BIOLOGY OF ROOT NODULES OF *PHASEOLUS* SPP. WITH REFERENCE TO THEIR ANATOMICAL STRUCTURES*

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Developing root nodules were studied on two very important pulse species, i.e., *Phaseolus mungo* and *Phaseolus aureus*. The first sign of nodulation was observed on the second day of seedling emergence. The general features of nodules of these species were more or less similar. A very young nodule appeared white and gradually turned pinkish with subsequent development. The maximum nodulation coincided with the onset of flowering indicating best period of nitrogen fixation. Histologically, a fully mature nodule comprised of four distinct zones : cortical zone, vascular strand, nodule meristem and central bacteroid zone. The bacteroid cells contained only few rhizobia in very young nodule which became completely filled when mature. Degeneration of mature nodule, however, started soon after flowering–approximately after 5–6 weeks in *P. mungo* and 8–9 weeks in *P. aureus* following the browning of nodules. These nodules finally disintegrated and became a part of the surrounding rhizosphere.

INTRODUCTION

The nodules produced by the symbiotic nitrogen-fixing bacteria of the genus *Rhizobium* have been studied during past many years. Most of the earlier studies were made on the anatomical structure of mature nodules in woody leguminous species (Fred *et al.* 1932; Bieberdorf 1938; Allen and Allen 1940; Arora 1956*a*, 1956*b*; Bergersen 1955; Harris *et al.* 1949 and Nutman 1965). In fact, there has been no record on the various developmental stages of nodulation. Besides, these two cultivated species of the genus *Phaseolus* were not studied earlier. This paper deals chiefly with the well-defined developmental stages of nodule morphogenesis in *P. mungo* and *P. aureus*, as a pre-requisite for electron microscopy.

MATERIALS AND METHODS

The nodules of black gram (*Phaseolus mungo* var. T_9) and green gram (*P. aureus* var. NP 28), diploid species, were collected from the Applied Botany Section of this Institute. The specific rhizobial strain was isolated locally from nodules formed on young vigorously growing plants.

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Prior to sowing, the seeds were treated with mercuric chloride (1:1000) solution for 3 to 5 min followed by a short dip in 95 per cent alcohol and washed in sterile water. The seeds were then dried in air and sown in pots containing sterile vermiculte + sand mixture, after inoculation with the isolate, and grown under glass-house. Another lot of seedlings was grown under field conditions where natural inoculum was present. The potted seedlings were irrigated with modified Bonner's nutrient solution at suitable intervals. The samples were collected on 2, 5, 15 and 50 days of seedling emergence. In each case, sufficient number of seedlings were examined for morphological studies with special emphasis on the anatomical structure of nodules.

Nodule samples of different age groups were fixed in Fleming's Osmic-chrom-acetic solution for 6–8 hr, dehydrated and embedded in paraffin wax (60-62°C). The sections were cut at 8 μ thick and stained in Heidenhain's haematoxylin.

Plastic sectioning—The nodule samples were embedded in plastic mixture following the method of Mollenhauer (1964). The embedded nodules were made into tiny blocks and mounted on the lucite rod and trimmed into smaller faces of 2×1 mm; 2-3 μ thick sections were cut on a LKB-Ultratome using glass knives. The sections were placed on clean slide and stained with a drop of Paragon multiple stain for a short time, washed and air-dried. Finally, sections were examined microscopically.

