Factors Influencing Indole-3-Acetic Acid Biosynthesis of Root-Nodule Bacteria Isolated from Various Leguminous Plants

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Abstract

Indole-3-acetic acid (IAA) biosynthesis was monitored in 50 isolates of root-nodule bacteria from 10 leguminous plants. The isolate DASA12010 from *Sesbania rostrata* and DASA23008 from *Desmanthus virgatus* produced the highest amount of IAA in Tris-TMRT medium supplemented with L-tryptophan at 25.16 ± 0.81 µg/ml and 23.70 ± 1.32 µg/ml, respectively. Five isolates producing the highest amount of IAA were selected to examine growth curve and IAA production. In all isolates tested, growth and IAA production started simultaneously. The highest levels of IAAwas produced in the stationary phase of growth. The influence of factors on IAA production was examined during a10-day cultivation. D-glucose (1.00 mg/ml), D-fructose (1.00 mg/ml), lactose (1.00 mg/ml), L-asparagine (1.00 mg/ml), glutamic acid (1.00 mg/ml) and ABA (0.01 mg/ml) decreased IAA production in some isolates, but increased IAA production in some isolates.While SDS (1.00 mg/ml) decreased IAA production in all isolates. The partial sequences of the 16S rDNA indicated high homology with members of *Rhizobium* sp., *Sinorhizobium* sp. and *Mesorhizobium* sp.

Keywords: Indole-3-acetic acid, colorimetric assay, root-nodule bacteria, *Rhizobium* sp., *Sinorhizobium* sp. and *Mesorhizobium* sp.

1. Introduction

Indole-3-acetic acid (IAA) is a naturally occurring auxin [1]. Production of IAA is widespread among plant associated bacteria [2]. Production of IAA, a plant hormone that does not apparently function as a hormone in bacterial cells, may have evolved in bacteria because it is important in the bacterium-plant relationship [3]. IAA alone or in conjunction with other plant hormones might be involved in several stages of the symbiotic relationship [4, 5]. Furthermore, IAA has long been assumed to play a role in one or more aspects of nodule growth and development [6]. Root-nodule bacteria such as *Rhizobium* species have been shown to produce IAA in culture. These species were isolated from various leguminous plants including *Roystonea*

regia [5], *Desmodium gangeticum* [7], *Dalbergia lanceolaria* Linn. F. [8], *Alysicarpus vaginalis* DC. [9], *Cajanus cajan* [10] and *Phaseolus mungo* [11]. There is only restricted knowledge regarding factors that may influence the IAA biosynthesis of these root-nodule bacteria. Factors including $ZnSO_4$ [7, 9], $NiCl_2$ [10], L-asparagine [7, 8, 9], glutamic acid [10], nicotinic acid [7], flavonoids [12, 13], mannitol [8], glucose [10], SDS [8, 9], and biotin [8] have been reported to increase IAA production in *Rhizobium*. Since these factors were tested with *Rhizobium* strains isolated from only a limited number of host plant species, their effect on IAA production of root-nodule bacteria from some other hosts have not been investigated. Therefore, the purpose of this study is to examine the production of IAA among 50 root-nodule bacteria isolated from 10 leguminous plants and compare the effect of some factors on IAA biosynthesis by the selected strains from various host plants. Additionally, we identified genus of these symbiants based on sequence analysis of the 16S rDNA.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Root nodules of 10 leguminous plants grown in Thailand were collected from different geographic origins. Bacteria were isolated from root nodules and the purity of isolates was ensured as described previously [14]. Forty-three isolates from Thailand and other 7 rhizobial strains from NIFTAL and IRRI used in this study are listed in Table 1. Bacteria were cultured in Yeast-Mannitol (YM) medium [15] at 28°C. Pure cultures were maintained on Yeast-Mannitol Agar (YMA) slants at 4°C and frozen in 50% glycerol at -80°C.

2.2 Quantification of IAA Production

The isolates were propagated in Tris-TMRT broth supplemented with L-tryptophan [16] at 28°C in the dark for 7 days. The IAA concentration in the cultures was determined by colorimetric assay [17]. 2.00 ml of 0.01M $FeCl_3$ in 35% $HClO_4$ was added into 1.00 ml of supernatant of the culture. The reaction was incubated in the dark at 30°C for 25 min. The absorbance at 530 nm was measured. The concentration of IAA was determined by comparison with a standard curve.

