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Accumulation of RNA in the senescing root nodular cells of soybean (*Glycine max* (L.) Merrill) – Histochemical evidence

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Abstract

Accumulation of RNA in the senescing soybean nodules formed by *Bradyrhizobium* as evidenced by histochemical studies is reported in this paper. Using RNA/DNA specific stain and digesting the RNA with RNase enzyme confirmed the accumulation of RNA. Such accumulation of RNA was not found in the young cells of the nodule. Accumulation of RNA was found to have a negative relationship with haemoglobin content, ARA activity and total soluble protein content of the cell. Positive relationship between the age and RNA accumulation was noticed. It appears that the RNA was more likely from the plant cell but not from bacteroids. The accumulation of RNA was found very close to the nucleus of the cell. The volume of accumulated RNA was as large as that of nucleus itself. We report the possible involvement of translation inhibition in the senescing nodular cells in the older nodule tissues. We believe, this is the first report of unusual accumulation of RNA in the symbiotic association.

Key words: RNA accumulation, senescence, soybean, root nodule, histochemical.

Introduction

Accumulation of RNA in a cell is a rare phenomenon. It does not occur under normal circumstances. When the plant is subjected to stress, probably to overcome that stress the plant adopts several mechanisms and one such mechanism is accumulation of RNA[1]. Accumulation of RNA has been reported in sorghum leaves by subjecting the plants to salinity stress[2]. Hermesmeir et al.[3] have reported large scale changes in the defence mechanism of plant during a pest attack and one such mechanism was mRNA accumulation. Similarly Vasquez et al. have studied the changes in mRNA accumulation pattern by subjecting the plants to cold treatment[4]. All these studies point out that the accumulation of RNA is a sort of expression of a cell under stress conditions. It is well known that the nitrogen fixation in a root nodule of a leguminous plant is also a stress to the plant. Eight electrons and 16 ATPs are required for the reduction of N_2 to NH_4 indicating that it is an energy intensive process[5].

It is very well established that root nodulation and N_2 fixation is strain on the plant and causes stress to the plant. Whether there could be accumulation of RNA in the nodular cells also is a matter of concern. But there is no report on this aspect; hence a field study and glasshouse study was initiated with an objective to investigate into the details of RNA accumulation in the soybean root nodule using histochemical techniques. For the study, ten soybean genotypes comprising short and long duration were selected and trials were conducted in three successive seasons.

Materials and Methods

Source of nodules: Ten soybean genotypes namely Monetta, KB-79, KB-92, NRC-2, PK-1125, Bragg, Hardee, MAUS-53-2, MACS-124, KHSb-2; the first five being short duration genotypes requiring less than 100 days for crop maturity and the last five being long duration genotypes requiring more than 100 days for crop maturity were selected. Authentic seeds were procured from the Co-ordinator, All India Co-ordinated Research Project on Soybean, GKVK, Bangalore. The seeds were treated with *Bradyrhizobium japonicum* symbiont culture obtained from Department of Agricultural Microbiology, College of Agriculture, GKVK, Bangalore. The plants were grown in the main field on red loamy soils having a pH of 5.4, low in nitrogen, phosphorus and cation exchange capacity and medium in potassium. The same soils were used for growing plants in a pot in greenhouse. The plants were grown for 6 and 7 weeks in case of short and long duration genotypes respectively for collection of nodules. The root system was uprooted carefully, washed in tap water and processed for further studies.

Classification of nodules: Two types of nodules were collected at the time of collection from roots. The nodules, close to the main tap root in the collar region that were large and bold in size were collected separately and designated as old nodules. The nodules away from the tap root relatively small in size, mostly anchoring the secondary or tertiary roots were collected separately and designated as young nodules. The objective of

collecting nodules separately was to study the comparative changes in older and younger nodules.

Acetylene reduction assay (ARA): Nitrogen fixing ability of older and younger nodules was determined using acetylene reduction assay technique. Amount of C_2H_2 reduced to C_2H_4 is considered as the ability of fixing nitrogen from atmosphere[6] and the same was monitored using a gas chromatograph (NUCON, India). Higher the amount of C_2H_2 reduction, greater is the N_2 fixing ability of the nodules.

Repetition of study: The plants were grown once in the field during Aug – Sept 1996 and the nodules were collected and subjected for examination. Similarly the plants were grown for nodules in a pot culture twice during November – December 1996 and June – July 1997 for confirmation of results using the same field soil.

