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Research article

UHPLC-MS/MS and QRT-PCR profiling of PGP agents and *Rhizobium* spp. of induced phytohormones for growth promotion in mungbean (*var.* Co4)

Chaitanya S. Mogal [a], Vanrajsinh H. Solanki [b], Rohan V. Kansara [b], Sanjay Jha [c,*], Susheel Singh [b], Vipulkumar B. Parekh [d], B.K. Rajkumar [e]

[a] *Department of Plant Molecular Biology and Biotechnology, Navsari Agricultural University, Navsari, 396450, Gujarat, India*
[b] *Food Quality Testing Laboratory, N. M. College of Agriculture, Navsari Agricultural University, Navsari, 396450, Gujarat, India*
[c] *ASPEE Shakilam Biotechnology Institute, Navsari Agricultural University, Surat, 395007, Gujarat, India*
[d] *Department of Basic Science and Humanities, ACHF, Navsari Agricultural University, Navsari, 396450, Gujarat, India*
[e] *Main Cotton Research Station, Navsari Agricultural University, Surat, 395007, Gujarat, India*

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ABSTRACT

In present study, five potential strains with different plant growth promotion (PGP) characteristics were used. By considering various PGP properties of different bacterial strains, several treatments based on various combinations were developed and studied on mungbean (*var.* Co4). The quantification of the phytohormones was performed on ultrahigh-performance liquid chromatograph coupled to heated electrospray ionization tandem mass spectrometry (UHPLC/HESI-MS/MS). Indole 3-acetic acid (IAA) and Indole 3-butyric acid (IBA) were quantified in positive ionization mode while Gibberellic acid (GA_3) and salicylic acid (SA) were quantified in negative ionization mode. Among all the treatments two penta combinations of consortia 1 (*Rhizobium* + *Azospirillum* + *Pseudomonas* + *Bacillus spp.* + *Bacillus licheniformis*) and consortia 2 (*Rhizobium* + *Azotobacter* + *Pseudomonas* + *Bacillus spp.* + *Bacillus licheniformis*) were found most effective. Higher amount of IAA (1.043 μg g^{-1}), IBA (0.036 μg g^{-1}), GA_3 (1.999 μg g^{-1}) and SA (0.098 μg g^{-1}) Fresh weight (FW) were found in treated adolescent root tissues of consortia 2 as compared to consortia 1. Moreover, transcriptional level of the plant hormones were 2–4 fold higher in the relative gene expression study of three genes: *ARF* (Auxin responsive factors), *ERF-IF* (Ethylene-responsive Initiation Factors) and *GAI* (Gibberellic-Acid Insensitive) in consortia 2, on the 15th, 30th and 45th day using quantitative real time-Polymerase chain reaction (qRT-PCR). Furthermore, Yield attributing characters like, the number of nodules plant^{-1}, number of pods plant^{-1}, weight of nodule and seed yield plant^{-1} were also increased as compared to the control. As a result, the current research elucidated that penta combinations consortium of *Rhizobium* sp. and rhizobacteria can be developed as a single delivery system biofertilizer for enhancing mungbean productivity.

1. Introduction

Plants and microorganisms are known to interact; the rhizosphere is a critical area that stimulates crop development and increases yield. The rhizosphere is a complex and combative habitat for plant–microbe interactions aimed at extracting necessary major and minor nutrients from nutrient resources. In recent years, several plant growth promoters have been identified, including *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Serratia*, *Enterobacter*, *Bacillus*, and *Paenibacillus* (Muresu et al., 2008; Gururani et al., 2013; Kumari et al., 2018). Plant growth promoting rhizobacteria (PGPR) promotes plant growth by producing phytohormones, nitrogen fixation, and phosphorus solubilization (Tabassum et al., 2017). Auxins, gibberellins, cytokinins, ethylene, and abscisic acid are some of the phytohormones that PGPR can produce to mediate plant cell enlargement, division, and extension in both symbiotic and non-symbiotic roots (Goswami et al., 2016).

Pulses are important food crops with high protein content that are frequently utilised in mass feeding, but per acre production is extremely low. There is need to synchronise the process of colonisation and survival for successful plant growth promoting rhizobacteria (PGPR) interactions with legumes, since rhizobia and bradyrhizobia commonly persist in the plant rhizosphere, which highlights the necessity to supplement with Plant growth promoting (PGP) agents (Joshi and Bhatt 2011; Andy et al., 2020).

* Corresponding author.
E-mail address: sanjayjha874@gmail.com (S. Jha).

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The presence of PGP bacteria in the rhizosphere is known to promote root hair development, shoot and root growth, plant hormone regulation, root colonisation, nitrogen fixation, pathogen suppression and mineral solubilization these all are important steps for the beneficial bacteria-plant reciprocity (Lucas et al., 2009; Gopalakrishnan et al., 2010; Deshwal and Kumar 2013). Researchers are focused on creating a more effective *Rhizobium* consortium with other PGP agents (Naseri and Hemmati 2017). Several researches have been done over the last few decades to demonstrate the efficacy of co-inoculation of *Rhizobium* with PGPRs on legumes (Naseri and Younesi 2021). A wide range of PGPRs, including *Bacillus* and *Pseudomonas* species, are widely found in the rhizosphere of legume and non-leguminous crops (Muresu et al., 2008; Abbasi et al., 2010; Okazaki et al., 2016; Khati et al., 2018). Simultaneously, co-inoculation of bacterial species such as *Rhizobium* sp., *Azotobacter* sp., *Pseudomonas* sp., *Bacillus* sp. has evidenced a various benefits in plants and cultivation systems (Korir et al., 2017; Kalantari et al., 2018).

Phytohormones are class of naturally occurring, organic compounds which, at low concentrations, stimulate physiological processes. Despite being a minor component of the metabolome, phytohormones serve a critical function in the regulation of germination, growth and development (Tian et al., 2020). However, it is uncertain how volatile organic compounds (*i.e.* phytohormones) impact on plant growth. They are generated by PGPRs and stimulate the density and length of root hairs, resulting in an overall increase in a plant's root surface area, as well as to directly promote plant development by activating plant defence systems, which leads to enhanced nutrient absorption and growth (Tsegaye et al., 2017; Liu et al., 2018). Certain phytohormones, including auxin, gibberellin (GA) and salicylic acid (SA), play critical role in the maintenance of stem-cell systems in shoot meristems and have complicated functional relationship (Xiong et al., 2020).

At the cellular level, auxin responses are influenced by auxin response factors (*ARF genes*) that are identified based on their ability to bind to promoter elements that confer auxin responsive gene. *ARF genes* are transcription factors that bind to TGTCTC-containing auxin response elements (*AuxREs*) present in the promoters of primary/early auxin response genes and mediate auxin response (Li et al., 2016). The *ERF-IF* gene (Ethylene response factor) is activated by the binding of *EIN3/EIL* in the main ethylene response element (*ERE*) located in the promoter of *ERF-IF*, which is engaged in the ethylene signal transduction pathway and functions as a positive regulator of ethylene response in different cropping system (Zemlyanskaya et al., 2018). With increased understanding of inorganic fertilizer-based agricultural techniques, it has become critical to search for region-specific imminent microbial inoculants to obtain desirable crop yield (Ramesh et al., 2014). The ability to optimise the effectiveness of the bean-*Rhizobium* symbiosis for agricultural sustainability will require a thorough study of agro-ecological variables regulating rhizobial nodule growth during the growing season (Tabandeand and Naseri, 2019).

