strains further confirmed their evolutionary relationships with their respective relevant in Genbank data base (Supplementary Fig. 1).

Both strain KPS-10 and KPS-14 were metabolically diverse as they utilized 47 and 44 carbon sources out of 71 and showed resistance against 19 chemicals respectively in Biolog GEN III microplate assay.

Both *Serratia* spp. strains KPS-10 and KPS-14 solubilized inorganic phosphate forming halo zones on tri-calcium containing Pikovskaya's agar plates yielding solubilization index 2.71 and 2.96 respectively (Fig. 1A). Quantification analysis showed that P-solubilization ability of KPS-10 was significantly higher (128.53 ± 16.67 μg ml^{-1}) than KPS-14 (48.61 ± 3.50 μg ml^{-1}). HPLC analyses of cell-free supernatant of bacterial strains revealed that both KPS-10 and KPS-14 produced gluconic acid (2.99 ± 0.99 and 7.23 ± 1.39 μg ml^{-1} respectively). Lactic acid (16.74 ± 2.32 μg ml^{-1}) was detected only in culture supernatant of *S. plymuthica* strain KPS-10. Phytase activity was positive for only one bacterial isolate KPS-14 (Fig. 1B) with a P-mineralization index of 1.34 ± 0.128 and phytase activity of 1.98 10^{-10} kcat.

Analysis of *pqq*E gene sequence of *S. plymuthica* KPS-10 strain showed 96.8 % similarity with *Serratia* sp. KPS-8 (accession No LN998254) and 94 % similarity with *S, plymuthica* strain (accession No CP015613) *pqq*E gene (Supplementary Fig. 2).

Of two, only KPS-10 showed production of IAA as well as antifungal activity against *F. oxysporium* (Fig. 1C). The IAA production was recorded up to 8.84 ± 1.55 μg ml^{-1}.

3.2. AHLs assay and ESI-MS/MS analysis

Both strains produced purple color on LB-medium when overlaid with biosensor strain CV026 indicating the production of AHLs (Fig. 2A). Purified AHLs from cell free supernatant confirmed by plate overlay assay (Fig. 2 B) were run on RP-TLC. RP-TLC of AHLs also showed positive result with CV026 by producing violacein (purple color pigment) (Fig. 2C) and showed the presence of different AHLs. Comparison was done with *Rhizobium* sp. A34 containing PRL1J1 as reference for AHLs.

The ESI-MS/MS analysis of AHLs (Fig. 3A) showed that KPS-14 produced C6-HSL, 3OHC6-HSL, C8-HSL, 3oxoC9-HSL, 3OHC9-HSL 3oxoC10-HSL and 3oxoC12-HSL (Fig. 3A, Table 1).While in case of KPS-10 the identified AHLs included;C6-HSL, 3OHC5-HSL, 3OHC6-HSL, C10-HSL, 3oxoC10-HSL and 3oxoC12-HSL(Fig. 3B, Table 1). In KPS-10 high amount of 3oxoC10-HSL + H_2O observed while in KPS-14 3OHC9-HSL produce in high amount and absent in KPS-10. Structures of these AHLs were confirmed by MS/MS analysis; the daughter ions produced are either the loss of CO, HCOH, H_2O, O, ethylene side chains or the hydrolysis of amide linkage. Most of the daughter ions produced were assigned their degradation schemes are drawn as illustrated (Fig. 3C) and representing the tandem MS of *m/z* 288 $[M+H]^+$ of 3oxo-C10-HSL + H_2O.

3.3. In vitro and in vivo plant inoculation experiments

3.3.1. Exp.1: Inoculation response and rhizosphere survival in potato under P-deficient condition

In potato rhizosphere, the population density of both bacterial strains changed differently after inoculation depending upon the plant growth stage. Up to 20 days after inoculation (DAI) *S. plymuthica* KPS-10 maintained reasonably higher (7.24 log CFU g^{-1} of rhizosphere sand) population density when compared with *Serratia* sp. KPS-14 (5.10 log CFU g^{-1} of rhizosphere sand). Both *S. plymuthica* KPS-10 and *Serratia* sp. KPS-14 showed a gradual decline in population density till 60 days post inoculation. After 60 days post inoculation, the population densities recorded was 3.64 and 3.67 log CFU g^{-1} of rhizosphere sand respectively, for KPS-10 and KPS-14.

Observations regarding effect of bacterial inoculation on potato growth parameters showed that the inoculated plants performed significantly well under P-deficient conditions. Percent increase of 14 and 30 was observed in KPS-10 and KPS-14 inoculated plants. In case of shoot length, percent increase was 70 and 47 in KPS-10 and KPS-14 inoculated plants. Percentage increase for shoot fresh weight was more than 200 in both inoculated plants over control (Fig. 4A). Similarly, root and shoot fresh as well as dry weights were significantly higher in treated plants (Fig. 4B).

3.4. Ultrastructural studies

Root colonization of both bacterial strains with potato roots was authenticated by ultrastructure studies using TEM. Photomicrographs showed presence of bacterial cells in potato root hair folding, root hair polysaccharidal material and rhizosphere (Fig. 5).

