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CHEMOTACTIC RESPONSES OF SWEET FLAG (*Acorus calamus* L.) ROOT EXUDATES AND EVALUATION OF INOCULATION EFFECTS ON ITS GROWTH

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ABSTRACT. Root exudate is an important source of nutrients for microorganisms in the rhizosphere and it plays a major role in the early colonization including chemotactic responses and adsorption of rhizospheric bacteria. In this study, we characterized the root exudates from sweet flag under hydroponic conditions and assessed their effect on plant growth. In the present study, the crude root fractions of sweet flag recorded a maximum yield of 520.6 µg plant^{-1} followed by cationic, anionic and neutral fractions. Among the qualitative and quantitative analysis of different fractions, the cationic fraction recorded a maximum of 90 µg plant^{-1} for glutamic acid, followed by aspartic acid, glycine, serine and proline. In the anionic fraction, malic acid recorded a maximum of 78.0 µg plant^{-1} followed by oxalic, succinic, citric and glutamic acid fractions. The neutral fractions included different saccharides, among which, fructose recorded a maximum of 42.5 µg plant^{-1}, followed by glucose, maltose, ribose and arabinose. The relative chemotactic response (RCR) of PGPB (plant growth-promoting bacteria) strains towards different root exudate fractions of *Acorus calamus* was recorded and it was observed that the combination of Anionic + Cationic + Neutral fraction recorded maximum chemotactic response for PGPR strains. The adsorption of PGPR strains in the root of the *Acorus calamus* was recorded in three different phases of growth and among these, Log phase bacterial cells exhibited maximum colonization of 7.65×10^{-6} cells g^{-1} with *A.venilandii* (ACAzt-2). Inoculation effect of PGPB strains on the root exudate of *Acorus calamus* and its growth was evaluated and it was observed that the treatment T_5 – Consortium recorded maximum plant height and root growth of *Acorus calamus*, followed by T_2. Our results indicate that sweet flag root exudates induce chemotactic responses of PGPR strains and promoted their growth.

Introduction

The rhizosphere is best defined as the volume of rhizome around living roots, which is influenced by root activity and root exudate quantity and the number of individual components of the root exudates (Hiltner, 1904). This is a densely populated area in which plant roots must compete with the invading root systems of neighbouring plants for space, water and mineral nutrients, and with other soil-borne organisms, including bacteria for their energy requirements. Plants mediate both positive and negative interactions in the rhizosphere via root exudates (Bais *et al.*, 2006; Philippot *et al.*, 2013). (Ryan, Delhaize, 2001; Bais *et al.*, 2004). The ability to secrete a wide range of compounds in the rhizosphere is one of the most remarkable metabolic features of plant roots, with around 5–21% of total photosynthetically fixed carbon being transferred into the rhizosphere through root exudates (Whipps, 1990; Marschner, 1995; Derrien *et al.*, 2004). Friman *et al.* (2020) reported that the plant interaction with the above and below ground plants parts results in changes in the traits of the plant in terms of defence response against the insect herbivorous.

Root exudation includes the secretion of a diverse array of carbon-containing primary metabolites, such as saccharides, amino acids and phenolic acids, as well as more complex secondary compounds that are involved in plant defence and in stimulatory or inhibitory interactions with other soil organisms (Bertin *et al.,* 2003; Jones *et al.,* 2004; Bais *et al.,* 2006). Soil microorganisms play a crucial role in the sustainability and functioning of soil-based ecosystems because of their involvement in key processes, such as mineral nutrition cycling, organic matter turnover, soil structure formation and toxins removal (van Elsas *et al.,* 2006; Brussaard *et al.,* 2007). Root exudates are considered as one of the most important factors that affect soil microorganisms (Bais *et al.,* 2006; Yao, Allen, 2006). In continuous monocropping systems, crop roots repeatedly release the same types of exudates for many years, occasionally resulting in significant colonization and infection by certain beneficial or pathogenic microorganisms that utilize these substrates. Thus, root exudates persisting during the planting season of a monoculture crop could be responsible for soil sickness, and their allelopathy should be better understood in terms of soil microbial ecology (Inderjit, 2005; Kaur *et al.,* 2009). Root exudates are one of the most poorly quantified compounds of the belowground C cycle as they only occur in the narrow rhizosphere and are rapidly absorbed by different soil components and/or assimilated by soil microorganisms (Neumann *et al.,* 2009; Paterson, 2003; Phillips *et al.,* 2008; Pavankumar *et al.* 2019). Sharma *et al.* (2020) reported that the root exudates play a major in plant growth-promoting bacterial biofilm formation and colonization in *Brachypodium.*

