

The Ascorbic Acid Production in Root, Root Nodule and in Culture by *Rhizobium* sp. Isolated from the Legume *Cajanus cajan* (L.) Millspaugh

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The root nodules of *Cajanus cajan* (L.) Millspaugh, a leguminous medicinal plant, was determined to contained high amount of ascorbic acid (AsA). A Glucose pool present in the root nodule might serve as precursor for AsA production. A symbiont was isolated from root nodules of the plant and was identified as a *Rhizobium* sp. based on biochemical and 16S rDNA based molecular phylogenetic approach. The symbiont produced large amount of AsA (640 µg/ml) from glucose supplemented basal medium. The production of AsA by the symbiont was increased to a greater extent than over control when the glucose (1.0 %) supplemented mineral medium was enriched with biotin (20 µg/100ml), thiamine hydrochloride (20 µg/100ml) and L-glutamic acid (0.2%). Our result supported the modern concept that AsA was also synthesized in root nodules of legume and refute the earlier concept that root nodules can not synthesize AsA.

Key words: *Cajanus cajan*, *Rhizobium* sp., Ascorbic acid (AsA), Root nodules.

L-Ascorbic acid (vitamin C) is a major soluble antioxidant and cellular reductant in plants and animals¹. However, humans, other primates, guinea pigs, bats, some birds and fishes are unable to synthesize ascorbic acid and need to incorporate it in the diet. This is because they lack a functional gene encoding L-gulonono-1, 4-lactone oxidase, the enzyme that catalyzes the last step of ascorbic acid

synthesis in animals. In contrast, the last step of the D-Mannose / L-Galactose² and D-galacturonic acid³ pathways for ascorbic acid synthesis in plants are catalyzed by L-galactono-1, 4-lactone dehydrogenase (GalLDH), which is localized in the mitochondrial inner membrane⁴.

Reactive Oxygen Species (ROS) are continuously produced as a result of aerobic metabolism or in response to biotic and abiotic stresses⁵. ROS are not only toxic by-products of aerobic metabolism, but are also signaling molecules involved in plant growth and environmental adaptation. Antioxidants such as ascorbic acid (AsA) can protect the cell from

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oxidative damage by scavenging the ROS and play an important role in optimizing cell function by regulating cellular redox state and modifying gene expression⁶.

Regulation of ascorbic acid (AsA) production and their role in scavenging oxidative stress of plant system is one of the new focusing sites in root nodule symbiosis^{7,8}. Earlier, it was thought that the legume root nodules were unable to synthesize AsA or the young root nodule of some leguminous plants was able to synthesize AsA and this capacity was lost early in the root nodule development⁹. The AsA was imported from the shoot or the root in the plants. Matamoros *et al.* (2006)¹⁰ reported for the first time by in situ hybridization that the GalLDH mRNA was particularly abundant in the infected zone of indeterminate and determinate nodules. This refuted the previous hypothesis that ascorbic acid is not synthesized in nodules and lend support to a previous conclusion that ascorbic acid in the infected zone is primarily involved in the protection of host cells against peroxide damage. So the ascorbic acid was produced by *Rhizobium* sp. in nodule play an important role in different metabolic function particularly in stress condition.

The present study involved mainly the production of AsA in root, root nodule and in culture by symbionts isolated from the root nodules of the plant pigeon pea (*Cajanus cajan* L.) and to get an explanation for the nodular AsA. Attempt was made to optimize the cultural requirements for production of AsA. The isolated symbiont was identified by biochemical and 16S rDNA based molecular phylogenetic approach.

MATERIAL AND METHODS

The Plant and the symbiont

The certified seeds of selected medicinal pulse shrub pigeon pea [*Cajanus cajan* (L.) Millspaugh] were purchased from an authentic seed-selling center in this city. The plants were grown for 40 to 45 days in experimental plots. The mature, fresh root nodules and young roots were collected from the plant to perform the experiments. The symbiont was isolated from the mature, fresh root nodules on slightly modified yeast extract mannitol (YEM) medium¹¹. A number of colonies were appeared on YEM plate. The bacteria were

numbered as P1 to P25 randomly to designate well. The productivity of AsA among all the isolates was tested and the strain P2 was selected for maximum level of AsA production in the medium. The bacterium was grown in YEM medium and growth was measured turbidometrically using a Shimadzu UV-Vis double beam Spectrophotometer (Model-190) at 540nm.