2.3 IAA Production During Growth of the Selected Bacteria

Five isolates producing the highest amount of IAA were selected to examine growth curves and IAA production profiles. Bacteria were grown at 28°C at 200 rpm for 5 days and used as inoculum. The total cell counts of inoculum was examined by the standard plate count methods and inoculated into Tris-TMRT broth supplemented with L-tryptophan [16]. The initial cell number of each strain was 6.00×10^5 CFU/ml. Cultures were grown at 28°C at 200 rpm in the dark for 10 days. The cell numbers was measured by the standard plate count method and the IAA concentration in cultures was monitored as described above.

2.4 Effects of Factors on IAA Production of the Selected Bacteria

Effects of factors including 3 kinds of sugars (D-glucose, D-fructose and lactose), 2 kinds of amino acids (L-asparagine and glutamic acid), a plant hormone (abscisic acid: ABA) and a surfactant (sodium dodecyl sulfate: SDS) on IAA production of the selected strains, were determined. The inoculum was prepared and the IAA concentration in the cultures was monitored as described above when Tris-TMRT medium was supplemented with D-glucose (1.00 mg/ml), D-fructose (1.00 mg/ml), lactose (1.00 mg/ml), L-asparagine (1.00 mg/ml), glutamic acid (1.00 mg/ml), ABA (0.01 mg/ml) and SDS (1.00 mg/ml).

2.5 Sequence Analysis of Partial 16S rDNA

Genomic DNA of the selected bacteria was extracted from exponentially grown culture by using the Wizard^{R} genomic DNA purification kit (Promega, WI). Partial 16S rDNA was amplified using universal primers UN16S 926f (5' AAACTYAAAKGAATTGACGG 3') and UN16S 1392r (5' ACGGGCGGTGTGTRC 3') [18]. PCR reaction was done as described previously [19]. Negative controls (no DNA added) were included in all sets of reactions. The presence and size of the amplified frag- ments was determined by agarose (1% in TBE buffer) gel electrophoresis and purified using a QIA Quick Gel Extraction kit (Qiagen, Valencia, CA). The purified PCR product was sequenced by Macrogen, Korea. The nucleotide sequences (approximately 500 bp) of the 16S rDNA of the selected strains were aligned using BLASTN (http://www. ncbi.nlm.nih.gov).

2.6 Statistical Analyses

Experimental data were compared by using the SPSS program version 13.0 (SPSS Inc., Chicago, IL).

3. Results and Discussion

3.1 Quantification of IAA Production

Fifty bacterial isolates from 10 leguminous plants produced the amount of IAA ranging between 0.60 ± 0.14 to 25.16 ± 0.81 µg/ml (Table 1). The isolates DASA12010 from *Sesbania rostrata* and DASA23008 from *Desmanthus virgatus* produced the highest amount of IAA at 25.16 ± 0.81µg/ml and 23.70 ± 1.32µg/ml, respectively. While the isolates DASA 12039 and DASA12033 from *Sesbania aculeata* produced the lowest amount of IAA at 0.60 ± 0.14 µg/ml and 0.78 ± 0.22 µg/ml, respectively. Production of IAA is widespread among plant associated bacteria [2]. Both rhizobia and bradyrhizobia have been reported to produce IAA in culture [6, 20, 21, 22]. It has been hypothesized that production of IAA that does not apparently function as a hormone in bacterial cells, may have evolved in bacteria because it is important in the bacterium-plant relation- ship [3]. Among root-nodule bacteria, IAA has long been assumed to play a role in one or more aspects of nodule growth and development [6]. Here we report the IAA production by root-nodule bacteria isolated from various leguminous plants.

Table 1 IAA production by 50 bacterial isolates from 10 leguminous plants.

