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TEM STUDIES OF NODULES ELICITED BY *SINORHIZOBIUM MELILOTI* PLEIOTROPIC MUTANT DEFECTIVE IN EXOPOLYSACCHARIDE SYNTHESIS

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Abstract: **Five purine auxotrophic mutants of *Sinorhizobium meliloti* strain Rmd 201(WT) were isolated using NTG mutagenesis. Mutants elicited white, small, spherical, determinate, and Fix$^-$ pseudonodules on *Medicago sativa*. These nodules lacked bacteroids and one mutant Rmd 1102(A_2) exhibited Exo$^-$ phenotype due to production of defective exopolysaccharide, particularly lacking xylose sugar. Cross feeding with xylose as sole carbon source in presence of parental strain restored the wild-type phenotype. Light microscopy revealed aborted infection tubes, bacteroid-devoid cortical cells, and bacterial lysis. Transmission electron microscopy showed that the mutant Rmd 1102 Exo$^-$ Pur$^-$ formed infection tubes but bacteria lysed within infection tube and could not transform into bacteroids. The mutant also accumulated large sized starch granules, suggesting a defect in starch utilization. Abundant lipid droplets and absence of PHB granules was noted in mutant cells. This pleiotropic mutant exhibited Exo$^-$ and Fix$^-$ phenotypes, affecting purine biosynthesis and exopolysaccharide production, disrupting normal nodule development and bacteroid production.**

Keywords: ***Sinorhizobium meliloti*****, NTG mutagenesis, Exopolysaccharide, Purine auxotrophic mutants, *Medicago sativa* nodules.**

1. INTRODUCTION

Rhizobium-legume symbiosis is a crucial process for nitrogen fixation. Leguminous plants release flavonoids, attracting rhizobia from the soil. Flavonoids activate Nod proteins, triggering rhizobial nodulation genes to produce Nod factors. These factors bind to plant receptors, causing root hairs to curl and trap rhizobia. Infection threads are formed as rhizobia enter the roots. Inside root hairs, they stimulate root cortex cell division and nodule primordia formation. Rhizobia are released into symbiosomes within nodules, differentiating into bacteroids. Bacteroids convert atmospheric nitrogen into ammonia, a vital nutrient for the plants growing in nitrogen deficient soils. In return, the plant provides carbon and energy sources, fostering rhizobial growth within root nodules, ensuring a mutual symbiotic relationship. *Sinorhizobium meliloti* forms indeterminate nodules on *Medicago sativa*, characterized by a persistent meristem that remains active throughout nodule development (Concha and Doerner 2020). Within these nodules, newer plant cells progressively become infected by rhizobia. Mature nodules contain several distinct zones. They include the apical nodule meristem, invasion zone, nitrogen-fixing zone, senescence and saprophytic zone (Dart 1977). In the invasion zone, bacteria are released from infection threads, while in the nitrogen-fixing zone, bacteria that have transformed into bacteroids actively fix nitrogen

(Pérez Guerra, Coussens et al. 2010). Importantly, bacteroids formed are terminally differentiated and cannot revert to viable bacteria. Importantly, bacteroids formed are terminally differentiated and cannot revert to viable bacteria. Both determinate and indeterminate nodules exhibit changes in gene expression and metabolism during this process (Mergaert, Kereszt and Kondorosi 2020).

Many researchers have observed symbiotic defect in laboratory experiments involving various rhizobial strains and host plants, particularly in the context of rhizobium purine auxotrophs. Ultrastructural studies of other purine auxotrophs isolated in our laboratory clearly exhibited infection tube in intercellular regions of prefixation zone. Infection tube did not advance to cortical cells and lysed. Bacteria lysed either before release or after release but could not transform into bacteroids. In *S. meliloti* and alfalfa symbiotic studies several researchers have documented that purine auxotrophy specifically effects the infection process, resulting in formation of ineffective nodules. These nodules induced by purine auxotrophs of *S. meliloti* do not perform nitrogen fixation, rendering them ineffective for their intended purpose (Dickstein, Scheirer et al. 1991); (Kerppola and Kahn 1988), (Scherrer and Dénarié 1971); (Swamynathan and Singh 1992); (Djordjevic, Ridge et al. 1988); (Kim, Okuda et al. 1999); (Newman, Diebold et al. 1994); (Vincent 1980). Although, these nodules can form normal infection threads (Swamynathan and Singh 1992), they fail to successfully infect host plant cells (Dickstein, Scheirer et al. 1991). A similar defect is noted in *Sinorhizobium meliloti* Pur^- mutants, which induce empty, ineffective nodules on alfalfa (Davies and Walker 2007). These mutants are defective in lipopolysaccharide production. Further, it has been demonstrated that pyrimidine auxotrophs of *Sinorhizobium meliloti* Rmd201 generated by Tn5 random mutagenesis also induce symbiotically ineffective nodules (Vineetha, Vij et al. 2001). This clearly indicates the importance of pyrimidine/purine biosynthetic pathway or their intermediates in bacteroid transformation and nodule development. Additionally, rhizobium purine auxotrophs, specifically strain ANU280 (a derivative of NGR234), can induce fully developed nodules on clover hosts, but these nodules are Fix^- and exhibit symbiotic defects (Djordjevic, Ridge et al. 1988). Notably, the above defects cannot be complemented by adding purine/pyrimidine or their intermediates to the growth medium, and the auxotrophic mutants experience multiple adverse effects at the level of protein synthesis and overall metabolism. In conclusion, it is evident that purine/pyrimidine intermediates or by products of their biosynthetic pathway play a significant role for establishment of effective symbiosis between rhizobial strains and their host plants.

