

Isolation and Selection of Indigenous *Azospirillum* spp. and the IAA of Superior Strains Effects on Wheat Roots

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Abstract: IAA produced by bacteria of the genus *Azospirillum* spp. can promote plant growth by stimulating root formation. Native *Azospirillum* spp., isolated from Iranian soils had been evaluated this ability in both qualitative and quantitative methods and registered the effects of superior ones on morphological, physiological and root growth of wheat. The roots of wheat seedling responded positively to the several bacteria inoculations by an increase in root length, dry weight and by the lateral root hairs.

Key words: *Azospirillum* spp. % isolation % laboratory % qualitative % quantitative % wheat

INTRODUCTION

Some microorganisms of soil, like *Azospirillum* sp. *Azotobacter* sp; *Enterobacter* sp. etc. have shown to encourage plant growth, by promoting the outbreak of secondary roots. Bacteria of the genus *Azospirillum* have been isolated from the rhizosphere and roots of a variety of plants including cereals and grasses [1-4]. Several reports have described the beneficial effect of *Azospirillum* inoculation on plant growth; hence these organisms have been attracting interest [2, 3, 5]. Inoculation with indigenous *Azospirillum* strains is an important procedure when studying their inherent capacity to benefit crops. In some cases, indigenous strains can perform better than introduced strains in promoting the growth of crops due to their superior adaptability to the environment. Inoculation with *A. brasilense* and a local strain clearly improved growth and increased the yield of three cultivars of wheat in different areas of Israel [6].

Azospirillum grown in culture are known to produce growth promoting compounds, such as gibberellin-like and cytokinin-like substances and auxins such as IAA from tryptophan [4, 7, 8]. The general belief exists that *Azospirillum* increases root mass and function and changes root and root hair morphology [9-13]. Horemans and Vlassak [14] demonstrated that *A. brasilense* could produce IAA

in the absence of tryptophan when grown aerobically in the presence of NH₄, while De Francesco *et al.* [15] showed that the highest levels of auxin were produced in both N₂-fixing condition and limiting ammonia stationary cultures of *A. brasilense* strain Sp6. The pH has a significant effect on the amount of IAA produced [16]. Vitamins may also play a role in the regulation of IAA synthesis in *A. brasilense*. Very low levels of the B vitamins, especially pyridoxine and nicotinic acid, increased production of IAA in *A. brasilense* [17].

It is possible to mimic the effects of *Azospirillum* using IAA which increases root hairs and branching [16]. Kolb and Martin [18] have shown that spraying a solution of 10⁻⁶ g l⁻¹ IAA on roots of wheat growing in root boxes resulted in a significant increase in root length which mimicked *Azospirillum* inoculation. Spraying an inoculum of *A. brasilense* strain FT-326 on roots of *Beta vulgaris* resulted in significant increases in both root length and number of laterals. Similarly, a cell-free supernatant of *A. brasilense* Cd applied to soybean plants induced the highest number of roots and increased root length [19]. The work of Jain and Partriquin [20] strongly suggests that IAA is responsible for root hair branching in wheat, since it is affected by both plant and bacterial genomes. These genome effects can be explained by differential plant sensitivity to IAA and differing abilities of bacteria to produce IAA.

The possibility that roots produce plant growth-promoting substances in response to bacterial cell surface components or pectic enzymes of *Azospirillum* needs to be investigated but it is known that the effect of *Azospirillum* on the formation of root hairs and lateral roots is due not only to IAA but, probably, to still unidentified phytohormones or substances.

The first objective of this study was to isolate indigenous *Azospirillum* spp. from several dry lands on Tehran, Golestan and Khuzestan regions, Iran and to screen their IAA production ability (with both qualitative and quantitative methods). The second was to determine the effectiveness of superior strains, grown in different cultures - in promoting wheat root growth and dry matter, using two methods, in order to select several promising strains for further experiments.

MATERIALS AND METHODS

Isolation of Indigenous *Azospirillum* spp.: The media used in this study were those recommended by Baldani and Dobereiner [21] and Rodriguez Caceres [22].