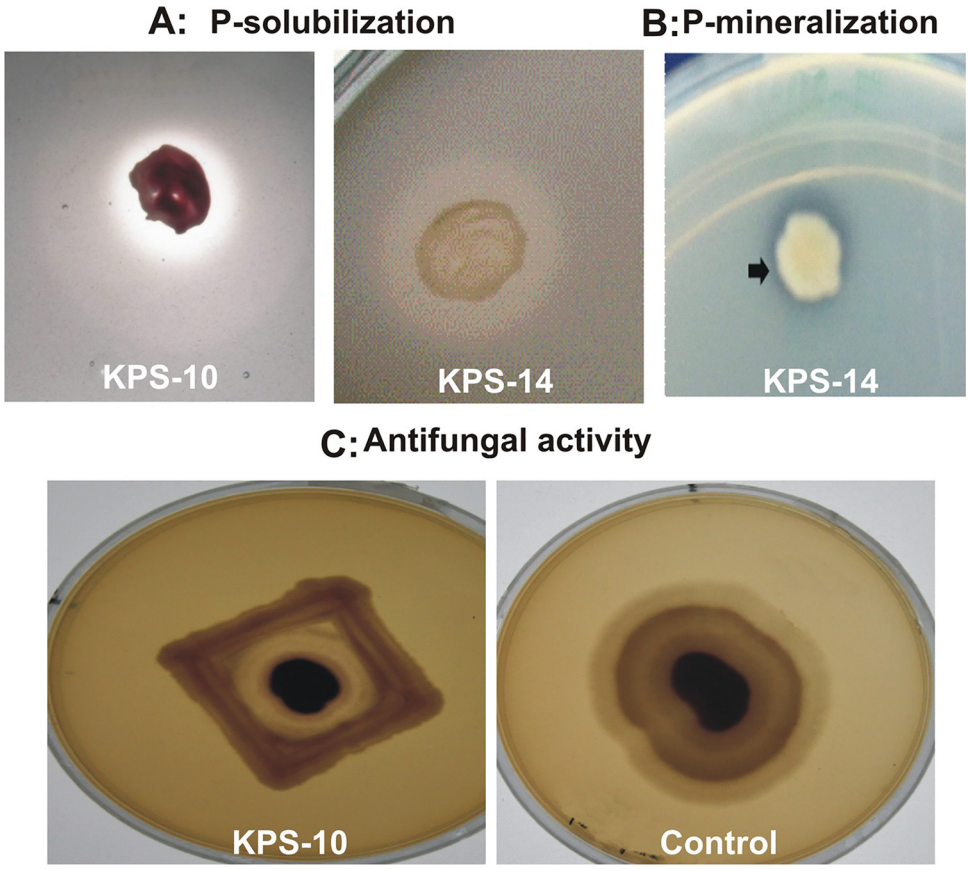

Fig. 1. plate assays of *Serratia* spp. KPS-10 and KPS-14; showing solubilization of tri-calcium Phosphate (A), phytate activity (B) and antifungal activity (C) against *Fusarium oxysporum*.

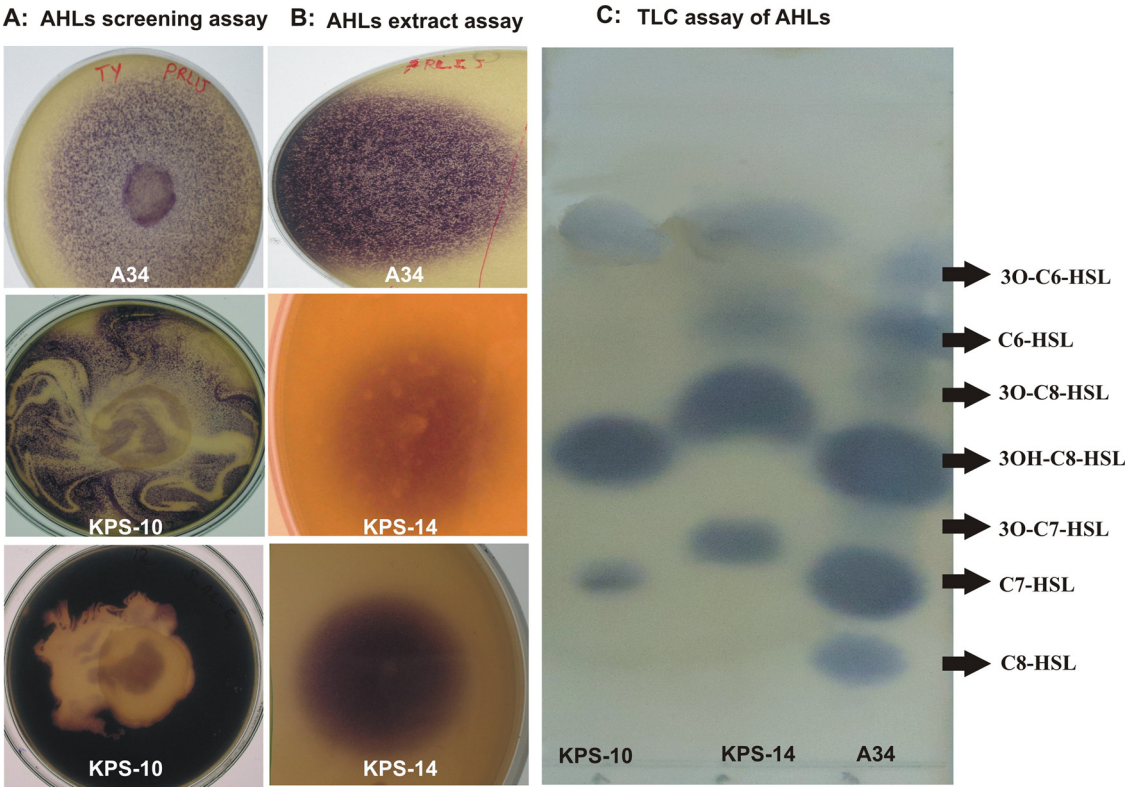

Fig. 2. Plate assay for detection of AHLs using biosensor strain *Chromobacterium violaceum* CV026 (A) and confirmation of extracted and purified AHL through plate assay (B) and profiling of the AHLs through thin layer chromatography (C).