In present study, several purine auxotrophs were isolated using NTG mutagenesis. Interestingly one purine auxotroph Rmd 1102(A_2) appeared to be pleiotropic mutant because it exhibited Exo^- Pur^- phenotype and was forming symbiotically defective, small, white and determinate pseudonodules that lacked detectable nitrogenase activity on *Medicago sativa* (Swamynathan and Singh 1995). Rmd 1102(A_2) elicited normal infection threads, shepherd crook formation, normal infection but nodules could not fix nitrogen. Prototrophic revertants showed restoration of symbiotic ability and mutation of Rmd 1102(A_2) was a point mutation (Swamynathan and Singh 1992). Rmd 1102(A_2) showed a block at the step of conversion of AICAR (amino imidazole carboxamide ribonucleotide) to IMP (inosine monophosphate) in purine biosynthetic pathway (Fig. 1). This suggests that point mutation in Rmd 1102(A_2) is either at the step of conversion of AICAR (amino imidazole carboxamide ribonucleotide) to FAICAR (5-formamido-4-imidazole carboxamide ribonucleotide) or conversion of FAICAR (5-formamido-4-imidazole carboxamide ribonucleotide) to IMP (inosine monophosphate). This indicates that point mutation could be in PurH, PurJ, PurP or Pur0 genes or some other gene affecting these genes (Zhang, Morar and Ealick 2008). However point mutation in Rmd 1102(A_2) is a pleiotropic mutation that is simultaneously effecting exopolysaccharide synthesis, purine biosynthesis and forming ineffective determinate nodules. External supplementation of plant growth medium with AICAR or IMP at the time of inoculation of the seedlings with bacterial strains did not cause the formation of nitrogen fixing nodules. Mutant bacteria could be obtained from nodules of 30-day-old seedlings. Detailed analysis of wild type strain Rmd 201 and mutant Rmd 1102(A_2) Pur^- Exo^- was carried out using both light and electron microscopic studies in order to detect the exact step of block during nodule organogenesis.

2. MATERIALS AND METHODS

Bacterial strains: The wild type strain Rmd201 is a streptomycin resistant derivative of AK 631 which forms effective indeterminate nodules on host *Medicago sativa* and the mutant Rmd 1102(A_2) formed pseudonodules which are not able to fix nitrogen. Rmd 201(WT) can grow well on minimal media. The purine auxotroph Rmd 1102(A_2) was isolated using NTG mutagenesis and could grow on minimal media only when supplemented with adenine.

Media and culture conditions: Complete medium (MSY) and minimal medium (RMM) for Rhizobium were used (Vincent 1980).

Light and transmission electron microscopy: Dipped freshly harvested nodules in Karnovsky's fixative for 3-5 hrs at room temperature. Rinsed the nodules with 0.1M phosphate buffer twice and stored overnight at 4°C. Post-fixation was done by treating with 2% OsO_4 in 1M phosphate buffer for 10 min. After passing through the graded series of alcohol (70%-100%), nodules were embedded in Epon Araldite mixture (Mollenhauer 1964).

3. RESULTS

Microscopic examination of infection process: Normal infection thread formation and root hair bending was seen in both Rmd 201(WT) and Rmd 1102(A_2) exopolysaccharide defective purine mutant (Fig. 2). Rmd 201(WT) induced normal indeterminate nodules on roots of *Medicago sativa* and Rmd 1102(A_2) induced determinate pseudonodules (Fig. 3).

Light microscopy studies of nodules induced by WT strain and mutant strain:

Light microscopic studies of transverse sections of nodules of Rmd 201(WT) clearly exhibited bacteroids (B), prefixation zone (P_Z), meristematic zone (MZ), interzone (IZ), and nitrogen fixing zone (NiZ). Empty cells (Ec) surrounding the filled cells can also be seen (Fig. 4). Light microscopic studies of transverse sections of nodules of Rmd 1102(A_2) mutant showed infection tube (It) bordering the prefixation zone (Fig. 5). Empty cells of interzone are filled with starch granules.

TEM studies of nodules induced by WT strain and mutant strain:

Transverse sections of nodules elicited by wild type Rmd 201(WT) revealed many bacteroids in theinterzone region. There is branching of infection tube (Fig. 6) and infection tubes proceed in cells through middle lamella in between cell walls.