Root exudate collection presents significant challenges due to difficulties associated with i) accessing the rhizosphere without disturbance or damage to plant roots as a result of the collection system; ii) selecting a suitable collection medium that does not affect root physiology and exudate recovery; and iii) spatial and temporal variations in root and rhizosphere environment (Phillips *et al.,* 2008). Various approaches have been used to collect exudates either directly from nutrient solutions, where plants are grown or through accumulation in solid media (generally sand or glass beads) or recovery through different flushing or extraction procedures (Gransee, Wittenmayer 2000; Sandnes *et al.,* 2005; Tang, Young 1982). However, the recovery of exudates by such approaches exert various physiological effects on the plant and incomplete leaching or adsorption of exudates by the solid media (Gransee, Wittenmayer 2000; Neumann, Römheld, 2007; Sandnes, *et al.* 2005).

Since root exudates are mainly derived from photosynthesis, this results in significant carbon (C) cost for plants as root exudates are believed to have important functions in the regulation of plant growth (both directly or indirectly), although most of these functions are just beginning to be investigated (Bertin *et al.,* 2003; Walker *et al.,* 2003).

In this study, the quantification of different fractions of the root exudates and chemotactic responses of the *Acorus calamus* medicinal plant and their effects of plant growth-promoting rhizobacteria on its growth was evaluated.

Material and methods

Collection of root exudates from *Acorus calamus*

For the collection of root exudates of *Acorus calamus* Linn., the wide-mouthed glass bottles with an aluminium mesh at the bottom for supporting the seeds were used. The lids were provided with holes fitted with a cotton plug for easy flow of filtered air. About 500 ml of Fahraeus nutrient solution of 1 mM $CaCl_2$, 2 mM K_2SO_4, 1 mM $MgSO_4$, 1 mM Fe(III)-citrate, 1 mM KNO_3, and the microelements (Fåhraeus, 1957) was added in the bottle and the entire set was wrapped in papers, followed by sterilization in an autoclave.

Five *Acorus calamus* transplanted plant showing no contamination on nutrient soft agar were transferred to the bottles and placed in the solution. The bottles were kept in the sunlight for three weeks and afterwards, the plants were removed and the solution containing root exudates was carefully collected, centrifuged at 300 g for 10 min and filtered through a 0.45 μm fritted glass filter, freeze-dried and weighed.

Fractionation of purified root exudates

The purified freeze-dried crude root exudate was dissolved in sterile distilled water and made up to the original volume. Then it was passed through 2.5 × 1.0 cm columns of Dowex-1 (200–400 μm mesh, formate form) and Dowex-50 (200–400 mesh, chloride form) (Sigma Chemical Company, USA) resins respectively to fractionate the exudates into anionic, cationic and neutral fractions.

The Dowex-50 cation exchange resin column was eluted with 1 M NH_4OH to obtain the cationic fraction of *Acorus calamus* root exudates and the eluate was evaporated to dryness until no ammonia odour was detected. The Dowex-1 anion exchange resin column was eluted with glacial acetic acid to get the anionic fraction of the same. The effluent that passed through the cation and anion exchange resin column constituted the neutral fraction. The different fractions obtained were freeze-dried and weighed.

Qualitative and quantitative analysis of the cationic fraction of the root exudates

The freeze-dried, cationic fraction of the root exudate was redissolved to the original volume in sterile distilled water and bidimensional paper (Whatman No. 1) chromatography was carried out to separate the amino acids using the following solvent systems:

- Butanol : acetic acid : water (4:1:5 v/v),
- Phenol : water (3:1 w/v).

Aliquots of the cationic fraction (20 μL) were spotted on two papers separately and both papers were subjected to chromatography under identical conditions. After developing the paper in the above-mentioned solvent system for 14 hr, the positions of amino acids were determined by spraying one paper

with ninhydrin reagent (2 g of ninhydrin in 25 ml acetone: 25 ml of 0.2 M acetate buffer in pH 5.5). The spots were identified by co-chromatography with authentic amino acids.

On the other paper, positions corresponding to the ninhydrin positive spots were marked, cut into several small segments and eluted into 8 ml of 80 per cent ethanol for 45 min., two ml of the ninhydrin reagent were added to the eluant (8 ml) and the tubes were kept in boiling water bath for 15 min. The pink colour developed was measured by Spectronic 20 colourimeter at 550 nm. The concentration of each amino acid present in the cationic fraction was determined by comparing their absorbance against the standard graph prepared with known quantities of different amino acids.

