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Indole acetic acid and flavonoids production by rhizospheric bacteria isolated from *Medicago sativa* L. rhizosphere

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Abstract

Rhizospheric region of the roots is richly supplied with the secondary metabolites that orchestrate all sort of rhizospheric interactions and hence communicate plant root with their below-ground competitors. A chemical dialogue is established between the microbes and plant roots that create an atmosphere of complex and dynamic interactions flourishing either symbiosis or pathogenicity. A wide range of secondary metabolites are found there in rhizospheric region but particularly flavonoids and auxins are documented to be the most important signalling elements in plant-microbe interactions. The present work aims to isolate some of the best growth promoting rhizo-bacteria from the rhizosphere of *Medicago sativa* L. and their potential for secondary metabolites. A total number of 15 different rhizospheric bacterial strains were isolated from the rhizosphere of *Medicago sativa* L. using L-agar medium. All of these strains were tested for the secretion of Indole acetic acid (IAA) and flavonoids. Three strains were known to be the best producer of IAA and flavonoids. Fifteen different strains in the rhizosphere of just one plant suggest that how diverse they are, and their production of useful secondary metabolites show their importance for the plants.

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Introduction

Rhizosphere, the zone around plant roots, is occupied by enormous amount of micro-organisms and the chemicals exuded by the plant roots (Hartmann *et al*., 2008). The length of the rhizosphere is variable and depends on the soil structure, texture, size of particle, content of water and capacity of buffer (Nye, 1984; Darrah, 1993; Junk, 2002;).

The soil consists of dynamic populations of micro-organisms (bacteria, cyanobacteria, actinomycetes, fungi, algae, protozoa , nematodes) which play an important role for the plant in the form of enhancing soil fertility (biological N_2 fixation), increasing the availability of nutrients in the rhizosphere (phosphate solubilization, siderophore production), phytohormones production (Indole acetic acid, Gibberellin, Cytokinin), inducing increase in root surface area, enhancing beneficial symbiosis of the host with other microbes and combination of some positive modes of action (Khan *et al*., 2009; Ahemad and Khan, 2012; Khan *et al*., 2006. They also play significant role in the maintenance of soil structure, detoxification of hazardous chemicals and pathogens control (Elsgaard *et al*., 2001; Filip, 2002; Giller *et al*., 1998).

Plant root exudates and lysates (rhizodepositions) provide a niche and attract the soil microbial population. Quite often up to 15% of the root surface may be covered by microbial colonies, majority of them belongs to variety of bacterial strains. In the rhizosphere, bacterial population densities may be very high (up to 100 times higher) than in the bulk soil. Bacteria use plant exudates as nutrients for their growth and release some metabolites into the rhizosphere which in turn act as signaling molecules for the rest of microbial populations as well as for root cells of the host plant (Van Loon and Bakker, 2003; Bais *et al*. 2004; Gray and Smith, 2005; Kiely *et al*., 2006). *Rhizobium*, a legume symbiont, is the most worked out example of signal exchange between host plant and bacteria. Flavonoids exuded by plant roots, act as signal compounds which induce transcription of nodulation genes (Nod genes) necessary for infection, attraction of bacteria chemo-tactically and enhancement of bacterial cells growth rate, while bacteria release Nod factors, which help in nodule formation in legume plants. Nod factors are proteins, perceived by plant root hairs, which function in a hormone like fashion to initiate nodules in host plant. In this symbiotic association *Rhizobium* provide fixed N_2 to the host plant for the biosynthesis of amino acids and proteins, while the host give carbohydrates to bacterium (Brencic and Winans, 2005; Gray and Smith, 2005).

For each symbiotic association the specific flavonoids may be different as the case studied for three symbiotic pairs. Exudates from three plants (alfalfa, clover and soybean) were collected from the rhizosphere and identified for signaling compounds (flavonoids). The identified flavonoids were daidzein and genistein for soybean-*Bradyrhizobium japonicum* (Kosslak *et al*., 1987), luteolin for alfalfa-*Rhizobium meliloti* (Peters *et al*., 1986) and 4', 7-dihydroxyflavone for clover-*Rhizobium trifolii* (Redmond *et al*., 1986). Similarly nodulation genes of *Rhizobium leguminosarum,* which is responsible for the formation of nodules on *Vicia sativa* and *Pisum sativum,* were found to be activated by hesperitin anderiodictyol (Firmin *et al*., 1986; Zant *et al*., 1987). The source of Rhizobium nod D gene, responsible for the specific responses, correlates to various flavonoids and in some cases determines host range (Horvath *et al*., 1987; Spaink *et al*., 1987). Moreover, several of the recent reports suggest that Indole Acetic Acid (IAA) can also function as a signaling molecule in rhizobacteria and therefore, may have a direct effect on bacterial physiology (Spaepen *et al*., 2007). In the present study rhizosphere of *Medicago sativa* L. was studied to determine the presence of different bacterial strains and to analyze their filtrates for the determination and quantification of flavonoids and auxin.