Histochemical studies: The nodules were washed free of adhering soil particles and transferred to bottles (25 ml capacity, BOROSIL) containing Carnoy's fixative (6 parts ethyl alcohol, 3 parts chloroform and 1 part of glacial acetic acid) for 6 hours at room temperature (30°C). The nodules were dehydrated using series of alcohol (70, 80, 90 and 100%). The nodules were kept in alcohol series for about 24 hours but for 4 hours in absolute alcohol. The nodules were then treated with butanol (25, 50, 75 and 100%, diluted with alcohol) for hardening of tissues. Similarly as in case of alcohol the nodules were retained in butanol for 24 hours in all concentrations. Flakes of paraffin wax (melting point 58 – 60°C) were dropped into 100% butanol and then transferred into oven maintained at 60 ± 2°C for easy infiltration of wax into tissues. The wax infiltration is possible only in presence of butanol hence the wax and butanol is allowed for 48 hours. The wax is changed for 4 times. Subsequent changes are effected every 24 hours. The last change is given by mixing bee's wax at 20% to get a continuous ribbon of sections in microtome. Later the nodules were removed from oven and embedded in wax and these wax embedded blocks were used for microtomy.

Microtome: Rotary microtome (ERMA, Japan) was used to cut thin sections (8 µm) of nodules. The sections were spread on pre-cleaned slides. The sections that represent the centre portion of the nodule were only selected for staining purposes. 6 to 8 sections were spread on each slide and fixed on to slide using gelatine solution (0.1%) as adhesive. The sections were flattened by warming the slides at 45°C, air dried for 72 hours in dust free environment before they were used for staining.

Deparaffinisation: Before staining the nodular sections they were deparaffinised by dipping them in xylene for 10 minutes. Xylene treatment was repeated till traces of paraffin wax from the material was dissolved and removed. Later it was dipped in butanol for stabilisation of tissues. This step is considered as a crucial step since the wax inhibits the entry of stain into the nodular tissue. After deparaffinisation the nodular sections were stained for different parameters as per standard procedures.

Staining for RNA/DNA: The nodular sections were first stained for presence of RNA/DNA structures using methyl green pyronin stain – the specific stain for nucleic acids. A solution of 0.1% methyl green and 0.1% Pyronin G (prepared in distilled water) were prepared separately and then mixed in 1:1 ratio to obtain the stain. The magenta colour inside the nodule represented the RNA and dark blue colour represented the DNA. The nodule sections were observed under light microscope. For reconfirmation of presence of RNA, RNase enzymatic test was also performed. The deparaffinised sections were dipped in RNase enzyme (Sigma, 10 mg per ml) for 45 minutes for complete digestion of RNA. The complete absence of RNA after digestion confirmed the presence of RNA.

Staining for insoluble polysaccharides: The nodular sections were stained for studying the density and distribution of insoluble polysaccharides within the nodule using periodic acid – Schiff's stain. The pink colour dots in the intercellular spaces denote the presence of insoluble polysaccharides.

Staining for proteins: The nodular sections were stained with mercuric bromophenol blue for studying the concentration of soluble protein within the nodule. This was done to signify the nitrogen fixation in cells indirectly.

Results and Discussion

Accumulation of RNA in senescing cells: In the present study the accumulation of RNA has been evidenced. This accumulation has been noticed in the older cells (senescing cells) of a large size nodule probably considered as senescing nodule. This is not accidental because the phenomenon was observed in all the ten genotypes in all the three seasons. Hence it is concluded that it is a regular phenomenon. The accumulation of RNA was confirmed by staining techniques and presented through plates 1 and 2. The presence of RNA was reconfirmed by enzymatic tests. Application of RNAse enzyme over the nodular sections dissolved the RNA completely indicating that the magenta colored structure in the nucleus was none other than RNA. This is further shown in plates 3 and 4. However we have not characterised the RNA and such work is needed to be taken up.