N-free semisolid malate medium (NFb medium): (g l⁻¹ D.W.) DL-malic acid, 5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; KOH, 4; NaCl, 0.1; CaCl₂, 0.02; agar, 1.75; trace element solution, 2 ml; alcoholic solution of Bromothymol Blue (5%), 2 ml; Fe EDTA, 4 ml; vitamin solution, 1 ml; NaOH to adjust the PH to 6.8. The trace element solution contained: 200 mg Na₂MoO₄·2H₂O; 235 mg MnSO₄·H₂O; 280 mg H₃BO₃; 8 mg CuSO₄·5H₂O; 24 mg ZnSO₄·7H₂O; 200 ml D.W. The vitamin solution contained: 10 mg biotin; 20 mg pyridoxine; 100 ml D.W.

Congo Red Agar (CRA) medium: (g l⁻¹ D.W.) DL-malic acid, 5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; KOH, 4.5; NaCl, 0.1; agar, 15-20; yeast extract, 0.5; FeCl₃·6H₂O, 0.015; 15 ml Congo red solution (0.25 %); NaOH to adjust the PH to 7.0.

Azospirillum spp. were isolated from roots of wheat (*Triticum aestivum*.), maize (*Zea mays*.), barley (*Hordeum vulgare*.), rye (*Secale cereale*.) grass (*Poa* sp.) and some weeds (*Cynodon dactylon*, *Dactylis* sp., *Lolium* sp., *Digitaria* sp.). Root samples were collected from Tehran (Karaj, Qazvin and Varamin), Golestan and Khuzestan regions, Iran. Fresh root samples were washed in rapidly running tap water for 5 min to remove the soil particles adhering to the root surface. The roots were rinsed in sterile water, then cut into pieces (5-8 mm), which were macerated with forceps and introduced

into semisolid NFb medium (10 ml medium in a 20 ml-vial). After 72 h incubation at 33°C, the white halo formed 3-6 mm below the media surface was a sign of nitrogenase activity. When the cultures exhibited a positive nitrogenase activity, they streaked out on CRA plates. Typical pink, often wrinkled colonies were picked out and transferred into semi-solid NFb medium. Pellicle formation in this medium indicated successful isolation. Purified colonies were transferred to a nutrient agar slant for storage and use for further studies.

Qualitative IAA production ability test: This experiment was carried out by the method proposed Bric *et al.* [23]:

Growth media: Luria-Bertani (LB) agar medium was used containing: (g l⁻¹ D.W.) Bacto-Tryptone (Difco), 10; yeast extract, 5; NaCl, 5; agar, 20. The PH was adjusted to 7.5 with 1 N NaOH before autoclaving. LB, amended with 5 mM L-tryptophan, (LBT) (1.2115 g l⁻¹ LB).

Assay conditions: Plates (9-cm diameter) containing LBT medium were divided into a grid pattern by a fine-marker. Grid plates were inoculated by 50 indigenous strains of *Azospirillum* spp. in two replications, using sterile tooth picks. Each inoculated plate was overlaid with an 82 mm diameter nitrocellulose membrane. Plates were overlaid immediately after inoculation and incubated until colonies reached 0.5 to 2 mm in diameter. After an appropriate incubation period, the membrane was removed from the plate and treated with Van Urk Salkowski reagent (2% 0.5M FeCl₃ in 35% perchloric acid) [24]. Membranes were saturated in a petri dish by overlaying on a reagent-saturated filter paper (whatman no. 2). The reaction was allowed to proceed until adequate color developed. All reagent incubations were conducted at room temperature at 30°C. Bacteria producing IAA were identified by the formation of a characteristic pink to red halo within the membrane immediately surrounding the colony. Halo color and diameter were recorded after 30 min and 2 h (Fig. 1).

Standard assays: A dilution series with concentrations of IAA (0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 2, 4 and 8 nmol) per 10 micro liter aliquot was applied directly onto nitrocellulose membrane and were assayed on whatman no. 2 filter pads saturated with Salkowski reagent. The halo colors were compared with those of bacteria.

