

# Ultrastructural Studies on Root Nodules of *Pithecellobium dulce* (Roxb.) Benth. (Fabaceae)

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## Summary

Ultrastructural studies were conducted on *Pithecellobium dulce* (Roxb) Benth. root nodules collected from trees growing under natural conditions. Rhizobial infection on root surface of *P. dulce* started with curling of root hair. Both curled and straight root hairs were observed. The internal structure of a mature nodule showed an epidermis, cortex, vascular region and a bacteroid region. Vascular bundles were amphicribal. A distinct periderm consisted of sclereid tissue could be observed in the cortex outside the vascular tissue. The bacteroid region contained infected and un-infected (interstitial) cells intermingled with each other. Infected cells of developing nodules as well as of mature nodules were vacuolated. Infection threads were also observed in the bacteroid zone. The rhizobia were released from the infection thread into the host cytoplasm form rhizobial (unwalled) droplets. Rhizobia were also observed in the intercellular spaces between infected cells. Starch grains were observed in the interstitial cells.

## Key words

root nodule, *Pithecellobium dulce*, ultrastructural studies

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## Introduction

*Pithecellobium dulce* is a mimosoid tree legume that grows naturally in Pakistani soils (Ali, 1973a). The tree is used extensively as a shade or shelterbelt tree with a great tolerance of arid and harsh sites. Its pods locally known as Jungle jalebi are eaten as fruit. It coppices readily and can be managed as a hedge. *P. dulce* is noted for their tolerance of heat, drought, salinity and impoverished soils. *P. dulce* forms nodules with rhizobia. Nodulation is common in all types of soils, but quantitative data on fixation has not been reported.

The indigenous woody legumes and their root nodule bacteria play an important role in the overall nitrogen increment of Pakistani soils (Mahmood, 1999). The old classification of nodule-forming genera in the family *Rhizobiaceae* has been revised. Six genera of leguminous nodule-forming bacteria: *Rhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Synorhizobium* have been recognized. These genera are included in the family *Rhizobiaceae* and are collectively known as rhizobia (Amarger, 2001; Vessey et al., 2004). The process of nodule formation is closely related with the infection of roots by appropriate rhizobia. Rhizobia enters the root via root hairs in majority of legumes and trigger the process of nodule development (Libbenga and Harkes, 1973; Dart, 1977; Rubina and Mahmood, 1992; Qadri and Mahmood 2003, 2004). Structural studies on nodules of tree legumes have been conducted on *Sesbania sesban* (Mahmood and Jamal, 1977), *Prosopis glandulosa*, (Baird et al., 1985). *Andira Spp.* (Faria, et al., 1986), *Leucaena leucocephala* (Rubina and Mahmood, 1992), *Anadenanthera peregrina* (Gross et al., 2002), *Dalbergia sissoo* (Qadri and Mahmood, 2002, 2004), and *Samanea saman* (Qadri and Mahmood, 2003, 2004). Survey of literature shows that structural studies on root nodules of *P. dulce* have not been carried out in the past. This paper describes mode of infection, nodule development, and structure of nodules in *P. dulce* with particular reference to the mechanism of release of rhizobia in the cytoplasm of infected cells.

## Materials and methods

Nodules of *P. dulce* were collected from plants growing at the Karachi University campus. For the light microscopy the nodules and roots were fixed in F.A.A. (Formalin-aceticacid-alcohol) for 18 h. Pieces of nodules (1-2 mm) were dehydrated in ethanol series and infiltrated with L.R. (London resin) white resin at room temperature and polymerized at 60°C for 24 h. Serial sections (0.5-2 µm) were cut with a glass knife using a Sorral J.B-4 ultra microtome and transferred to glass slides in a large drop of water. The sections were dried on a hot plate at 40°C, stained with

aqueous toluidine blue (in 1.0% borax, pH 4.4) and mounted in Canada balsam (Faria et al, 1986).

For transmission electron microscopy, small pieces of nodules (1-2 mm) were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7) for 4 h. The tissues were then transferred to 1% aqueous osmium tetroxide for 2-4 h at room temperature (Faria et al., 1986). The fixed material was processed for transmission electron microscopy as described by (Qadri and Mahmood, 2004).