The accumulation of RNA was not a general phenomenon in all cells but it was a phenomenon occurring in the older cells/ senescing cells of an older nodule. Probably this could be a

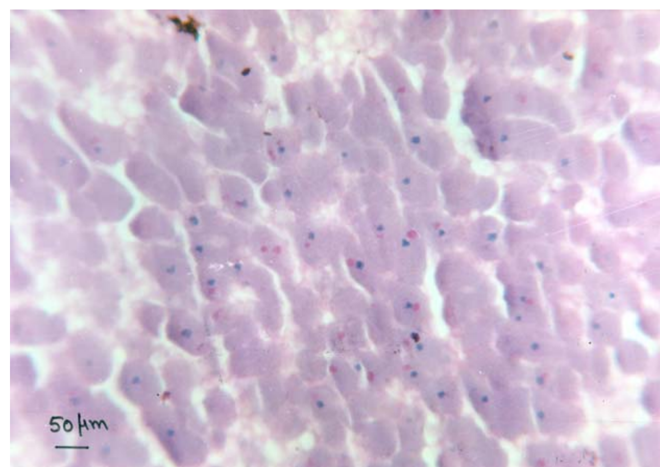

Plate 1: Transverse section of a soybean root nodule indicating RNA accumulation in the center of an old nodule in few senescing cells.
Note: The magenta colour represents the RNA and the deep blue colour DNA. the magnification is 100 x (10x eye piece, 10x objective).

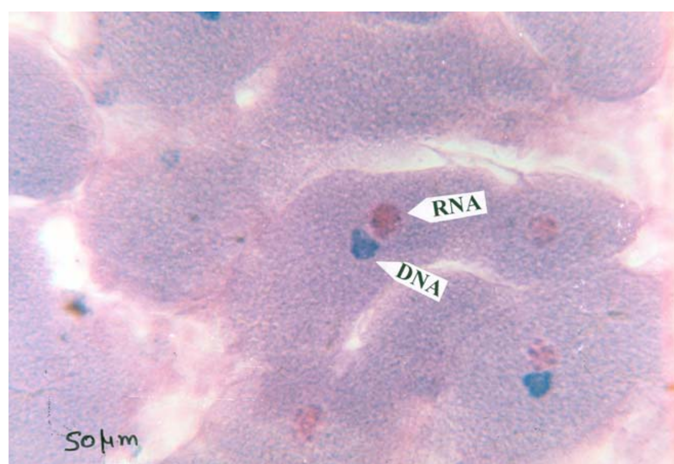

Plate 2: The details of this plate are similar to that of plate 1 except for the magnification. The magnification is 400x(10x eye piece, 410x objective).

phenomenon occurring associated with senescence of cells because the younger cells of the same old nodule did not exhibit the accumulation of RNA. Similarly the cells of younger nodules did not show accumulation of RNA suggesting that RNA accumulation does not take place in younger cells but does accumulate in older or senescing cells of the root nodule. The reasons for accumulation of RNA are still not clear. The stress may be one of the reasons for RNA accumulation[2,3,4]. Discrepancies in phosphorylation[7] or variations in levels of auxins[8] may lead to accumulation of mRNA. Further it is reported that the cytoplasmic control of mRNA expression is as important as transcriptional and nuclear post-transcriptional levels of regulation. Hence the status of mRNA is presumably determined by interactions with special cytoplasmic proteins that regulate its translation. The factors that may inhibit the translation are specific translational repressors; mRNA masking proteins; and proteins that affect mRNA stability[9]. In the senescing nodule any of these factors may be operating suppressing the translation thus leading to accumulation of RNA.

In this study we have noticed the accumulation of RNA in senescing cells. Pla et al.[10] also have noticed the accumulation of RNA in older senescing cells, our observations are in accordance with the published reports. However the reports are not in resonance with the results of Suga et al.[11] because they have reported mRNA accumulation in the hypocotyl and growing tip region of radish (*Raphanus sativus* L.).

Leghaemoglobin content: Leghaemoglobin content in young and old nodules was assessed comparatively in all genotypes by cutting the nodules transversely and exposing them to atmosphere. The results suggested that the cut surface across the full area of young nodules turned pink quickly indicating the presence of leghaemoglobin throughout the nodule. On the other hand the older nodules did not turn pink but most of them were green in colour. This indicates that the older nodules were not having sufficient leghaemoglobin content (Table 1).

Acetylene reduction assay: Older and younger nodules were analysed for N_2 fixing ability using acetylene reduction technique. The results indicated that the younger nodules were more efficient than older nodules in N_2 fixation (Table 2). The differences across genotypes were also significant.

Stress in cells: There could be several types of stress in a cell. We have investigated the stress arising due to supply of reserve

Table 1. Comparative evaluation of young and older nodules for leghaemoglobin content.