Infected cells adjoining empty cells showed starch granules near cell wall that were either electron dense or showed unstained texture. In nitrogen fixing zone some cells showed freshly released rhizobia but many cells have active bacteroids (Fig. 6). Young bacteroids showed peribacteroid membrane. When the bacteria had transformed into bacteroids, no PHB granules could be observed. All organelles in cytoplasm were in active stage. Cytoplasm showed ribosomes,small lipid droplets and enlarged nucleus with irregular shapes.

Transverse sections of nodules elicited by Rmd 1102(A_2) showed infection tube in intercellularspace of prefixation zone. Total number of infection tubes in mutant were much less as compared to those in wild type strain. Infection tube shows tube matrix with lysed bacteria (**). Infection tube was unable to release bacteria. The mitochondria shows enlarged cristae. Nuclei were enlarged. Many vesicles were seen around cell wall. Endoplasmic reticulum was short and fewer in number. No nitrogen-fixing zone could be observed and no released bacteria were present. Bacteria did not transform into bacteroids. Observed starch granules (S) that appeared to have originated from nearly degenerated amyloplasts in prefixation zone. Infection tube did not release the bacteria and bacteria had degenerated (Fig. 7).

4. DISCUSSION

The present study involves a pleiotropic purine mutant strain, Rmd 1102(A_2), exhibiting Exo^- Pur^- phenotype and eliciting ineffective nodules on host *Medicago sativa*. Other purine auxotrophs isolated in our laboratory TEM studies clearly exhibited infection tube in intercellular regions of pre-fixation zone. Infection tube did not advance to cortical cells and lysed. Bacteria lysed either before release or after release but couldnot transform into bacteroids. This advocates that purine auxotrophy is related to ineffective nodule formation. Notably, spontaneous revertants exhibit a phenotype similar to the wild type strain Rmd 201, implying a single gene mutation with pleiotropic effects (Vincent 1980). In *Sinorhizobium meliloti*, the purine pathway is intricately linked to exopolysaccharide synthesis and both appear to be prerequisite for effective symbiosis to be established though the molecular pathway linking both is still not understood. Exopolysaccharides (EPS) are vital components for the successful establishment of an effective symbiosis between alfalfa and rhizobia. Rhizobial mutants producing defective EPS are unable to elicit effective nodules on the host plants (Castellani, Luchetti et al. 2021). Impairment in nucleotide sugar diphosphate production can lead to defective exopolysaccharides. Rmd 1102(A_2) purine mutation disrupts this pathway and potentially effects exopolysaccharide synthesis. While different exopolysaccharides can compensate for each other, this mutation seems to involve a distinct exopolysaccharide, as it is not complemented by genes for EPS I synthesis. This clearly proves purine mutation and defective exopolysaccharide production in mutant Rmd 1102(A_2) impaired development of nitrogen fixing nodules. Altered nodules were devoid of bacteroids and unable to fix nitrogen. Further research, including double mutant studies, is needed to confirm this distinction. *Sinorhizobium meliloti* likely produces multiple exopolysaccharides, possibly due to co-evolution with various legume hosts, suggesting the presence of several exopolysaccharides in this bacterium.

During interaction between plants and invading bacteria, it was noted that when bacteria enter root cells, they trigger the cells to transform into meristematic cells, which then undergo differentiation into specific zones, ultimately forming nodule structures (Dart 1977). In this process, the bacteria themselves undergo a transformation into nitrogen-fixing bacteroids. This transformation involves the expression of a plant protein and the synthesis of heme by bacteria, leading to the formation of leghemoglobin, a crucial component for efficient nitrogenase function. In *Sinorhizobium meliloti*, defective mutants display one of two primary traits: either they cannot initiate nodule formation (Nod^-) or they create nodules incapable of efficient nitrogen fixation (Fix^-). Genetic characterization of these Fix^- mutants has revealed that their mutations are located on the pSym mega plasmid (Zimmerman, Szeto and Ausubel 1983), a large genetic element of over 700 kb (Rosenberg, *et. al.* 1981), carries both nod and fix genes. Interestingly, the expression of these nod and fix genes depends on the host plant. Typically, the blockage of Fix^- nodules occurs when bacteria are released (Dart 1977); (Vincent 1980); (Truchet, Barker et al. 1989) from the infection thread stage or when leghemoglobin and bacterial nitrogenase (Zimmerman, Szeto and Ausubel 1983) reach normal levels.

Two types of bacterial infection thread advancement processes can been observed: one involves infection thread penetration through the middle lamella of the cell wall, leading to infection tube formation, while the other entails direct penetration of root epidermal cell walls by the bacterium, by passing through root hairs. In some cases, infection threads are not essential for cell proliferation (Finan, Hirsch et al. 1985) and nodule development.

In nodules infected with wild type bacteria, no amyloplasts were present in populated cells butstarch granules were bordering populated cells (Fig. 6) and its metabolites pass through plasmodesmata into populated cells. The role of starch is to supply energy rich material for maintenance of nodule metabolism. In wild type, nodule transverse sections such starch was not found in nitrogen fixing zone and inter zone indicating its full utilization to some extent. In Rmd 1102(A_2) almost all starch grains remained unutilized (Fig. 7). The differential degree of starch utilization in the mutant cells is either due to lysis of mutant cells or their utilization to some extent to repress host immune response. Amyloplasts of nodular origin were present in mutant cells but lacking in wild type cells. Mitochondria were seen in both wild type and mutant cells but in the mutant cells, they were elongated.