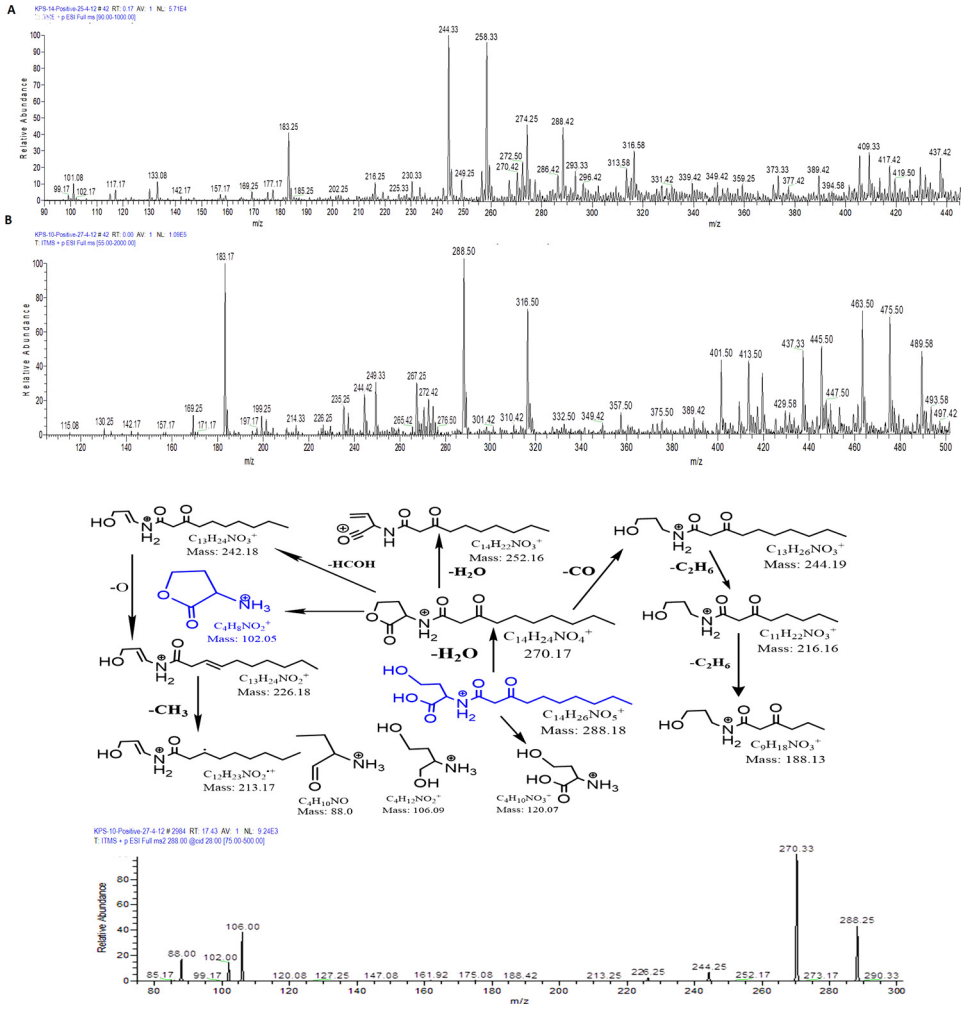

Fig. 3. (A,B): Mass spectrometry chromatogram (Full MS) with peaks at $[M+H]^+$, $[M+H+H_2O]^+$ and $[M + Na]^+$ of AHLs extracted from strain KPS-14 (A) and KPS-10 (B). Protonated AHLs $[M+H]^+$ peaks were at very low intensity. Peaks were identified as $[M+H+H_2O]^+$ and $[M + Na]^+$ for the presence of AHLs by adding water molecule or formation of sodium adducts. C: Fragmentation of AHLs 3oxoC10-HSL *m/z* 288 All daughter ions generated from the fragmentation of *m/z* 288 are unambiguously assigned (above) and Mass spectrometry chromatogram (Full MS) with peaks at $[M+H]^+$, [M+H+H2O]+ and $[M + Na]^+$ of AHLs extracted from strain KPS-10 (below). Protonated AHLs $[M+H]^+$ peaks were at very low intensity. Peaks were identified as $[M+H+H2O]^+$ and $[M + Na]^+$ for the presence of AHLs by adding water molecule or formation of sodium adducts.

3.5. Exp.2: Confocal microscopy analysis for root colonization

The individual bacteria inoculated into LB show bacterial attachment to the glass surface that show the potential of these bacteria to develop biofilm (Fig. 6A–D).

Confocal laser scanning microscopic analyses of inoculated potato, rice, wheat, maize and rapeseed roots after 15 days of inoculation showed the presence of bacterial aggregates on root hair and the junctions between the primary and the lateral root surface, which indicated the strong colonization potential of both strains on different hosts (Figs. 6 and 7).

In potato roots, inoculated with KPS-10, bacteria were attached to the entire root surface forming a biofilm like structure while in roots inoculated with KPS-14, the bacteria were tightly associated onto the root hair and also found scattered in the rhizosphere. The non-inoculated control plants were totally devoid of any bacteria attached on the roots (Fig. 6 E,F,G).

In non-homologous host, both strains showed significant colonization potential forming tight associated bacteria onto the root surface (Fig. 7A, B, D and E). Bacteria were mainly colonized to the surface of primary root, root hairs and root tip, the zone of elongation and differentiation. In case of maize, soybean and rapeseed the bacteria show very little colonization. Very few bacteria were found associated to the roots of these plants (Fig. 7 C,F).

Table 1
LC–MS/MS analysis of AHLs in spent culture supernatant of KPS-14 and KPS-10.

AHLs $[M+H]^+$	Relative abundance of isolated ions*		Daughter ions
	KPS-14	KPS-10	
C6-HSL 200	+	+	182,172,158,146,118102,88,
3OHC5-HSL 202	–	+	184,174,159,146,123,119,102
3OHC6-HSL 216	+ +	+ +	198.173,159,132,115,102,84
3oxoC9-HSL 256	+ +	–	238,228,214,200,185,172,159,102,
C10-HSL 256	–	+	238,228,192,172,102.88
3OHC9-HSL 258	+ + +	–	240,130,200,186,172,159,102
3oxoC10-HSL 270	+ +	+ +	252,226,200,185,172,159,102,88
3oxoC10-HSL + H2O 288	+ +	+ + +	270,252,244,226,213,188,120,106,88
3oxoC12-HSL 298	+	+	280,270,256,242,228,200,186,180,172,159,102,88

*Relative abundance of isolated ions in values of E1-3.

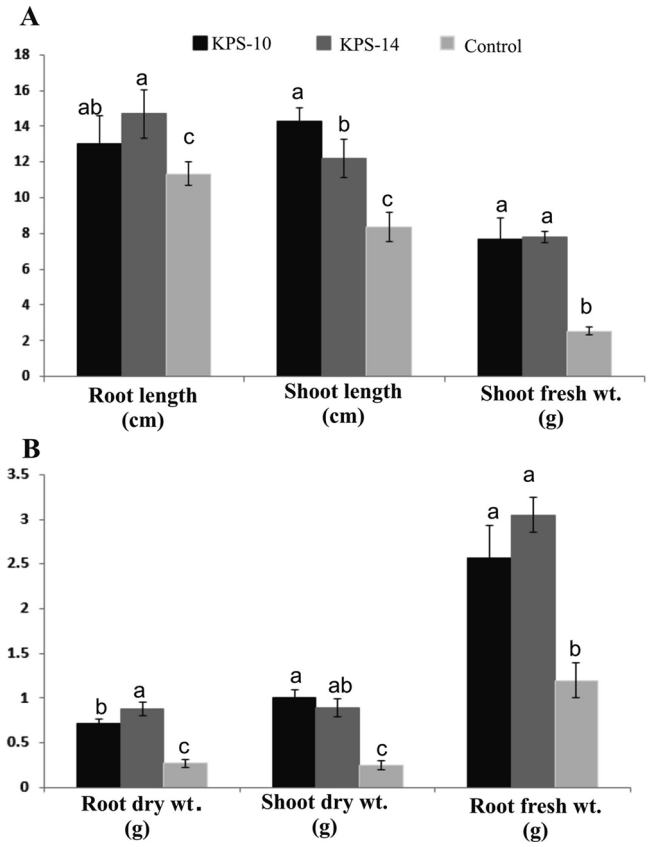

Fig. 4. Effect of inoculation on potato growth grown in sterilized sand. The data is average of 5 replicates each.

3.6. Exp.3: Effect of purified AHLs on seedling growth and root hair development

Inoculation of purified AHLs to different seeds show increased seedling growth (Fig. 8A and B) and induction of root hair development by addition of AHLs to the growth medium as compared to those grown on control agar plates (Fig. 8D and F) when comparison was done with those seedlings which were grown without AHLs supplementation (Fig. 8C and E).

3.7. Exp.4: Inoculation response on non-homologous hosts under axenic conditions

In planta detection of *Serratia* spp. KPS-10 and KPS-14 inoculated seedlings (Fig. 9) show that AHLs were produced on the growing roots of rice and maize. Similar observations were made when other non-homologous roots were analyzed (data not shown). The non-inoculated roots did not show the production of any purple color on both rice and maize roots.