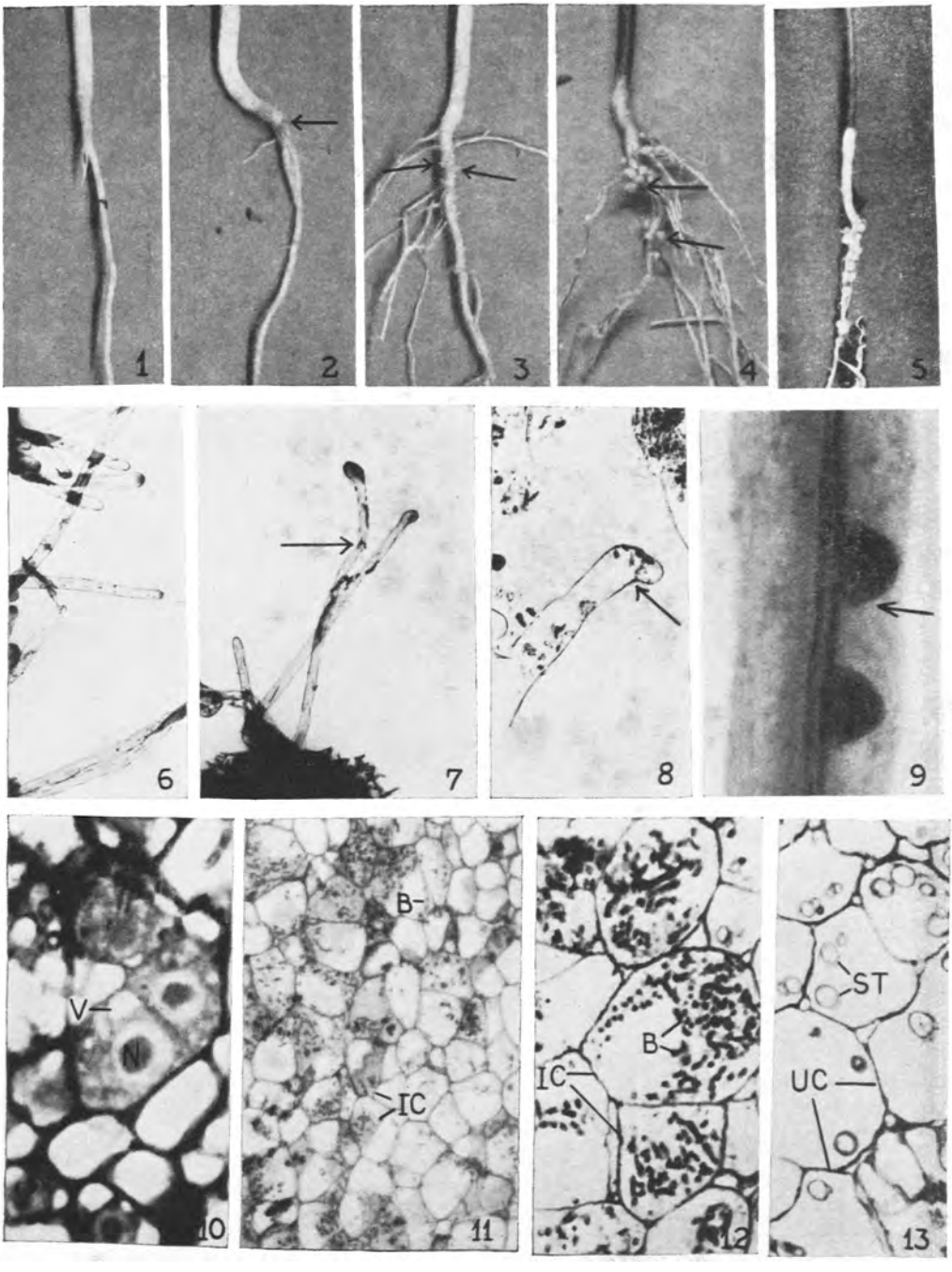
Preparation of suspension—Smear preparations were made from developing nodule sap and from the culture medium. These were stained in Gram's stain for gross bacterial morphology.

Results and Discussion

(A) *External morphology and inoculation studies*

The seedlings on the day of emergence do not show any sign of nodule formation but on the second day very small semi-spherical white protuberances could be noticed at few points when examined with a stereomicroscope (Figs. 1 and 2). Later, these protuberances become more and more prominent (Fig. 3) with the considerable increase in size. Within days the nodule is well formed (Fig. 4) with