Quantitative IAA production ability test: To determine the amounts of IAA produced by each isolate, a colorimetric technique was performed using the Van Urk Salkowski

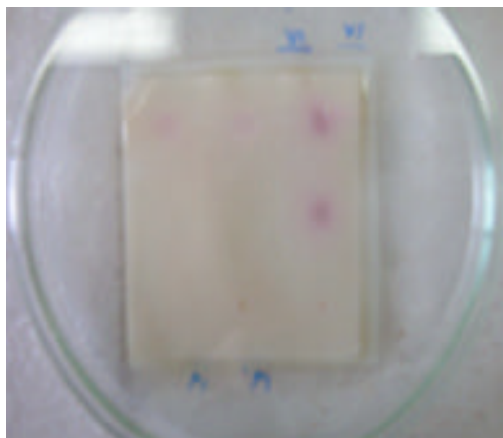


Fig. 1: Visualization of colonies of *Azospirillum* on nitrocellulose membrane after reaction of a membrane with the Salkowski reagent

reagent. Isolates were grown in no-agar LBT medium and incubated at a 30°C temperature during 24 h in a rotary shaker (90 rpm). After that time they were centrifuged (5000 rpm, 25 min). The supernatant liquid was mixed with salkowski reagent (2:1) and the color was measured by spectroscopy at 530 nm after 30 min and 2 h.

Using LB medium, concentrations of IAA (0, 5, 10, 15, 20, 25, 30, 40, 60, 80, 100, 150, 200 and 300 ppm) were prepared, treated with Salkowski reagent as above and the developed color was measured.

Assays to test the stimulatory effects of *Azospirillum* spp. on wheat root growth:

Organisms and growth: The ten superior *Azospirillum* spp. strains were grown at 30°C for 24 h in 100 ml Erlen mayors containing 4 different mediums: (g l⁻¹ D.W.) I) K₂HPO₄, 0.78; KH₂PO₄, 0.61; MgSO₄·7H₂O, 0.2; FeSO₄·7H₂O, 0.00625; EDTA(=Tiriplex III), 0.0093; Na₂MoO₄·2H₂O, 0.02; MnSO₄·H₂O, 0.01; DL-malic acid, 5; NaOH to adjust the PH to 7.0; KNO₃, 2.0. II) KNO₃ was replaced by 0.625 g l⁻¹ NH₄Cl. III, IV) the first and the second mediums were supplemented with D,L tryptophan (100 mg l⁻¹). Sterile solution of biotin (10G⁵ g l⁻¹) was added to the cultures.

Seed test: Eight native and commercial varieties of spring and winter wheat (*Roshan*, *Adle jaded*, *Adle ghadim*, *Bezostaya*, *Sardari*, *Inia*, *Ataei*, *Naz*) were obtained from the Gene Bank of the Agronomy and Plant Breeding Center. The seeds were tested for germination speed, uniform growth and number of radicles and the suitable one was selected for the experiment.

Experiments testing the influences of the bacteria on wheat root growth:

Seeds (variety *Roshan*) were surface sterilized by Ethanol (96%) for 10 seconds, Sodium hydrochloride (5%) for 10-12 min and were washed with sterile distilled water at least 7 times, then incubated at 25°C for germination. Germinated grain was placed in an Eppendorf plastic tube of which the tip had been excised. The plastic tube was placed in a 30 ml test tube on top of 10 ml of Hoagland Solution. The test tubes were supplemented with 100 micro liter of a 2 day old culture of *Azospirillum* spp. strains with the same bacterial solution (McFarland). In the second method; one third of a 30 ml test tube was filled with dry sand and 10 ml of Hoagland Solution was added into it. After autoclaving the grains were thrown in the tubes and inoculated with *Azospirillum* spp.

In order to compare the ability of the bacteria to produce IAA, a dilution series with concentration of IAA (0.01, 0.02, 0.04, 0.06, 0.08, 0.1 and 0.2 nmol) were added to the Hoagland Solution. All manipulations were performed under sterile conditions. After incubating at 28°C for 14 days (14 h in the light, 10 h in the dark per day), number of roots, root length and dry weight were determined.

RESULTS AND DISCUSSION

Isolation of Indigenous *Azospirillum* spp.: The inoculation of crop plants with associative N₂-fixing bacteria of the genus *Azospirillum* was proposed in the mid-1970s as a new approach to provide fixed N and to reduce fertilization requirements or to increase yield [25]. Also the plant-growth-promoting abilities of *Azospirillum* have aroused interest in its use as bacterial fertilizer [26].

The initial parameter for the quality determination of inoculum formulations of agricultural use is the isolation and identification of the guaranteed microorganisms. About fifty *Azospirillum* strains were isolated from different parts of Tehran, Golestan and Khuzestan regions. All 50 strains were isolated from plant roots.