Numerous PGP agents as well as *Rhizobium* spp. have been found to have strong plant growth promoting effects in soil, but the molecular mechanism by which most of these bacteria interact with plants is unknown. This was the first attempt to quantify phytohormones with a combination of PGPR and a specific *Rhizobium* spp., using UHPLC-MS/MS (Ultra-high performance liquid chromatography tandom mass spectrometry) due to its high sensitivity, efficacy and low detection limits as compared to other spectroscopic techniques as well as quantitative real time-Polymerase chain reaction (qRT-PCR) profiling in mungbean (*var.* Co4) for yield attributing characters and growth promotion by activating phytohormones related genes for better nutrient acquisition.

2. Materials and methods

2.1. Plant material

The Mega Seed Pulses and Castor Research Unit, Navsari Agricultural University, Navsari 396450, Gujarat, India, provided certified seeds of chosen pulse shrub mungbean (*var.* Co4). The plants were grown in pots for 70–75 day's and adolescent roots and mature fresh nodules were harvested at 35th day.

2.2. Chemicals and reagents

Authenticated materials of Indole 3-acetic acid (IAA), Indole 3-butyric acid (IBA), Gibberellic acid (GA_3), and salicylic acid (SA) with a purity of 99.9% were procured from Sigma-Aldrich Pvt. Ltd. Sodium chloride, sodium sulphate, magnesium sulphate, sodium acetate, chloroform and isopropanol (all from Merck in Darmstadt, Germany), acetonitrile, methanol, acetone and water (all MS-grade) were from Darmstadt (Germany). The primary secondary amines (PSA) were purchased fromSupelco Sigma Aldrich (Germany). The polyvinylidene difluoride (PVDF) syringe filters (diameter: 0.22 μm) were obtained from Thermo Scientific, USA.

2.3. Standard preparation

Stock solutions (2 mg L^{-1}) of all four phytohormones were prepared in amber coloured volumetric flask (50mL) by using methanol and kept at -20 °C. The intermediate standard (250 μg L^{-1}) was prepared by using stock solutions and then sequentially diluted with methanol: water (80:20, v/v) to achieve concentrations of 100, 50, 25, 10, 5, 2.5 and 1 μg L^{-1}.

2.4. Apparatus

A heavy-duty variable speed homogenizer (SRK Instruments, Gujarat), centrifuge (Eppendorf, Germany) and Turbovap (Caliper life science, PerkinElmer, USA) were used to process the plant samples. The IAA, IBA, GA_3 and SA were determined using an LCMS-QqQ (Triple Quadrupole), TSQ Quantum Access Max® equipped with a UHPLC (Thermo Scientific, USA). In addition, the relative gene expression of three genes *ARF* (Auxin responsive factors), *ERF-IF* (Ethylene-responsive Initiation Factors) and *GAI* (Gibberellic-Acid Insensitive) were studied using the CFX96 quantitative real time PCR System (qRT-PCR) from Biorad, USA.

2.5. Characterization of microbes used and their maintenance

Five well-known PGPR bacteria *viz., Rhizobium spp. LSMR1, Azotobacter chroococcum, Azospirillum brasilense, Pseudomonas fluorescens and Bacillus spp* were obtained from the Department of Agriculture Microbiology, University of Agricultural Science (UAS, Bangalore). These bacteria were grown on Yeast Extract Mannitol Agar (YEMA), Azotobacter Agar, Azospirillum Medium w/o Agar, Pikovskaya Agar and Nutrient Agar medium (Himedia, India). All microbes used in the experiments were subjected to morphological, biochemical, plant growth promoting characterization and taxonomic identification based on their 16S rRNA gene sequence accession number (available at https://www.ncbi.nlm.nih.gov, National Center for Biotechnology Information, USA) (Table 1).

2.6. Experimental setup

Soil of Pulses and Castor Research Unit farm (20°55′53.7″N 72°53′41.1″E) was collected, sieved through a 10mm mesh sieve and autoclaved properly. The soil was placed in 5 kg polythene-lined clay pots. The pot experiment was carried out with thirteen treatments of a distinct mixture of PGPR (1×10^8 ml) in mungbean, as mentioned in Table 2. Mungbean (*var.* Co4) seeds were treated with a recognised standard culture @ 10 ml kg^{-1} seed and dried in a shed for 30 min before planting. The dosages of single bio-inoculants were lowered in the combination of culture treatments such that the total volume of the culture remained constant, i.e. 10 ml kg^{-1} of seed in seed treatment. Ten prepped seeds and control (uninoculated seeds) were planted in each container. Three plants were kept in each container after the first leaf

Table 1. Biochemical characters of PGP agents and *Rhizobium spp.*

Characteristic of the test organism	*Rhizobium* spp.	*Pseudomonas fluorescens*	*Bacillus* spp.	*Azotobacter chroococcum*	*Azospirillum brasilense*
Gram's reaction	-ve	-ve	+ve	-ve	-ve
Shape	Rods	Rods	Rods	Rods	Rods
Pigment	-	+	-	-	-
Pigment colour	Translucent	Fluorescent green	off-white	White	off-White
Starch hydrolysis	+ve	+ve	+ve	+ve	+ve
Catalase production	+ve	+ve	+ve	+ve	+ve
Methyl red test	-ve	-ve	-ve	-ve	-ve
Nitrate reduction	+ve	+ve	+ve	+ve	+ve
IAA Production (μg/ml)	73.32	92.71	92.49	96.68	99.06
GA Production (μg/ml)	83.3	101.1	103.4	110.3	102.1
Nitrogenase activity (n moles C_2H_4/h^{-1} $culture^{-1}$)	679.26	–	–	645.48	600.02
Accession numbers 16S rRNA gene	KR072691	KF054767.1	JF513170.1	HQ018746.1	HQ018756.2

+ve positive, *-ve* negative, *IAA* Indole 3- acetic acid, GA_3 Gibberellic acid, *μg/ml* microgram per ml, *n moles* C_2H_4/h^{-1} *culture*$^{-1}$ nano moles ethylene per hour per culture, Accession numbers of 16S rRNA gene sequence were obtained from NCBI (https://www.ncbi.nlm.nih.gov).

Table 2. Treatments details for pot experiment with PGP agents and *Rhizobium spp.*

No.	Treatment Details
T_1	Absolute control
T_2	Seed + *Rhizobium* spp. (1×10^8 ml) @ 10 ml/kg seeds
T_3	Seed + *Azotobacter chroococcum* (1×10^8/ml) @ 10 ml/kg seeds
T_4	Seed + *Azospirillum brasilense* (1×10^8/ml) @ 10 ml/kg seeds
T_5	Seed + *Pseudomonas fluorescence* (1×10^8/ml) @ 10 ml/kg seeds
T_6	Seed + *Bacillus spp.*(1×10^8/ml) @ 10 ml/kg seeds
T_7	Seed + *Azospirillum* + *Rhizobium* (1×10^8/ml) @ 10 ml/kg seeds
T_8	Seed + *Azotobacter* + Rhizobium (1×10^8/ml) @ 10 ml/kg seeds
T_9	Seed + *Pseudomonas* + Rhizobium (1×10^8/ml) @ 10 ml/kg seeds
T_{10}	Seed + *Bacillus spp.* + Rhizobium (1×10^8/ml) @ 10 ml/kg seeds
T_{11}	Seed + *Bacillus licheniformis* (1×10^8/ml) @ 10 ml/kg seeds
T_{12}	Seed + Consortia 1 ($T_2+T_3+T_5+T_6+T_{11}$) (1×10^8/ml) @ 10 ml/kg seeds
T_{13}	Seed + Consortia 2 ($T_2+T_4+T_5+T_6+T_{11}$) (1×10^8/ml) @ 10 ml/kg seeds

fully unfolded. As a control, uninoculated seeds were planted. The all PGPR and *Rhizobium* spp. strains were used for experimentation during *Rabi* season for two years 2017–18 and 2018–19. Randomizations of pots were done as per completely randomised design (CRD).