Qualitative and quantitative analysis of the anionic fraction of the root exudates

The freeze-dried anionic fraction of the root exudates was redissolved in the original volume of sterile distilled water and one-dimensional paper (Whatman No. 1) chromatography was carried out to separate the organic acids using the following solvent system: (n-pentanol: 5M formic acid 1:1, v/v). Aliquots of the anionic fraction (about 20 µl) were subjected to chromatography under identical conditions. After developing the paper for 15 hr, the position of organic acids was determined by spraying with bromothymol blue reagent (100 mg of bromothymol blue dissolved in 1 ml of 0.02 M NaOH and then raised to 250 ml with distilled water). The spots were identified by using reference chromatograms, with known organic acids, developed in the same solvent system. On the other paper, positions corresponding to these bromothymol blue positive spots were marked, cut out into smaller segments and eluted in 8 ml of distilled water and the concentration of each organic acids present in the anionic fraction was determined by titrating the eluant against 0.0144 M NaOH with phenolphthalein as the indicator.

Qualitative and quantitative analysis of the neutral fraction of the root exudate

The freeze-dried neutral fraction of the root exudate was redissolved to the original volume in sterile distilled water and bidimensional paper (Whatman No. 1) chromatography was carried out to separate the saccharides using the following solvent system:

- 1. n-propanol : Ethylacetate : water (70:10:20, v/v),
- 2. n-butanol : Acetic acid : water (100:22:50,v/v).

Aliquots of 10 µl of neutral fraction were spotted on two papers separately and both papers were subjected to chromatography under identical conditions. After developing the papers for 40 hr, the positions of saccharides were ascertained by spraying one paper with aniline hydrogen phthalate reagent (1.7 g phthalic acid; 1 ml aniline, 90 ml ethanol; 5 ml glacial acetic acid, 5.0 ml 40 per cent trichloroacetic acid). The spots were identified by chromatography with authentic saccharide. On the other paper, positions corresponding to these spots were marked, a paper was cut into smaller segments and eluted in 8 ml distilled water in a test tube. Reducing saccharides were estimated by the Somsgyi-Nelson method (Somogyi, 1952) and fructose (after hydrolysis with 0.1 M oxalic acid) by the method of Bacon and Bell (1948).

Chemotaxis by capillary assay

In vitro chemotaxis was assayed according to the method described by Rao and Johri (1989) with a slight modification. The root exudate was introduced into capillary tubes of 15 mm length and 0.25 mm diameter and tubes were placed into the cell suspensions of each of *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas* isolate for 30 minutes. Then capillaries were rinsed with distilled water and the content of capillaries was ejected onto plates containing the yeast extract glucose agar medium. Five replicates were maintained for each kind of organisms. The relative chemotactic response was calculated by the number of cfu per capillary containing the root exudate divided by the number of cfu in capillaries containing sterile distilled water (Eq. 1).

Determination of the number of cells adhered to the root

Acorus calamus plants were surface sterilized by immersion into 95 per cent ethanol for 1 min., followed by incubation for 20 min in 1 per cent of NaOCl. After rinsing five times with sterile distilled water, the sterilized seeds were placed on the surface of 1 per cent water agar on Petri plates (9 cm diameter at the rate of five transplanted plant exudates plated). Plants were incubated in an inverted position for 10 days at room temperature to allow growth. The plates were sealed with wax to avoid agar drying during germination. After transplanting, the seedlings were transferred onto semisolid Weaver's medium maintained in a test tube, at the rate of one seed per tube. The PGPB isolates, namely *A. lipoferum* (ACAzs-5), *A. venilandii* (ACAzt-2), *B. cereus* (ACPb-3) and *P. fluorescens* (ACPf-4), were harvested at lag, log and stationary phases, separately. One ml of a culture of each of the three different growth phases was inoculated in tubes maintained at 30 ± 2 °C alternating 10 hr in light and 14 hr in darkness for 48 hr. Five replication were maintained for each treatment.

After 48 h of inoculation, the roots from each treatment were excised separately into 10 ml of sterile distilled water. To enumerate the rhizoplane population of the root, serial dilutions were made and an indirect viable count was made on nitrogen-free malate agar, Waksman's base medium 77, Pikovskaya's and King's B media to enumerate populations of *A. lipoferum* (ACAzs-5), *A. venilandii* (ACAzt-2), *B. cereus* (ACPb-3) and *P. fluorescens* (ACPf-4) strains, respectively.

$$Relative\ chemotactic\ response = \frac{The\ number\ of\ colonies\ per\ capillary\ tube\ with\ root\ exudates}{The\ number\ of\ colonies\ per\ capillary\ tube\ with\ distilled\ water} \quad (1)$$

Evaluation of its growth parameters in *Acorus calamus* as influenced by Inoculation of PGPR strains

The *A. calamus* plants were raised in wide-mouthed glass bottles and inoculated with PGPR strains such as *A. lipoferum* (ACAzs-5), *A. venilandii* (ACAzt-2), *B. cereus* (ACPb-3) and *P. fluorescens* (ACPf-4) at 5 ml of each and consortium is the combination of all organisms. The plant growth-promoting effect of the PGPR strains was evaluated by determining their plant height and root length were recorded on the 21st day after planting.