Host plants	Bacterial isolates	Geographic origins	IAA (µg/ml)
Glycine max	DASA01001	NIFTAL/University of Hawaii	2.69 ± 0.25
	DASA01002	Chiang Mai/Thailand	0.79 ± 0.02
	DASA01003	Chiang Mai/Thailand	1.50 ± 0.10
	DASA01004	Chiang Mai/Thailand	1.29 ± 0.16
	DASA01005	Chiang Mai/Thailand	1.38 ± 0.15
	DASA01006	Chiang Mai/Thailand	2.75 ± 0.13
	DASA01011	Lampang/Thailand	2.78 ± 0.08
	DASA01013	Lampang/Thailand	1.86 ± 0.65
	DASA01014	Chiang Mai/Thailand	1.81 ± 0.19
	DASA01015	Chiang Mai/Thailand	2.94 ± 0.09
	DASA01017	Chiang Mai/Thailand	5.03 ± 0.08

Values are the means of three replicates ± SD.

Table 1 (continued) IAA production by 50 bacterial isolates from 10 leguminous plants.

Host plants	Bacterial isolates	Geographic origins	IAA (µg/ml)
	DASA01059	Loei/Thailand	2.77 ± 0.09
	DASA01207	Loei/Thailand	1.30 ± 0.14
	DASA01214	Khon Kaen/Thailand	10.28 ± 0.08
	DASA01234	Udon Thani/Thailand	1.18 ± 0.02
	DASA01240	Surin/Thailand	1.66 ± 0.27
Vigna radiata	DASA02008	Saraburi/Thailand	6.19 ± 0.75
	DASA02009	Chainat/Thailand	1.58 ± 0.36
	DASA02010	Chainat/Thailand	4.02 ± 0.23
	DASA02011	Chainat/Thailand	4.01 ± 0.05
	DASA02068	Sukhothai/Thailand	3.12 ± 0.09
	DASA02074	Pijitr/Thailand	1.43 ± 0.14
	DASA02077	Pijitr/Thailand	2.67 ± 0.33
	DASA02082	Petchaboon/Thailand	2.81 ± 0.05
	DASA02087	Petchaboon/Thailand	1.47 ± 0.09
	DASA02095	Petchaboon/Thailand	1.11 ± 0.14
Sesbania rostrata	DASA12006	Lampang/Thailand	1.53 ± 0.71
	DASA12007	Nonthaburi/Thailand	2.06 ± 0.55
	DASA12009	Satun/Thailand	2.48 ± 0.17
	DASA12010	Udon Thani/Thailand	25.16 ± 0.81
Sesbania speciosa	DASA12028	Kanchanaburi/Thailand	18.16 ± 0.59
Sesbania aculeata	DASA12033	IRRI/Philippines	0.78 ± 0.22
	DASA12037	IRRI/Philippines	3.84 ± 0.43
	DASA12039	IRRI/Philippines	0.60 ± 0.14
	DASA12052	IRRI/Philippines	2.91 ± 1.00
Neptunia sp.	DASA21001	Nongbualampoo/Thailand	13.30 ± 4.20
	DASA21002	Nongbualampoo/Thailand	11.79 ± 0.92
	DASA21004	Suratthani/Thailand	17.47 ± 2.46
	DASA21005	Suratthani/Thailand	16.78 ± 5.45
Desmanthus virgatus	DASA23008	Nakhon Ratchasima/Thailand	23.70 ± 1.32
	DASA23010	Nakhon Ratchasima/Thailand	15.21 ± 1.58
	DASA23011	Nakhon Ratchasima/Thailand	3.99 ± 0.70
	DASA23017	Nakhon Ratchasima/Thailand	2.04 ± 0.64
	DASA23024	Khon Kaen/Thailand	14.33 ± 1.30
	DASA23028	NIFTAL/University of Hawaii	1.43 ± 0.58
	DASA23032	NIFTAL/University of Hawaii	14.99 ± 0.39
Acacia mangium	DASA35030	Prachinburi/Thailand	5.76 ± 0.33
Pterocarpus macrocapus	DASA37187	Phetchabun/Thailand	2.64 ± 0.39

Values are the means of three replicates ± SD.

Table 1 (continued) IAA production by 50 bacterial isolates from 10 leguminous plants.

Host plants	Bacterial isolates	Geographic origins	IAA (µg/ml)
Mimosa invisa	DASA54001	Nakhon Ratchasima/Thailand	12.61 ± 6.47
	DASA54005	Trat/Thailand	9.78 ± 1.26

Values are the means of three replicates ± SD.