Materials and methods

Host plant, bacterial strains, culture medium and growth conditions

Alfalfa (*Medicago sativa* L.) plants were collected from the lawn of University College of Science Shankar, AWKUM. The plants were dig out with fine

roots intact. The roots were well shaken to remove the soil particles. Then the roots were suspended in 100 ml autoclaved distilled water. Serial dilution was done up to 10^{-6}. The screening and isolation of rhizospheric bacteria was carried out on L-agar medium plates. A 100µl of 10^{-6} diluted serial was poured on the surface of petri plate containing L-agar medium and spread on the plate surface with the help of inoculation loop. The bacterial colonies were purified using streaking method.

Isolation of rhizospheric bacteria

A total number of 31 strains were isolated using L-agar medium in sterilized petriplates. Out of these 15 were known to be different and were selected for further analysis. These 15 strains were coded as, F5-w1, F5-w2, F6-y, F3-w, F4-w, F3-w-rd, F6-w, F5-p, F5-br, F1-w-rd, F1-w-br, F2-lw, F6-p, F4-w and F5-y.

IAA extraction and quantification

Bacterial culture filtrates (BCF) of all 15 strains were screened for the presence of IAA using Salkowski reagent (Table 2) (Benizri *et al.,* 1998). Bacterial samples of all 15 strains were inoculated in 50 ml of broth medium. After overnight incubation in the dark, samples were centrifuged in appendorfs at 10000 rpm for 5 minutes. 1 ml of BCF was mixed with 2 ml of Salkowski reagent and kept in the dark at room temperature for 30 minutes. After 30 minutes the pink color developed was checked for the determination of IAA using PerkinElmer Lambda 25 spectrophotometer.

Calibration curve for IAA

Different IAA ratios were made to obtain a standard curve. These different ratios of IAA were given in Table 1. These different ratios of IAA were then used to determine the amount of IAA present in the culture filtrates of bacterial strains. This procedure was repeated three times.

Calibration curve for flavonoids

Different flavonoid ratios were made to obtain a standard curve. These different ratios of flavonoid are given in Table 2. These different ratios of flavonoid were then used to determine the amount of flavonoid present in the culture filtrates of bacterial strains. The procedure was repeated three times.

Results

IAA standard curve

10 different concentrations of pure IAA were made starting from 10 µg/mL to 100 µg/mL. Their optical density was measured with Perkin Elmer Lambda 25 spectrophotometer. Standard curve of IAA wasstraight with $R^2 = 0.970196$.

Table 1. Concentrations of IAA to make standard curve.

S. NO.	Concentrations (µg/mL)	Optical density (OD)
1	10	0.4091
2	20	0.6119
3	30	0.9994
4	40	1.06
5	50	1.36
6	60	1.57
7	70	1.62
8	80	1.8
9	90	1.95
10	100	2.03

Table 2. Salkowski reagent for colorimetric assay of IAA

S. NO.	Solution	Components	Quantity
1	A:0.5M Ferric chloride	$FeCl_3$	0.8125 ($10ml^{-1}$ W/V)
2	B: 35% Perchloric Acid	$HClO_4$	50 ($100ml^{-1}$ V/V)

1 ml of solution A was mixed with 50 ml of solution B to prepare Salkowski (Benizri *et al*., 1998).

Flavonoids standard curve

5 different concentrations of pure flavonoid were made starting from 0.1 µg/mL to 1000 ng/mL. Their optical density was measured with PerkinElmer Lambda 25 spectrophotometer. Standard curve of flavonoid was straight with R^2 = 0.99.

Table 3. Concentrations of flavonoid to make standard curve.