Lipid droplets were found in abundance in Exo^- mutant cells of Rmd1102(A_2) in prefixation zone (Fig. 8). In wild type cells, lipid droplets were observed to some extent in prefixation zone and nitrogen fixing zone. Utilization of lipids is essential for another very important pathway in bacteria to generate reserve energy material in the form of PHB granules. In wild type PHB granules supplied material for peribacteroid membrane of the bacteroids and the functional bacteria utilized starch. The extra energy is stored in form of lipid droplets. Exo^- mutant, lacked PHB granules and bacteria lysed, so lipid droplets remained unutilized. In Exo^- mutant modified form of exopolysaccharide might have given signals not to generate lipid droplets by the host. Overall, the mutant exhibits alterations in infection thread development specially after advancement and gets lysed. Bacteria lyse without release and bacteroids are absent. Starch production and utilization, as well as the absence of lipid droplets is also noted. Rmd 1102(A_2) also displays differences in bacteroid differentiation, with nuclear activity remaining mostly normal but concentrated near the cell wall.

5. CONCLUSION

The study focuses on the purine mutant strain Rmd 1102(A_2) in *Sinorhizobium meliloti*, which exhibits an Exo^- Pur^- phenotype and elicits ineffective nodules. The mutation disrupts the purine pathway and modifies exopolysaccharide synthesis critical for effective nodule formation. Different exopolysaccharides can compensate for each other, but this mutation appears to involve a distinct exopolysaccharide not complemented by EPS I genes. In the interaction between bacteria and plant roots, Fix^- mutants with mutations on the pSym mega plasmid are unable to initiate efficient nodule formation due to variations in gene expression depending on the host plant. TEM studies clearly indicate that in mutant Rmd 1102(A_2) infection tube gets lysed without bacterial release and totally devoid of bacteroids. In addition, there is a differential degree of starch utilization, possibly due to mutant cell lysis or utilization to repress host immune responses. Mitochondria showed elongation in the mutant cells. Lipid droplets are abundant in the Exo^- mutant, indicating a lack of PHB granules and bacterial lysis. This suggests that modified exopolysaccharide in the mutant may influence host signals and energy utilization. Additionally, the mutant showed altered starch utilization, differences in bacteroid differentiation, and the absence of PHB granules, suggesting that mutation altered intricate process of nodule formation in *Sinorhizobium meliloti* and simultaneously affected many pathways.

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ABBREVIATION

NTG	N-methyl-N'-nitro-N-nitrosoguanidine
Rmd	*Sinorhizobium meliloti* strain Rmd 201
Exo-	Exopolysaccharide-deficient phenotype
Fix-	Nitrogen fixation-deficient phenotype
A2:	Mutant strain designation
TEM-	Transmission electron microscopy
Pur-	Purine auxotrophic phenotype
Nod	Nodulation
Nod factors	Signaling molecules produced by rhizobia in response to flavonoids
Nod proteins	Proteins activated by flavonoids to trigger rhizobial nodulation genes
S. meliloti	Sinorhizobium meliloti
Tn5 random Mutagenesis	Transposon 5 random mutagenesis
ANU280	A specific strain, a derivative of NGR234
NGR234	Another strain of rhizobium
AICAR	Amino Imidazole Carboxamide Ribonucleotide
IMP	Inosine Monophosphate
FAICAR	5-Formamido-4-Imidazole Carboxamide Ribonucleotide
PurH, PurJ, PurP, PurO	Genes involved in the purine biosynthetic pathway
AK 631	Original strain designation
MSY	Modified Schenk and Hildebrandt medium
RMM	Rhizobium Minimal Medium
OsO4	Osmium tetroxide, a chemical used in electron microscopy
Ec	Empty cells
B	Bacteroids
Pz	Prefixation zone
MZ	Meristematic zone
IZ	Interzone
NiZ	Nitrogen fixing zone
Nod^-	Nodule initiation-deficient phenotype
Fix^-	Nitrogen fixation-deficient phenotype
PHB granules	Polyhydroxybutyrate granules, a storage form of carbon and energy in bacteria
EPS I genes	Exopolysaccharide I genes
pSym mega plasmid	Symbiotic mega plasmid

