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ASCORBIC ACID PRODUCTION IN ROOT, ROOT NODULE AND IN CULTURE BY *RHIZOBIUM UNDICOLA* ISOLATED FROM THE AQUATIC LEGUME *NEPTUNIA OLERACEA* LOUR.

PALLAB KUMAR GHOSH[1], PRASHANTA KUMAR BHATTACHARYYA[2], TARUN KUMAR DE[3] AND TUSHAR KANTI MAITI[1]*

[1]Microbiology Laboratory, Department of Botany, The University of Burdwan, Golapbag, Rajbati, Burdwan-713104, West Bengal, India,
[2]Taxonomic laboratory, The University of Burdwan, Golapbag, Rajbati, Burdwan-713104, West Bengal, India.
[3]Department of Marine Science, Calcutta University, 35, B.C.Road, Kolkata-700019, India

ABSTRACT

The root nodules of *Neptunia oleracea* Lour. aquatic legume contained higher amount of ascorbic acid than root. A symbiont was isolated from root nodule of the plant and was identified as *Rhizobium undicola* based on biochemical and 16S rDNA sequence based molecular phylogenetic approach. The symbiont produced large amount of ascorbic acid (224µg/ml) from glucose supplemented basal medium. The production of ascorbic acid by the symbiont was increased to 187 % over control under optimized nutrient condition. The higher amount of ascorbic acid in nodule might be produced by *Rhizobium* sp. to meet its metabolic requirements, particularly in facilitating root infection for nodulation and delay nodule senescence. Results of the study supported the hypothesis of synthesis of ascorbic acid in root nodules of legume plant.

KEY WORDS: ***Neptunia oleracea*, *Rhizobium undicola*, Ascorbic acid (AsA), Root nodules**

TUSHAR KANTI MAITI
Microbiology Laboratory, Department of Botany, The University of Burdwan, Golapbag, Rajbati, Burdwan-713104, West Bengal, India,

INTRODUCTION

L-Ascorbic acid (vitamin C), a water soluble antioxidant and cellular reductant in plants and animals is commonly indispensable for physiological functions.[1] It is an essential nutrient for humans, non-human primates[2] as it cannot synthesize, due to lack of functional gene encoding l-gulono-1,4-lactone oxidase that catalysing the last step of ascorbate biosynthesis.[3, 4] The idea of inability to synthesize ascorbic acid (AsA) by the root nodules even in their young state was reversed by study of Matamores et al.[5] The presence of low concentrations of ascorbic acid in legume nodules positively correlated with nodule effectiveness.[6] The most important beneficial properties of ascorbic acid in nodules was established by the application of exogenous ascorbic acid to soybean plants. By direct infusion of ascorbic acid into stems resulted a 4-fold increase in nitrogenase activity and marked delay in nodule senescence.[7] Matamoros et al.[8] further observed that the L-galactono-1, 4-lactone dehydrogenase (GalLDH) mRNA was abundant in the infected zone of indeterminate and determinate nodules. This enzyme is an important enzyme in ascorbate biosynthesis. So, this finding refute the previous hypothesis that ascorbic acid is not synthesized in nodules, on the contrary, it supports to a previous conclusion that ascorbic acid in the infected zone is primarily involved in the protection of host cells against peroxide damage. The regulation of ascorbic acid (AsA) production and their role in scavenging oxidative stress i.e. reactive oxygen species (ROS) is one of the new focusing area in root nodule symbiosis.[9, 10] Besides, nodule senescence happens to be a delayed response of the plant due to the presence AsA in nodule. [11] Therefore, the ascorbic acid of *Rhizobium* sp. in nodule plays an important role in metabolic functions particularly in stress condition. The ascorbic acid production by the nodule symbionts in nodules were investigated in certain pulse legumes.[12,13,14] However no works have been reported for ascorbic acid production by symbiont isolated from aquatic legume plant. The present study describes the production of AsA in root, root nodule and in culture by symbiont of root nodules of aquatic legume plant *Neptunia oleracea* Lour. The isolated symbiont designated as N37 and identified by physio-biochemicals and 16S rDNA sequence based molecular phylogenetic approach. Attempt was also made to optimize the cultural requirements for production of AsA in culture to obtain an explanation of the nodular AsA.

MATERIALS AND METHODS

Sources of Plant

The plant collected from the pond of village Koichor, District Burdwan, West Bengal, India and identified as *Neptunia oleracea* Lour.The plant is a tropical, annual, aquatic, floating herb belongs to the subfamily Mimosoideae of family Fabaceae. This legume is unusually developed buoyant floating stems and adventitious roots arising from nodes with numerous root nodules.