Sl. No.	Genotypes	Younger nodules	Older nodules
1.	Monetta	Pink	Brown
2.	KB-79	Pink	Brown
3.	KB-92	Pink	Brown
4.	NRC-2	Pink	Pink
5.	PK-1125	Pink	Pink
6.	Bragg	Pink	Light pink
7.	Hardee	Pink	Light pink
8.	MAUS-53-2	Pink	Light pink
9.	MACS-124	Pink	Light pink
10.	KHSb-2	Pink	Light pink

Pink – The cut surface of nodule turned pink within 4 minutes due to leghaemoglobin
Brown – The cut surface turned brown due to poor leghaemoglobin content
Green – The tissues were green indicating no leghaemoglobin content

Table 2. Acetylene reduction assay (ARA) values of soybean root nodules in older (senescing) and younger (active) nodules of soybean.

Genotypes	ARA values expressed as n moles of C_2H_2 reduced to C_2H_4 per gram nodule weight per hour	
	ARA values of older nodules	ARA values of younger nodules
1. Monetta	5974	7863
2. KB-79	6975	8954
3. KB-92	5979	7836
4. NRC-2	6254	7756
5. PK-1125	8658	7856
6. Bragg	9326	9235
7. Hardee	7209	8754
8. MAUS-53-2	13107	15376
9. MACS-124	11023	13785
10 KHSb-2	6027	7893
SEM±	663	744
CD@ 0.05	1989	2232

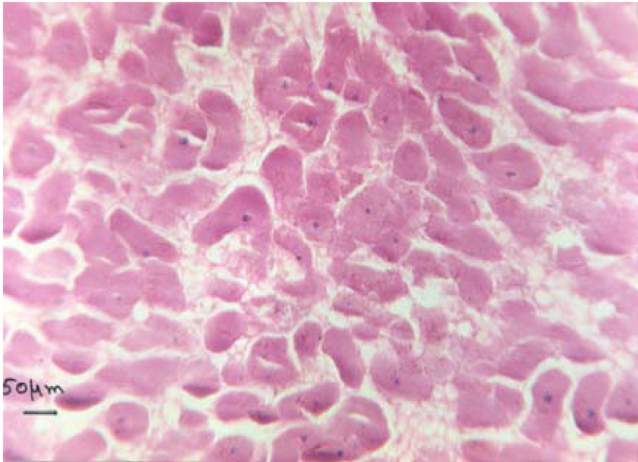

Plate 3: RNase enzyme test: The RNA that was found accumulated in the cell was digested using RNase enzyme. The nodular sections stained after enzyme treatment showed only DNA but not RNA signifying the accumulation of RNA.
Note: Absence of magenta colour indicating digestion of RNA by RNase enzyme. The magnification is 100x (10xeye piece, 10x objective).

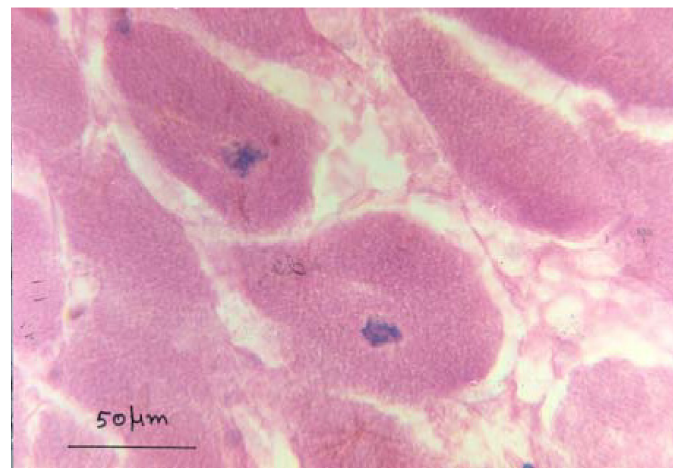

Plate 4: The details of this plate are similar to that of Plate 3 except for magnification. The magnification is 400x (10x eye piece, 40x objective)