2.7. Sample extraction and cleanup

The samples were processed and evaluated at the Food Quality Testing Laboratory NAU, Navsari, Gujarat, India. For aldolesecent roots, sample was evaluated using the modified QuEChERs (Quick, Easy, Cheap, Effective, Rugged, and Safe) method (AOAC 2007).

The adolescent root samples of mungbean (*var.* Co4) were minced and homogenised using a heavy duty variable homogenizer and a representative sample (15 ± 0.1 g) was taken in 50 mL capacity polypropylene centrifuge tubes. Then 1% acetic acid in acetonitrile (15 mL) was added as an entracting solvent to the sample and placed in a deep freeze for 20–30 min. Therafter, $MgSO_4$ (6.0 g) and sodium acetate (1.5 g) were added and shaken for 1.0 min. The contents were centrifuged at 2205 g for 2.0 min. Later, the supernatant (6.0 mL) was transferred into 15 mL polypropylene tubes containing anhydrous $MgSO_4$ (0.9 g) and PSA (0.3 g), vortexed for 1.0 min and centrifuged at 1125 g for another 2.0 min. For further analysis, an aliquot (2.0 mL) was transferred to 15 mL capacity test tubes and evaporated to dryness with nitrogen gas using TurboVap. Before being injected into the appropriate instrument, the samples were filtered through syringe filters (0.22 μm pore size) (Saran et al., 2020).

2.8. LC-MS/MS analysis

UHPLC coupled with heated electrospray ionization tandem mass spectrometry (UHPLC/HESI-MS/MS) system was used to analyze phytohormones IAA, IBA, GA_3 and SA. The optimization of parameters on UHPLC and MS/MS of all four phytohormones were studied in both positive and negative ion modes (Table 3). The LCQUAN™ 2.9 QF1 software was used to process the data. The phytohormones limit of detection (LOD) was defined as the lowest sample concentration that could be detected (signal-to-noise ratio = 3). The limit of quantification (LOQ) was defined as the lowest sample concentration that can be determined quantitatively with sufficient precision and accuracy (signal-to-noise ratio = 10). The various concentrations (especially in the linear dynamic range) were recorded to determine the analytical method's limit of detection (LOD) and limit of quantitation (LOQ). The recovery of

Table 3. Optimized parameters of phytohormones on UHPLC-MS/MS.

Parameters	IAA	IBA	GA_3	SA
MS Parameters				
• Source of ionization:	Heated Electrospray Ionization (HESI)			
• Capillary voltage:	4500V			
• Ion mode	Positive	Positive	Negative	Negative
• Vaporizer temperature:	350 °C			
• Sheath gas (N_2):	48 arbitrary unit			
• Aux gas (N_2):	18 arbitrary unit			
• Capillary temperature:	325 °C			
• Tube lens:	52V	60V	62V	30V
• Precursor ion (m/z)	176.0	204.0	345.1	137.0
• Product ion (m/z):	77.2 (40)	130 (40)	143.1 (42)	59.5 (42)
• Collision energy (eV)	130.1 (17)	186.1 (17)	239.1 (19)	109.0 (19)
UHPLC Parameters				
• Column:	Hypersil Gold® C18 column 150 × 4:6 mm; 5 μm particle size			
• Mobile Phase:	Solvent A: Water with 0:1 % formic acid Solvent B: Methanol with 0:1 % formic acid			
• Flow:	Gradient			
• Flow rate:	0.3 ml/min			
• Gradient profile:	(t (min), %A): (0, 95), (1, 95), (3, 55), (9, 90), (13, 95), (15, 95)			
• Retention time (RT)	6.2 min	6.6 min	6.0 min	6.4 min

V volts, *°C* degree Celsius, *(m/z)* mass-to-charge ratio, *IAA* Indole 3- acetic acid, *IBA* Indole 3-butyric acid, GA_3 Gibberellic acid, *SA* salicylic acid.

Table 4. Description of primers for qRT-PCR analysis.

Sr. No	Gene name	Gene Function	Primer sequence (5′-3′)	GC content (%)	cDNA amplicon length (bp)
Indigenous genes					
1.	*β-Actin* F	Makes up the structural framework inside cells	TTCGCAGCAACAAACAT	41.2	371
2.	*β-Actin* R		TAAGCGGTGCCTCGGTAAGAAG	54.5	
3.	*VrTUB*F	Plays a critical role in directing the deposition of cellulose microfibrils during plant cell wall formation.	CTTGACTGCATCTGCTATGTTCAG	45.8	422
4.	*VrTUB* R		CCAGCTAATGCTCGGCATACTG	54.5	
Relative genes					
5.	*ARF* F	Regulate gene expression in auxin signalling transduction	GGAAGATCCTGTGTGAGGTTATG	47.8	140
6.	*ARF* R		CTCCTCAGTAGAGCCGTTATCT	50	
7.	*ERF-IF* F	Acts as a positive regulator of ethylene response in plants	TCCATCGCCTGATCCCTTTG	55	210
8.	*ERF-IF* R		GAAGCAAGCAAACCAAGCCA	50	
9.	*GAI* F	Transcriptional activator or co-activator of GA signaling	GGATCCAAATCCCAACCTATCC	50	245
10.	*GAI* R		GTACTCGCGCTTCATGATCTC	52.4	

β-TUB Beta tubulin, *ARF* Auxin response factor, *ERF-IF* Ethylene response factor, *GAI* Gibberellic acid insensitive receptor, *F* Forward. Primer, *R* Reverse Primer, *GC* Guanine Cytosine content, *bp* base pair.

phytohormones was used to assess the accuracy and precision (Kansara et al., 2021).

2.9. Quantitative analysis through quantitative real time-PCR (qRT-PCR).

2.9.1. RNA extraction

The transcriptional level was examined in adolescent root tissues of mungbean (*var.* Co4) on the 15th, 30th and 45th day after treatment. Total RNA was isolated from each treatment's adolescent root samples using a modified *TRIzol*^{TM} (Invitrogen, USA) method. About 0.1 g roots were pulverised in liquid nitrogen using a sterile pestle and mortar for each sample. Then the powder was transferred to a 1.5 ml tight-capped centrifuge tube with 1 ml *TRIzol*^{TM} reagent and incubated for 5 min at room temperature. After vigorously shaking for 15 s, a double amount of chloroform (0.2 ml) was added and incubated at room temperature for 2–3 min.