Figs. 1–13. 1–4, representative infected seedlings of *Phaseolus mungo* at different stages of development. ×1; 1, one-day-old seedling showing complete absence of any visible nodule growth ; 2, two-day-old seedling showing the first appearance of nodule as small specks (arrow) as seen under the stereomicroscope; 3, five-day-old seedling showing a number of small nodules on the main tap root (arrows); 4, ten-day-old seedling showing numerous nodules of different sizes (arrows); 5, representative ten-day-old infected seedling of *Phaseolus aureus*. ×1. 6–8, light micrographs of root-hair cells of control and infected seedlings; 6, root-hair cells of control showing straight profiles. × 400; 7–8, curled and deformed (arrows) root-hair cells of infected seedlings. × 450; 9, light micrograph of unsectioned part of secondary root with nodule initial stained with aceto-carmine–vascular bundles are seen in the central region. × 50; 10–13, light micrographs of the young, mature and degenerating nodules studied from plastic sections; 10, section through a two-year-old nodule showing a group of meristematic cells containing large nuclei (N) and dense cytoplasm (also seen are highly vacuolated cells around). ×1650; 11, section through a five-day-old nodule—infected cells (*IC*) characterised by the presence of few bacteria (*B*) in the cytoplasm. ×1500. 12, enlarged view of an infected cell showing the degree of packing of bacteria. × 2000; 13, a group of highly vacuolated uninfected cells (*UC*) containing starch grains (*ST*). × 2000.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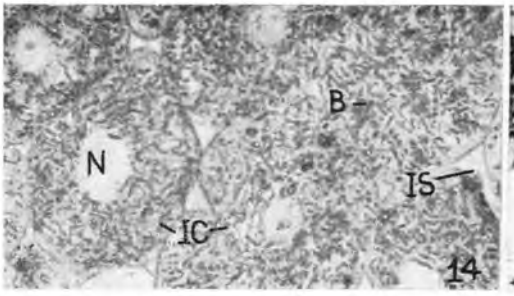
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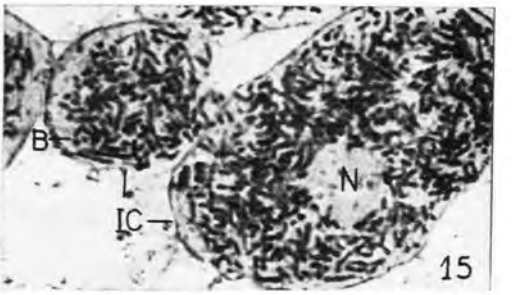
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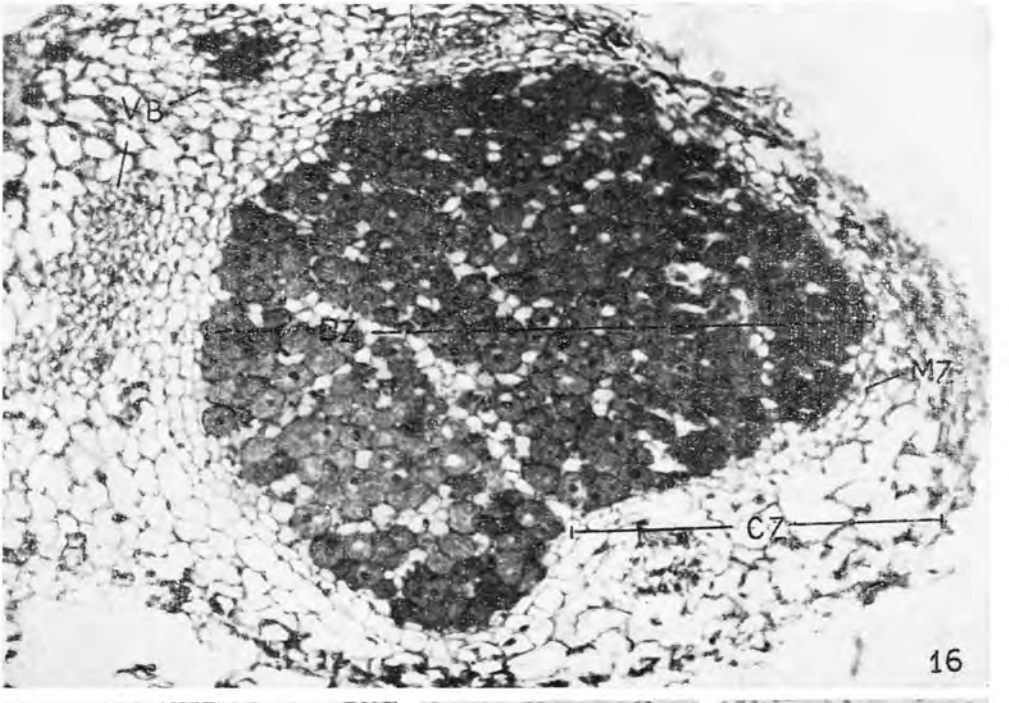
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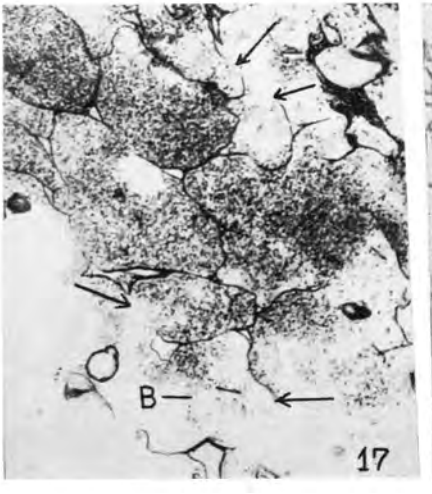
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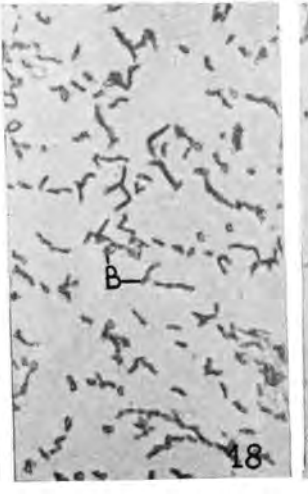
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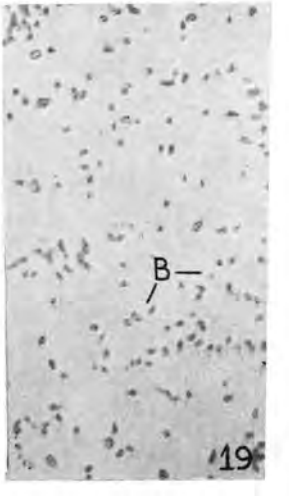
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respect to its shape, size and central bacteroid tissue content. Moreover, various parts of the main tap and secondary roots are always subject to fresh infections. Control seedlings when grown aseptically do not show any nodule growth. Milky-white colour of the young nodules gradually turns pinkish with subsequent development and finally dirty brown when the nodules start degeneration. The maximum nodulation is attained in 5-6 weeks in *P. mungo* and 7–9 weeks in *P. aureus* which coincide with the onset of flowering.