In wheat, rice, maize and rapeseed, the increase in seedling shoot was very obvious visually (Fig. 10A). Wheat, rice, maize and rapeseed inoculate with the KPS-10 and KPS-14 strains observed that the shoot length was significantly increased in case of KPS-10 with respect to control. In KPS-14 inoculated wheat and rice, shoot length was significantly increase while in rapeseed and maize the shoot length was less affected. Maximum increase was observed in the shoots inoculated with KPS-14. In case of seedling root length, the inoculation effect was more pronounced in seeds inoculated with KPS-14 followed by KPS-10 (Fig. 10B). The fresh roots of wheat, maize, rice and rapeseed were investigated for colony forming units. The CFU inoculated with KPS-14 for wheat, maize and rice was significantly higher than KPS-10 treatment. While in rapeseed the CFU is significantly higher for KPS-10 treatment. No. of colony forming units attached to the fresh roots of these crops are tabulated (Table 2).

Microscopic observations of inoculated roots show that bacterial strains altered the primary tap root architecture to more branched exploratory system (Fig. 11). AHLs produced by *Serratia* spp. promoted the lateral root formation and root hair in wheat (Fig. 11B and C), rice (Fig. 11E and F), maize (Fig. 11H and I) and rapeseed (Fig. 11K and L). Root hair parameters were studied after seven days of inoculation. Untreated control seedlings showed the cellular typical organization of primary root tip and root hairs were developed at a distance from the root tip. As compared to control, in inoculated seedlings root hairs were formed on the entire root surface and thickening of root hairs were also observed (Fig. 11).

4. Discussion

The rhizosphere management for efficient nutrient (N, P) utilization by plants is gaining interest day by day due to increasing demand as well as cost of the fertilizer along with environmental safety aspects. Different types of bacteria have the ability to mobilize soil fixed phosphorus for plants uptake (Richardson and Simpson, 2011; Ahemad and Kibert, 2014) and produce AHLs (signaling molecules) to interact and architect the rhizosphere. The study presented here, describes the

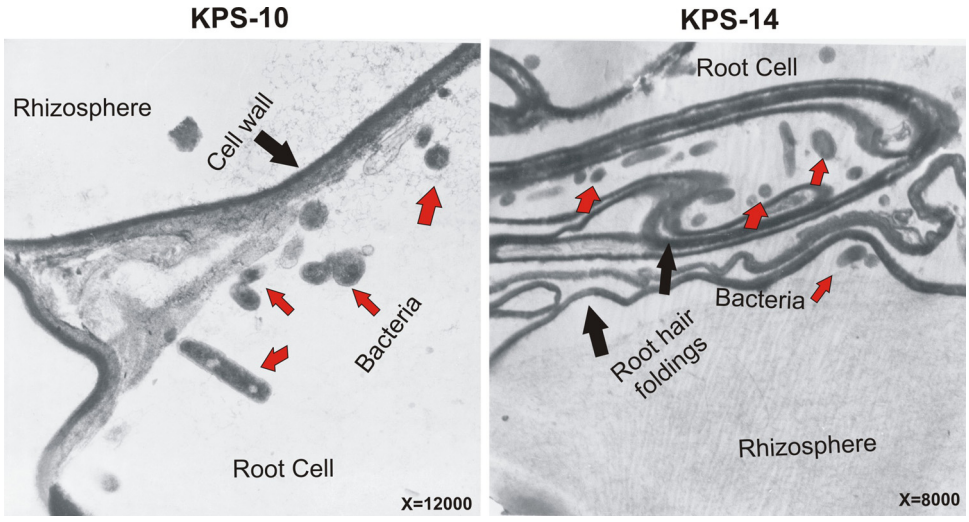

Fig. 5. Transmission electron micrograph of ultrastructre localization and root colonization of potato inoculated with *Serratia* spp. KPS-10 and KPS-14.

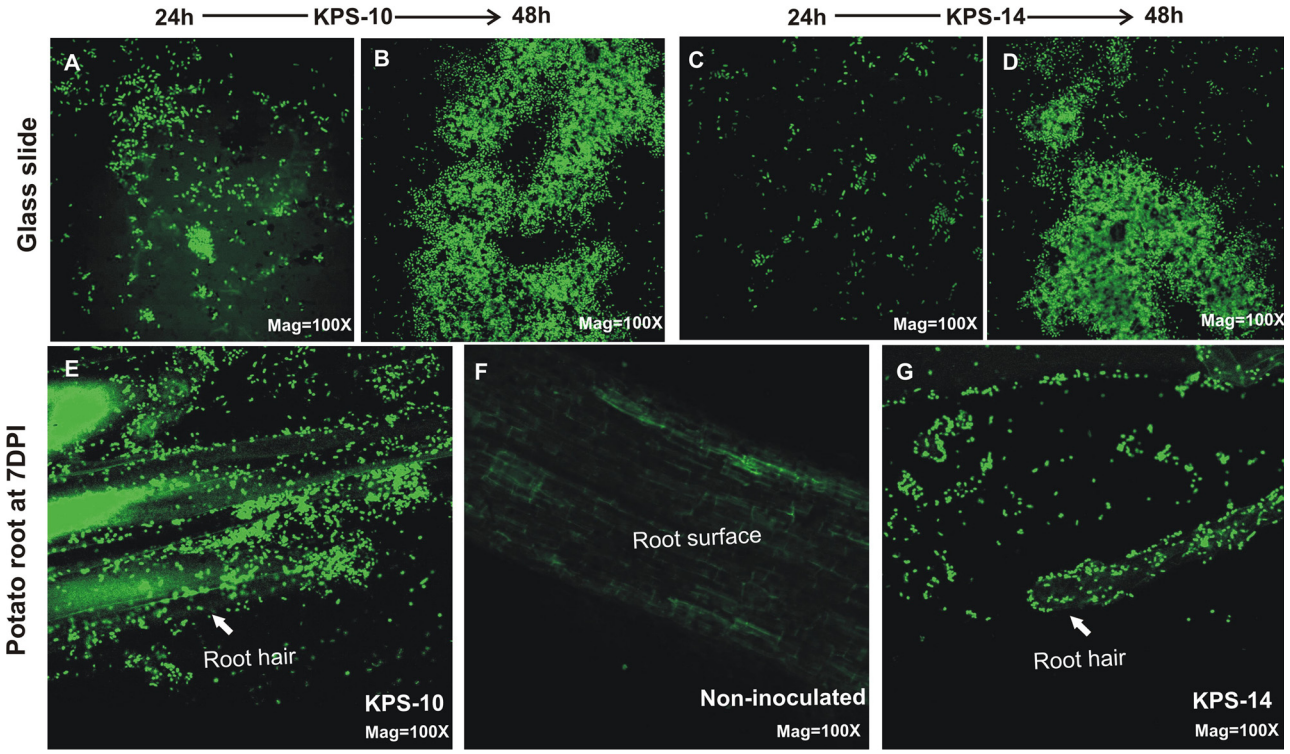

Fig. 6. Confocal laser scanning micrographs showing a gradual increase in the biofilm development by yfp-labelled *Serratia* spp. KPS-10 (A, B) and KPS 14 (C, D) on glass slide *in vitro.* and potato root colonization grown in sterilized sand inoculated with yfp-labelled *Serratia* spp. KPS-10 (E) and KPS 14 (G). The non-inoculated control is also shown (F).