S. NO.	Concentrations (ng/mL)	Optical density (OD)
1	0.1	0.00012
2	1	0.00109
3	10	0.01301
4	100	0.13201
5	1000	1.42011

Table 4. Optical density of bacterial culture filtrates for the determination and quantification of IAA

S. NO.	Sample ID	Optical density	IAA (µg/mL)
1	F5-wl	0. 1112	4.83
2	F5-w2	0.1325	5.76
3	F6-y	0.0946	4.11
4	F3-w	0.1179	5.12
5	F4-w	0.1189	5.16
6	F3-w-rd	0.1509	6.56
7	F6-w	0.0662	2.87
8	F5-p	0.0298	1.29
9	F5-br	0.2786	12.11
10	F1-w-rd	0.2773	12.05
11	F1-w-br	0.0504	2.19
12	F2-lw	0.7028	30.55
13	F6-p	0.0549	2.38
14	F4-w	0.0755	3.28
15	F5-y	0.1038	4.51
16	Blank	-0.0300	-1.30

Table 5. Optical density of bacterial culture filtrates for the determination and quantification of flavonoids

Sample No	Sample ID	Optical Density	Flavonoids (ng/mL)
1	F5-w1	0.3297	235.5
2	F5-w2	0.3708	264.85
3	F6-y	0.3533	252.35
4	F3-w	0.0437	31.21
5	F4-w	0.0625	44.64
6	F3-w-rd	0.2173	155.21
7	F6-w	0.1734	123.85
8	F5-p	0.1958	139.85
9	F5-br	1.0779	769.92
10	F1-w-rd	1.1000	785.71
11	F1-w-br	0.1425	101.78
12	F2-lw	1.1564	826
13	F6-p	1.0168	726.28
14	F4-w	1.3977	998.35
15	F5-y	0.0053	3.78
16	Blank	0.0053	3.78

Discussion

Rhizobium, a legume symbiont, is the most worked out example of signal exchange between host plant and bacteria. Flavonoids, exuded by plant roots, are known to act as signalling compounds which induce transcription of nodulation genes (Nod genes) necessary for infection, which attract bacteria chemotactically as well as enhance bacterial growth rate, while bacteria release Nod factors, which help in nodule formation in legume plants (Brencic and Winans, 2005; Gray and Smith, 2005). In most of the studies carried out on rhizosphere, root exudates were analyzed for the secretion of secondary metabolites but little is known about bacteria to be also a valuable source of these important secondary metabolites.