Complete nodules and free hand section of nodules were fixed for scanning by electron microscope for TEM. They were dehydrated in 100% ethanol followed by an ethanol: acetone series to 100% acetone (Faria et al., 1986). The specimens were then dried using a Polaron critical point drier (BIO-RAD), coated with gold in coating unit (JFC-1100), and examined with (Jeol T-20) scanning electron microscope.

## Results and discussion

The nodules were distributed singly as well as in clusters on the main and lateral roots. Young nodules were globose in all the examined species of *P. dulce*. Mature nodules were elongated, branched and coralloid (Fig. 1). Bacteria entered the root via root hairs (Fig. 2). Both curled and straight root hairs were noted in *P. dulce* (Fig. 2). The entry of rhizobia inside the roots is not limited to root hair infection, other modes of entry such as their spread through intercellular spaces exist (Sprent and Faria, 1989). General structure of nodules showed a cortex containing vascular bundles which surrounded the infected tissues. The cortex was surrounded by a thick periderm comprised of sclereid tissue (Fig. 3). Vascular bundles were amphicribal (Fig. 4). There was a single vascular strand connecting the nodule vascular supply with the vascular supply of parent root (Fig. 3). The center of nodules contained both infected and uninfected (interstitial) cells (Figs. 3, 6, 7) intermingled with each other. Nodule formation was initiated by the entry of rhizobia into root tissue. Rhizobia enter the root via root hairs in majority of legumes (Dart, 1977; Rubina and Mahmood, 1992; Gross et al., 2002; Vessey et al., 2004). The infection thread was formed inside the root hair by multiplication of rhizobia which lie end to end within the thread. Similar observations were made in *Leucaena leucocephala* (Rubina and Mahmood, 1992) and *Samanea saman* (Qadri and Mahmood, 2004). Bacteroid region is the most prominent region of the nodule and occupies the central part of the nodule. Composition of this region may vary in different legumes. In the present study bacteroid region consisted of infected and uninfected (interstitial) cells intermingled with each other. Similar observations have been made for many leguminous species



Figure 1. The distribution of nodules on main and lateral roots of *P. dulce*

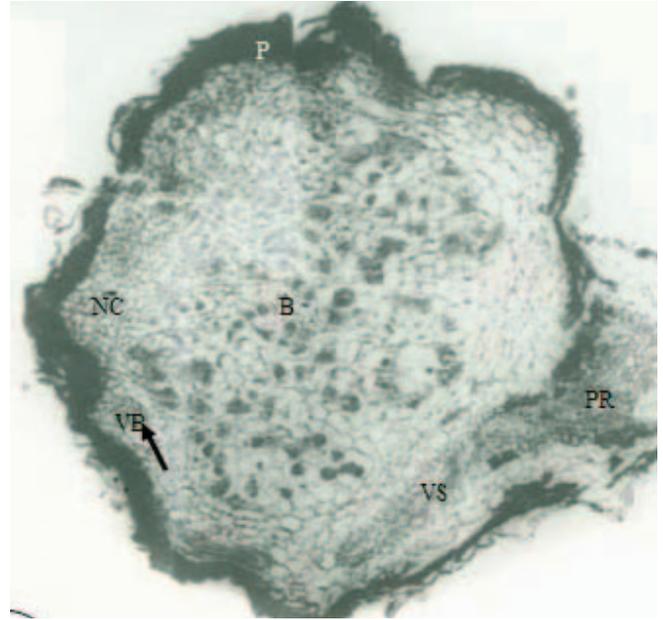


Figure 3. Light micrograph of transverse section of root nodule of *P. dulce* showing prominent periderm (P), nodule cortex (NC), bacteroid region (B), vascular bundles (VB), and vascular strand (VS) of nodule showing connection with the parent root (PR) x 450