Qualitative and Quantitative analysis of IAA production by *Azospirillum* spp.:

One of the principal mechanisms of promoting plant growth is related to the capability of *Azospirillum* to produce plant-growth-promoting substances [27]. Sixteen strains of *Azospirillum* spp. whose colonies were immobilized on a nitrocellulose membrane and then treated with salkowski reagent, produced a pink to red halo within the membrane surrounding the colonies (Table 1) and other bacteria not producing IAA effected no color change in the membrane

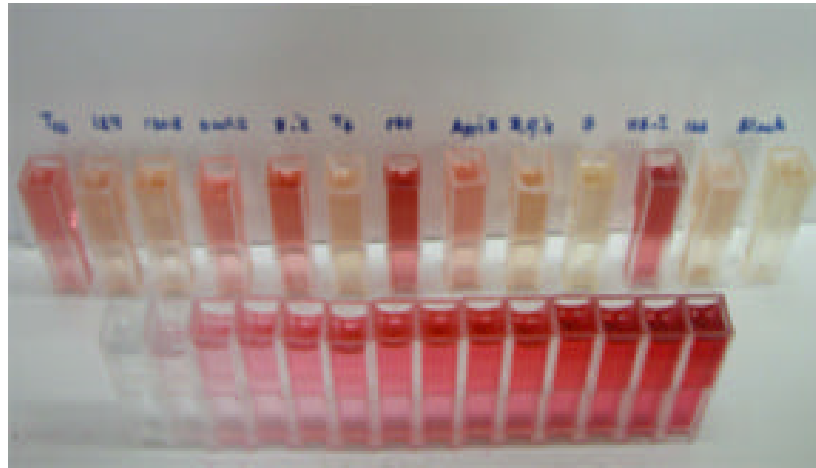


Fig. 2: Samples of the attained solutions of indole-3-acetics acid comparing with standards

Table 1: The results of qualitative and quantitative methods of producing IAA by bacteria of the genus *Azospirillum*

Bacteria	Qualitative method		Quantitative method (ppm)	
	Diameter of colony	Color	30 min	120 min
T 4	1.00	A	123.2	48.2
T 6	2.66	C	385.0	139.2
T 8	-	-	221.0	86.5
T 10	1.00	B	404.0	157.0
T 11	1.33	C	266.0	96.0
T 12	-	-	288.0	112.4
T 17	-	-	273.2	249.0
T 20	1.50	C	520.0	202.0
<i>Bromus</i> -I	-	-	190.0	74.0
<i>Dactylis</i> -I	1.66	B	235.0	92.0
72 L	-	-	75.5	29.4
118-I	1.66	C	761.0	297.0
Agri-II	1.00	B	463.0	181.0
166	1.00	A	193.9	75.5
122	-	-	337.0	131.5
7	1.60	C	51.0	20.0
171-I	1.50	C	183.0	71.4
130-II	2.00	C	169.0	66.0
161-II	-	-	379.0	262.6
Kh-44	-	-	498.0	194.0
Kh-8	1.13	B	446.0	174.0
Kh-9-II	-	-	313.0	122.0
124	1.40	C	29.0	11.0
171	1.50	C	293.0	114.0

A: light pink, B: pink, C: light red

and were easily distinguishable by visual inspection (Fig. 1). Color development was first visible at the highest IAA concentration within minutes and continued to

increase in intensity for a period of 30 min. Concentrations not visible at 30 min, did not develop upon further incubation.

Twenty five strains were selected for the quantitative assays including IAA producers and those which did not produce Indolic compounds in the previous method. All 25 treated strains in a culture medium containing DL-Tryptophan source, produced IAA, as detected by the salkowski reagent under colorimetry, in the range 29 mg l⁻¹ to 761 ppm. Figure 2. shows samples of the attained solutions of this compound comparing with standards.