3.2 IAA Production During Growth of the Selected Bacteria

In the previous studies, IAA biosynthesis were tested with *Rhizobium* strains isolated from only a limited number of host plant species including *Roystonea regia* [5], *Desmodium gangeticum* [7], *Dalbergia lanceolaria* Linn. F. [8], *Alysicarpus vaginalis* DC. [9], *Cajanus cajan* [10] and *Phaseolus mungo* [11]. Therefore this study was intended to compare IAA biosynthesis by the selected strains from various host plants. Five isolates including DASA12010 from *Sesbania rostrata*, DASA12028 from *Sesbania speciosa*, DASA21004 and DASA21005 from *Neptunia* sp. and DASA23008 from *Desmanthus virgatus*, which produced high amount of IAA, were selected to examine growth curve and IAA production. Besides isolates diversity, a high amount of IAA was considered as the selection criterion. The isolates which produced a little amount of IAA may have low expression of genes involved in the synthesized pathways and can not be stimulated by factors. Additional, it is reasonable to find out some factors for maximum IAA production for further agricultural application. In all selected strains, the same trend of IAA biosynthesis was observed. Growth and IAA production started simultaneously. Maximum production of IAA was detected during the stationary phase of growth. The result is consistent with previous reports [8, 9]. Fig. 1-5 shows the growth curve and IAA production of isolates DASA12010, DASA 12028, DASA 21004, DASA21005 and DASA23008, respectively.

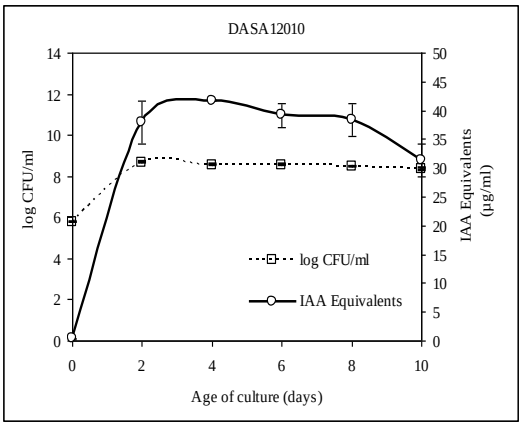

Fig. 1 Growth curve and IAA production of DASA12010. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

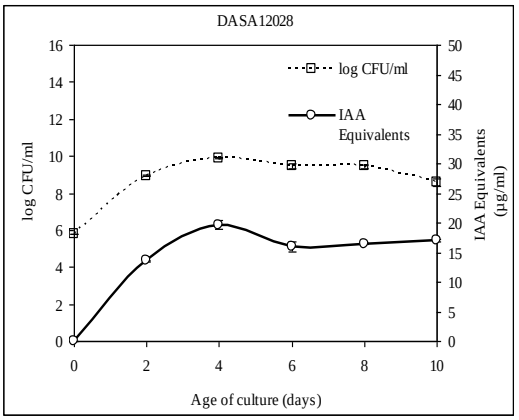

Fig. 2 Growth curve and IAA production of DASA12028. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

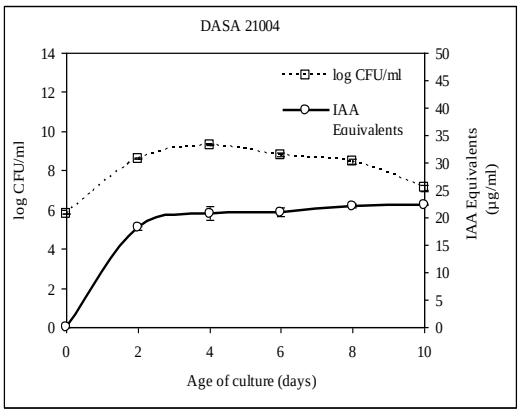

Fig. 3 Growth curve and IAA production of DASA21004. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

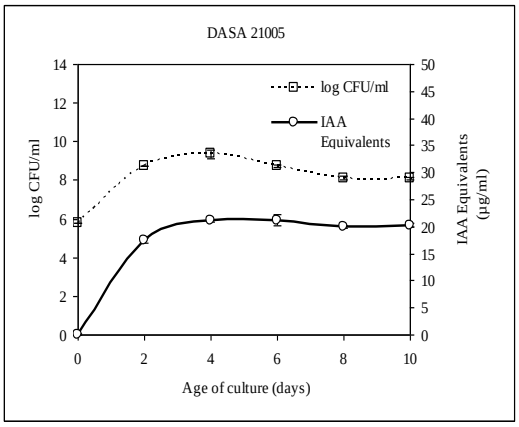

Fig. 4 Growth curve and IAA production of DASA21005. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