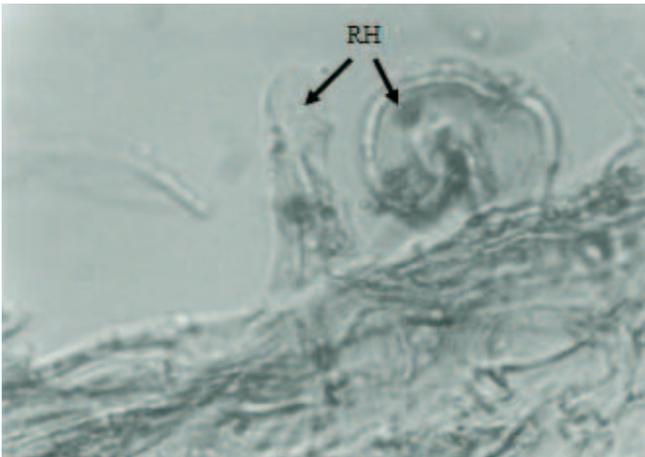


Figure 2. Curled and straight root hair (RH) of *P. dulce* showing infection (x330)

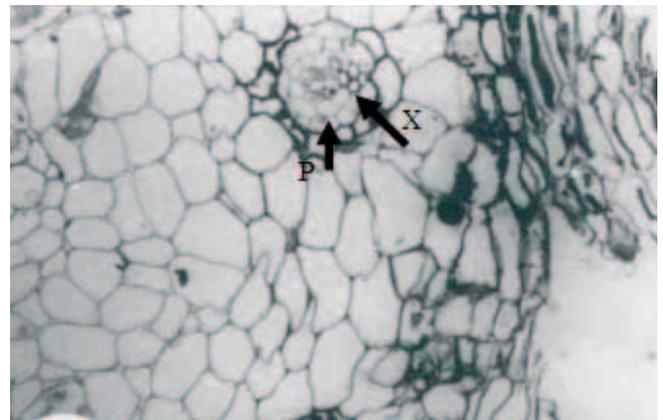
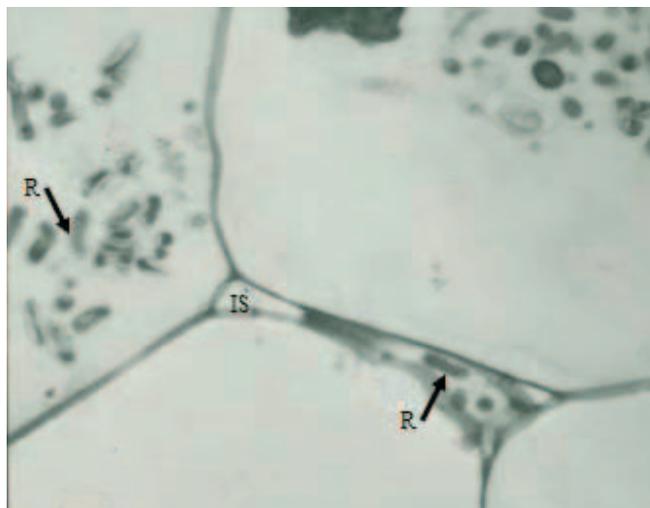


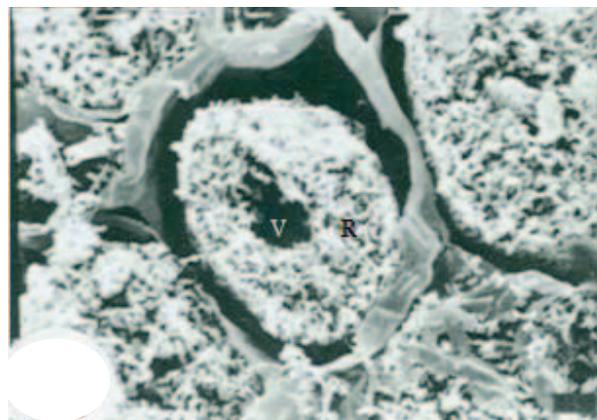
Figure 4. A single amphicribal vascular bundle shown enlarged from Fig.3 showing xylem (X) and phloem (P) (x260)

viz. *Sesbania grandiflora* (Harris et al., 1949), *Cyamopsis tetragonoloba* (Narayana, 1963), *Albizia* spp. (Dart, 1977), *Sesbania sesban* (Mahmood and Jamal, 1977), *Parasponia andersonii* (Trinick, 1979), *Leucaena leucocephala* (Rubina and Mahmood, 1992) and *Dalbergia sissoo* (Qadri and Mahmood, 2002, 2004). The infected region of the nodule