In *P. mungo*, a large number of nodules were seen to be localised around the upper region of the hypocotyl, whereas other nodules were distributed randomly throughout the length of the root system. Similar observations were noted in *P. aureus* (Fig. 5). The concentration of nodules on the upper region of the hypocotyl, in fact, corresponds to the zone of root hairs thereby increasing the loci of rhizobial infection. Nodules in *P. aureus*, in general, are larger and higher in number than in *P. mungo*. The number of ineffective nodules in these species is very sparse.

Isolation of culture and inoculation—Inoculum from young nodule produced good colony growth in Ashby's mannitol phosphate agar medium. Ideal colonies were noted in 20 to 24 hr of incubation at $29\pm1°C$. Inocula isolated from nodules of these two species were one and the same and proved equally efficient.

(B) *Light microscopy*

Entry of rhizobia through root-hair cells forms the primary mode of infection in these species. This was also evident from the 'curling' of root hairs on the day of seedling emergence (Figs. 6, 7 and 8). About 90–96 per cent of the root hair cells showed marked curling to various degrees. Yao and Vincent (1968), however, explained this effect as very specific reaction of the rhizobia at the time of primary infection. Apparently the exact causes of its effects are still not well understood.

The infection threads were traced in the initial stages of invasion from the root-hair cells down to several layers of cortical cells. Occasionally, disomatic cells have also been detected as of the spontaneous origin in the root cortex of very young seedlings. It is, however, difficult to accept these as the only preferential sites for the dissemination of rhizobia from the infection threads leading to the nodule formation as suggested by Wipf and Cooper (1940). The contention that somatic doubling may be the result rather than cause of infection is acceptable as proposed by Bhaskaran and Swaminathan (1958). Even from the latest available literature