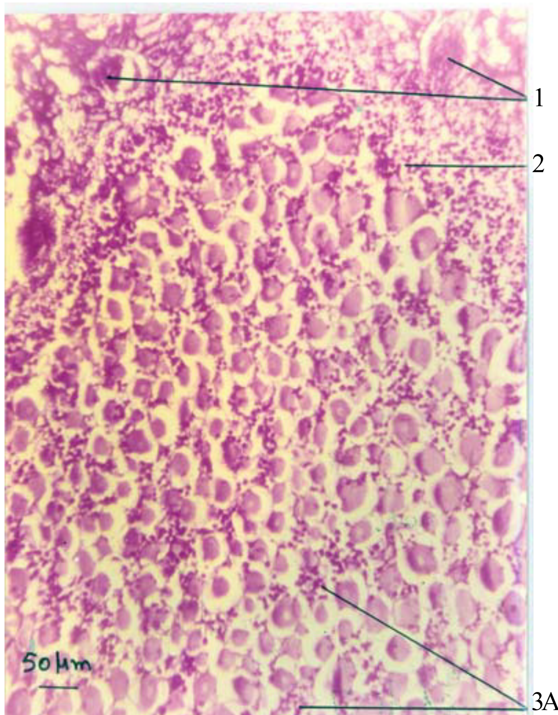

Plate 5- Young nodule
Density and distribution of insoluble polysaccharide graniles (photosynthates as visualised by PAS stain in a soybean root nodule. Note: Pink colour dots in intercellular spaces indicate the polysaccharide granules. The magnification is 100x (10x eye piece, 10x objective).

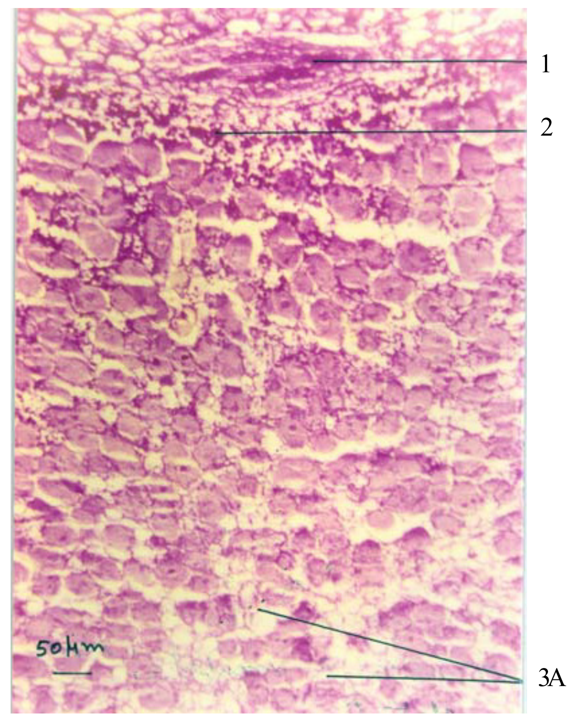

Plate 6 - Old (senescing) nodule
The details of this plate are similar to that of the Plate 5 except for senescing nodule.
Note: The sparse distribution of polysacharide granules in an older nodule. The magnification is 100x (10x eye piece, 10 x objective).

1. Vascular bundles; 2.High density of polysaccharides; 3A. Even distribution of polysaccharides; 3B. Uneven distribution of polysaccharides.

food in the nodule. The studies on distribution of photosynthates (reserve food material of the nodule) suggested that the older cells of the older nodules were devoid of photosynthates. The distribution of photosynthates in both young and older nodules is presented in Plates 5 and 6 respectively. The younger cells of the same older nodule were having ample supplies of photosynthates indicating that there is some type of auto regulation or a sort of competition for photosynthates between younger and older cells. The photosynthates supply was more or less uniform throughout the nodule in younger nodules without any competition between young or old cells suggesting that there was no stress in a younger nodule. The large distance between the vascular bundles and the older cells located in the centre may be another reason for no or less supply of photosynthates resulting in the stress for older cells.

This type of stress in senescing (older) cells of an older nodule

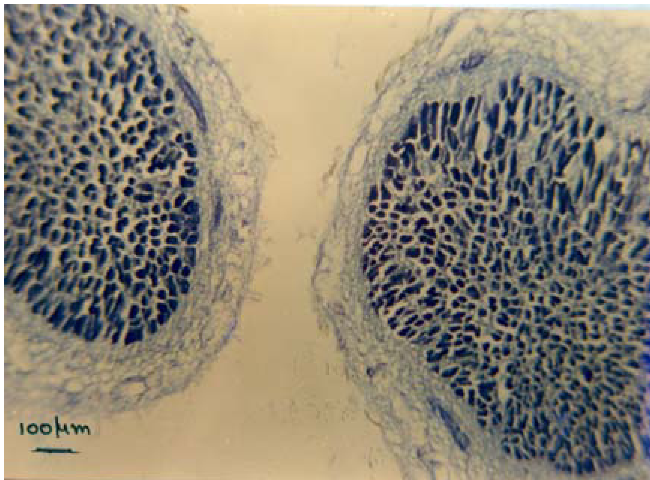

Plate 7 - Young nodule
Protein concentration as stained by mercuric bromophenol blue in a root nodule of soybean. Note:Dark staining indicates higher protein concentration. The magnification is 100x (10x eye piece, 10x objective).