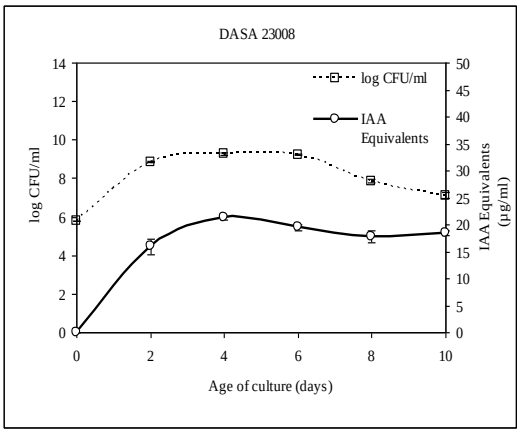

Fig. 5 Growth curve and IAA production of DASA23008. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

3.3 Effects of Factors on IAA Production of the Selected Bacteria

Effects of various factors including 3 kinds of sugars (D-glucose, D-fructose and lactose), 2 kinds of amino acids (L-asparagine and glutamic acid), a plant hormone (abscisic acid: ABA) and a surfactant (sodium dodecyl sulfate: SDS) on IAA production of the selected bacteria, were studied during a 10-day incubation period. Fig. 6-10 presents the influence of factors on IAA production by isolates DASA12010, DASA 12028, DASA21004, DASA21005 and DASA 23008, respectively. The amount of IAA decreased in all isolates when cells were grown in the presence of 1.00 mg/ml SDS. Effects of other factors varied in different isolates. D-glucose, D-fructose, lactose, L-asparagine, glutamic acid and ABA decreased IAA production by isolate DASA12010 during 2-10 days of culture. D-glucose, D-fructose and ABA could both increase and decrease the IAA production, while lactose, L-asparagine and glutamic acid were found to stimulate the IAA production by isolate DASA12028 during 2-10 days of culture. Three kinds of sugars increased IAA production during day 2-4, then decreased IAA during day 6-10 of the cultivation by DASA21004. This might be due to catabolic conjugation pathways that may be up-regulated in response to elevated IAA. Most IAA in higher plants is conjugated to sugars, amino acids or peptides [23]. However, the only microbial IAA-conjugating enzyme from *Pseudomonas savastanoi* pv. *savastonoi* has been reported to conjugate IAA to lysine [24]. Two kinds of amino acids stimulated the amount of IAA, and ABA decreased the amount of IAA produced by this isolate during 2-10 days of culture by DASA21004. The IAA biosynthesis in isolate DASA21005 was increased by D-glucose, lactose, L-

asparagine and glutamic acid, but decreased by D-fructose during 2-10 days of culture. ABA decreased the amount of IAA produced by DASA12010, DASA21004 and DASA23008 during 2-10 days of incubation, but increased the amount of IAA produced by DASA12028 and DASA21005 at the 2nd day of incubation. Three kinds of sugars and 2 kinds of amino acids were found to stimulate the IAA production in strain DASA23008, while ABA was found to inhibit the production of IAA in this isolate. There is only limited qualitative knowledge regarding factors that may influence the IAA biosynthesis of root-nodule bacteria. The IAA production by *R.* sp. isolated from *Cajanus cajan* could be increased up to 653.3% over the control by supplementing the medium with glucose (5.00 g/l) [10]. L-asparagine has been reported to increase the IAA production by *R.* sp. from *Desmodium gangeticum*, *R.* sp. from *Dalbergia anceolaria* Linn. f. and *R.* sp. from *Alysicarpus vaginalis* DC. up to 37%, 270.8%, and 70% over the control, respectively [7, 8, 9]. In this study, the result shows that D-glucose both increased and decreased IAA production depending on strains and phase of growth, and L-asparagine increased the IAA production in all isolates tested except DASA12010. Our results implied that the influence of factors on the IAA production varied in strains and phase of growth. While the previous studies [8, 10] have reported that SDS (1.00 μg/ml) increased the IAA production by *R.* sp., our result shows that SDS (1.00 mg/ml) could decrease the IAA production in all isolates tested, 84.74% to 93.37% over the control. However, the IAA level at day 0 of all isolates tested in the presence of 1.00 mg/ml SDS was significantly higher than that in the presence of other factors. As a cell wall affecting agent or surfactant, SDS might increase the release of tryptophan degrading enzymes as well as the conversion of trypto- phan to IAA. After a longer exposure, SDS might inhibit the activities of enzymes, leading to a decrease in IAA bioproduction.