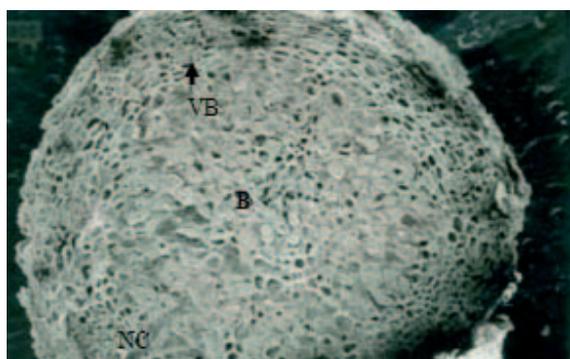
contained rhizobia which were of two types i.e. small coccoid forms and large elongated cells (Fig. 10). A few infection threads could also be observed in the infected region (Fig. 10). The rhizobia were also present in the intercellular spaces between infected cells (Fig. 5) suggesting that infection could also spread through intercellular spaces as described by Sprent and Faria (1989). Occurrence of rhizobia in intercellular spaces between infected cells have been observed by a number of investigators namely



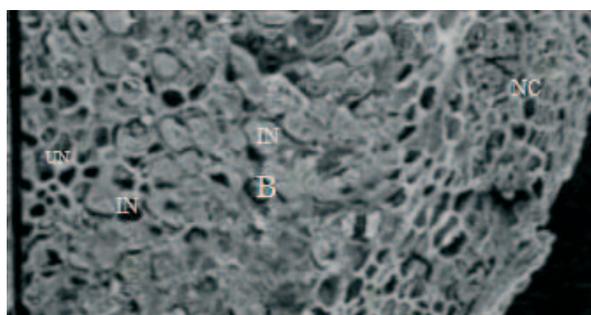
**Figure 5.** A transmission electron micrograph of *P. dulce* nodule showing rhizobia (R) in the intercellular space (IS) between infected cells of young nodules (x3000)



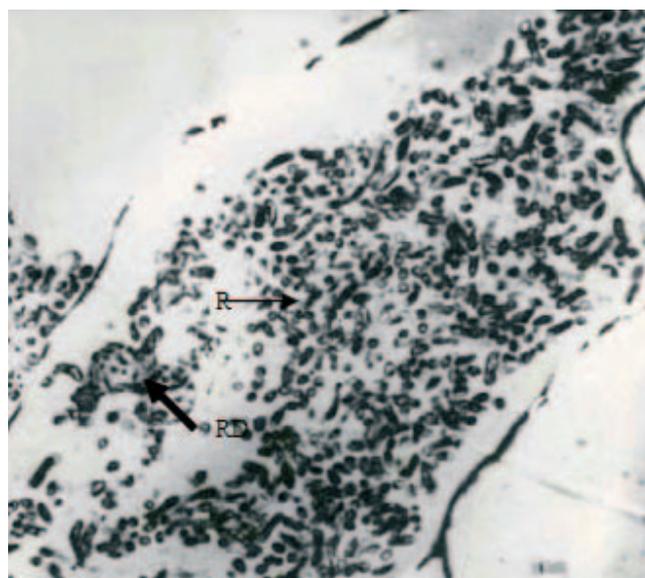
**Figure 8.** An enlarged view of an infected cell from Fig. 7 showing central vacuole (V); Rhizobia (R) can be seen around the vacuole (x 2500)



**Figure 6.** A scanning electron micrograph of transverse section of root nodule of *P. dulce* showing central bacteroid region (B), and nodule cortex (NC) vascular bundle (VB) (x125)



**Figure 7.** An enlarged portion from Fig. 6 showing bacteroid region (B) nodule cortex (NC); showing infected (IN) and uninfected cell (UN) cells (x320)



**Figure 9.** A transmission electron micrograph of an infected cell of root nodule of *P. dulce* showing rod shaped bacteroids (R) and rhizobial droplets (RD) (x1700)

(Lawriem, 1983) in *Aotus ericoides*, (Faria et al., 1987) in *Andria racemosa*, and (Qadri and Mahmood, 2005) in *Albizia lebbeck* nodules.