Microorganism, medium and growth conditions

Mature and fresh root nodules were selected in order to isolate the symbiont from *Neptunia oleracea* Lour. following the method of Ghosh et al. [13] and transfer the bacteria into axenic culture. The symbiont was grown in yeast extract mannitol medium (YEM) with 1% mannitol and 0.01% $CaCl_2$ $2H_2O$ instead of NaCl and $CaCO_3$ (pH 7.0) [15] in three replicates at 30±2°C on a rotary shaker for 24 h. A loop full of the symbiont was added to the medium and growth was measured turbidometrically by a Spectrophotometer at 540 nm.

Genomic DNA extraction, PCR amplification and sequencing of 16S rRNA gene

For 16S rRNA gene sequence analysis, DNA was isolated according to Sambrook and Russell.[16] The 16S rRNA gene of the strain N37 was amplified by the method described earlier Panday et al.[17] Primers used for the amplification of 16S rRNA were 5'-GAG TTT GAT CCT GGC TCA G-3' (forward primer) and 5'-AGA AAG GAG GTG ATC CAG CC-3'

(reverse primer) adopted from Das et al.[18]. 16S rRNA amplification was performed with a Thermal Cycler, Model PCT-200 (M.J. Research, Waltham, MA, USA). After amplification, the PCR products were purified using the QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using a CEQ Dye terminator cycle sequencing kit in an automated DNA sequencer (Model CEQ 8000; Beckman Coulter, Fullerton, CA, USA). The nucleotide sequences obtained were assembled using the sequence alignment editing program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The 16S rRNA gene sequence obtained was compared using the EzTaxon server.[19] Phylogenetic tree was constructed according to the Jukes-Cantor model [20] using the MEGA 5.1 software package (The Biodesign Institute, Arizona, USA). The statistical significance of branch points was calculated by 1000 bootstrap re-samplings of the data.[21]

Ascorbic acid production medium

The AsA production medium of legume symbiont was [13]: (g/L^{-1}) Glucose 8.0, K_2HPO_4 0.5, $MgSO_4$, $7H_2O$ 0.2, NH_4Cl 3.0, Riboflavin 0.005, of distilled water at pH 7.0. This was used as the basal medium for optimization experiments.

Extraction of Ascorbic acid from plant tissue and culture

AsA was extracted (with minor modification) and estimated following [22] using a standard curve prepared from AsA. Glucose present in that extract was estimated following Dubois et al.[23] Fresh sample (1g), young roots and mature nodules was homogenized separately with 10 ml 6% trichloroacetic acid. The homogenate was centrifuged at 5000 rpm for 10 minutes to eliminate plant debris. 5 ml supernatant was mixed with 3 ml of 2.5% 2-4-dinitrophenyl hydrazine solution prepared in 9 (N) H_2SO_4, to which 2-3 drops of 10% thiourea solution in 70% ethanol was added to prevent oxidation of AsA. The mixture was boiled in a water bath for 25 minutes and cooled to room temperature then 5 ml H_2SO_4(80%) was added to it. After 30 minutes, optical density was estimated at 530nm using a Shimadzu UV-VIS double beam Spectrophotometer (Model-190). After centrifugation 5 ml cultural filtrate was treated in the same way as it was performed in case of plant tissues to estimate the amount of AsA produced in culture.

Statistical analysis

Values are the mean ± SEM of 3 replicates. All data were subjected to students't-test analysis with significance level of $P<0.05$ using SPSS software package.

RESULTS AND DISCUSSION

Neptunia oleracea Lour. is commonly known as water sensitive plant or water mimosa. The stems of the plant is creeping, floating, nodules are formed at the base of the lateral and adventitious roots. Nodules were small aggregated deep pink in color, mostly elongated to oval shaped sometimes branched found in nodes. Each nodule contains plenty of bacteria as bacteroids. The Strain N37 isolated from the nodule of this aquatic plant was identified as *Rhizobium undicola* on the basis of biochemical characteristics and 16S rDNA sequence homology result. The strain N37 showed 99.859% sequence similarity with *R.undicola (Allorhizobium undicola)*(Y17047).[24] In the maximum likelihood phylogenetic tree (Fig. 1) of the strain N37 was formed a cluster with strain of *Rhizobium undicola* LMG11875^T (Y17047), *Rhizobium undicola* OURAN110 (HQ895844) and sequence similarity as 99.859% and 99.8% respectively (Table 1). On the basis of more than 99% sequence homology of 16S rDNA, and biochemical characteristics confirms the species status of the strain N37 as *Rhizobium undicola.* The 16S rDNA sequence was deposited to NCBI and the sequence accession number is KF309665. The strain N37 showed the following physiological and biochemical characteristics: positive for catalase activity, nitrate reductase, citrate utilization, Voges-proskauer test, and negative for 3-keto lactose production, lysine decarboxylase activity, indole test and showed very little growth in glucose peptone medium. The strain produced acid gas from glucose,