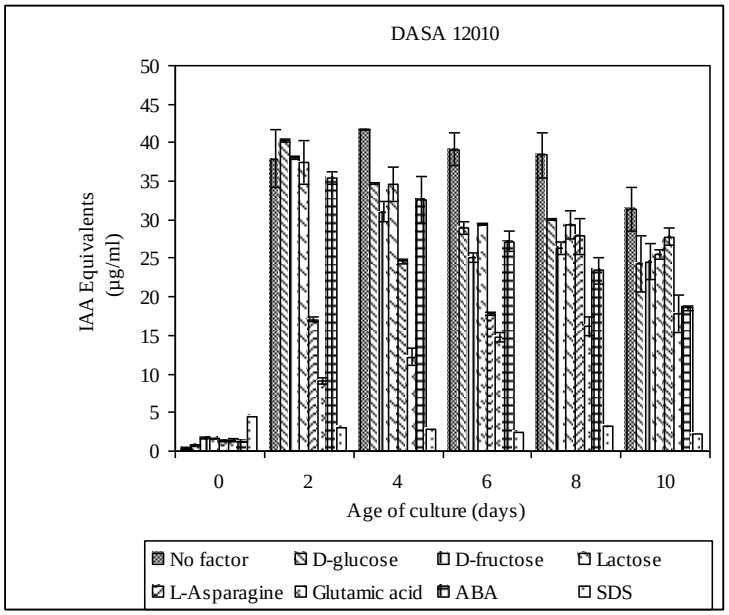

Fig. 6 The influence of factors on the IAA production by strains DASA12010. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

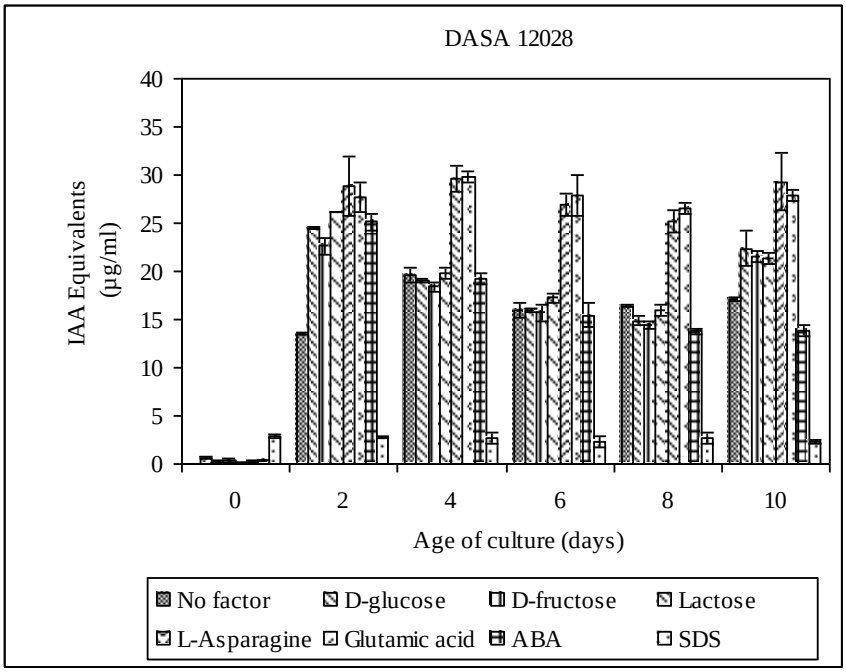

Fig. 7 The influence of factors on the IAA production by strains DASA12028. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