In the early stages of nodule development rhizobial droplets (unwalled droplets) were observed in the infected region (Figs. 9, 10, 12, 13). In the later stages of nodule development rhizobial droplets expanded in volume (Fig. 11, 14). Unwalled droplets (UD) is a descriptive term used by (Newcomb, 1976) in place of the term “vesicle” previously used for such membrane bound thread matrix structures released or associated with the infection thread in legume root nodules. Unwalled droplets (UD) are the sites of bacte-

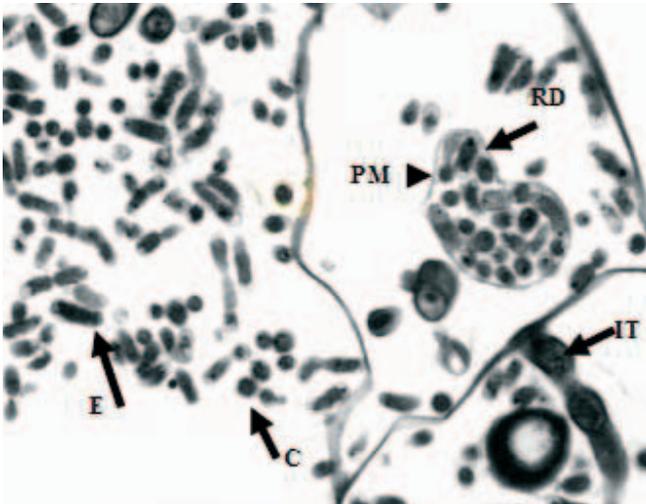


Figure 10. A transmission electron micrograph of an infected cell of *P. dulce* showing coccoid (C) and elongated (E) forms of bacteroids, rhizobial droplets (RD), and peribacteroid membrane (PM) around (RD) shown by arrowhead (x 2750)

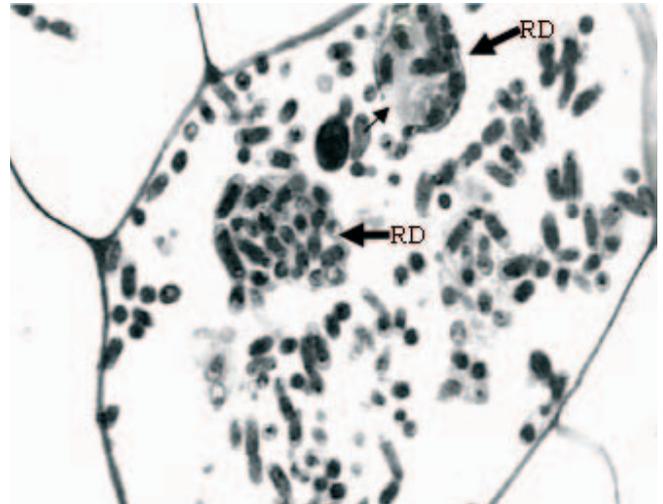


Figure 12. A transmission electron micrograph of an infected cell of *P. dulce* root nodule showing rhizobial droplet (RD) (x2750)

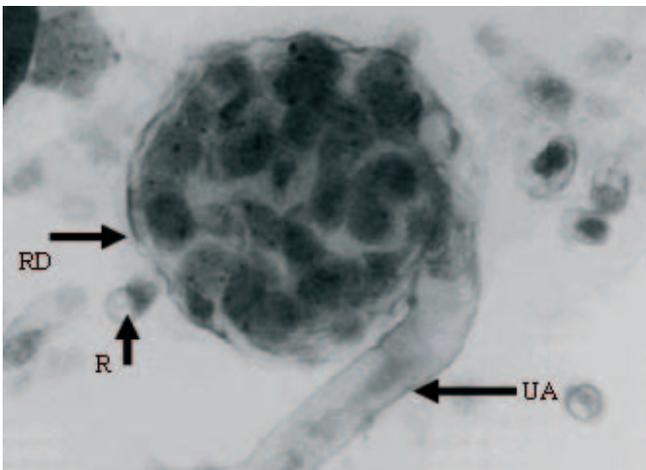


Figure 11. A transmission electron micrograph showing rhizobial droplet (RD) that is unwalling. Rhizobium (R) is coming out from the weaker portion of (RD). Note that RD is attached to an unbranched appendage (UA) which is non-septate. The rhizobia are vacuolated (x7670)