Microcentrifuge tubes were left at room temperature for 10 min before being centrifuged at 12000 rpm at 4 °C for 15 min. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol for 5 min at 4 °C at 7500 rpm. Pellets were air dried until the

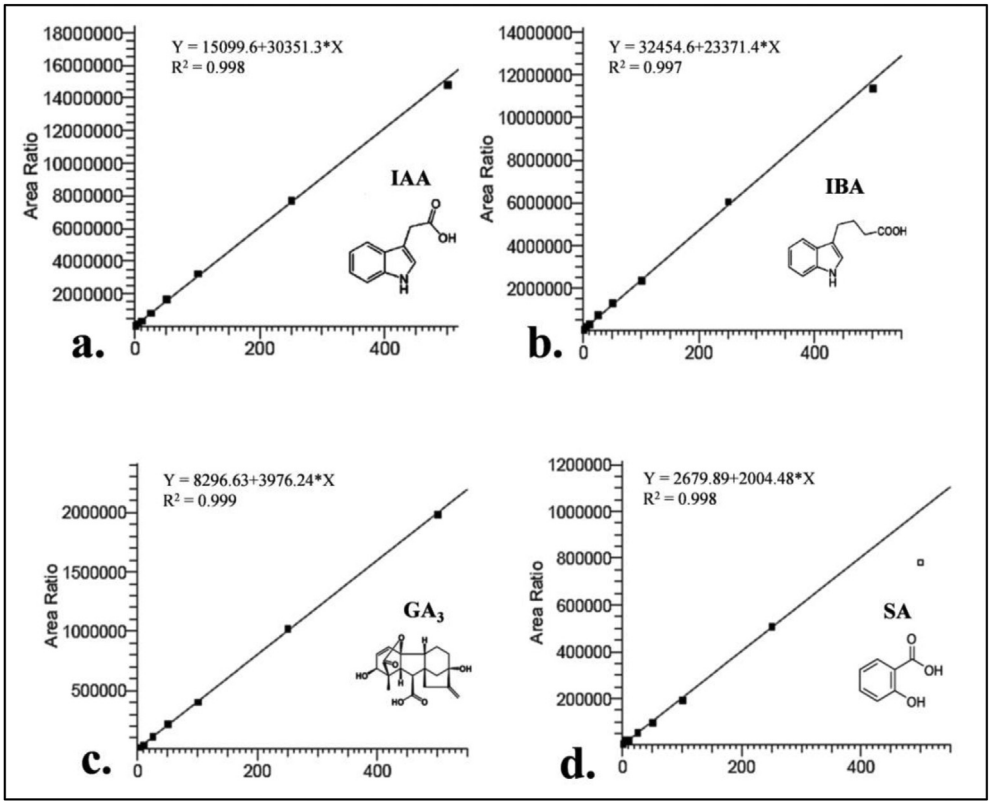

Figure 1. Optimization of chromatographic separation of plant hormones on UHPLC-MS/MS. Linearity and chemical structure of (a) Indole-3-acetic acid-IAA, (b) Indole-3-byutric acid-IBA, (c) Gibberellic Acid-GA_3 and (d) Salicylic Acid-SA.

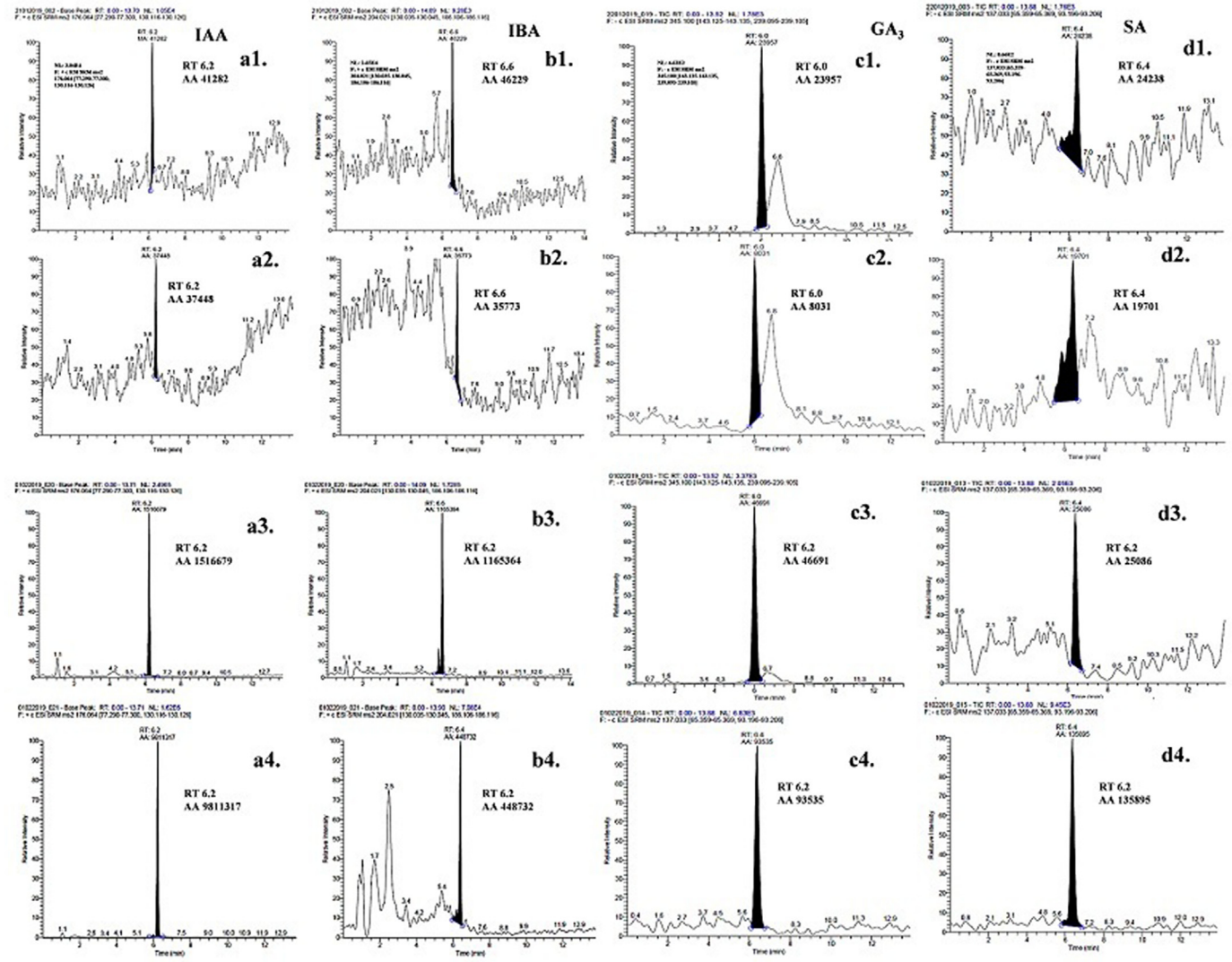

Figure 2. UHPLC–MS/MS chromatograms of plant hormones (A) Indole Acetic Acid, (a1) at standard 0.001 ppm, (a2) in control, (a3) Consortia 1 and (a4) Consortia 2; (B) Indole Butyric Acid, (b1) at standard 0.001 ppm, (b2) in control, (b3) Consortia 1 and (b4) Consortia 2; (C) Gibberellic Acid, (c1) at standard 0.001 ppm, (c2) in control, (c3) Consortia 1 and (c4) Consortia 2; (D) Salicylic Acid, (d1) at standard 0.001 ppm, (d2) in control, (d3) Consortia 1 and (d4) Consortia 2.

Table 5. Quantification of phytohormones in mungbean on UHPLC-MS/MS.