Statistical analysis

Experimental results were analysed by one or two-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Significant difference among different treatments was considered at $P < 0.05$.

Results

Collection and fractionation of the root exudates of *Acorus calamus*

The root exudates of *Acorus calamus viz.* were collected and the constituents of different fractionations are studied as shown in Fig. 1. The crude root exudate of *Acorus calamus* (520. 6 µg plant^{-1}), the cationic fraction (198.3 µg plant^{-1}), the anionic fraction (145.2 µg plant^{-1}) and the neutral fraction (102.8 µg plant^{-1}). The quantity of cationic fraction was higher than that of anionic and neutral fractions ande the anionic fraction was weightier than the neutral fraction in the root exudate of the *Acorus calamus*. The cationic fraction included different amino acids, the anionic fraction included different organic acids and the neutral fraction included different sugars.

Quantitative and qualitative analysis of different root exudates of *Acorus calamus*

Five different amino acids, *viz.* aspartic acid, glutamic acid, glycine, serine and proline were detected in the cationic fractions of the root exudates of *Acorus calamus*. The relative occurrence of amino acids was in the descending order of content: glutamic acid > aspartic acid > glycine > serine > proline (Table 1).

Five organic acids, *viz.*, malic acid, oxalic acid, succinic acid, citric acid and glutaric acid were detected in the anionic fractions in the root exudates of *Acorus calamus*. The organic acids present were in the descending order of content: malic acid > oxalic acid, > succinic acid > citric acid > glutaric acid.

Five different saccharides, *viz.*, fructose, glucose, maltose, ribose and arabinose were detected in the neutral fractions of the root exudates of *Acorus calamus*. The saccharides present were in the descending order of content: fructose > glucose > maltose > ribose > arabinose (Table 1).

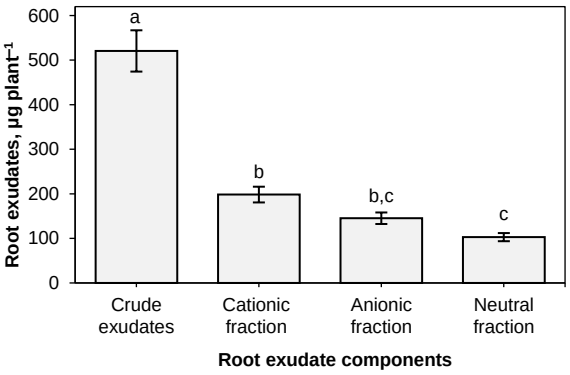

Figure 1. Fractions of the root exudates of *Acorus calamus*. Values represent a mean of six replications ± SD; Standard deviation. Different letters (a-c) after values indicate a significant difference among treatments at ($P < 0.05$)

Table 1. Qualitative and quantitative analysis of different fractions of the root exudates of *Acorus calamus*

Cationic fraction		Anionic fraction		Neutral fraction	
Amino acid	Quantity, µg plant^{-1}	Organic acid	Quantity, µg plant^{-1}	Sugar	Quantity, µg plant^{-1}
Aspartic acid	57.5 ± 3.1^{b}	Malic acid	78.0 ± 4.0^{a}	Fructose	42.5 ± 1.5^{a}
Glutamic acid	90.0 ± 5.0^{a}	Oxalic acid	39.5 ± 4.5^{b}	Glucose	24.5 ± 2.5^{b}
Glycine	23.5 ± 4.5^{c}	Succinic acid	17.5 ± 3.5c	Maltose	15.0 ± 2.0^{c}
Serine	16.5 ± 3.5^{d}	Citric acid	13.5 ± 2.5^{c}	Ribose	12.5 ± 2.0^{c}
Proline	10.5 ± 3.7^{d}	Glutaric acid	6.5 ± 1.5^{d}	Arabinose	7.5 ± 1.5^{d}

Values represent a mean of six replications ± SD (standard deviation).
Different letters after values indicate a significant difference among treatments at ($P < 0.05$)

Table 2. Relative chemotactic response of PGPR strains towards different root exudate fractions of *Acorus calamus* Linn.