Genomic DNA Extraction and PCR Amplification of 16S rRNA gene

Genomic DNA from the bacterial strain was extracted following Mavnum (1961)¹² and was used as template for PCR. Amplification of 16S rRNA genes was performed with conserved universal primers for bacterial 16S rDNA primers SSU 16S-F (5' AACTCC TAC GGG AGG CAG CAG 3') and SSU 16S-R (5' AAG GAC TAC CAG GGT ATC TAA TCC 3') which yielded PCR products of about 0.6 Kb¹³. Amplification was carried out in a 50 µl reaction mixture containing 1.5 mM MgCl₂, 0.2 mM each dNTPs, 10 pmole each primers, 10 ng DNA template and 1 U Taq polymerase with reaction buffer. Hot start PCR was performed at 95°C prior to addition of DNA Taq polymerase. PCR amplification was performed with a Master Cycler gradient. The Cycling parameters of initial 3 minutes denaturation at 94°C followed by 30 Cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 minute were employed. PCR products were examined by Gel Electrophoresis on 1 % Agarose gel in 1X TAE buffer.

Nucleotide sequencing

The strain was identified as *Rhizobium* sp. by 16S rRNA gene sequence analysis. A continuous stretch of 389 nucleotides of 16S rRNA gene was used for phylogenetic analysis. The 16S rRNA gene sequences were subjected to thorough analyses for the determination of its affiliation and phylogeny. Initially the near related sequences were determined using BLAST program of NCBI (www.ncbi.nlm.nih.gov/Blast/nblast). The online programs from RDP database (<http://rdp.cme.msu.edu/>) like "Classifier" and "Sequence Match" were used to assess phylogenetic locus of culturable bacterial isolates. The "Hierarchy Browser" was used to retrieve 16S rRNA gene sequences from type strains of various *Rhizobium* species. During sequence analysis, the sequence similarity between any two sequences were calculated manually after aligning it in

CLUSTAL_X¹⁴ and then looking for mismatches versus total number of nucleotides used in such comparison. For phylogenetic analysis 16S rRNA gene sequences from type strains of different species of *Rhizobium* showing sequence identity greater than 96% with the strain P2 were considered in this study. All these sequences were retrieved from GenBank database and or from RDP database, aligned with CLUSTAL_X and edited manually. Distance based neighbour-joining trees was constructed by TREECON software¹³ using 100 replications and by using Jukes and Cantor (1969)¹⁵ correction.

Ascorbic acid production medium

The medium for AsA production was formulated by several trial and errors method, which contained Glucose 8 g, K₂HPO₄ 500 mg, MgSO₄·7H₂O 200 mg, L-Glutamic acid 2 g, thiamine hydrochloride 200 µg and Biotin 200 µg per 1000 ml of distilled water at pH 7.0.

Extraction of Ascorbic acid

AsA was extracted (with minor modification) and estimated following (Oser 1979)¹⁶ using a standard curve prepared from AsA. Glucose present in that extract was estimated following (Dubois et al 1956)¹⁷. 1g of each raw tissue material (fresh young roots and mature nodules) was homogenized separately with 10 ml 6% trichloroacetic acid. The homogenate was centrifuged at 5000 rpm for 2 minutes to eliminate plant debris. The volume of the filtrate was mixed with 3 ml of 2.5% 2-4-dinitrophenyl hydrazine solution prepared in 9 (N) H₂SO₄, 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 0°C and then 5 ml 80% H₂SO₄ 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 soft were package.