Sr. No.	Treatments	IAA (μg g^{-1} FW)	IBA (μg g^{-1} FW)	GA_3 (μg g^{-1} FW)	SA (μg g^{-1} FW)
1.	Absolute control	0.003 ± 0.048	0.005 ± 0.049	0.786 ± 0.048	0.008 ± 0.047
2.	*Rhizobium* spp.	0.005 ± 0.042	0.007 ± 0.044	0.922 ± 0.043	0.016 ± 0.045
3.	*Azospirillum*	0.027 ± 0.055	0.006 ± 0.056	1.040 ± 0.057	0.043 ± 0.058
4.	*Azotobacter*	0.033 ± 0.052	0.004 ± 0.051	1.546 ± 0.053	0.047 ± 0.054
5.	*Pseudomonas*	0.038 ± 0.053	0.008 ± 0.055	1.644 ± 0.054	0.053 ± 0.052
6.	*Bacillus spp.*	0.049 ± 0.044	0.018 ± 0.048	1.749 ± 0.047	0.055 ± 0.046
7.	*Azospirillum* + *Rhizobium*	0.052 ± 0.048	0.018 ± 0.049	1.793 ± 0.051	0.060 ± 0.052
8.	*Azotobacter* + *Rhizobium*	0.323 ± 0.055	0.019 ± 0.058	1.872 ± 0.057	0.064 ± 0.056
9.	*Pseudomonas* + *Rhizobium*	0.836 ± 0.056	0.033 ± 0.061	1.902 ± 0.058	0.078 ± 0.060
10.	*Bacillus spp.* + *Rhizobium*	0.386 ± 0.054	0.032 ± 0.058	1.887 ± 0.059	0.068 ± 0.057
11.	*Bacillus licheniformis*	0.413 ± 0.063	0.028 ± 0.065	1.953 ± 0.068	0.079 ± 0.062
12.	Consortia 1	0.979 ± 0.065	0.033 ± 0.048	1.980 ± 0.064	0.085 ± 0.058
13.	Consortia 2	1.043 ± 0.057	0.036 ± 0.056	1.999 ± 0.059	0.098 ± 0.053

Data are mean ± standard deviation (±), Consortia 1(T_2 + T_4+ T_5 + T_6 + T_{11}), Consortia 2 (T_2 + T_3+ T_5 + T_6 + T_{11}), *IAA* Indole 3- acetic acid, *IBA* Indole 3-butyric acid, *GA_3* Gibberellic acid, *SA* salicylic acid, *FW Fresh weight.*

ethanol evaporated before being dissolved in 25 μl of Diethyl pyrocarbonate (DEPC, Invitrogen, USA) treated water. For 10 min, tubes were maintained at 60 °C in a dry bath. To assess the quality of the isolated RNA, it was put onto a 1 % agarose gel (Sambrook and Russell, 2001; Solanki, 2016; Srivashtav et al., 2019).

2.9.2. Gene profiling

Three phytohormones genes (*ARF*, *ERF-IF*and *GAI*) were screened using genomic DNA and cDNA from adolescent root samples of mungbean (*var*. Co4). In quantitative real time-PCR (qRT-PCR) experiment, gene-specific primers were employed to amplify a set of mungbean phytohormones and housekeeping genes, and their details are presented in Table 4. For indigenous primers, *VrTUB* and *β-Actin* primers were chosen in accordance with Chang et al. (2010) and Sairam et al. (2009) respectively. The gene-specific primers (*ARF*, *ERF-IF* and *GAI*) were chosen in accordance with Tao et al. (2009) and Hao et al. (2011), PCR amplification was carried out in a 25 μl reaction volume containing 12.5 μl Top Taq master mix (QIAGEN), 0.5 M per primer, 10.5 μl nuclease-free water and 1 μl genomic DNA/cDNA (80 ng).

Initial denaturation at 94 °C for 3 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55–61 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. On 2.5% agarose gel, the amplified products were resolved. The reaction was carried out with the DyNAmo Colorflash SYBR green qPCR kit and ROX as a passive reference dye (Thermo Scientific, USA). Each 20 μl reaction volume comprised 80 ng cDNA, 200 nM Forward (F) and Reverse (R) primers, and 2x Master mixes. In 96-well optical reaction skirted plates, samples were initially denatured by heating at 95 °C for 3

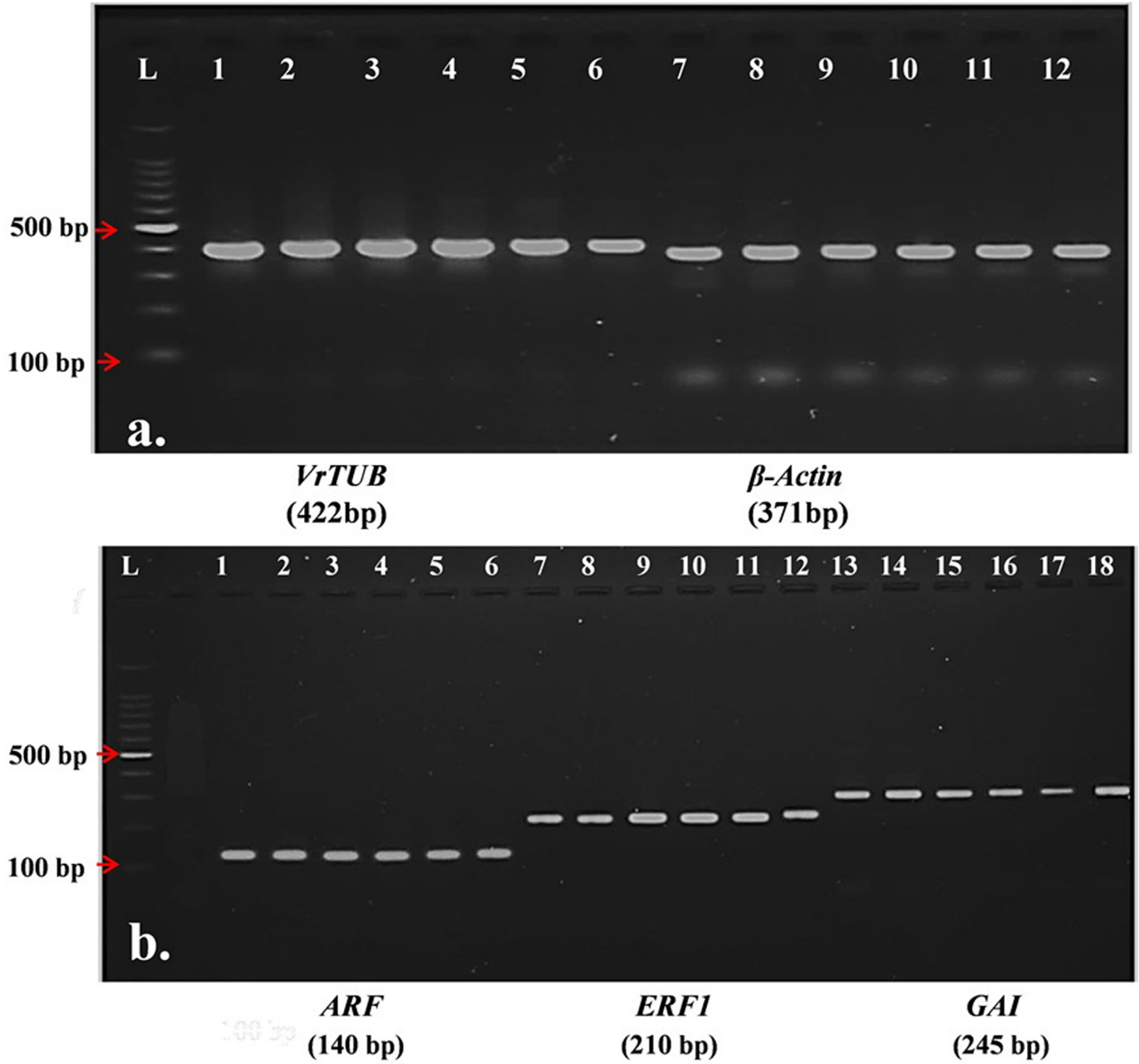

Figure 3. qRT-PCR analysis (a) Screening of reference genes and validated based on size by 1 kb gene ladder (L) [Lane L: 1 kb ladder; Lane 1–6: *Beta-tubulin* (*VrTUB* size: 422 bp); Lane 7–12: *β-Actin,* size: 371 bp]; (b) PCR product of relative genes *ARF, ERF-IF, GAI*. [Lane L: 1 kb ladder; Lane 1–6: Auxin response factor (*ARF* gene, size: 140bp); Lane 7–12: Ethylene response factor (*ERF-IF* gene, size: 210 bp); Lane 13–18: Gibberellic acid insensitive receptor (*GAI* gene, size: 245 bp)].

min, followed by a 40-cycle amplification and quantification protocol consisting of denaturation (95 °C for 10 s), annealing (56 °C for 10 s) and extension (70 °C for 20 s) (Thermo Scientific, USA).