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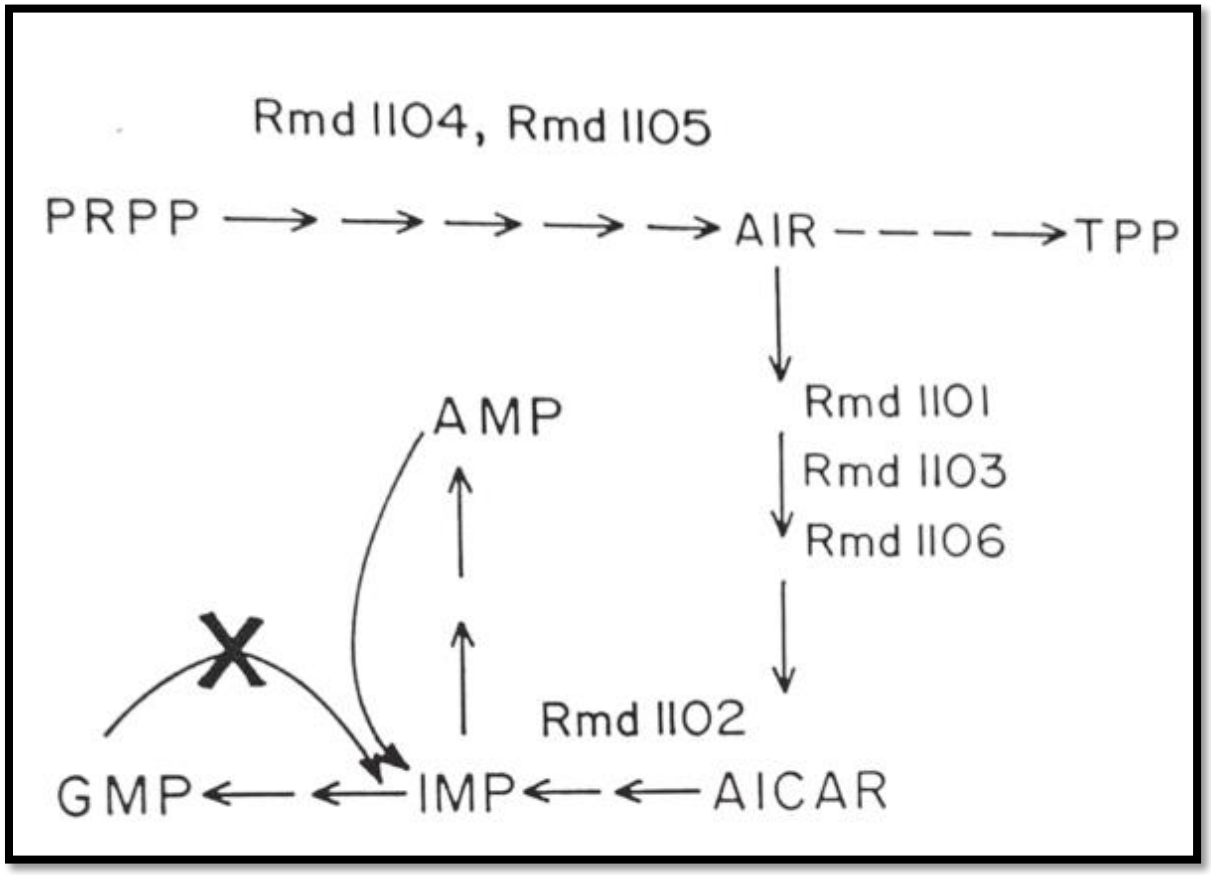

Fig. 1. Schematic diagram representing *de novo* purine biosynthetic pathway in *Sinorhizobium meliloti* along with relevant intermediates. Rmd 1102 (A_2) Exo^- Pur^- exhibits a block at the step of conversion of AICAR to IMP. PRPP- phosphor ribosyl pyrophosphate, AIR- amino imidazole riboside, AICAR- amino imidazole carboxamide riboside, IMP- inosine mono phosphate, AMP- adenosine mono phosphate, GMP- guanosine mono phosphate. Note GMP is not converted to IMP in *Sinorhizobium meliloti.*

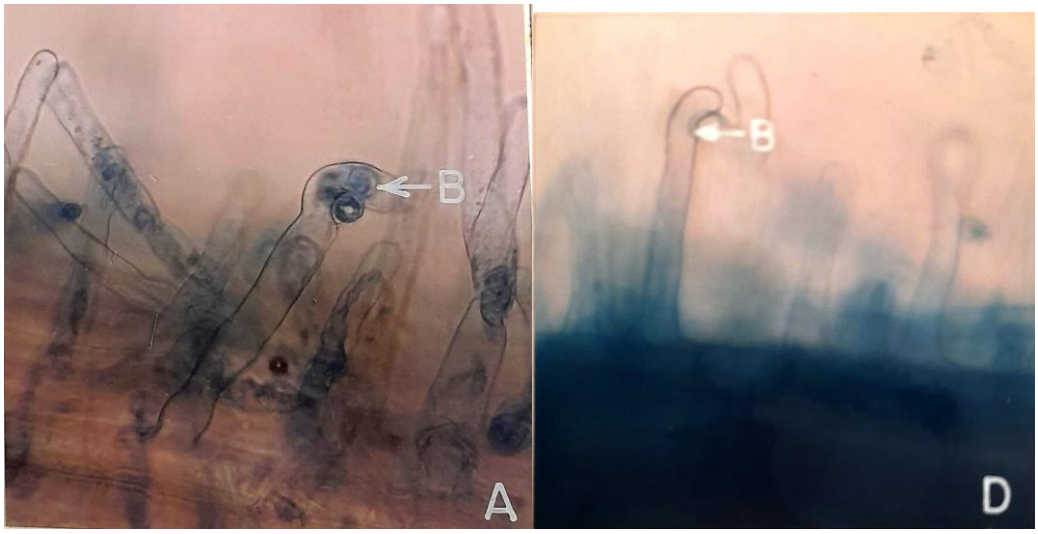

Fig. 2. (A) Normal infection thread formation and root hair bending (labelled B with an arrow) elicited by Rmd 201(WT) cells. **(D)** Normal infection thread formation and root hair bending (labelled B with an arrow) elicited by mutant Rmd1102(A_2).