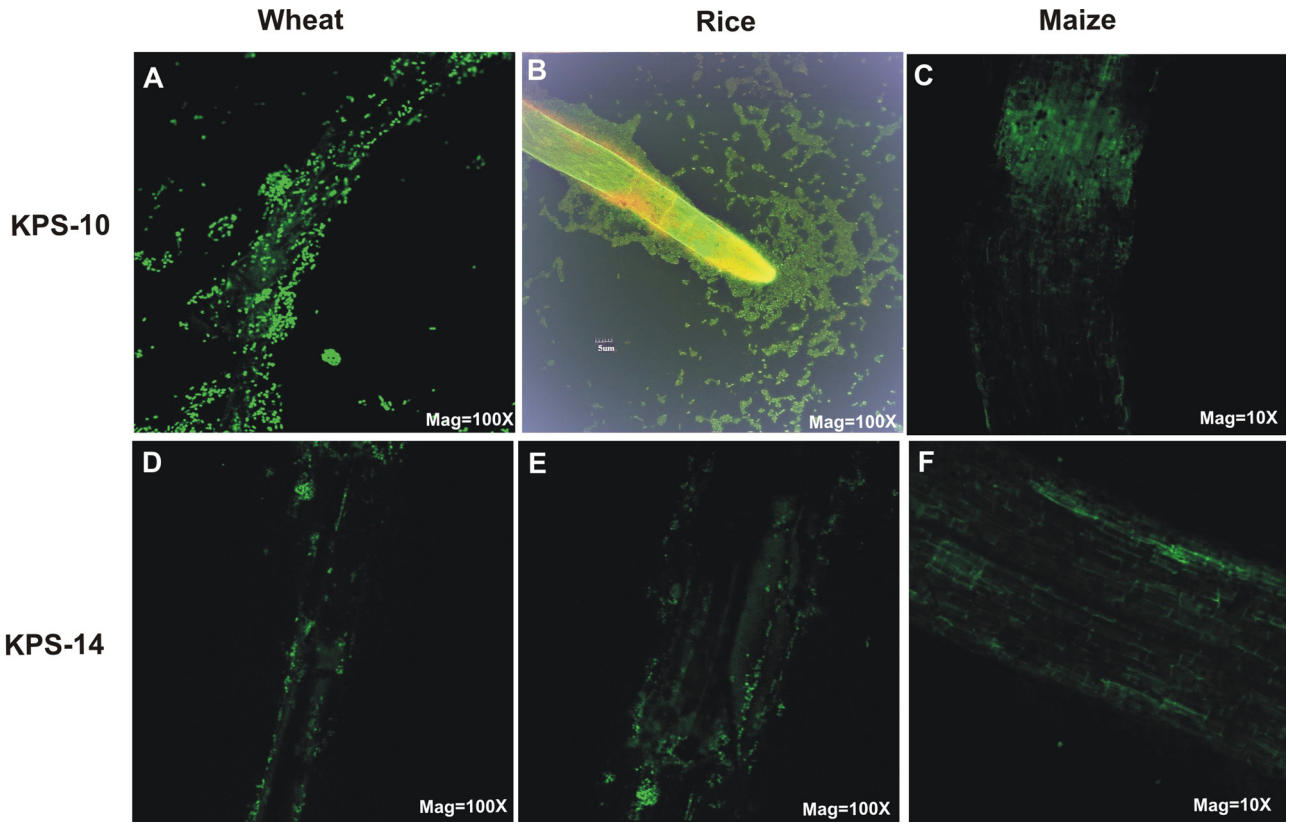

Fig. 7. Confocal laser scanning micrographs showing root colonization of yfp-labelled *Serratia* spp. KPS-10 (A, B,C) and KPS 14 (D, E,F) on *in vitro* grown wheat (A,D), rice (B.E) and Maize (C, F) under axenic conditions.