To verify amplification of a single product, a melting curve study was performed. For each primer pair, a PCR without a template served as a control. Melting curve analysis (60–95 °C) after 40 cycles confirmed the specificity of amplicons. For quantitative real-time PCR analysis, three biological replicates were employed for each sample, and three technical replicates were examined for each biological replication.

2.10. Statistical analysis

Completely Randomised Design (CRD) was used to carry out the pot experiment. The advanced SPSS 16.0 software was used for all statistical analyses and calculations of experimental data. All statistical results were presented as the mean of three replications ±standard deviation (SD) of at least three repetitions of each experiment and they were analysed using analysis of variance (one-way ANOVA).

3. Results and discussion

3.1. Method validation

The linearity studies of all four phytohormones (*i.e.* IAA, IBA, GA_3 and SA) at different levels (0.001, 0.005, 0.010, 0.025, 0.050 and 0.100 μg g^{-1}) in 9:1 v/v methanol: water on LC-MS/MS showed a linear response. The correlation coefficient (R^2, n = 5) values of IAA, IBA, GA_3 and SA were 0.998, 0.997, 0.999 and 0.998, respectively (Figure 1 a, b, c and d). The obtained values were in accordance with the acceptable limit of $R^2 \geq$ 0.99. The sensitivity of the analytical method was measured in terms of

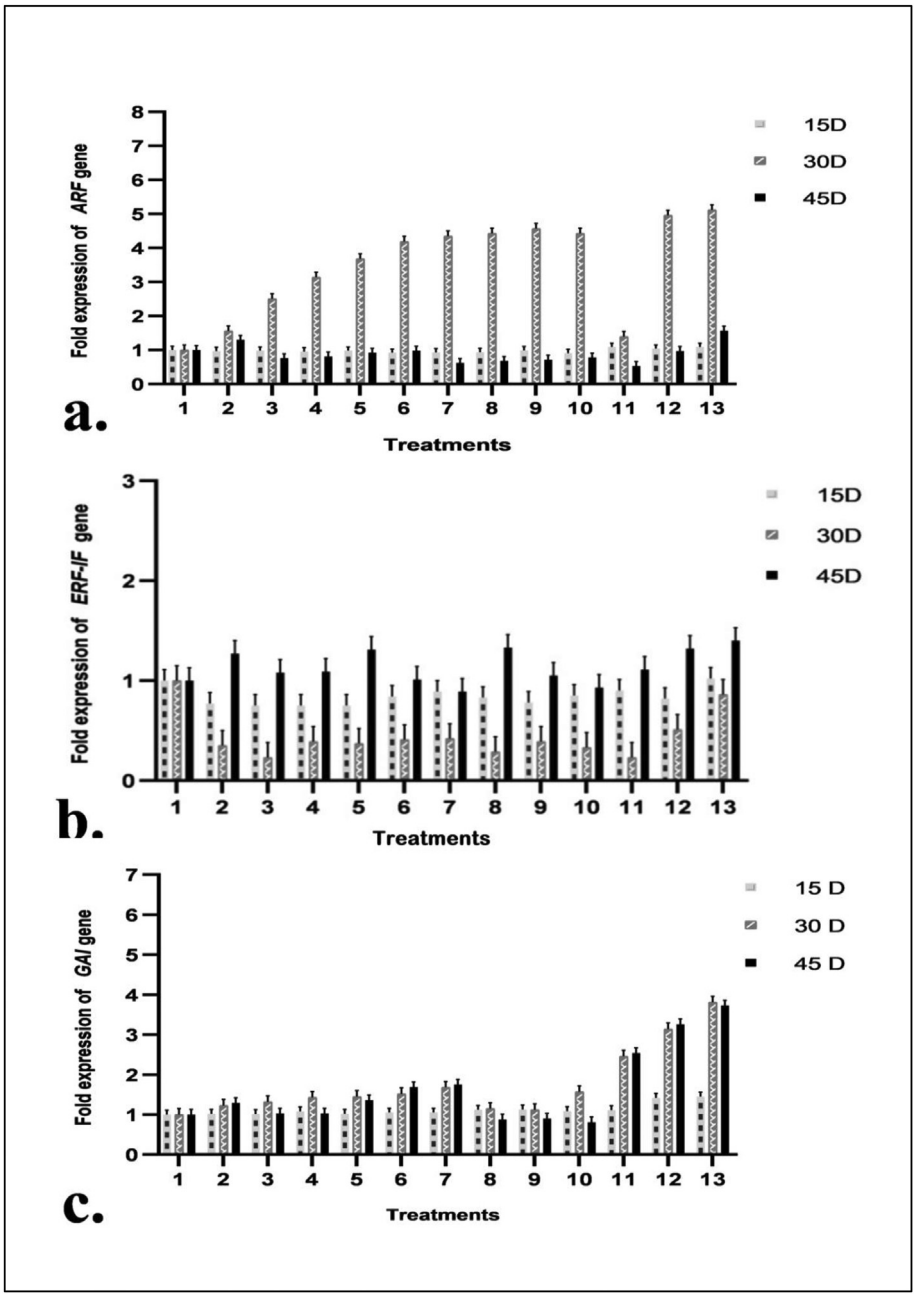

Figure 4. Fold expression level of responsive genes (a) Auxin response factor (*ARF* gene), (b) Ethylene response factor (*ERF-IF* gene), (c) Gibberellic acid Insensitive receptor (*GAI* gene) in mungbean; 15D: 15th Day, 30D: 30th Day, 45D: 45th Day.

limit of detection (LOD) and limit of quantification (LOQ), with a proper signal-to-noise ratio considered. The derived values of LODs and LOQs were for IAA (0.001 and 0.002 μg g^{-1}), IBA (0.001 and 0.002 μg g^{-1}), GA_3 (0.003 and 0.007 μg g^{-1}) and SA (0.200 and 0.006 μg g^{-1}). All of the results of recoveries and RSDs were obtained within the acceptable criteria of SANTE guidelines (SANTE, 2017), *i.e.* recovery (70–120 %) and RSD (≤20 %).

3.2. Determination of phytohormones on LC-MS/MS

Precursor and product ions for IAA (176.0 and 77.2, 130.1 m/z), IBA (204.0 and 130.0, 186.1 m/z), GA_3 (345.1 and 143.1, 239.1 m/z), and SA (137.0 and 59.5, 109.0 m/z) were optimized (Table 3). These main product ions were used to identify and quantify phytohormones (IAA, IBA, GA_3 and SA) from adolescent root tissues. Considering the peak shape and its resolution from the sample noise, the chromatographic conditions were contrived by optimizing the mobile phases and column conditions as per Table 3.

For the UHPLC-MS/MS analysis of all four phytohormones, MS-grade methanol and water, acidified with 0.1 % formic acid was used to achieve the high-resolution peak of the target compound. The use of acidified mobile phase, particularly aqueous methanol over aqueous acetonitrile, has significantly decreased baseline noise and increased the ionization efficiency of the phytohormones (Kite et al., 2007). The chromatograms of a standard solutions of IAA (6.2 min), IBA (6.6 min), GA_3 (6.0 min) and SA (6.4 min) with retention time (RT) were established under the aforementioned circumstances (Figure 2 a1, b1, c1 and d1). All four phytohormones were found to be linear in the range of 0.003–1.998 μg g^{-1}.