Fraction of root exudates	RCR root exudates fraction of *Acorus calamus* PGPR strains*			
	ACAzs-5	ACAzt-2	ACPb-3	ACPf-4
Crude	1.0 ± 0.16^{a}	3.8 ± 0.22^{a}	2.4 ± 0.13^{b}	3.0 ± 0.10^{a}
Anionic	0.5 ± 0.25^{b}	3.0 ± 0.22^{b}	1.5 ± 0.86^{bc}	2.5 ± 0.26^{ab}
Cationic	0.4 ± 0.66^{b}	1.5 ± 0.15^{c}	1.0 ± 0.40^{c}	1.3 ± 0.15^{b}
Neutral	0.4 ± 0.35^{b}	2.0 ± 0.26^{c}	1.5 ± 0.42^{bc}	1.7 ± 0.42^{b}
Anionic + Cationic	0.8 ± 0.46^{ab}	3.4 ± 0.42^{b}	2.1 ± 0.76^{b}	3.0 ± 0.68^{a}
Anionic + Neutral	0.8 ± 0.20^{a,b}	3.2 ± 0.75^{b}	2.0 ±0.45^{b}	2.7 ± 0.65^{a,b}
Cationic + Neutral	0.6 ± 0.50^{b}	3.0 ± 0.05^{b}	1.8 ± 0.65^{b}	2.3 ±0.87^{a,b}
Anionic + cationic + Neutral	1.0 ± 0.89^{a}	4.3 ± 0.19^{a}	3.1 ± 0.97^{a}	3.8 ± 0.42^{a}
Control	0.01^{c}	–	–	0.01^{c}

A. lipoferum ACAzs-5; 2- *A. venilandii* ACAzt-2; 3- *B. cereus* ACPb-3; 4- *P. fluorescens* AC*Pf*-4.
Values represent a mean of six replications ± SD (standard deviation).
*At 1×10^{6} CFU ml^{-1} inoculant level. Different letters after values indicate a significant difference among treatments at $P < 0.05$. RCR-Relative chemotactic response.

Relative response of PGPR strains towards different root exudate fractions of *Acorus calamus*

Relative chemotactic responses of PGPR strains of *Acorus calamus viz.* were used as such (crude) as well as their fractionated compounds either singly or in combinations to study their chemotactic activity and to determine the relative chemotactic response of four selected efficient PGPR strains *viz.*, *A. lipoferum* ACAzs-5, *A. venilandii* ACAzt-2, *B. cereus* ACPb-3 and *P. fluorescens* AC*Pf*-4 (Table 2).

Among the three fractions tested individually, all the four strains showed higher and lower RCR values to anionic, cationic and neutral fractions followed by crude root exudate as such and a combination of the fraction of any two and individual fractions. Chemotactic response of *A. calamus* to all the four-strain although higher towards recombined fractions than the other fraction, the RCR value obtained was in the order of *A. lipoferum* ACAzs-5 (1.0 ± 0.89), *A. venilandii* ACAzt-2 (4.3 ± 0.19), *B. cereus* ACPb-3 (3.1 ± 0.97) and *P. fluorescens* AC*Pf*-4, (3.8 ± 0.42). Chemotactic response to all the four strains although lowest towards cationic fractions than the other fraction, the RCR value obtained was in the order of *A. lipoferum* ACAzs-5 (0.4 ± 0.66), *A. venilandii* ACAzt-2 (1.5 ± 0.15), *B. cereus* ACPb-3 (1.0 ± 0.40) and *P. fluorescens* AC*Pf*-4, (1.3 ± 0.15) Table 3.

Table 3. Adsorption of PGPR strains to the roots of *Acorus calamus*

Crop	Growth phase	Root PGPR isolates*			
		A. lipoferum ACAzs-5	*A. venilandii* ACAzt-*2*	*B. cereus* ACPb-3	*P. fluorescens ACPf-4*
Acorus calamus	Lag	3.00 ±0.03^{b}	4.32±0.23^{a}	3.22±0.43^{b}	3.30±0.43^{b}
	Log	6.24±0.32^{ab}	7.65±0.34^{a}	6.36±0.56^{b}	6.70±0.56^{ab}
	Stationary	3.56±0.26^{a}	4.00±0.60^{a}	3.66±0.54^{a}	3.85±0.25^{a}

*1×10^6 cells g^{-1} of the root. Values represent a mean of six replications ± SD (standard deviation).
Different letters after values indicate a significant difference among treatments at $P < 0.05$.