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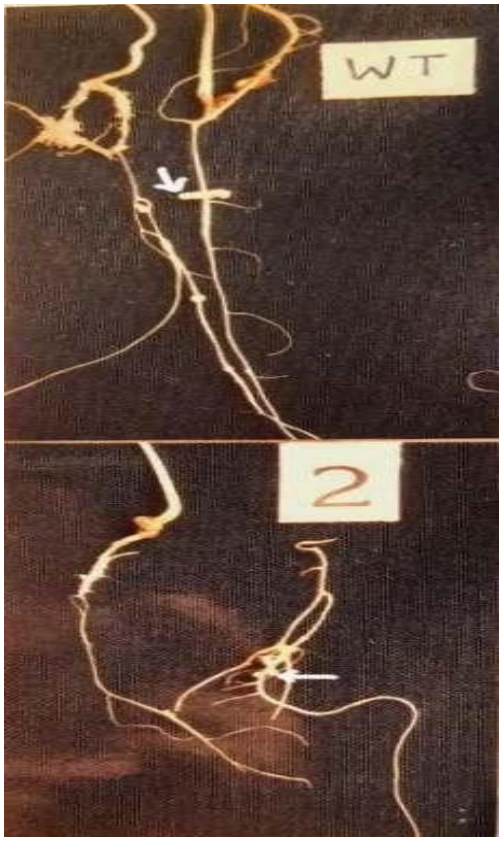

Figure 3: Label WT depicts morphology of indeterminate nodules elicited by WT strain *Rmd* 201. Label 2 depicts small and determinate nodules elicited by *Rmd* 1102 mutant Pur⁻and Exo⁻.

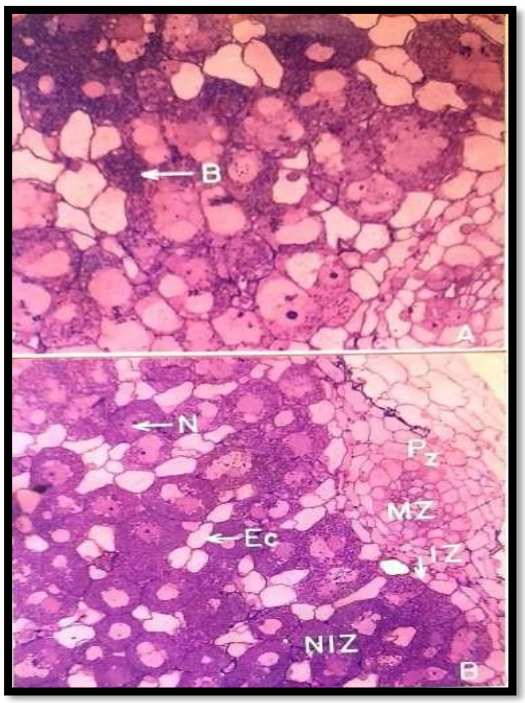

Figure 4: Light microscopy of semi-thin sections of *Rmd* 201(WT) nodules. The above figureshows bacteroides (B), prefixation zone (P_Z), meristematic Zone (MZ), interzone (IZ) , nitrogenfixing Zone (NiZ), enlarged nucleus (N), and Empty Cells (Ec). x1750.

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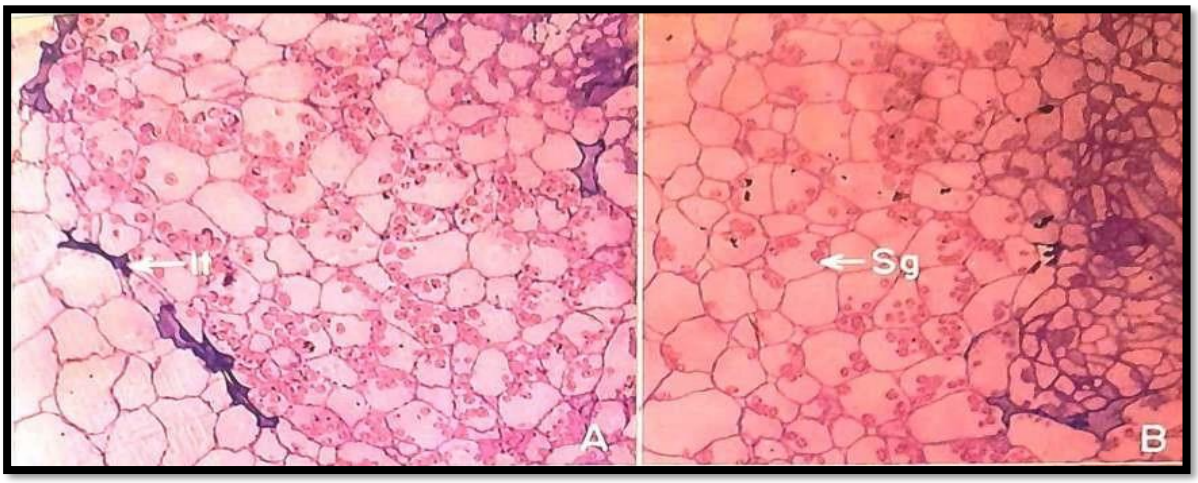