The multiple treatments trials were carried out on mungbean (*var.* Co4) and the highest concentration of IAA, IBA, GA_3 and SA in adolescent root tissue were obtained 1.043, 0.036, 1.999, and 0.098 μg g^{-1} FW in treatment T_{13} Consortia 2 (Table 5). When compared to the absolute control, treatment T_{12} Consortia 1 was observed with second highest concentration of all four phytohormone among all the different treatments (Table 5 and Figure 2 a2-a4, b2-b4, c2-c4 and d2-d4).

Plant growth is stimulated by the presence of phytohormones, which impact the endogenous mechanism of plants. Iqbal et al. (2017) revealed similar findings in which phytohormones such as auxin, ethylene, jasmonates, salicylic acid and strigolactone (SL) were demonstrated to promote plant growth. Furthermore, ethylene is thought to be multifunctional phytohormones that control growth. Phytohormones generated by PGPRs enhance the density and length of root hairs, resulting in an increase in a plant's root surface area (Tsegaye et al., 2017). This improves its nutrient and water absorption.

3.3. Influence of inoculation on relative gene expression of target genes

The relative expression analysis of three genes *i.e. ARF* (Auxin response factors), *ERF-IF* (Ethylene-responsive Initiation Factors) and *GAI* (Gibberellic-Acid Insensitive), at transcriptional level were performed in the juvenile root tissues of mungbean (*var.* Co4) on 15th, 30th and 45th day after treatment, using qRT-PCR technique. Using a 1 kb gene ladder, reference genes were screened and verified based on size. The highly stable *Actin* gene was chosen for gene expression data normalization based on its NormFinder stability value (Fig.3a and 3b).

3.3.1. Auxin response factor (ARF)

As compared to control on 15thday old mungbean adolescent root tissue, 5.12 folds increase in *ARF* gene expression is reported in treatment T_{13}. While second highest expression (4.96 folds) of *ARF* gene was reported in treatment T_{12} on 30th day after treatment as compared to control (Figure 4a). An increase in *ARF* gene expression was observed by Stearns et al. (2012) in the plants which have an association with ACC deaminase producing bacteria and suggesting that, in the absence of ethylene, *ARF* signalling can progress may have an impact on plant IAA biosynthesis induction. The production of IAA by the bacteria induces *ACC synthase*, which this ethylene may interact with plant auxin response signalling to repress bacterially induced auxin effects in the plant (Glick et al., 1999).

3.3.2. Ethylene response initiation factor (ERF-IF)

As compared to control on 15th, 30th and 45th day old mungbean root tissue, the expression of *ERF-IF* gene (ethylene response initiation factor) was found well balanced (Figure 4b). *ERF- IF* activated by the binding of EIN3/EIL in the primary ethylene response element (PERE) present in the promoter of *ERF1* which is involved in ethylene signal transduction pathway and acts as a positive regulator of ethylene response in rice

Table 6. Effect of different treatments on yield attributing characters of mungbean under pot condition.

Sr. No.	Treatments	Yield attributing characters											
		NNP			NPP			WNP Fw (g)			SYP (g)		
		Y^a	Y^b	P	Y^a	Y^b	P	Y^a	Y^b	P	Y^a	Y^b	P
1.	Control	8.07	8.05	8.06	19.73	18.92	19.33	0.59	0.55	0.57	2.74	3.05	2.90
2.	*Rhizobium* spp.	9.99	9.67	9.83	21.73	20.65	21.19	0.96	0.87	0.91	2.94	3.33	3.13
3.	*Azospirillum*	9.17	9.15	9.16	20.73	19.49	20.11	0.71	0.68	0.70	2.86	3.18	3.02
4.	*Azotobacter*	9.42	9.40	9.41	21.07	19.84	20.45	0.73	0.71	0.72	2.92	3.22	3.07
5.	*Pseudomonas*	9.82	9.57	9.69	23.07	20.68	21.87	0.72	0.75	0.73	3.76	3.65	3.71
6.	*Bacillus spp.*	8.66	9.09	8.88	20.73	19.26	20.00	0.69	0.70	0.69	2.84	3.14	2.99
7.	*Azospirillum* + *Rhizobium*	10.41	9.97	10.19	22.07	21.04	21.55	0.94	0.89	0.92	3.12	3.44	3.28
8.	*Azotobacter* + *Rhizobium*	10.48	9.80	10.14	22.73	21.43	22.08	1.10	1.06	1.08	3.15	3.61	3.38
9.	*Pseudomonas* + *Rhizobium*	10.69	10.84	10.76	24.27	21.69	22.98	1.17	1.13	1.15	3.76	3.87	3.82
10.	*Bacillus spp.*+ *Rhizobiu*	10.11	9.73	9.92	22.07	21.67	21.87	0.89	0.86	0.88	3.03	3.39	3.21
11.	*Bacillus licheniformis*	8.59	8.65	8.62	20.73	19.24	19.99	0.68	0.70	0.69	2.82	3.12	2.97
12.	Consortia 1	12.85	11.50	12.18	24.73	22.60	23.67	1.52	1.50	1.51	3.82	4.08	3.95
13.	Consortia 2	15.85	14.84	15.35	25.73	23.76	24.75	1.60	1.58	1.59	4.47	4.57	4.52
	SEM	0.074	0.101	0.071	0.239	0.176	0.150	0.010	0.006	0.003	0.016	0.023	0.020
	C. D. (P = 0.05)	0.21	0.29	0.20	0.68	0.50	0.43	0.03	0.02	0.01	0.05	0.07	0.06

NNP Number of nodules plant^{-1}, *NPP* Number of pods plant^{-1}, *WNP* Weight of nodules plant^{-1}(g), *SY* Seed yield plant^{-1}(g), *Fw* Fresh weight (g).
Y^a Year 2017–18, Y^b Year 2018–19, *P* Pooled data, Consortia 1(T_2 + T_4+ T_5 + T_6 + T_{11}), Consortia 2 (T_2 + T_3+ T_5 + T_6 + T_{11}).
SEM: Standard error of mean, C. D: Critical difference (n = 3).