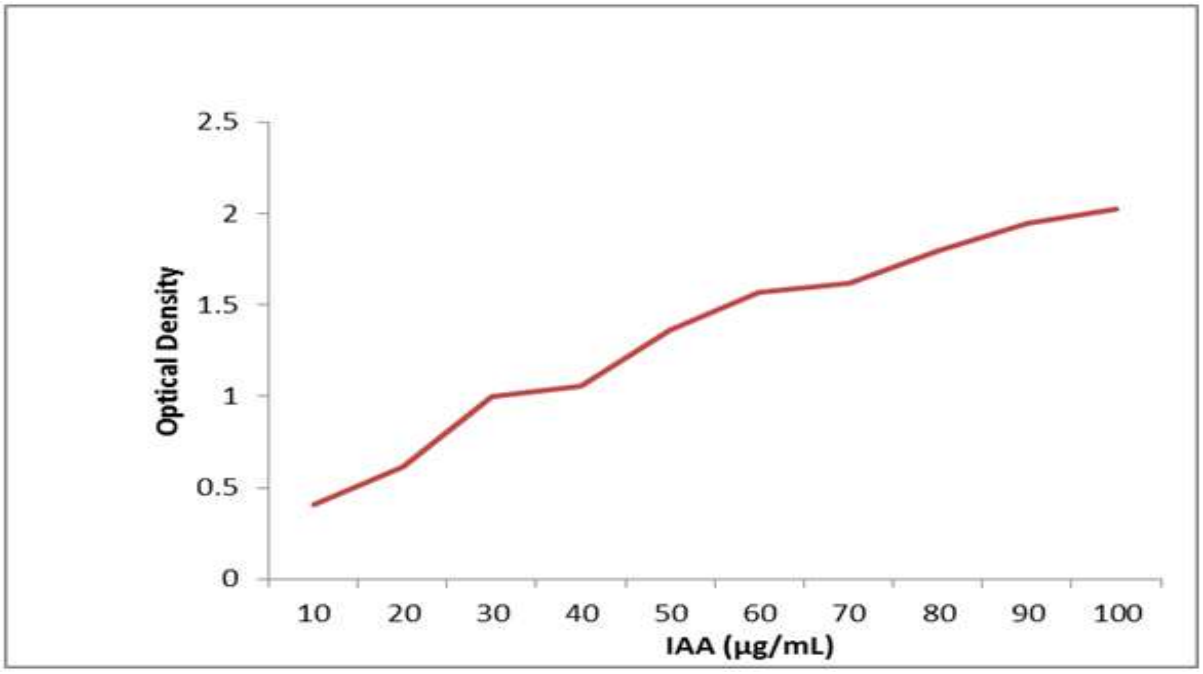

Fig. 1. Indole acetic acid (IAA) standard curve

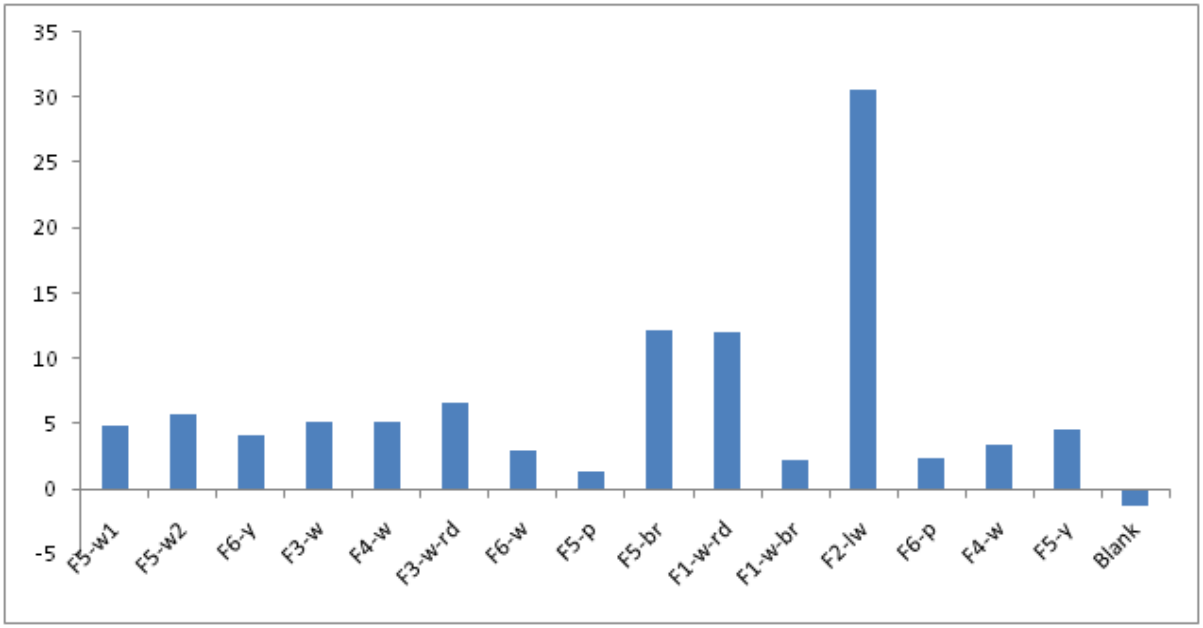

Fig. 2. Indole acetic acid (IAA) concentration of different bacterial strains isolated from the rhizosphere of *Medicago sativa* L. after incubation in L-agar medium for 24 hours at room temperature. Highest concentration of IAA (30.55µg/mL) was found in strain F2-lw.

In the present study 15 different bacterial strains were isolated from the rhizosphere of *Medicago sativa* and were tested for the secretion of IAA and flavonoids. Majority of these strains were found to be IAA and flavonoids producer. In terms of IAA quantification, the most important strains were F2-lw (30.55µg/mL), F5-br (12.11µg/mL), and F1-w-rd (12.05µg/mL) while F2-lw (826ng/mL), F5-br (769.92ng/mL), F1-w-rd (785.71ng/mL), F6-p (726.28ng/mL) and F4-w (998.35ng/mL) were the most flavonoids secreting strains of rhizospheric bacteria.