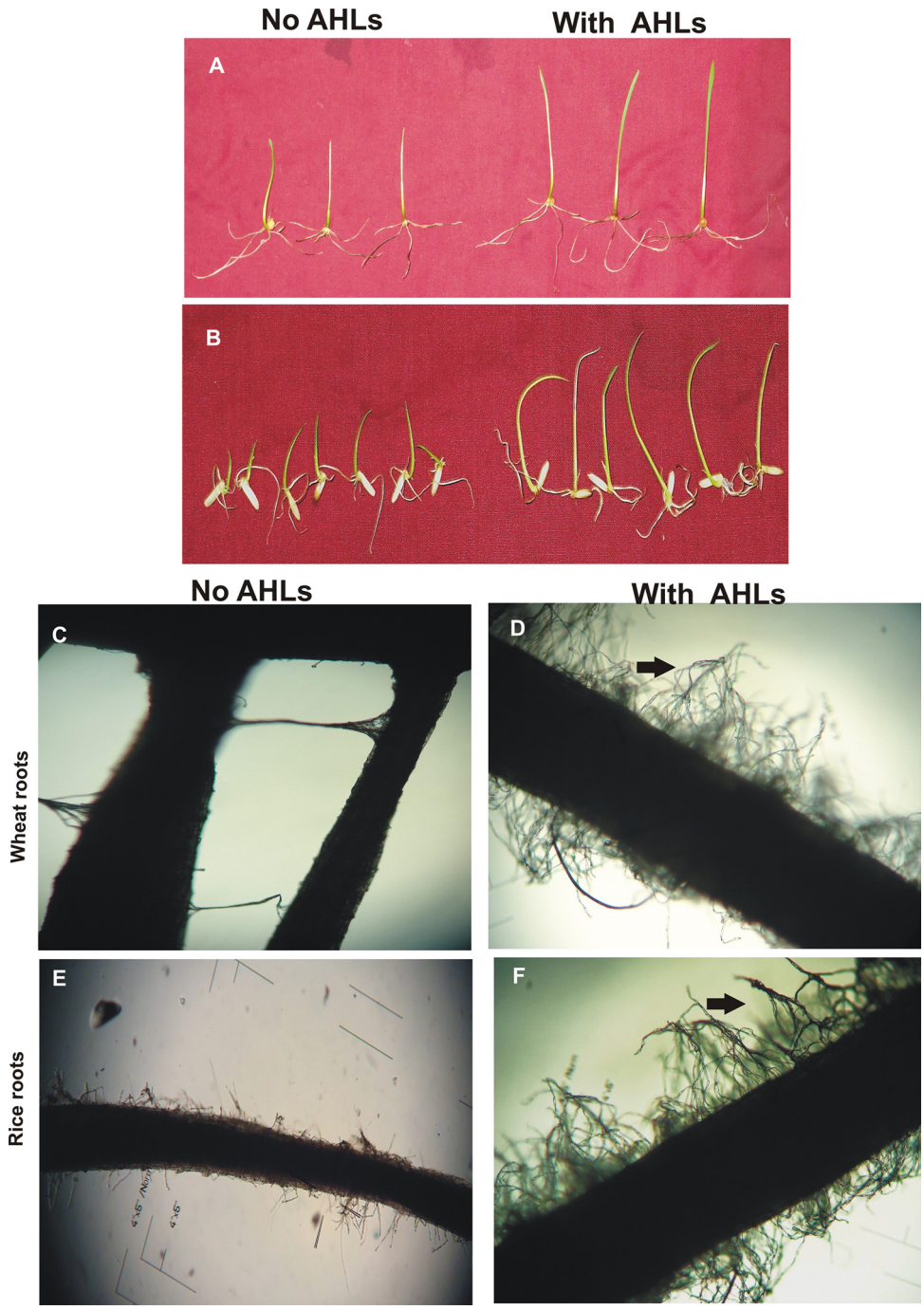

Fig. 8. Effect of addition of AHLs on seedling growth (A,B) and root hair development (C-F) of non-homologous hosts grown in water agar. The seedlings grown without AHLs are also shown for comparison. The root hair analysis were carried out under light microscope at 100X (C-F).

taxonomic affiliation of two AHL-producing PGP-bacteria isolated from potato rhizosphere and demonstration of their potential for root colonization and seedling growth of homologous host and non-homologous hosts. Both bacterial isolates were identified as *Serratia plymuthica* and phylogenetic analysis further confirmed their evolutionary relatedness to *Serratia plymuthica* strain HC27 where both strains positioned themselves in the same cluster in phylogenetic tree.

Both strains exhibited significant plant growth promoting traits which can improve the quality of plant growth directly and/or indirectly. Both were metabolically active and diverse in nutrient utilization which support the competency and adaptability of strains in the rhizosphere as metabolically versatile bacteria are most successful root colonizers and competitor in environment (Wielbo et al., 2007). The antifungal activity and IAA production (8.84 μg ml^{-1}) was exhibited only by KPS-10. IAA is a plant hormone that stimulate root development and proliferation and is a characteristic feature of any PGP-candidate specie. This trait helps plants to explore larger soil surfaces for extracting water and nutrients, directly contributing towards plant growth and development. Indirectly, this trait help plants by offering large habitat to attach and colonize bacteria on the root surfaces (Majeed et al., 2015; Hakim et al., 2020) which ultimately help in recycling various nutrients in the plant rhizosphere rendering them available for plant uptake. The antifungal activity further adds to the bacterial fitness as it does not allow pathogenic fungi to come in contact with plants, hence making them resilient to biotic stress and diseases (Ali et al., 2020). These abilities give any bacterium the ecological

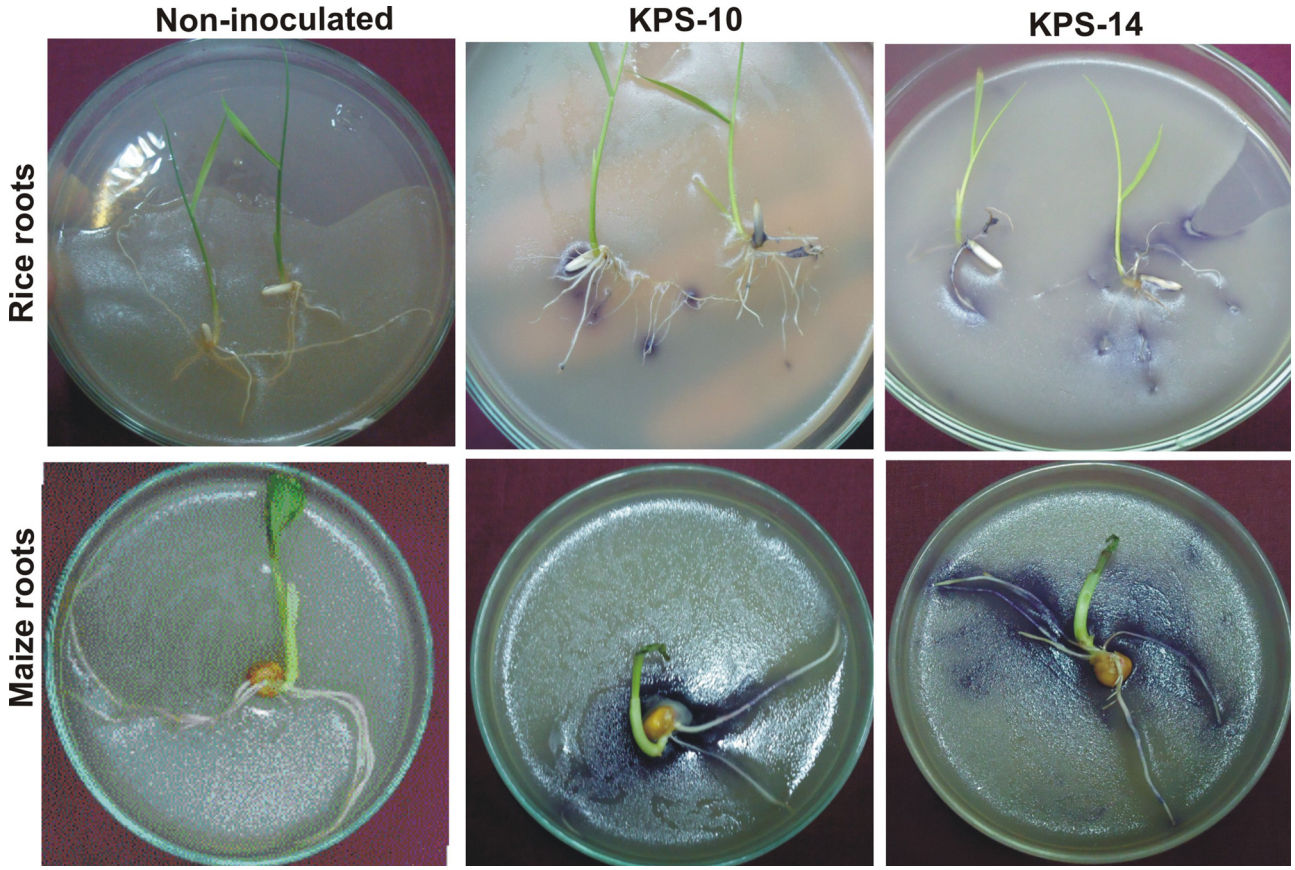

Fig. 9. *In planta* confirmation of AHLs production by bacteria during seedling growth and development on non-homologous hosts (only representative hosts are shown here).

advantage in the rhizosphere, which (due to its nutrient richness) works as microbial "hot-spot" for diverse interactions in the plant vicinity.