RESULTS AND DISCUSSION

The symbiont (strain P2) isolated from the mature root nodule of *Cajanus cajan* (L.) was identified up to genus level according to various biochemical tests and also on the basis of partial 16 S rDNA sequence analysis. The symbiont responded to many important physiological and biochemical characteristics which show the similar to the characters of *Rhizobium* spp. as stated in Bergey's Manual of Systematic Bacteriology¹⁸. The strain P2 showed the following physiological and biochemical characteristics: positive for catalase activity and Voges-proskauer test, indole production, 2% NaCl tolerance, amylase and protease activity tests; positive for 3-keto lactose production, citrate utilization and showed very little growth in glucose peptone medium. The strain produced acid from mannitol, glycerol, galactose, mannose, maltose, xylose, myo-inositol, raffinose, fructose, and sorbitol, but it produced no gas with sucrose, lactose, glucose, or arabinose. Growth in litmus milk resulted in an acidic reaction. The strain deposited in MTCC.

As evident from the neighbour-joining (NJ) phylogenetic tree (Fig.1), the strain P2 together with four other species of *Rhizobium* namely, *R. multihospitium*, *R. lusitanum*, *R. miluonense* and *R. hainanense* formed a cluster that is well separated from the *Rhizobium giardinii* and *Rhizobium etli-Rhizobium tropici* clusters. The strain P2 showed closest sequence similarity with

Table 1: Effect of different carbon sources on growth and AsA production by *Rhizobium* sp. in culture

Name of the Carbon source (0.1%)	OD for growth at 540 nm	AsA production (µg/ml)
Glucose	1.57±0.0115	47±0.8819
Galactose	1.14±0.0120	29±0.3333
Fructose	1.42±0.0115	23±0.5773
Sucrose	1.34±0.0120	25±0.8819
Lactose	1.09±0.0120	20±1.2018
Mannitol	1.47±0.0145	32±0.5773
Maltose	0.97±0.0033	21±0.5773
L-Arabinose	0.92±0.0145	17±0.6667
Ribulose	0.72±0.0115	14±0.8819

Data presented here are mean of three replicates.

Table 2. Effect of different nitrogen sources on growth and AsA production by *Rhizobium* sp. in culture

Nitrogen Sources (0.2%)	OD for growth at 540 nm	AsA production ($\mu\text{g/ml}$)
Control	0.32 \pm 0.0088	25 \pm 1.1547
Glycine	0.36 \pm 0.0033	90 \pm 0.6667
Sodium Nitrate	0.40 \pm 0.0088	100 \pm 1.2010
Potassium Nitrate	2.44 \pm 0.0057	300 \pm 0.6667
Ammonium Sulphate	1.44 \pm 0.0202	360 \pm 1.2010
Arginine	1.46 \pm 0.0057	366 \pm 0.8819
monohydrochloride		
L- asparagine	0.94 \pm 0.0145	528 \pm 0.5773
Ammonium Chloride	2.15 \pm 0.0202	538 \pm 0.5773
L-glutamic acid	2.44 \pm 0.0145	610 \pm 0.6667

The control set was devoid of any additional nitrogen source. In other cases, nitrogen sources were added individually. Data presented here are mean of three replicates.

Table 4. Increase in growth and AsA production by *Rhizobium* sp. with the most effective supplements

Supplement	Growth OD at 540 nm	AsA Production ($\mu\text{g/ml}$.)
Control	0.32 \pm 0.0066	25 \pm 1.1547
Control + biotin (20 μg /100ml)	1.13 \pm 0.0066	111 \pm 0.8819
Control + Thiamine hydrochloride (20 μg /100ml)	0.85 \pm 0.0088	81 \pm 0.8819
Control + biotin + Thiamine hydrochloride (20 μg /100ml)	1.20 \pm 0.0145	213 \pm 0.0066
Control + biotin + Thiamine hydrochloride + L-Glutamic acid (0.2%)	3.12 \pm 0.0145	640 \pm 1.7638

The control set was devoid of any additional supplements. Data presented here are mean of three replicates.