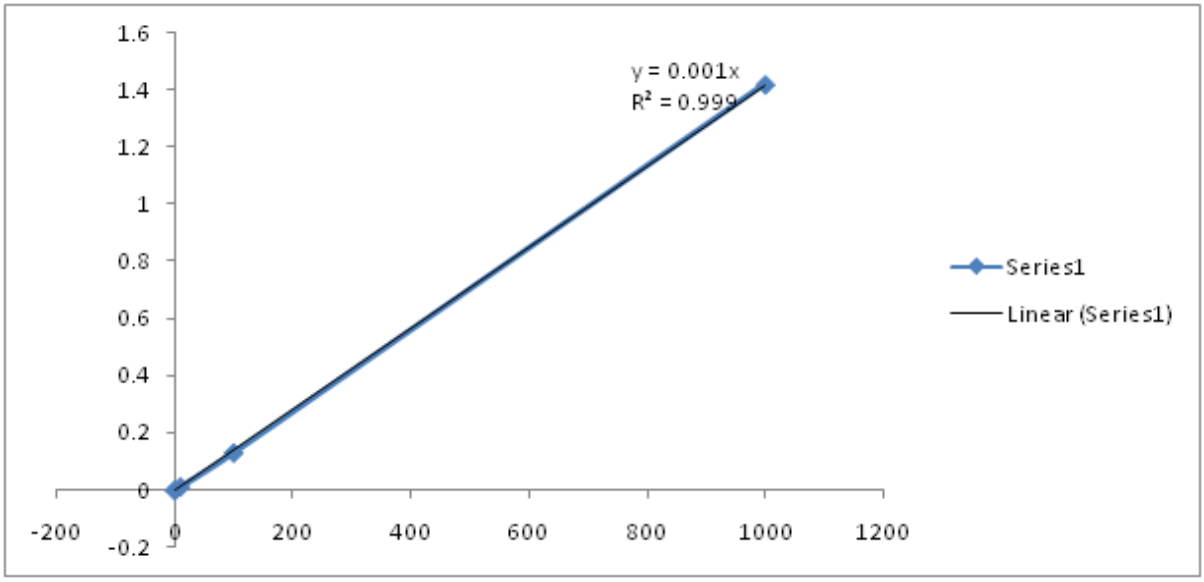

Fig. 3. Flavonoids standard curve

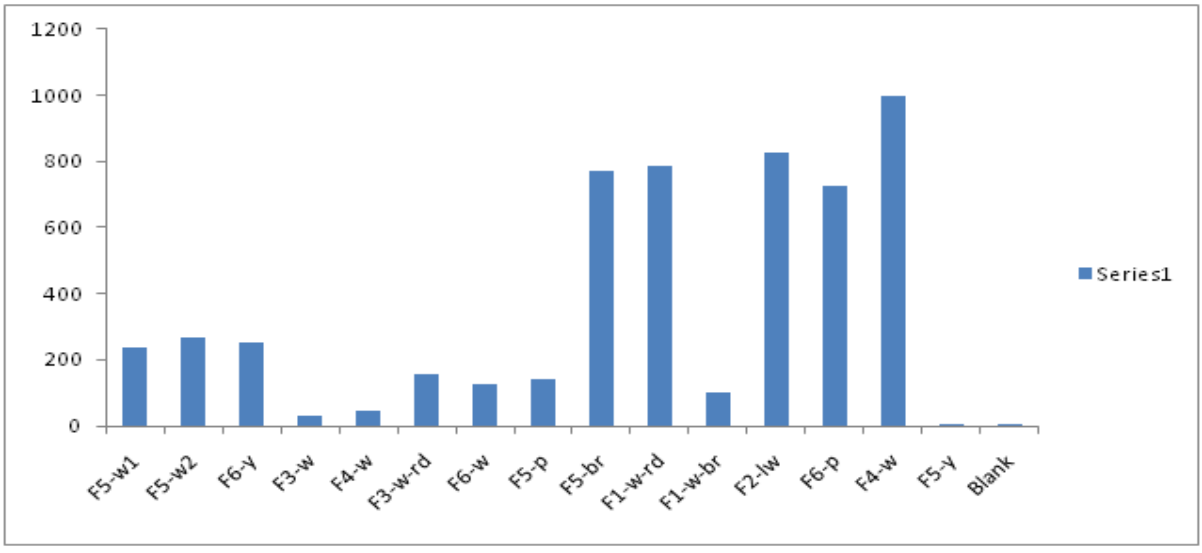

Fig. 4. Flavonoids concentration of different bacterial strains isolated from the rhizosphere of *Medicago sativa* L. after incubation in L-agar medium for 24 hours at room temperature. Highest concentration of flavonoids (998.35ng/mL) was found in strain F4-w.

Conclusion

This study indicates the diversity of rhizospheric bacteria and their secretion of some valuable secondary metabolites which not only function as growth promoting compounds (IAA) but also function as signaling chemicals (flavonoids) for the plants as well as other rhizospheric bacteria. Plant growth promoting bacteria are suggested to be used in the future as biofertlizers as it is an environment friendly and cheap source as compared to artificial fertilizers.

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