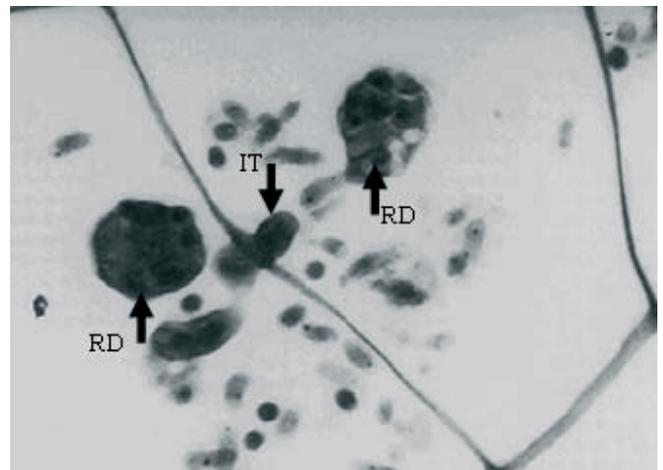
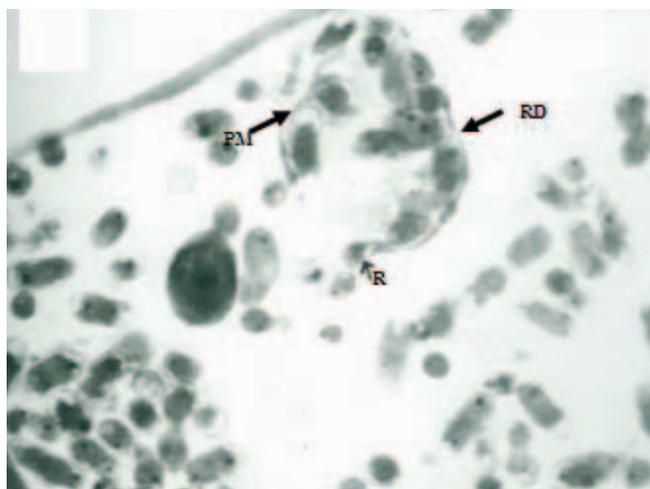


Figure 13. A transmission electron micrograph of *P. dulce* root nodule showing infection thread (IT) and rhizobial droplet (RD). Infection thread (IT) is crossing from one cell to the other by penetrating through the cell wall (shown by an arrow) (x3650)

rial cell release into the cytoplasm of host cell (Newcomb, 1976). Unwalling droplet formed an unbranched appendage which was non-septate (Fig. 11). UD is enclosed by host plasma membrane and contains numerous rhizobia (Figs. 10, 11) such structures have been shown by Newcomb (1976) and Ghalifour and Benhamou (1989) in pea nodules, Newcomb et. al. (1981) in *Vicia faba* nodules in pea nodules. The formation of the unwalling droplets and release of bacteria from the droplets are essential for the establishment of symbiotic inter relationship in pea root