galactose, sucrose, sorbitol, mannitol and glycerol (data not shown). Mature root nodules of the plant contained higher amount of AsA (37 µg/g of fresh tissue) than the non-nodulated roots (Table 2). The root nodules of *Phaseolus mungo* also contain higher amount of AsA than root.[13] Further, the high amount of AsA was detected at the apex of the nodules in *Phaseolus vulgaris* and this was found to be involved in the protection of host cells against peroxide damage that strongly suggest a participation of ascorbate in additional functions during symbiosis, possibly related to cell growth and division and molecular signaling.[11] The production of higher amount of AsA in nodule than root might be due to the presence of L-galactono-1,4-lactone dehydrogenase (GalLDH) enzymes. The nodular symbionts also help plant tissue to protect themselves, during initial infection and further protecting from biotic stress in the rhizospheric area. The higher amount of glucose in nodular tissue also supported the fact that nodule act as reservoir of glucose in *Rhizobium* sp. and eventually that serves as a precursor of AsA biosynthesis, which is supplied to the infected zone of plant tissues for further protection of plant from stress condition. So this result support the concept of synthesis of AsA in root nodule of legume, in contrary to the earlier concept that root nodule could not synthesize AsA.[8,13,14] Likewise, the high ascorbate levels in the nodules of *Glycine max* strongly suggest its additional function during symbiosis, possibly related to cell growth and division and to molecular signaling.[7] Ascorbate is required for the progression of the cell cycle and for cell elongation. This effect may play a critical role in nodule development along with polysaccharide. AsA has been shown to modulate expression of the genes involved in plant defense against biotic stress.[11] Potters et al.[29] and Vanacker et al. [30] also suggested the roles of ascorbate as ROS in nodule senescence in legume plants.

The isolate showed maximum production of AsA (140µg/ml) at 22 h (Fig. 2A). Normally the secondary metabolites produce during the stationary phase of growth, but in this case it is produced during the exponential phase of growth. The isolate produced AsA along it grew as AsA was involved in the protection of host cells against damage and also in cell growth and division. Further, it is observed that AsA production decreased during the late stationary phase of growth (Fig. 2A) and is correlated with the study of *Rhizobium* sp. From *Phaseolus mungo*.[13] To test the suitable carbon source in culture medium, it was found that maximum AsA produced (160µg/ml) (Table 3) in the presence of glucose (0.5%) (Fig.2C). Similar observation has also been noted in *Rhizobium* sp. isolated from *Phaseolus mungo*.[13] Different nitrogen sources were added separately in the nitrogen free medium (replacing NH_4Cl) to determine maximum AsA production by the symbiont (Table 4). Among them, NH_4Cl (control) was recorded as most suitable for AsA production (170µg/ml) although the bacteria used all of the nitrogen sources tested to produce AsA (Table 4). Vincent [30] observed *Rhizobium* sp. could use several nitrogenous compounds for growth. The optimum concentration of the preferred nitrogen source (ammonium chloride) for maximum AsA production by the *Rhizobium* sp. was 0.3 % and decreased thereafter (Fig.2D). Different vitamins were used to determine maximum production of AsA (Table 5). Among them riboflavin (0.5µg/ml) followed by B_{12} (1µg/ml) showed promotive effect for AsA production (Table 5). Ascorbic acid production enhanced to 187.17% over control when riboflavin supplemented in the optimized production medium (Table 6). All the supplements, which increased the production of AsA in culture, might have been available within the nodule for the utilization of the *Rhizobium* sp. from *Neptunia oleracea*. This might stimulate the *Rhizobium* sp. to produce more AsA, helping to promote infection, to enhance nodulation and also to regulate nodule senescence in the studied legume. It is there for ascertained that AsA production is the other beneficial aspect of symbiosis like hormone production and nitrogen fixation.[29]

Table 1
Molecular identification of isolates N37

Isolated strain	16S rDNA gene sequence length(bp)	Accession number	Closest relative and NCBI accession number	Similarity (%) on the basis of 16S rDNA
N37	1422	KF309665	*Rhizobium undicola* LMG 11875^T (Y17047), *Rhizobium undicola* OURAN110 (HQ895844), *Rhizobium undicola*Liujia-1(DQ648578)	99.8 99.8 99.7

Table 2
Contents of glucose and AsA in root nodule and root of Neptunia oleracea. Data presented here are mean of three replicates.