The effects of *Azospirillum* spp. on root growth:

Among the 25 strains, an IAA detection test was performed, 10 were selected for producing different concentrations of IAA. Tables 2 & 3 show the effect of different *Azospirillum* spp. culture supernatants on wheat root number, length and dry weight in the two methods (first: Hoagland, second: sand, assays). Data are means of three replicates. Means in each column followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range tests. Our results indicate that 14 day old wheat plants had formed much longer roots and more root hairs and lateral roots after the inoculation with *Azospirillum* spp. In all media, 10 super strains revealed different results. The dry weight for roots excised afterwards gave 10.63 mg for 14 d and 3.47 mg for negative controls and in the second method 39.87 and 20.00 mg, respectively for the same time (Tables 2 & 3). All ten strains produced concentrations that can stimulate the elongation of the root, parallel to the increase in IAA production, without

Table 2: Effect of different culture supernatants of *Azospirillum* spp. isolates on wheat root number, length and dry weight in Hoagland method. Data are means of three replicates. Means in each column followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range tests

		First Method		
Bacteria	Media	Root No.	Root Length (mm)	Root Dry Wt.(mg)
T6	I	7.33b-g	29.33n-q	4.43mn
	II	8.00a-e	29.00o-r	6.00g-i
	III	8.33a-e	46.00c-e	4.83j-n
	IV	6.67b-g	44.33d-f	4.43mn
Kh-8	I	5.67d-g	32.33lm-o	2.73q
	II	9.33a-c	30.33m-p	3.03q
	III	8.00a-e	38.33i-k	4.83j-n
	IV	9.33abc	37.00jk	4.77k-n
Agri-II	I	5.33e-g	32.67l-n	2.73q
	II	6.33b-g	33.00lm	3.10pq
	III	6.33b-g	36.33jk	4.90j-m
	IV	7.67b-f	39.67h-j	4.83j-n
Kh-9-II	I	6.33b-g	28.00p-t	5.67h-k
	II	6.00c-g	25.33s-u	6.57gh
	III	4.33fg	42.67e-h	4.43mn
	IV	8.00a-e	45.33c-e	5.73g-j
171	I	5.33e-g	25.67r-u	4.57l-n
	II	6.00c-g	26.67q-u	6.63 fg
	III	4.33fg	38.33i-k	5.50ij-l
	IV	4.00g	43.33d-g	4.77k-n
T10	I	8.33a-e	30.67m-p	4.63l-n
	II	6.00c-g	28.67p-s	6.30g-i
	III	4.33 fg	45.67c-e	8.60bc
	IV	4.00g	46.00c-e	7.70de
124	I	8.33a-e	21.33v	4.13m-o
	II	7.00b-g	23.67uv	4.27m-o
	III	7.33b-g	35.67kl	5.97g-i
	IV	9.33a-c	40.67g-i	7.63de
118-I	I	6.67b-g	41.67f-i	7.43ef
	II	7.67b-f	39.00i-k	9.27b
	III	6.00c-g	55.00b	10.60a
	IV	8.67a-e	60.33a	10.63a
<i>Dactylis-I</i>	I	9.00a-d	31.00m-p	4.80k-n
	II	8.67a-e	26.67q-u	6.23g-i
	III	9.67ab	52.67b	7.80c-e
	IV	11.33a	48.67c	8.40cd
166	I	8.67a-e	30.00m-q	3.90n-p
	II	7.33b-g	24.67tu	5.73g-j
	III	9.00a-e	44.00d-g	6.33g-i
	IV	9.33abc	46.33cd	7.43ef
Control		6.33b-g	17.33w	3.47opq

Table 3: Effect of different culture supernatants of *Azospirillum* spp. isolates on wheat root number, length and dry weight in sand, assys method. Data are means of three replicates. Means in each column followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range tests