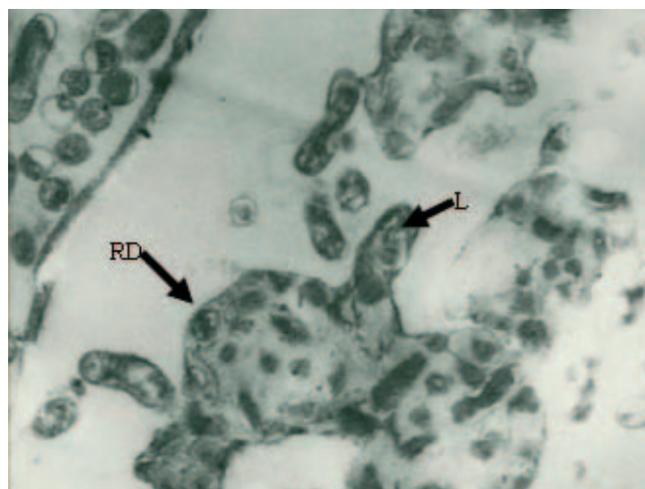
nodules (Newcomb, 1976). Infection threads crossed from one cell to the other intracellularly by penetrating the host cell wall (Fig. 13). Rhizobia came out from rhizobial droplets as single cells (Fig. 14) and were released into the host cytoplasm. Bacteria at the periphery of the droplet are often seen in an invagination formed by the pea cell plasma membrane (Newcomb, 1976). Subsequent invagination and pinching off the host cell membrane result in the release of a single bacterium within a surrounding membrane envelope into the host cytoplasm. Similar ob-



**Figure 14.** A transmission electron micrograph of an infected cell of *P. dulce* showing Rhizobia (R) coming out from the rhizobial droplet (RD). Note that the peribacteroid membrane (PM) is broken at this point (x7850)

servations were made for *P. dulce* (Fig. 10, 14). Thus the membrane envelope surrounding the bacteria is derived from the cell plasma membrane. At the later stages of nodule development in senescent infected cells UD showed lobed appearance (Fig. 12). The volumes of UD expanded and distortion of some bacteria also took place (Fig. 15). Similar structures have been reported by Lawrie (1983) for *Aotus ericoides*, a woody native legume.

Once the nodule has been formed it establishes connection with the vascular system of main root (Bond, 1948). In the present study the vascular system of the nodule surrounded the central bacteroid region without coming in direct contact with it. Such observations were recorded by a number of workers (Naz and Mahmood, 1976; Mahmood and Jamal, 1977; Wester and Hogberg, 1989; Sprent and Sprent, 1990; Rubina and Mahmood, 1992a). The vascular bundles found in the nodule cortex of *P. dulce*, were amphicribal. Amphicribal vascular bundles have been reported in a numbers of nodules of leguminous plants as reviewed by Qadri and Mahmood (2004). Vacuolated infected cells with a prominent central vacuole were also observed in the infected region of *P. dulce* (Fig. 7, 8). Sprent (1981) has reported vacuole in the infected cells of indeterminate nodules of tribe *Vicieae* that could act as temporary store for fixed nitrogen. Faria et al. (1987b) have surveyed this feature in tribes of subfamilies *Caesalpinioideae*, *Papilionoideae* and *Mimosoideae* and found that prominent central vacuole was found only in the genera *Cyclolobium* and *Poecilanthus* of the tribe *Tephrosieae*. Un-infected cells found in the bacteroid and cortical regions of *P. dulce* contained starch. It has been reported that nodules of several



**Figure 15.** A transmission electron micrograph of an older infected cell of *P. dulce*. Note the lobing (L) of rhizobial droplet (RD) during the senescent stage of nodule development. Bacteria are showing prominent vacuoles (x6000)

legume species store carbohydrates in the form of starch (Newcomb et al., 1981; Vaughn et al., 1982; Henson et al., 1986; Rubina and Mahmood, 1992; Qadri and Mahmood, 2004). Alfalfa nodules show conspicuous starch deposits in infected as well as uninfected cells (Vance et al., 1980; Vaughn et al., 1982). Hostak et al. (1987) found that uninfected cells of the nodules may prove an important source of carbohydrates for nodular tissues, because legume nodules require large amount of carbohydrates in order to generate adequate supply of energy needed in nitrogen fixing process.

In conclusion it may be said that the general structure of nodule of *P. dulce* in transverse sections conforms to the other woody legumes in having a cortex containing vascular bundles that surrounded the bacteroid region. In addition there was a pronounced sclereid layer in the cortex. The main feature of bacteroid zone was the presence of UD (unwalled droplets) through which release of bacteria into host cytoplasm occurred. In this respect *P. dulce* shows resemblance with herbaceous legumes such pea (Newcomb, 1976; Ghalifour and Benhamou, 1989) and *Vicia faba* (Newcomb et al., 1981).

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