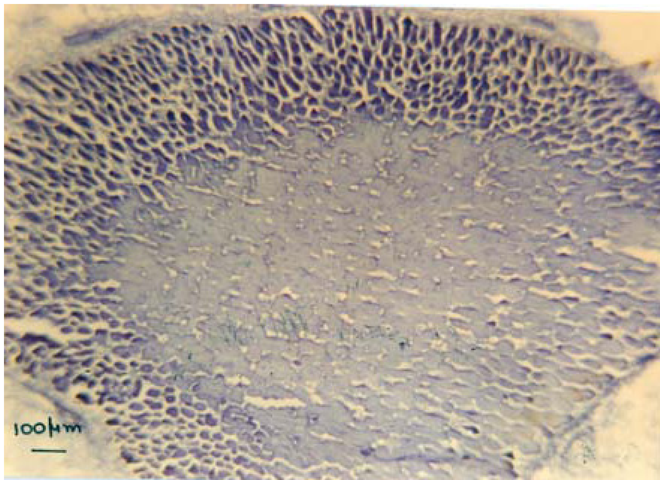

Plate 8 - Old (senescing) nodule
The details of this plate are similar to that of Plate 7 except for senescing nodule. Note: The light stain in older nodule showing lower protein concentration in an older nodule. the magnification in an older nodule. the magnification is 100x (10x eye piece, 10x objective).

has resulted in low efficiency of nitrogen fixation compared to younger cells. Leghemoglobin content test has proved this fact. As a consequence of this, the older cells have shown very low soluble protein concentration. On the contrary the younger nodule has shown very high concentration of protein suggesting efficient nitrogen fixation. Even the younger cells located on the peripheral region of the older nodule showed high protein concentration signifying efficiency in nitrogen fixation. These findings are depicted in Plates 7 and 8. The higher intensity of colour indicates higher protein content and vice versa.

Metabolic activity of cells was also evaluated using toluidine blue stain. It was found that the older cells of an older nodule were metabolically less active compared to the younger cells of the same older nodule. The younger nodule exhibited higher metabolic activity throughout the nodule. Based on these studies we conclude that older cells have less metabolic activity, hence they fix lower amount of atmospheric nitrogen. These observations are further supported by ARA values given in Table 2. The accumulation of RNA is further believed to come from the plant genes only and not from bacteroids since the accumulation is occurring just close to the nucleus. Further the bacteroids are distributed inside the nodular plant cell enclosed within a peribacteroid membrane[12] consisting of leghaemoglobin where heme portion is synthesised by the bacteroids interacting with plant genes. These genes are expressed only when they are in symbiotic association. Hence we believe the RNA is from plant source.

Hypothesis: Based on the above observations from this study we believe that this is the first report on RNA accumulation in the senescing nodular cells of soybean. We propose that accumulation of RNA is not an accidental phenomenon occurring in the senescing cells but a regular phenomenon. One of the major reasons (probably in addition to several other factors which we have not studied) is non-availability of food reserves (as photosynthates) to these senescing cells. As a result these cells are deprived of energy. The observations from this study clearly indicate that process of transcription was successful but onward process of translation was inhibited resulting in the accumulation of RNA. This translation is an energy intensive process. Since the food reserve was in short supplies thus the cells were not able to meet this process of translation successfully. The non-availability of food reserves to the senescing cells could be due to mechanical barrier (crossing several layers of cell) or it could be auto regulation mechanism of the nodule to deprive the senescing nodules and selectively supplying the most efficient N_2 fixing younger cells of the same nodule. Either one of them or both of them could be operating, but it is not clear to us. We believe that there could be a signalling mechanism between the plant cell and the microsymbiont for ceasing of N_2 fixation. Such a mechanism is already reported in blue green algae (BG algae) by Golden et al. [13]. In BG algae the heterocysts are the specialised structures for N_2 fixation and they receive photosynthates from the adjacent chain of vegetative cells. When the distance between the heterocyst and the photosynthates supplying vegetative cells is too long, the algal filament induces signal for one more centre cell to become heterocyst. Thus there is reason to believe such a mechanism may be operating in the root nodule also.

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