Plant parts	Glucose (µg/g fresh tissue)	AsA (µg/g fresh tissue)
Root nodule	730±1.52	37±0.57
Root	475±2.08	15±0.88

Table 3
Effects of different carbon sources on growth and AsA production by Rhizobium sp. in culture. The bacteria were grown in AsA-production medium for 24 hours at 30±2^oC with 2% inoculum dose. Data presented here are mean of three replicates.

Name of the Carbon Source(0.1%)	OD for growth at 540 nm	AsA production (µg/ml)
Control	0.42±0.005	00
Sorbitol	0.26±0.003	8±0.57
Lactose	0.32±0.006	10±0.57
Rhamnose	0.36±0.008	11±0.88
Galactose	0.50±0.008	30±0.66
Maltose	0.64±0.003	32±0.57
Mannose	0.50±0.002	35±1.15
L-Arabinose	0.41±0.002	38±0.88
Mannitol	0.75±0.006	49±1.45
Sucrose	0.53±0.008	54±1.15
Fructose	0.62±0.002	72±0.66
Glucose	0.92±0.003	82±0.57
Critical Difference at P=0.05	0.12	4.10

Table 4

Effects of different nitrogen sources on growth and AsA production by Rhizobium sp. in culture. The control set was devoid of any additional nitrogen source. In other cases, nitrogen sources were added individually. The bacteria were grown in AsA-production medium for 24 hours at 30±2°C with 2% inoculum dose. Data presented here are mean of three replicates.

Nitrogen Sources (mg/ml)	OD for growth at 540 nm	AsAproduction (µg/ml)
Control	0.84±0.011	78±0.88
Arginine monohydrochloride	1.40±0.026	100±1.20
L-Glutamic acid	1.30±0.017	110±0.88
Potassium nitrate	1.26±0.003	120±0.57
Sodium nitrate	1.44±0.020	130±1.45
Sodium citrate	1.34±0.020	130±1.45
Glycine	1.34±0.003	132±0.57
Ammonium nitrate	1.45±0.017	140±1.15
Ammonium sulphate	1.60±0.017	144±0.33
L-Asperaginine	1.56±0.011	146±0.66
Ammonium chloride	1.64±0.011	170±1.20
Critical Difference at P=0.05	0.10	4.28

Table 5

Effects of different vitamins sources on growth and AsA production by Rhizobium sp. in culture. The control set was devoid of any additional vitamin source. In other cases, vitamin sources were added individually. The bacteria were grown in AsA-production medium for 24 hours at 30±2°C with 2% inoculum dose. Data presented here are mean of three replicate.

Vitamin sources (0.5 µg/ml)	OD for growth at 540 nm	AsA production (µg/ml)
Control	1.40±0.010	96±1.20
Nicotinic acid	1.92±0.011	183±0.88
Thiamine hydrochloride	1.90±0.008	190±0.57
B_{12}	2.05±0.014	195±1.45
Biotin	2.20±0.012	200±0.66
Riboflavin	2.25±0.011	208±0.57
Critical Difference at P=0.05	0.07	5.40

Table 6

Increase in growth and AsA production by Rhizobium sp. with the most effective supplements. The bacteria were grown in AsA-production medium for 24 hours at 30±2°C with 2% inoculum dose. The control set was devoid of any additional supplements. Data presented here are mean of three replicates.