the four species with whom it produced a common cluster (all 99.5% identity) followed by *R. elii* (99.2% identity) and *R. tropici* (99% identity). Such high extent of sequence similarity and phylogenetic positioning indicates that the strain P2 is definitely a species of *Rhizobium*. However, information based on overall genome relatedness with type strains of all the species of *Rhizobium* showing 16S rDNA sequence identity of 97% or more would have given clear idea about species status of the strain P2¹⁹. Moreover, the phylogenetic deduction was based only on 389 nucleotide long 16S rDNA sequence. So, in absence of detailed phenotypic, chemotaxonomic and overall genome relatedness data, the strain P2 was concluded as *Rhizobium* sp., pending further investigation for species

Table 3. Effect of different vitamins sources on growth and AsA production by *Rhizobium* sp. in culture

Vitamin sources	OD for growth at 540 nm	AsA production ($\mu\text{g/ml}$)
Control	0.32 \pm 0.0066	25 \pm 1.1547
Nicotinic Acid	0.85 \pm 0.0088	81 \pm 0.8819
Riboflavin	0.94 \pm 0.0088	92 \pm 1.4529
Thiamine	1.08 \pm 0.0557	105 \pm 0.8819
Hydrochloride		
Biotin	1.13 \pm 0.0066	115 \pm 1.4529

The control set was devoid of any additional vitamin source. In other cases, vitamin sources were added individually. Data presented here are mean of three replicate.

status. The Genbank accession number of 16S rRNA gene from the strain P2 is HMO56031.

The bacteria showed growth and production of AsA at the same time in production medium and reached its maximum (540 $\mu\text{g/ml}$) at 24 h (Fig. 2A). Most of the secondary metabolites

Table 5. Content of glucose and AsA in root nodule and root of *Cajanus cajan*

Plant parts	Glucose ($\mu\text{g/g}$ fresh tissue)	AsA ($\mu\text{g/g}$ fresh tissue)
Root nodule	113 \pm 0.0066	53 \pm 0.8819
Root	93 \pm 0.8819	28 \pm 0.5773

Data presented here are mean of three replicates.

were produced during the stationary phase of growth, but this secondary metabolite was produced during the exponential phase of this bacterium. The bacteria produced AsA along it grew as AsA was involved in the protection of host cells against damage and also in cell growth and division. The level of AsA production decreased during the late stationary phase of growth (Fig. 2B). Similar observations have also been noted in *Rhizobium* sp. isolated from *Phaseolus mungo*²⁰.

Different carbohydrates were used as carbon source in culture medium to get maximum AsA production by the symbiont (Table 1). Among them glucose showed considerable high amount of AsA production. Maximum production of AsA was recorded (540 µg/ml) when the medium was enriched with 1.0% of glucose (Fig. 2C).

Different nitrogen sources were added separately in the nitrogen free medium (replacing NH₄Cl) to estimate maximum AsA produced by the

symbiont (Table 2). Among them, L-glutamic acid was recorded to produce maximum AsA (610 µg/ml) although the bacteria used all of the nitrogen sources tested to produce AsA (Table 2). According to Vincent (1974)²¹ *Rhizobium* sp. could use several nitrogen compounds for growth. The optimum concentration of the preferred nitrogen source (L-glutamic acid) for maximum AsA production by the *Rhizobium* sp. was 0.2 % (Fig. 2D). The level of AsA production decreased as L-glutamic acid concentration was above 0.2 % (Fig. 2D).

Different vitamins were used to determine maximum production of AsA (Table 3). Among them biotin (20 µg/ml) followed by thiamine hydrochloride (20 µg/ml) showed promotive effect for AsA production (Table 3). The production synergistically enhanced when biotin, thiamine hydrochloride, and L-glutamic acid were supplemented in production medium (Table 4).