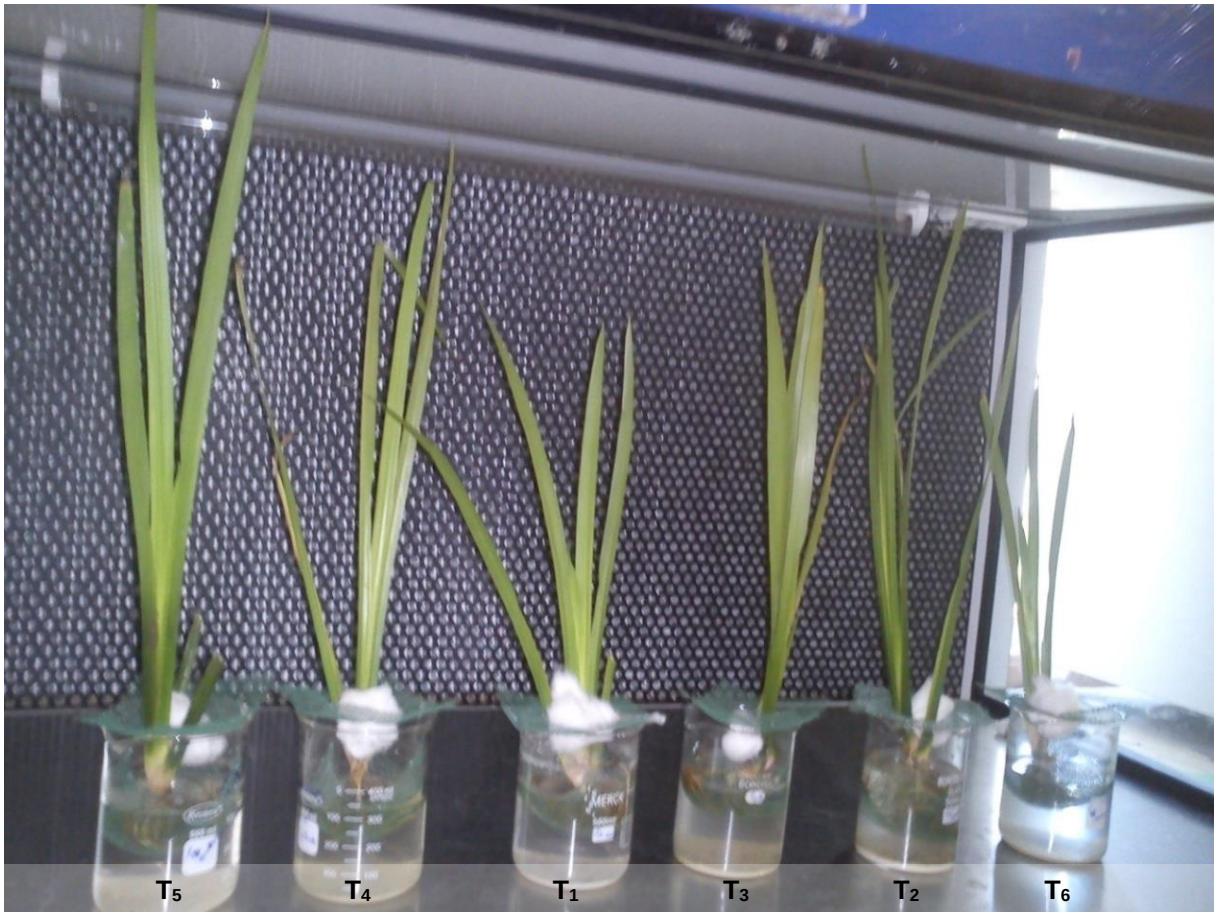

Figure 2. Plant growth as influenced by PGPR strains on the root exudates of *Acorus calamus* (T_1-*Azospirillum*, T_2-*Azotobacter*, T_3-*Bacillus* T_4-*Pseudomonas*, T_5-Consortium and T_6-Control)

Adsorption of PGPR strains to the roots of *Acorus calamus*

The adsorption of PGPR strains namely *A. lipoferum* ACAzs-5, *A. venilandii* ACAzt-2, *B, cereus* ACPb-3 and *P. fluorescens* ACPf-4 to Acorus calamus root as influenced by lag, log and stationary growth phase cells was studied and the results are presented in Table 2. Irrespective of strains, the log phase cells recorded the highest number of adsorbed bacteria to *A. calamus* roots. A log phase 6.24×10^6 for *A. lipoferum* ACAzs-5, 7.65×10^6 for *A. venilandii* ACAzt-2, 6.36×10^6 for *B. cereus* ACPb-3, and 6.70×10^6 for *P. fluorescens* ACPf-4, respectively were recorded in *A. calamus* roots.