Phosphorus is an important element required for plant growth but usually found as insoluble phosphates or organic phytates. Bacteria recycle these plant-unavailable P-forms and convert them into plant-available P-forms (Rodriguez and Fraga, 1999). Although both strains showed almost similar phosphate solubilization indices on plate but showed a significant difference in the capability to solubilize P-in liquid medium when quantification was done using standard method. Rodríguez and Fraga (1999) reported that the plate halo zone sometimes produces contradictory results. However, the range of P-solubilization was in the range already reported for other PGPR (Oliveira et al., 2009; Qian et al., 2010). Both strains produced gluconic acid but lactic acid was produce only by the strain KPS-10 (16.74 ± 2.32 μg ml^{-1}). We can relate the higher P-solubilization of KPS-10 with the presence of multiple organic acid that work collectively to reduce the pH and solubilize the inorganic phosphorus synergistically. These organic acids are known to lower the pH which results in the solubilization of the insoluble phosphorous (Qian et al., 2010). Both the P-solubilization and organic acid production are directly proportional to the time of incubation as described earlier (Shahid et al., 2012). Analysis of the pqqE gene and its high similarity with other *Serratia* species reveals the presence of co-factor which is responsible for the extracellular glucose oxidation into organic acids in the presence of GDH. This co-factor involves in the regulation and production of the organic acids and indirectly correlate with the different activities as the signaling and P-solubilization.

The strain KPS-14 also exhibited significant P-mineralization ability (phytate activity). Phytase activity is the ability of the bacterium to mineralize organic-P in the soil making them available for plant growth. Both these abilities (*i.e.*, P-solubilization and P-mineralization) enable the host plant to uptake P from different sources of bound-P present in the soil (inorganic and organic) (Kashif et al., 2014; Rasul et al., 2019).

Both of the *Serratia* spp. used in this study produced a range of AHL molecules. AHLs are signaling molecules produced by Gram-negative bacteria which aid in crosstalk with other prokaryotes (bacteria) and eukaryotes (host) for regulating numerous physiological processes (Gray et al., 1994). It has been observed that a large number of AHLs are produced by the beneficial rhizobacteria and used as signaling molecules as well as biocontrol activities (Dubuis et al., 2007). More particularly, *Serratia plymuthica* produced AHL molecule HRO-C48 regulate the production of pyrrolnitrin and exoenzymes, IAA production as well as colonization and biocontrol activity against grey mold (Liu et al., 2007; Muller et al., 2009; Pang et al., 2009). Our TLC data show strong signal for 3-hydroxy-HSL then previously reported (Ovadis et al., 2004; van Houdt et al., 2007). *S. fonticola* reported to contain three AHLs namely: C4-HSL, C6-HSL and 3-oxo-C6-HSL (Ee et al., 2014). The phenotypes that are regulated in *Serratia* spp. by means of AHLs are remarkably diverse and of profound biological and ecological significance, and often interconnected with other global regulators (Houdt et al., 2007). The results revealed that both the strains are diverse in production of AHLs molecules including the 3OHC9-HSL and 3oxoC10-HSL with strong signal; a feature that make them different from other *Serratia* species.

While in the rhizoshpere, the survival and persistence of inoculated bacteria is of paramount importance. Bacteria commonly colonize the plant roots, because root are rich in nutrients and provide larger surface area for bacterial attachment. Furthermore, in the field the inoculated bacteria have to compete with the indigenous bacteria to exert any significant plant growth promotion effect. The root colonization potential of *Serratia* spp. KPS-10 and KPS-14 in the homologous and non-

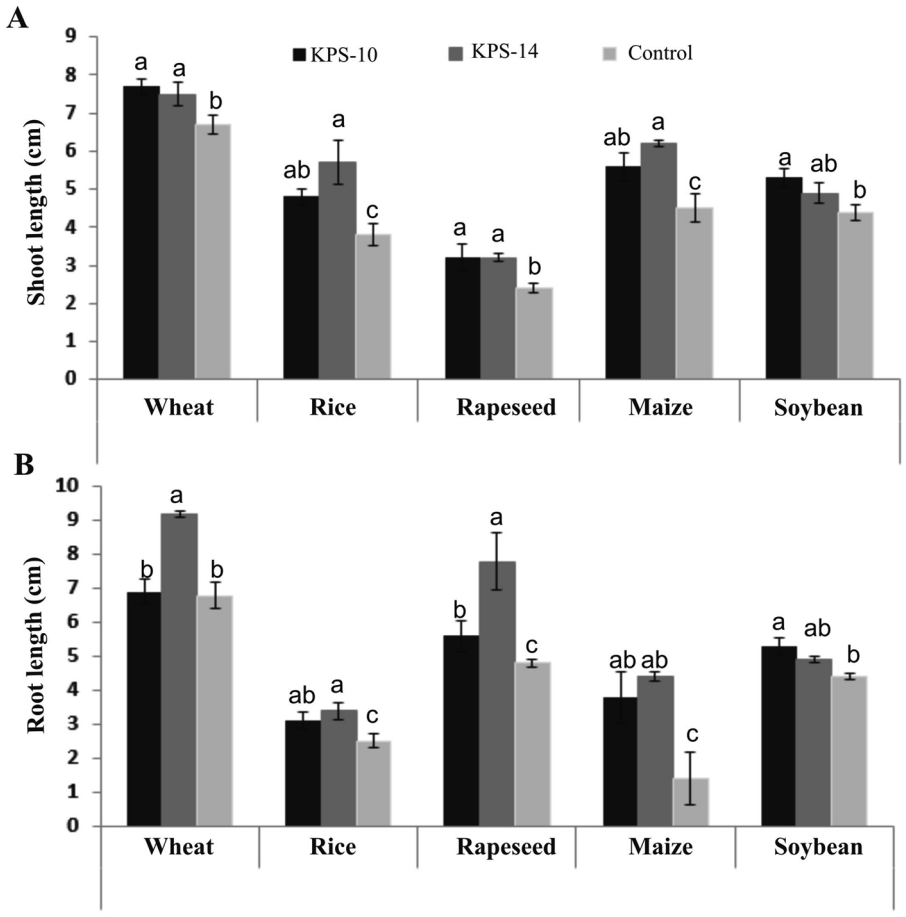

Fig. 10. Effect of inoculation with *Serratia* spp. on shoot length (A) and root length (B) of non-homologous hosts in axenic cultures.

Table 2
Number of colony forming units (log of CFU) attached to the roots of non-homologous hosts.