		Second Method		
Bacteria	Media	Root No.	Root Length (mm)	Root Dry Wt.(mg)
T6	I	5.00b-f	27.33r	19.17lm
	II	5.33a-f	50.67k	19.30lm
	III	4.67c-f	51.00jk	31.07d
	IV	4.33d-f	58.00d-f	26.17e-g
Kh-8	I	3.33f	41.00o	31.00d
	II	3.67ef	33.67p	22.13i-k
	III	4.33d-f	45.33mn	33.87bc
	IV	4.67c-f	55.00f-i	25.27f-h
Agri-II	I	6.00a-d	45.00mn	33.53b-d
	II	6.00a-d	46.33lm	34.37bc
	III	4.33d-f	50.33k	35.57b
	IV	3.33f	59.00c-e	25.83e-g
Kh-9-II	I	5.00b-f	24.33s	27.53ef
	II	6.33a-d	44.00m-o	19.03lm
	III	5.67a-e	46.33lm	27.03ef
	IV	5.00b-f	56.33e-h	24.10g-i
171	I	7.33a	24.00s	25.97e-g
	II	7.00ab	44.00m-o	14.93n
	III	6.00a-d	42.67no	25.70e-g
	IV	5.33a-f	51.33jk	23.07h-j
T10	I	5.67a-e	21.67st	18.60lm
	II	6.00a-d	52.33i-k	22.50i-k
	III	6.33a-d	52.33i-k	28.30e
	IV	5.33a-f	61.67bc	35.73b
124	I	4.67c-f	17.67u	17.60m
	II	6.00a-d	30.00qr	28.17e
	III	5.00b-f	43.67m-o	26.10e-g
	IV	5.67a-e	54.00h-j	31.97cd
118-I	I	6.67abc	31.67pq	20.47j-l
	II	5.67a-e	62.33bcd	28.50e
	III	5.67a-e	60.33bcd	30.93d
	IV	6.00abcd	75.33a	39.87a
<i>Dactylis-I</i>	I	6.00abcd	20.33tu	17.70m
	II	5.67a-e	49.33kl	26.73e-g
	III	6.67abc	54.67ghi	28.23e
	IV	6.00abcd	56.33efgh	38.27a
166	I	5.67a-e	19.67tu	14.77n
	II	7.00ab	46.33lm	22.60i-k
	III	6.00abcd	52.33ijk	27.43ef
	IV	5.00b-f	57.67defg	34.10bc
Control		6.00abcd	30.67q	20.00k-m

I: Cells grown with KNO₃, II: Cells grown with NH₄Cl.

III: Cells grown with KNO₃+L-TRP, IV: Cells grown with NH₄Cl+L-TRP

inhibiting it. The addition of DL-Tryptophan to the medium significantly enhanced the root growth due to the higher IAA production by the cells. Excised roots of wheat seedling respond positively to the addition of IAA (different con.) by an increase in root length, dry weight and by the formation of additional lateral roots. However, high concentrations of IAA inhibited the growth of wheat segments (data not shown). Cells grown on both nitrogen sources increased the root dry weight significantly, but NH_4^+ seemed to be more efficient than nitrate. Both root test methods were found to be appropriate for such experiments. However, the second method was more suitable for detecting stimulatory effects of *Azospirillum* on the lateral roots. Furthermore, root outgrowth, i.e. round, nodule-like tumours were observed on roots in some treatments.

Many PGPRs produce growth regulators such as auxins [28, 29]. In this study we analyzed one of the plant hormones, indole-acetic acid (IAA), in the bacterial culture of the *Azospirillum* spp. with both qualitative and quantitative methods and observed that strain 118-I produced the largest amount of IAA (285.51 mg l⁻¹) among the strains (data not shown). The assay proposed by Bric *et al.* [23] is a useful method for the detection and enumeration of *Azospirillum* and other bacteria for which IAA production is an appropriate criterion. However, using quantitative assays to increase the resolution and accuracy of the experiment seems to be inevitable. The use of the technique for the detection of IAA using the Van Urk Salkowski reagent is an important option for the obtaining of qualitative and semi-qualitative results that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants. In this study all *Azospirillum* isolates produced auxins in the presence and absence of DL-TRP although much variation was observed in the potential to produce auxins. Auxin production by all isolates increased when culture medium was supplemented with an auxin precursor, DL-TRP. depending on the microorganisms' kinetics stabilization. Several investigators have shown that inoculation with *Azospirillum* or the application of pure hormone substances induces the proliferation of lateral roots and root hairs [12, 29]. Results of this test showed that there was a significant increase in root elongation, root dry matter and development of lateral roots. The stimulus involved in the formation of additional root hairs and laterals needs to be identified. Also the possibility that bacteria produce and export more phytohormones under

the influence of root exudates cannot be excluded from consideration. Auxin produced by *Azospirillum* may particularly cause the formation of lateral roots as observed for several plants [30].

Many investigators Tchan and Kennedy [31] and Christiansen-Wengiger [32] have shown that bacterial-produced IAA caused the formation of nodule-like tumours at the root tips of non-legumes which is probably of interest to agriculture. The findings of this study provide significant evidence that IAA was produced by *Azospirillum* spp. and that it causes clear biological activity in root plants. The development of techniques for the utilization of plant-growth-promoting bacteria (including *Azospirillum*) in order to reduce rates of fertilizer application should be recommended for financial reasons and also to prevent environmental pollution by avoiding excessive applications of industrially produced fertilizers to cultivated fields.

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