Effect of PGPR strain on the growth of *A. calamus* plants

There were significant differences ($P < 0.05$) in plant heights of *Acorus calamus* plants treated with *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas* strains. The plant height of *Acorus calamus* plants increased significantly due to the inoculated PGPR strains on all 7, 14 and 21 (DAP). The maximum plant height of T_5 – consortium (29.0 ± 1.7 cm plant^{-1}) was followed by T_2. The minimum plant height was recorded in T_1 – *Azospirillum* (22.8 ± 1.7 cm plant^{-1}) Table 4, on 21 DAP when compared to the uninoculated control. (Figs. 2, 3).

Figure 3. Root growth of T_5 (consortium) as influenced by PGPR strains.

Table 4. Effect of PGPR strains on the growth of *A. calamus* in terms of a) plant height (cm) and b) root length (cm). Values represent a mean of six replications ± SD; Standard deviation. Different letters after values indicate a significant difference among treatments at $P < 0.05$.

Treatment	Plant height, cm	Root Length, cm
T_1 – *Azospirillum*	22.8 ± 1.7^b	17.0 ± 2.1^b
T_2 – *Azotobacter*	27.4 ± 2.3^a	23.9 ± 2.2^a
T_3 – *Bacillus*	26.7 ± 2.7^a	24.2 ± 1.7^a
T_4 – *Pseudomonas*	24.3 ± 3.2^{ab}	25.0 ± 3.2^a
T_5 – Consortium	29.0 ± 1.7^a	25.4 ± 2.9^a
T_6 – Control	18.6 ± 1.2^c	13.8 ± 1.9^c

There was a significant difference in root lengths of *Acorus calamus* plants of five replications treated with *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas*, obtained from the maximum plant height at all sampling periods of root exudates. The root length of the *Acorus calamus* significantly increased due to the inoculated PGPR strains on 7, 14 and 21 DAP. The treatment T_5 – was significantly over other treatments. The maximum root length of T_5 – consortium, (25.4 ± 2.9 cm plant^{-1}) followed by T_2 – *Azotobacter venilandii* (23.9 ± 2.2 cm plant^{-1}) was recorded. The minimum root length was recorded in T_1 – *Azospirillum* (17.0 ± 2.1 cm plant^{-1}).

Discussion

In the root exudates, fructose constitutes over 80% of carbohydrates, indicating that this saccharide residue is the main source of carbon in the rhizosphere of young *A. calamus* plants. Similar findings have been obtained during the growth of wheat, rice and *Catharanthus roseus* seedlings (Jones, Darrah 1993; Přikryl, Vančura, 1980; Bacilio-Jimenez *et al.* 2001; Karthikeyan *et al*, 2007; Karthikeyan *et al.* 2012; Karthikeyan *et al.* 2013).

The cationic fraction of *Acorus calamus* root exudate contained 90.0 µg of glutamic acid, 57.5 µg of Aspartic acid, 23.5 µg of glycine, 16.5 µg of serine and 10.5 µg of proline plant^{-1}. These appreciable quantities of secreted amino acids can initiate the chemotaxis of the PGPR strains. Somers *et al.* (2004) have shown that the amino acids and carbohydrates of the root exudates play a major role in chemotaxis on root surfaces. In the present study, different saccharides *viz.*, fructose, glucose, maltose, ribose and arabinose were obtained at varying quantities from the neutral fraction of the root exudate. These primary metabolites were found responsible for the chemotactic attraction of the plant growth-promoting bacteria.

The anionic fraction of *Acorus calamus* root exudate contained five organic acids *viz.*, malic, oxalic, succinic, citric and glutamic acid. Kloss *et al* (1984) reported the predominance of malic acid in the root exudates of a C4 plant.

The chemotactic response of PGPR strains was in the order of *A. venilandii* ACAzt-2 > *P. fluorescens* ACPf-4 > *Bacillus cereus* ACPB-3 > *A. lipoferum* ACAzs-5. The RCR values for all the PGPR strains towards recombined fraction were significantly higher ($P < 0.05$) than those of crude root exudates.

The *Azotobacter choroococcum* was reported to show *a* stronger response to sugars and amino acids, but weaker towards organic acids (Sood, 2003). Kloss *et al*, (1984) reported that the *P. fluorescens* was strongly attracted towards citric and malic acids, which were predominant in the root exudates of the tomato plant. Root exudates modulate the interaction between plant and plant growth-promoting rhizobacteria (Deweart *et al.* 2002). Mark *et al.* 2005 reported that behavioural changes in the bacteria were a result of altered gene expression elicited by the compounds present in root exudates. Vora *et al.* (2021) reported differential chemotaxis and biofilm formation behaviour in plant

growth-promoting rhizobium strains based on studies on inter- and monocropped plants.

The adsorption of PGPR strains in the roots of *Acorus calamus* was also recorded. *A. venilandii* recorded maximum populations in the logarithmic phase than other phases. The mucilages of the root secretions play a major role in enhancing of adsorption of the cells.