	CFU10^8/g of fresh root weight				
Treatments	Wheat	Rice	Maize	Rapeseed	Soybean
KPS-10	6.8	2.3	4.6	9.1	4.4
KPS-14	8.0	3.8	5.1	3.2	4.2

Values are average of four replicates each. The data was taken 10 days after inoculation (13 days old plants).

homologous hosts showed their rhizosphere fitness, survival and persistence. In potato, at 20 DAI the population density of KPS-10 (7.24 log CFU g^{-1}) was higher than KPS-14 (5.10 log CFU g^{-1}) which declined at crop maturity *i.e.*, 60 DPI (3.6 log CFU g^{-1} for both) This initial population density is sufficient enough to exert plant beneficial effect as previously described in wheat rhizosphere bacteria (Fischer et al., 2010). The gradual decrease in the bacterial population can be the effect of growth stage of plant and the interactions of microbes with other indigenous communities in the potato fields (Van Overbeek and Van Elsas, 2008). Photomicrographs showed the presence of the strains in the rhizoshpere, root hair folding and in the root hairs polysaccharide material. TEM revealed the strong associations and adequate number present in rhizoshpere due to the presence of enough nutrients and growth conditions that allow the bacteria to colonize (Guerrero-Molina et al., 2012). In the non-homologous host such as in wheat, maize, rice and rapeseed, the root colonization studies carried out by the confocal laser scanning microscope revealed that KPS-10 have higher potential of root colonization in different hosts and also observed the entry in roots of wheat while KPS-14 colonized on the surface of the roots, root hairs and root tip. The number of colony forming units is higher in KPS-14 in all the treatments than the KPS-10. This result correlates with the potato colonization that the KPS-14 survival rate is higher than the KPS-10 in all the non-homologous hosts except rapeseed (Fischer et al., 2010). This quality alongwith other plant beneficial traits make both these bacteria excellent candidate for development of inoculum production.

The inoculation assays exhibited a significant response in inoculated seedlings in potato, wheat, rice, maize and soybean. In potato, root length and shoot length increased 50 % and 45 % respectively than the non-inoculated control along-with a significant increase in plant fresh and dry weights. In non-homologous hosts, the change in root length was non-significant but shoot length significantly increased. The microscopic observations further revealed that the morphology of the roots significantly altered; many secondary roots developed from the primary tap root all covered with intensive root hair growth. The non-inoculated roots on the other hand show very thin root. This observation lead to the conclusion that the AHLs are significantly increase the root morphology by producing the lateral root development, root hairs and thickening of the roots.

5. Conclusions

The study concludes that *Serratia* spp. have plant growth promotion potential for different plant hosts. Use of these strains may reduce the input of chemical fertilizers (P) ultimately reducing the soil and water pollution in agricultural lands. Hence, both strains can be further exploited for large scale field testing. After biosafety analysis, the strains may be recommended for as general purpose (universal)

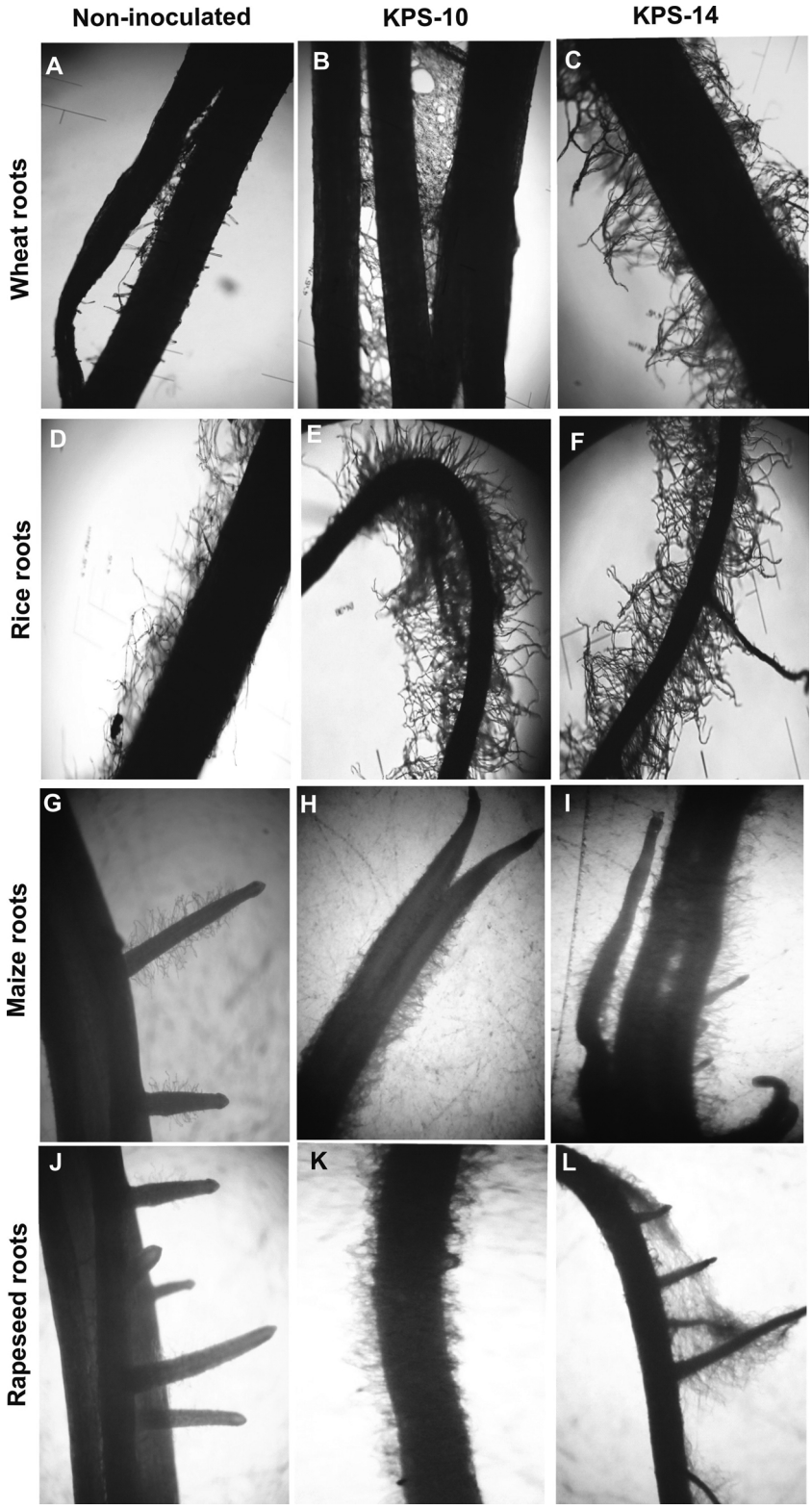

Fig. 11. Root hair development by the inoculation of *Serratia* spp. on non-homologous hosts in axenic cultures. The analysis were carried out under light microscope at 100X magnification (A-F) and stereomicroscope (G-L).

inoculum for growing different crops.

Consent for publication

All authors reviewed and agreed to the final version of the manuscript. The authors agree to the contents and the contribution.

Consent to participate

All agree.

Author contribution statement

MKH did all the basic experiments as part of his PhD studies,
KAM supervised the studies of MKH,
SH co-supervised MKH,
MJS helped in the write up,
Ayesha did all LCMS analysis as part of her MPhil studies,
KF did confocal and plate assay on non-homologous hosts as part of her MPhil studies,
TN helped in pot experiments and initial draft,
AM helped in strain transformation,
MJI did the TEM studies,
AI conceived the whole studies of Ayesha, KF, done confocal analysis, supervised the MPhil studies of Ayesha and KM, edited the initial draft and finalized the manuscript

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Ethics approval

Not applicable

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.micres.2020.126506.

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