FIGS. 14–19. 14, a group of 10-day-old mature nodule-infected cells (*IC*) packed with bacteroids (*B*) and centrally placed nuclei (*N*)—inter-cellular space (*IS*) is prominent. × 1600 ; 15, enlarged view of a mature infected nodule cell showing long rods of rhizobium. × 2000 ; 16, paraffin section through a 15-day-old nodule stained with Flemming's triple stain [the centrally located densely stained cells form the bacteroid zone (*BZ*) surrounded by the thin-walled cortical cells (*CZ*) and nodule meristem (*MZ*), also visible is vascular bundle (*VB*)]. × 150; 17, section through a degenerating nodule showing release of bacteria following the breakdown of cell walls (Arrows indicate the broken cell walls). × 1600; 18–19, light micrographs of rhizobial smears obtained from nodule cell-sap and young culture respectively—bacteria (*B*) show a marked difference in their morphology. × 2000.

it has not been possible to know exactly how long an infection thread remains functional once it has initiated from the root-hair cells. Immediately after the infection of few cortical cells, a meristematic tissue develops around it. The initiation of nodule primordium appears as darkly stained bulge embedded in the root cortex (Fig. 9). Anatomically, meristematic cells show dense cytoplasm with small degree of vacuolation (Fig. 10). The infected cells of 5-day-old nodules on the other hand, contain few rhizobia dispersed in the cytoplasm (Figs. 11 and 12). Moreover, these are surrounded by highly vacuolated parenchymatous cells containing abundant starch grains (Fig. 13). The vacuoles gradually diminish and finally obliterate. As the infected cells become packed with rhizobia, these cells enlarge significantly (Figs. 14 and 15) and the inter-cellular spaces increase considerably in this region. A section of the fully mature nodule is presented in Fig. 16.

The detailed anatomical structure of the developing nodules reveals an important fact that the division of the infected cells is complete in the first few days of infection following which only the cell growth and enlargement take place. Moreover, it was confirmed by autoradiographic studies (Prasad 1970) showing a complete absence of any mitotic figures in the infected cells of growing nodule.

The rhizobial cells are regularly, if not equationally, distributed, following each successive division of infected cells. These infected cells, however, show hypertrophic effect to cope with the multiplying rhizobia till the whole cell cytoplasm is fully packed. On the other hand, majority of the starch-containing uninfected cells are confined to the outer side of the nodule and only few are interspersed in the bacteroid zone. Initially, the number of starch grains is higher in young nodules which is reduced considerably at later stages. The presence of starch bodies probably serves as 'energy reserve' which is gradually used up as the central tissues grow with the rapidly multiplying rhizobia within.

Differentiation of vascular strands in the nodules begins soon after the meristematic activity is pronounced. These vascular strands originate from the main stele and develop towards the apex of the bulged area. The central zone may appear as entire or divided into two or three lobes. The general anatomy of a mature nodule comprises of four distinct zones, i.e. cortical zone, vascular strands, nodule meristem and the central bacteroid zone (Fig. 16). The general conclusions about the anatomy of mature nodule agree with its basic structure as proposed by Allen and Allen (1940), Bond (1948), Harris *et al.* (1949), Arora (1956 *a*, *b*) and Narayan (1962), excepting the sequential changes which have been observed for the first time.

Degeneration of nodules—Histologically, the study of these nodules reveals that the degeneration starts from the side and proceeds towards the centre. At an advanced stage of degeneration, the whole central bacteroid tissue appears disorganized with the disintegration of the inter-cellular walls resulting in the formation of giant cells (Fig. 17). Vascular strands become non-functional with the loss of content from the main stele.

Study of bacterial smear—Within the host cells, the rhizobia invariably show rods measuring 0.7–3.5 μ in length and 0.5–0.6 μ in diameter. The rhizobial cells are comparatively longer in the young developing nodules than at mature stages.

The growth, however, continues till the whole cytoplasmic components are broken down. Moreover, the finer aspects of such morphological differences have not been clearly understood and are open for discussion. There is very low record of the modified L, T or Y-forms of bacteroids. The study also reveals a sharp morphological difference between the bacteria obtained from the mature nodule sap and from the artificial culture medium. In the former case, bacterial cells were much larger, uneven rods; whereas in the latter these were much smaller and straight rod profiles (Figs. 18 and 19). No internal details could be seen in any case. It is really interesting to note that each particular strain of *Rhizobium* expresses different morphological characters in conditions of natural cell sap and the synthetic media.

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