Supplement	Growth OD at 540 nm	AsA Production (µg/ml)	Production increment (%)
Control (©)	0.84±0.011	78±0.88	00
©+Glucose (0.5%)	1.44±0.026	160±2.33	105.12
©+Glucose (0.5%) +Ammonium chloride (0.3%)	1.84±0.011	188±1.45	141.02
©+Glucose (0.5%) +Ammonium chloride (0.3%) + Riboflavin (0.5µg/ml)	2.34±0.012	224±2.02	187.17

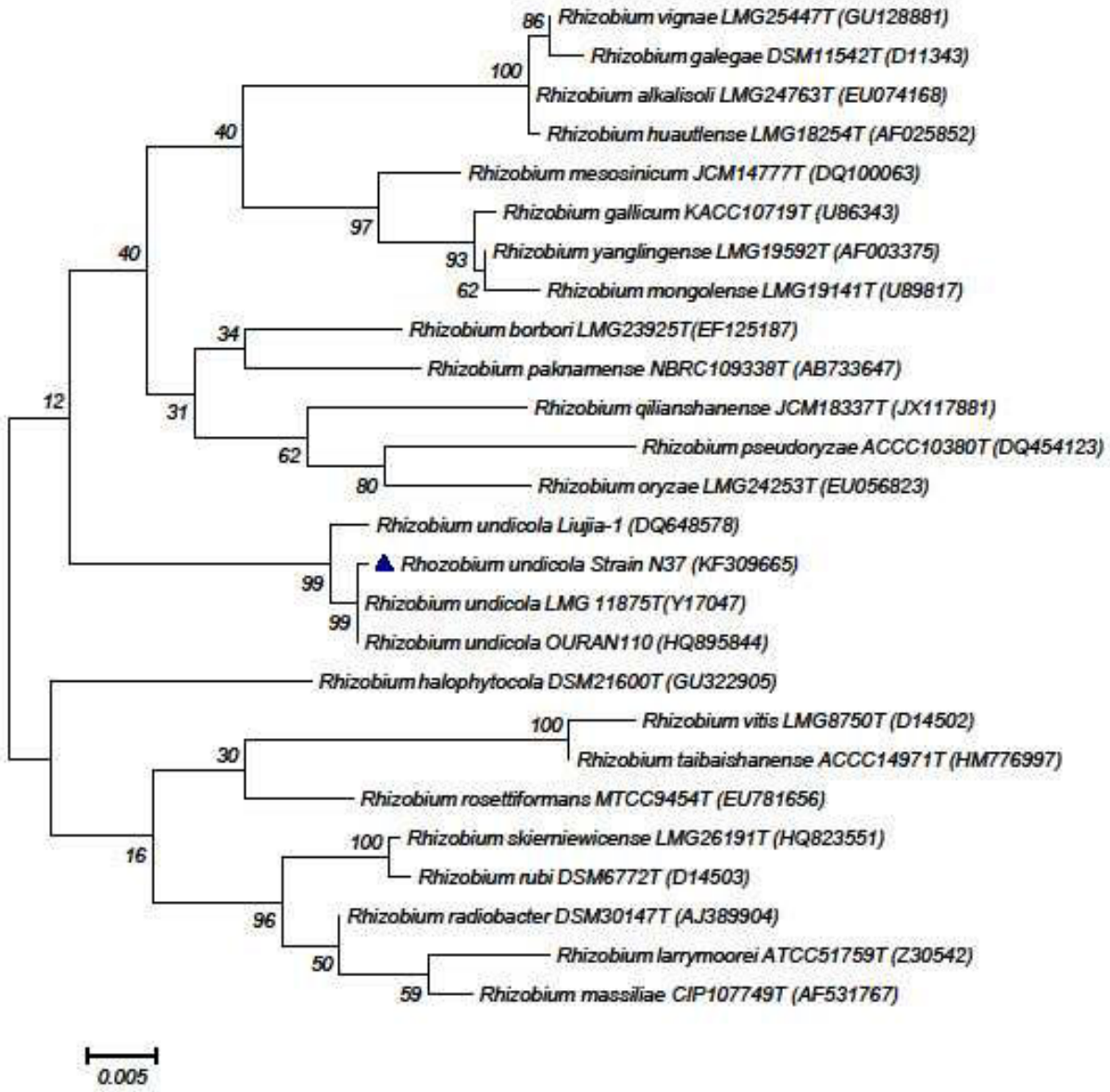

Figure 1

Maximum Likelihood method based on the Jukes-Cantor model phylogenetic tree showing the position of Rhizobium undicola strain N37 among the related taxa based on 16S rRNA gene sequences. Bootstrap values expressed as percentages of 1000 replications are given at branch points. Accession numbers are given in parentheses. Bar 5 substitutions per 1000 nucleotide position.