Figure 5: Light microscopic studies of semi- thin transverse sections of nodules of *Rmd* 1102(A 2) mutant. The above figure shows infection tube (It) bordering the pre-fixation zone in the mutant. Empty cells of interzone are filled with starch granules. x1750

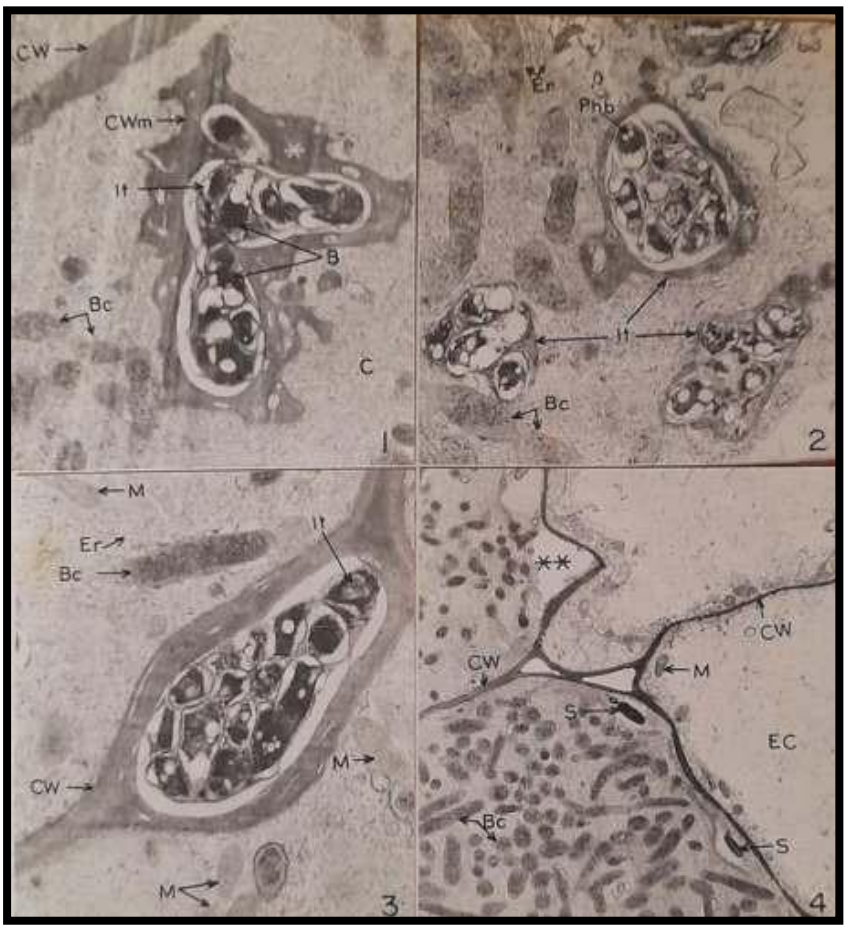

Figure 6: TEM studies of ultra-thin transverse sections of *Rmd* 201 (WT) nodules. In the abovefigure, label 1 and label 2, show Infection thread (It) with branching and bacteroides (Bc), cellwall (CW), cytoplasm (C), cell wall matrix (CWm), bacteria (B) within Infection tube (It), endoplasmic reticulum (Er), and poly hydroxy butyric acid granules (Phb). The cell wall material can be seen around infection tubes (*). (1) x 5,873 and (2) x 7,308. Label 3 reveals infectiontube proceeding in the cell through middle lamella. Endoplasmic reticulum (Er), mitochondria(M), and cell wall (CW). x 13,050. Label 4 depicts empty cells (Ec) in vicinity of cells filled with bacteroides (Bc), cell wall (CW), bacteroides (Bc), mitochondria (M), and starch granules (S) in populated cells. Intercellular spaces can be seen near Empty Cells (EC) and filled cells (**).x 5,090.