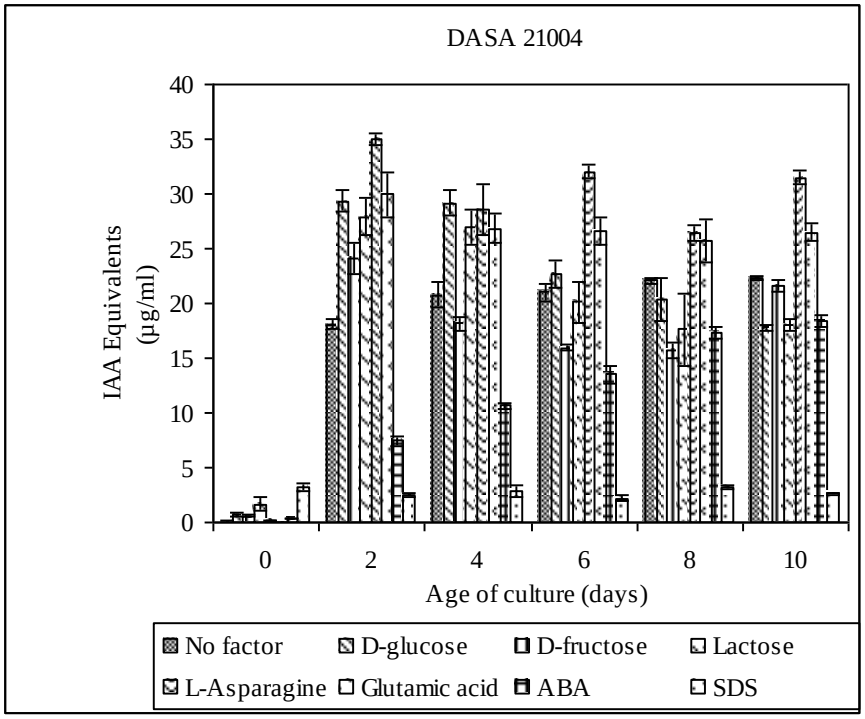

Fig. 8 The influence of factors on the IAA production by strains DASA21004. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

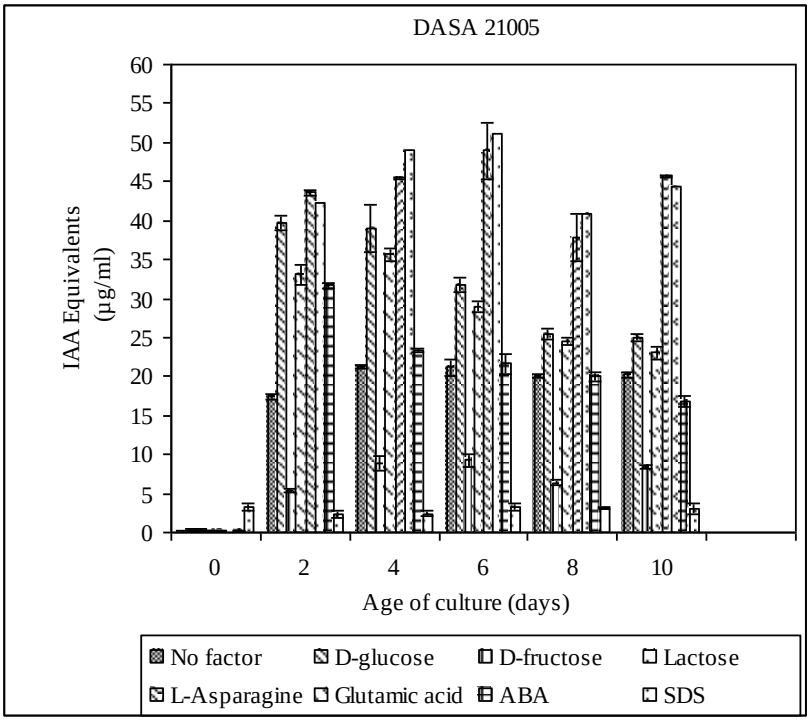

Fig. 9 The influence of factors on the IAA production by strains DASA21005. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