Mature root nodules of the plant

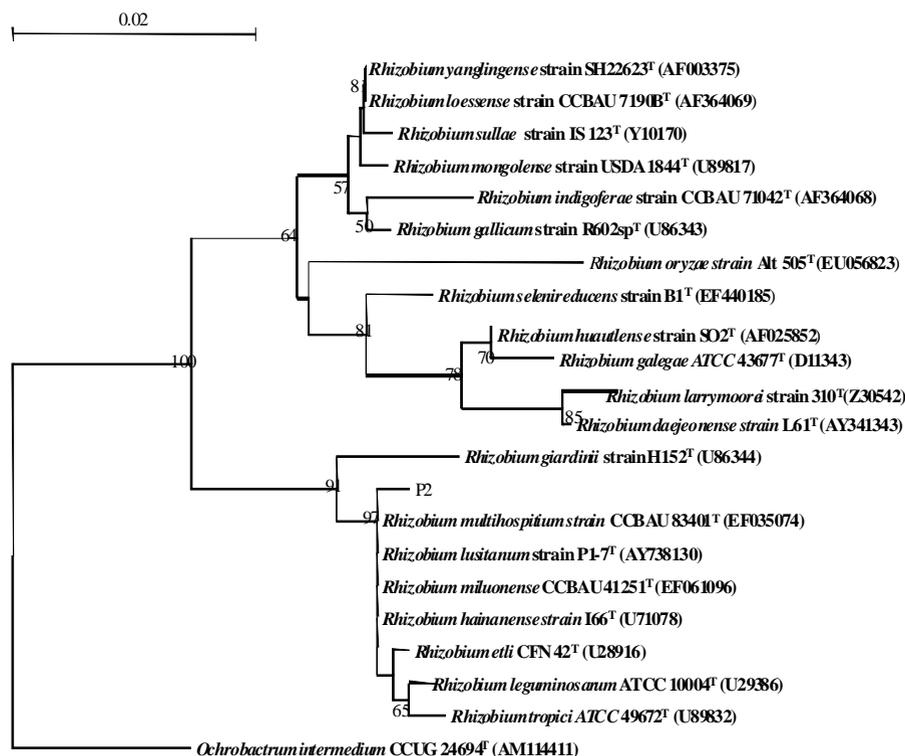
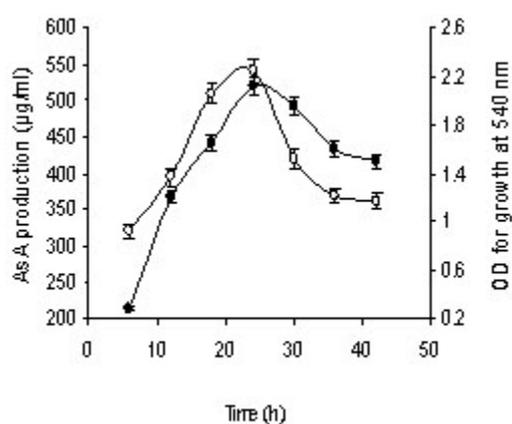


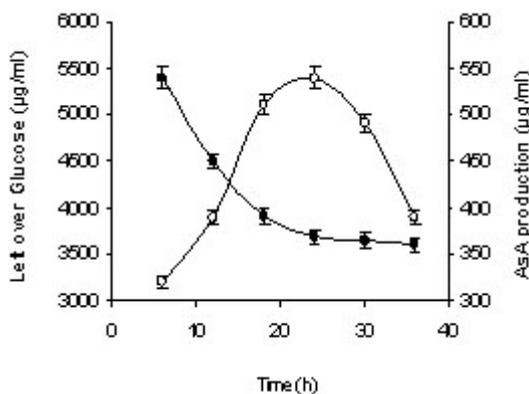
Fig. 1. Phylogenetic tree based on neighbour-joining (NJ) method, showing the relationships between strain P2 and other related species of the genus *Rhizobium*. Bootstrap values of 100 replications are shown at the branch. The tree was generated using TREECON (Van de Peer and Wachter, 1997) and Jukes and Cantor (1969) correction. Sequence from *Ochrobactrum intermedium* strain CCUG 24694^T was used as an out group

contained higher amount of AsA (53 $\mu\text{g/g}$ fresh tissue) than the amount of AsA (28 $\mu\text{g/g}$ fresh tissue) present in the non-nodulated roots (Table 5). The root nodules of *Phaseolus mungo* also contain high amounts of AsA than root²². 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 and strongly suggest a participation of ascorbate in additional functions