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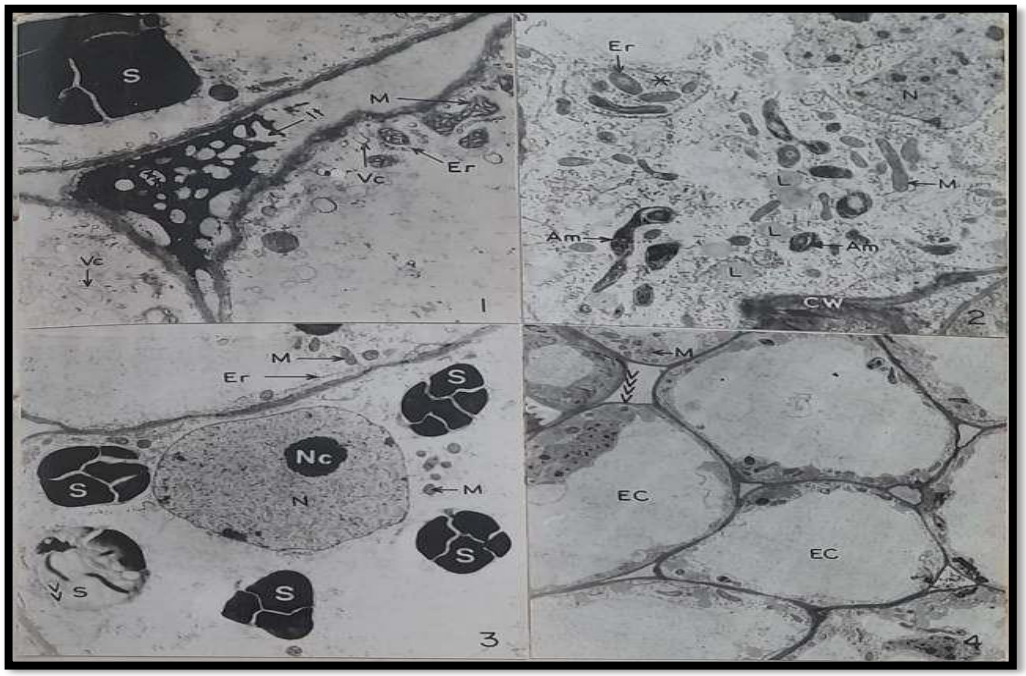

Figure 7: TEM studies of ultra-thin sections of *Rmd* 1102 (A 2) Pur$^-$ and Exo$^-$ mutant. Label 1depicts Infection tube (It) seen in intercellular space of prefixation zone. Infection tube showstube matrix with lysed bacteria (**). The mitochondria (M) shows enlarged cristae. Starch granule (S) has originated from nearly degenerated amyloplast in prefixation zone. x 13,050. Label 2 depicts, Infection tube could not release the bacteria they had degenerated. It showsabundant lipid droplets (L), Mitochondria (M), and distorted amyloplast (Am). Endoplasmic reticulum (Er) encircling some amyloplast can also be seen (*). x 16,313. Label 3 depicts abundance of starch granules (S) seen in the cells of prefixation zone. The starch granules are electron dense showing their metabolization. The granule (<<) shows initiation of metabolization. x 13,050. Label 4 shows that most cells of prefixation zone are empty cells (EC). Empty Cells also show intercellular space (<< <<). x 4,307.

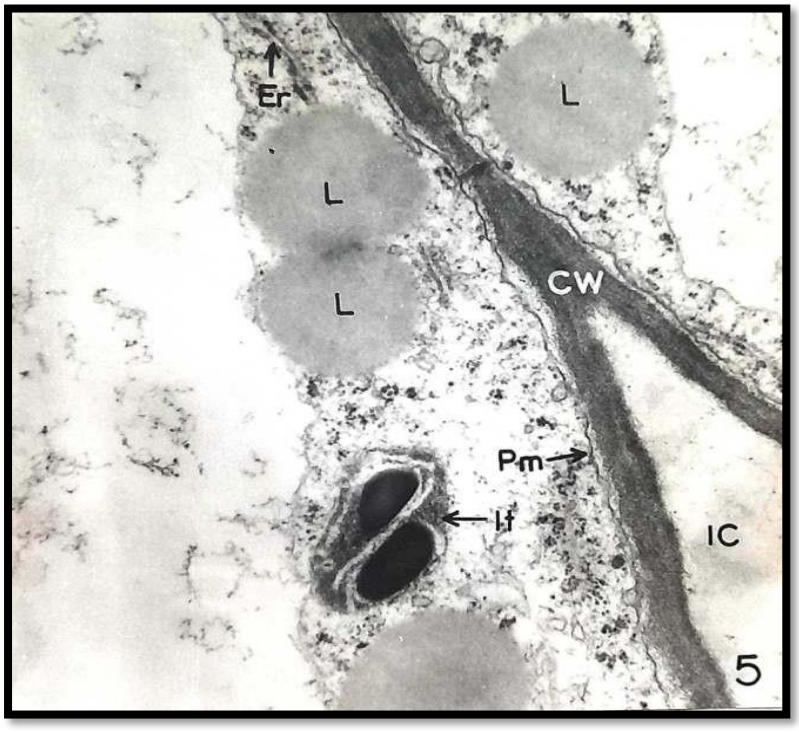

Figure 8: TEM studies of ultra-thin sections of *Rmd* 1102 (A 2) Pur$^-$ and Exo$^-$ mutant. Label 5 shows abundant Lipid droplets (L) and an infection tube (It) lysing without internal material of cell wall and less dense granular matrix. x 35,235.

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