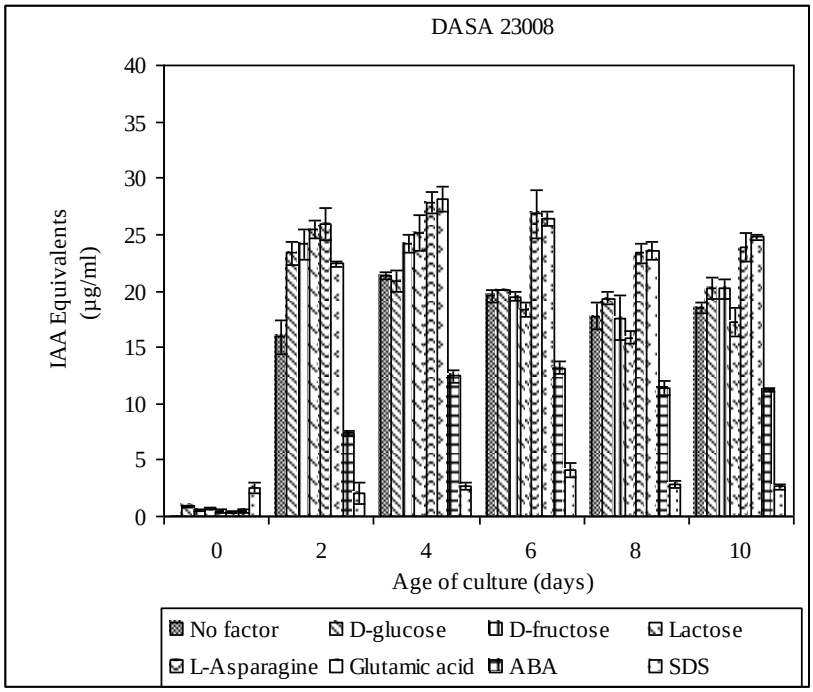

Fig. 10 The influence of factors on the IAA production by strains DASA23008. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations.

3.4 Sequence Analysis of Partial 16S rDNA

The partial nucleotide sequences of the 16S rRNA gene of the selected isolates DASA12010, DASA12028, DASA21004, DASA21005, and DASA23008 were amplified. PCR results indicated that each isolate had a single PCR product of about 500 bp (data not shown). Analysis of the sequence by BLASTN indicated all strains were closely related to strains of root-nodule bacteria. Four isolates (DASA12010, DASA21004, DASA21005 and DASA 23008) showed high homology with many strains of *Rhizobium* including *R.* sp. sc-w, *R.* sp. DV8, *R.* sp. DV4, *R.* sp. DV2, *R.* sp. DV1, *R.* sp. DV3, *R.* sp. BICC651R, *R.* sp. Ca-BZ, *R.* sp. EGY2, *R.* sp. TH-2005 and *R.* sp. TANU14 with 96%, 99%, 78% and 98% similarity, respectively. Even though the isolates DASA21004 and DASA21005 both were isolated from the same genus of host, Neptunia sp., these two isolates were not identical at the intraspecies level. The partial sequence 16S rDNA of DASA21004 showed 99% similarity with reference strains mentioned above. While the partial sequence 16S rDNA of DASA21004 showed 75% similarity to those reference strains. The alignment between the partial sequence 16S rDNA of DASA21004 and DASA21005 revealed that these two sequences had 88% homology to each other. The 16S rDNA sequence of the remaining isolate DASA12028 indicated high homology (81%) with different species of root-nodule bacteria such as *Sinorhizobium* sp. MA11, *Mesorhi-zobium* sp. BNC1, *Sinorhizobium medicae* WSM419, *S. medicae* RPA20, *S. medicae* RPA18, *S. kostiense* LMG14911, *S. meliloti* SEMIA103, *S. arboris* LMG14919 and *S. fredii* CCBAU01348. The partial nucleotide sequence of gene encoding for 16S rRNA gene of the selected isolates has been deposited in GenBank under accession number EU259507-259511.

4. Conclusion

Isolates of root-nodule bacteria from various leguminous plants were found to produce IAA during their growth. Isolates from the same host plant showed the different levels of the IAA production. The effects of D-glucose (1.00 mg/ ml), D-fructose (1.00 mg/ml), lactose (1.00 mg/ ml), L-asparagine (1.00 mg/ml), glutamic acid (1.00 mg/ml) and ABA (0.01 mg/ml) on the IAA production were varied in different isolates, even in isolates assigned to the same genus *Rhizobium* sp., whereas SDS (1.00 mg/ml) decreased IAA production in all isolates.

5. References

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