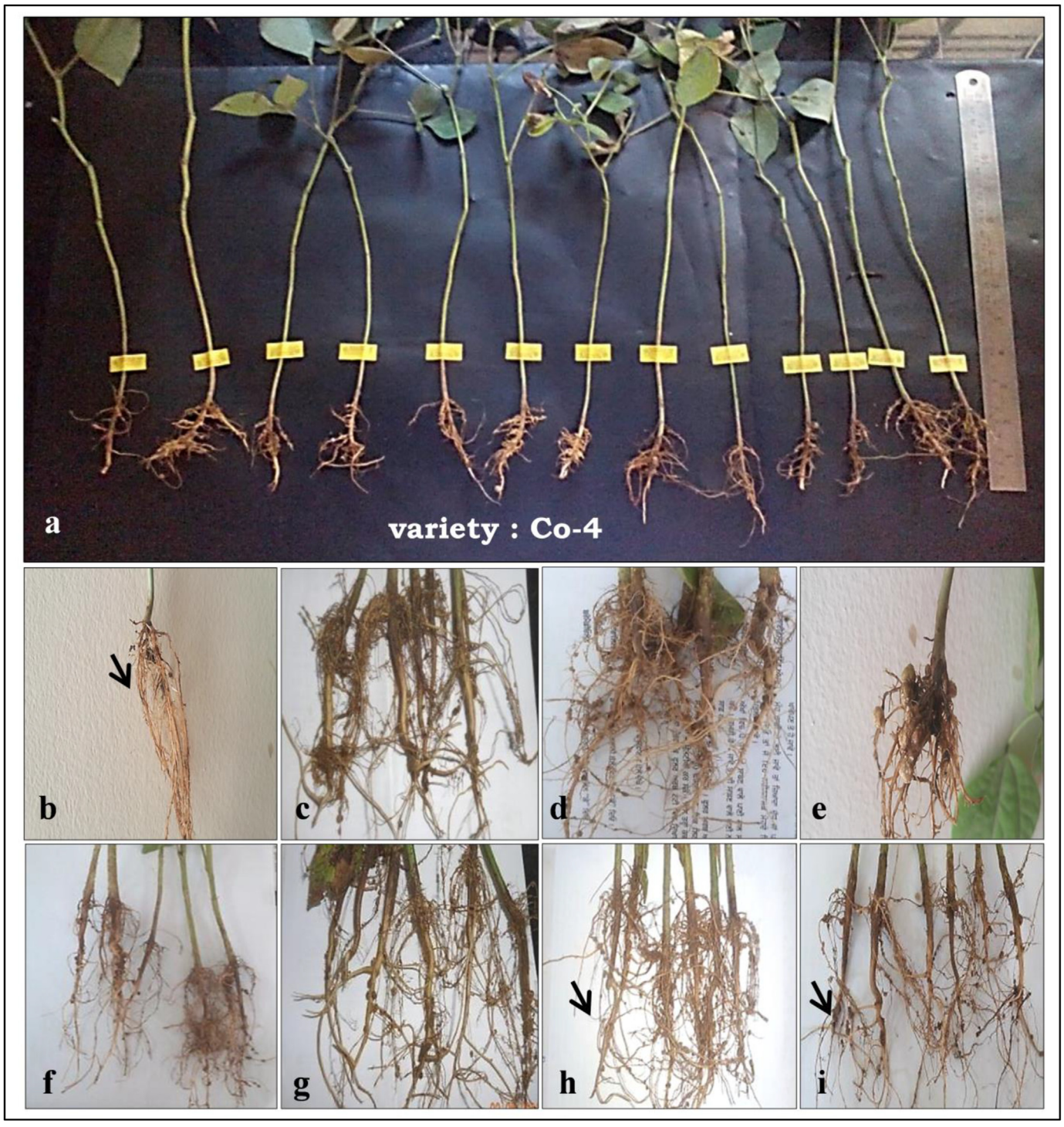

Figure 5. Yield attributing characters, no of nodules per plant of mungbean. (a) variety: Co-4, (b) Control, (c) *Rhizobium*, (d) *Azospirillum*, (e) *Azotobacter*, (f) *Pseudomonas,* (g) *Bacillus spp*., (h) Consortia 1 (i) Consortia 2.

(Alonso and Stepanova, 2004). It is well known that ethylene plays a role in the initiation of root hairs and induce the emergence of adventitious roots in rice at the same time the over expression of *OsERF* exhibit short root, coiled primary root and slightly short shoot phenotype and elevated response to exogenous ethylene but also improved the tolerance to stresses (Mao et al., 2005; Ito et al., 2006). In the present study, expression of ethylene response initiation factor *(ERF-IF)* gene found no variations in mungbean roots on penetration of microorganism.

3.3.3. Gibberellic acid insensitive (GAI) receptor

In case of *GAI* receptor expression, all treatments showed increase in gene expression after 30thday of sawing. Treatments T_{13} and T_{12} showed 3.87 and 3.26 fold increase in *GAI*expression as compare to control, after 45 day (Figure 4c). Fu et al. (2001) found high-level expression of *GAI* caused dwarfism and reduced GA_3 responses. *SLR1* a *GAI* gene, whose high level expression negatively regulate the plant responses to GA (Ikeda et al., 2001; Itoh et al., 2002).

3.4. Influence of inoculation on yield attributing characters

As compared to the uninoculated control, all the treatments significantly increased nodulation prominence and seed yield plant^{-1} (Table 6). Pre-sowing inoculation of mungbean seeds with penta combination Consortia 2 (T_{13}: *Rhizobium* + *Azotobacter* + *Pseudomonas* + *Bacillus spp.* + *Bacillus licheniformis*) demonstrated the highest nodulation status in terms of a number of nodule plant^{-1} (Figure 5) and the highest seed yield plant^{-1} in both *rabi* seasons (Year, 2017–2018 and 2018–2019), followed by penta combination Consortia 1 (T_{12}: *Rhizobium* + *Azospirillum* + *Pseudomonas* + *Bacillus spp.* + *Bacillus licheniformis*). Pooled analysis of two seasons (Table 6) revealed that inoculation with penta combination Consortia 2 resulted in substantially more nodules plant^{-1} (15.35) (Figure 5), pods plant^{-1} (24.75), fresh weight of nodules plant^{-1} (1.59 g) and seed yield plant^{-1} (4.52) than other treatments and the uninoculated control (Table 6 and Figure 5). However, the penta combination inoculation with Consortia 1 was only comparable in nodules plant^{-1} (12.18) and pods plant^{-1} (23.67) (Table 6).

Tri and Tetra combinations of rhizobacteria tested in a single medium exhibited more diverse PGP characteristics than a single inoculant in wheat (Kumar et al., 2021). Single inoculations with *Rhizobium*, PSB, or PGPR were found less efficient than combined inoculations. *Rhizobium* and PSMs (*Aspergillus awamorii* and *Pseudomonas striata*) as dual inoculants increased chickpea grain production in the field (Andy et al., 2020). Valverde et al. (2006) demonstrated that co-inoculation of *Pseudomonas jessenii* PS06 and *Mesorhizobium ciceri* C-2/2 improves chickpea nodulation, growth, and seed production in both greenhouse and outdoor trials. Similarly, *Mesorhizobium* and PGPR were able to significantly improve nodulation and root and shoot dry matter in chickpea (Verma et al., 2010; Verma and Yadav, 2012). The enhanced yield of mungbean in combination inoculation might be due to plant growth stimulation. Furthermore, PGPRs are known to produce a range of secondary metabolites, which may be contributed significantly to plant defence and production.

PGPRs are also known to increase levels of flavonoid-like compounds in legume roots, which may be an additional role in nodule development during seed bacterization (Sharma et al., 2005). PGPR and PSB are known to promote Biological Nitrogen Fixation (BNF) by increasing nodulation and inhibiting the growth of harmful microorganisms in the root system. At the blooming stage, similar drift was seen for all growth metrics compared to the uninoculated control.

4. Conclusions

Based on the findings, it is possible to infer that these standard known strains of plant growth promoting rhizobacteria (PGPR) have the capacity to be utilized as biofertilizers agents. The unique capability of different isolates in the consortia has attributed plant growth promotion. In mungbean (*var.* Co4), penta combination of Consortia 2 ($T_2 + T_4 + T_5 + T_6 + T_{11}$) and Consortia 1 ($T_2 + T_3 + T_5 + T_6 + T_{11}$) were the most robust in terms of growth promotion, yield and yield attributing characteristics via modulating phytohormones and improved nutrient uptake over the control (T_1). Furthermore, it induces the gene expression of the *Auxin response factor (ARF)* at the same time the auxin redistribution is controlled by stimulating plants growth and lateral initiation in plant shoots and roots. Thus, the utilization of PGPR in pulses could provide alternative platform to farmers minimize modern agriculture challenges.

Declarations

Author contribution statement

Chaitanya S. Mogal: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Vanrajsinh H. Solanki: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rohan V. Kansara: Performed the experiments; Wrote the paper.

Sanjay Jha: Conceived and designed the experiments; Analyzed and interpreted the data.

Susheel Singh: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Vipulkumar B. Parekh; Rajkumar B. K.: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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