The migration of two rhizosphere beneficial bacteria in the soil towards living wheat plants or towards synthetic attractants is known to occur *in vivo* (Rovira, 1969), and the migration of the *A. brasilense* and *Pseudomonas fluorescens* towards wheat roots occur in the soil (Bashan, 1986). Furthermore, results from soft-agar, capillary tube, and soil chemotaxis assays indicate the attraction of rhizobacteria to seed and seedling root exudates (Begonia, Kremer, 1999). *A. brasilense* exhibits positive chemotaxis towards a large number of organic compounds such as amino acids, saccharides, organic acids, typical for plant root exudates. Malate, succinate, and fructose have been the most effective attractants for *A. brasilense* (Zhulin *et al.*, 1988). The better colonization by *A. brasilense* and *Bacillus* sp., to plant roots may be attributed to chemical effectors, which might have favoured better root colonization. The capacity of root exudates to attract bacteria could be attributed to some of their components (Jin *et al.*, 2019). Rekha *et al.* (2020) reported the role of *B. subtilis* RR4 in the enhancement of root exudation of malic acid and salicylic acid, which serve as plant growth promoters and stress alleviators in rice plant.

Several bacteria were described as presenting positive chemotaxis toward different molecules exuded by plants, including sugars, amino acids, various dicarboxylic acids such as succinate, malate and fumarate, and aromatic compounds such as shikimate, quinate, protocatechuate, vanillate, acetosyringone, gallate, catechol and luteolin (Brencic, Winans, 2005).

Root exudates could supply rhizobacteria with precursors needed for phytohormone synthesis. An interesting report describes the mapping of sugar and amino acid availability in *Avena barbata* root exudates (Jaeger *et al.*, 1999). This study highlighted the availability of tryptophan mainly in the root tip region. Tryptophan is the precursor for indole 3-acetic acid, a major auxin, suggesting that rhizobacteria could exploit root exudate pools for various growth regulator precursors (Zhang *et al.*, 2019).

Plants also exude aminocyclopropane-1-carboxylic acid (ACC), which is an ethylene synthesis precursor and can be used as carbon and nitrogen sources by rhizobacteria, as recently shown by acdS expression mainly by root exudates assimilating bacteria and those inhabiting root tissue (Haichar *et al.*, 2012; Karthikeyan *et.al.*, 2012). The use of ACC by ACC-deaminase-producing rhizobacteria reduces the amount of ACC outside the plant and equilibrated the ACC level outside and inside. Plants release more ACC and therefore produce less ethylene, which inhibits root elongation (Glick *et al.*, 1998).

As mentioned above, plants exude a high variety of saccharides, such as glucose, fructose and sucrose, which are also suggested to be involved in the production of exopolysaccharides (EPSs) by the rhizospheric bacteria. The EPSs are the main contributors in legume-rhizobia interactions, leading to nodulation and nitrogen fixation. They have also other functions such as root-adhering soil structuring, non-legume plant growth promotion or evasion from the legume defence response during crack entry in roots (Alami *et al.*, 2000).

The root exudation can have a major impact on the nutrient acquisition by plants. Further, the PGPR strains are inoculated on the plants and were analysed for their growth parameters. The treatment T_5 (consortium) was recorded the maximum plant height and root length of *Acorus calamus* on all three sampling periods. This was due to the action by PGPR strains, which enhance the plant growth-promoting activity. In this study, we found that the composition and concentration of sugars, amino acids, and organic acids from sweet flag root exudates exert great attraction of PGPR strains. This may provide them with a clear advantage over PGPR strains. Our results strongly suggest that the chemoattractant characteristics of PGPR strains probably favour the *Acorus calamus* growth and could induce the PGPR strains colonies to improve the plant growth.

Conclusions

This study leads to the conclusion that concerning the characterization of root exudates and chemotactic responses from the sweet flag on their effects of PGPR inoculations. The rhizosphere microbes play an important role in improving the medicinal values of medicinal plants. This increases the interest in the research of interaction between medicinal plants and the rhizosphere microbes for the improvement in terms of plant growth and yield of phytochemical constituents of medicinal plants. The inoculation of PGPR is a technology to enhance the quantity and quality of medicinal compounds. Therefore future research is recommended for a better understanding of the diversity and functions of rhizosphere bacteria to improve the yield of *Acorus calamus.*

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

PP – study conception and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision and approval of the final manuscript;
BK – study conception and design, acquisition of data, analysis and interpretation of data, critical revision and approval of the final manuscript;
MMJ – study conception and design, critical revision and approval of the final manuscript.

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