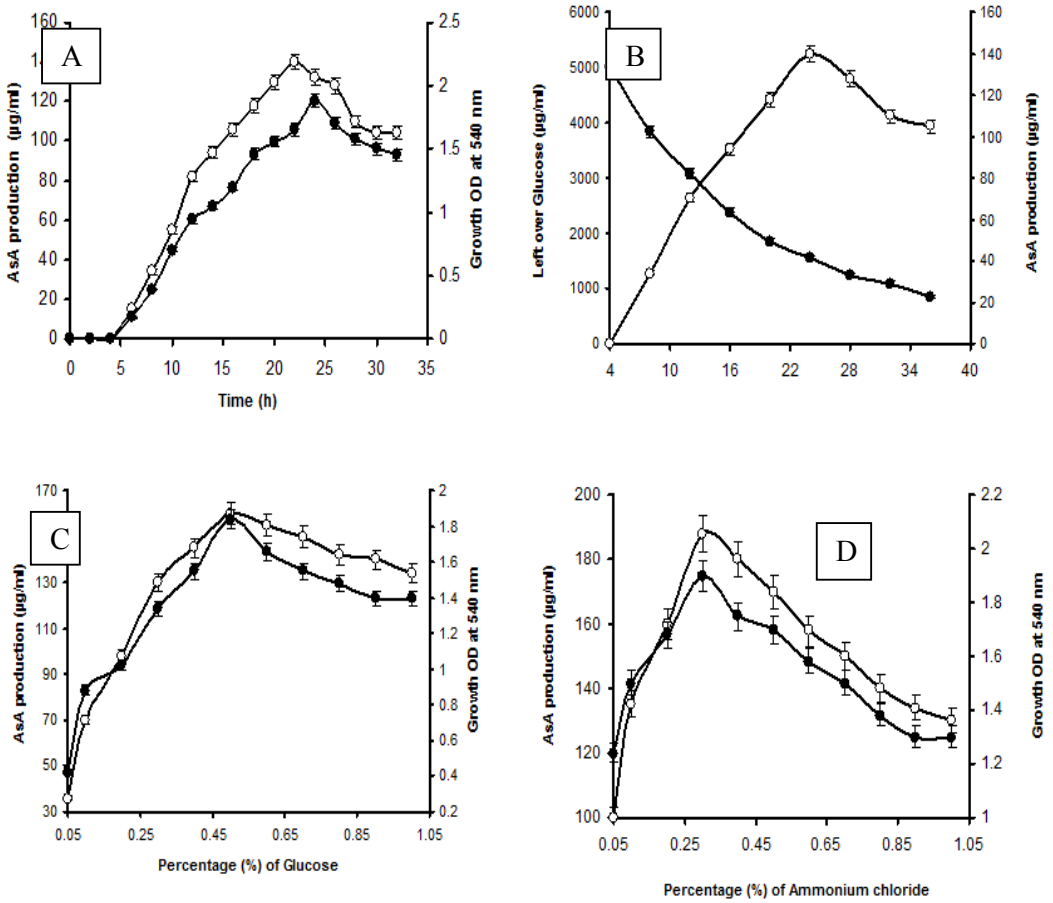

Figure 2

Growth (closed circles) and AsA production (open circles) by Rhizobium sp. (strain N37) in culture. The bacteria were grown in AsA-production medium at 30±2°C. (A) Growth curve and (B) Consequent decrease in glucose level of the medium. (C) Effect of different concentrations of glucose. The bacteria were grown in AsA-production medium for 24 hours at 30±2°C with 2% inoculums dose. (D) Effect of different concentrations of ammonium chloride. The bacteria were grown in AsA-production medium for 24 hours at 30 ± 2°C with 2% inoculums dose. Y-axis bar shows standard error value up to ±3%.

CONCLUSION

From the above result it may be concluded that AsA (vitamin C) is a reductant and low molecular weight antioxidant.The symbiont isolated from this aquatic legume identified as *Rhizobium undicola* on the basis of biochemical characterization and 16S rDNA homology. Considerable evidence suggested that AsA has central importance in plant biology particularly in hormone synthesis, gene expression, cell division and growth.Mature root nodules of this legume contained higher amount of AsA than that of the non-nodulated roots. Thus it indicated that the AsA in nodule is synthesized by the symbiont. Further, it can be assumed that the AsA is involved in the protection of host cells against peroxide damage that strongly suggest its participation of ascorbate in additional functions during symbiosis, possibly related to cell growth and molecular signaling. Further, the delayed response in nodule senescence might be the effect of

the nodule symbionts producing ascorbic acid.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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