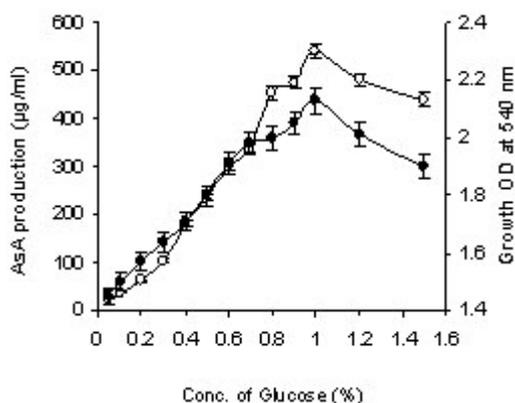
during symbiosis, possibly related to cell growth and division and molecular signaling⁸. The production of higher amount of AsA in nodule than root is due to presence of L-galactono-1, 4-lactone dehydrogenase (GalLDH) enzymes that are synthesized by gene present in *Rhizobium* sp. The gene responsible for this enzyme is controlled by surrounding environmental condition of *Rhizobium* sp. as well as biotic stress and different molecular signaling of plant tissues. The



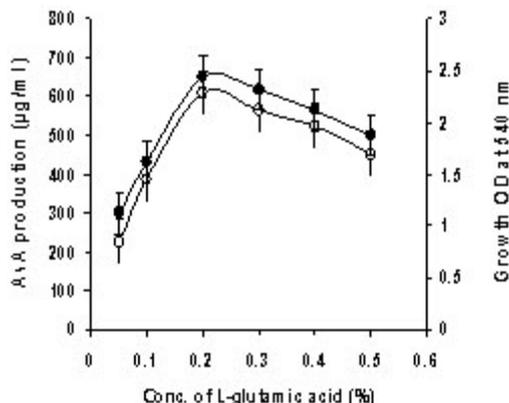
A) Growth (closed circles) and AsA production (open circles) by *Rhizobium* sp. (strain P2) in culture. The bacteria were grown in AsA-production medium at $30\pm 2^\circ\text{C}$. Y-axis bar shows standard error value up to $\pm 5\%$



B) Production of AsA (open circles) by *Rhizobium* sp. (Strain P2) in culture and consequent decrease in glucose level of the medium (closed circles). The bacteria were grown in AsA-production medium at $30\pm 2^\circ\text{C}$. Y-axis bar shows standard error value up to $\pm 5\%$



C) Effect of different concentrations of glucose on growth (closed circles) and AsA production (open circles) by *Rhizobium* sp. (strain P2) in culture. The bacteria were grown in AsA-production medium for 20 hours at $30\pm 2^\circ\text{C}$ with 10% inoculum dose. Y-axis bar shows standard error value up to $\pm 5\%$.



D) Effect of different concentrations of L-glutamic acid on growth (closed circles) and AsA production (open circles) by *Rhizobium* sp. (strain P2) in culture. The bacteria were grown in AsA-production medium for 20 hours at $30\pm 2^\circ\text{C}$. Y-axis bar shows standard error value up to $\pm 5\%$

Fig. 2. Growth of the *Rhizobium* and production of AsA in Culture

nodular symbionts also help plant tissue to protect themselves, during initial infection and further protecting from biotic stress in the rhizospheric area²³. 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 modern concept^{1, 9}, that AsA also synthesized in root nodule of legume and which refute the earlier concept that root nodule can not synthesize AsA⁸. Likewise, the high ascorbate activity levels found in the nodules of *Glycine max* strongly suggest a participation of ascorbate in additional functions during symbiosis, possibly related to cell growth and division and to molecular signaling⁷.

So ascorbate in the infected zone of legume plant is primarily involved in the protection of host cells against peroxide damage²⁴. 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 stress²³.

All the supplements, which increased the production of AsA in culture, might have been available within the nodule to the *Rhizobium* sp. of *Cajanus cajan* from the soil and root leachate as and when require. This might stimulate the *Rhizobium* sp. to produce more AsA, helping to promote infection, enhance nodulation and also regulate nodule senescence in legumes. It may be concluded that AsA production is another beneficial aspect of symbiosis like hormone production and nitrogen fixation²⁵.

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