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ESTABLISHMENT OF SYMBIOSIS *IN VITRO*, BETWEEN *RHIZOBIUM* AND PEA (*PISUM SATIVUM*) ROOT CALLUS

V. RANGA RAO
Department of Botany, Sri Venkateswara College, Delhi University, Delhi
SUDHIR SOPORY
School of Life Sciences, Jawaharlal Nehru University, New Delhi
AND
N. S. SUBBA RAO
Division of Microbiology, Indian Agricultural Research Institute, New Delhi

ABSTRACT

Pea (*Pisum sativum*) root callus was inoculated with *Rhizobium leguminosarum* and studied for the establishment of symbiosis. Infection thread-like structures were observed penetrating the callus intercellularly and bacteria and bacteroid-like bodies were seen in the cells of the callus tissue. Nitrogenase activity was detected in some samples of calli indicating the nitrogen fixing ability of the infected callus tissue.

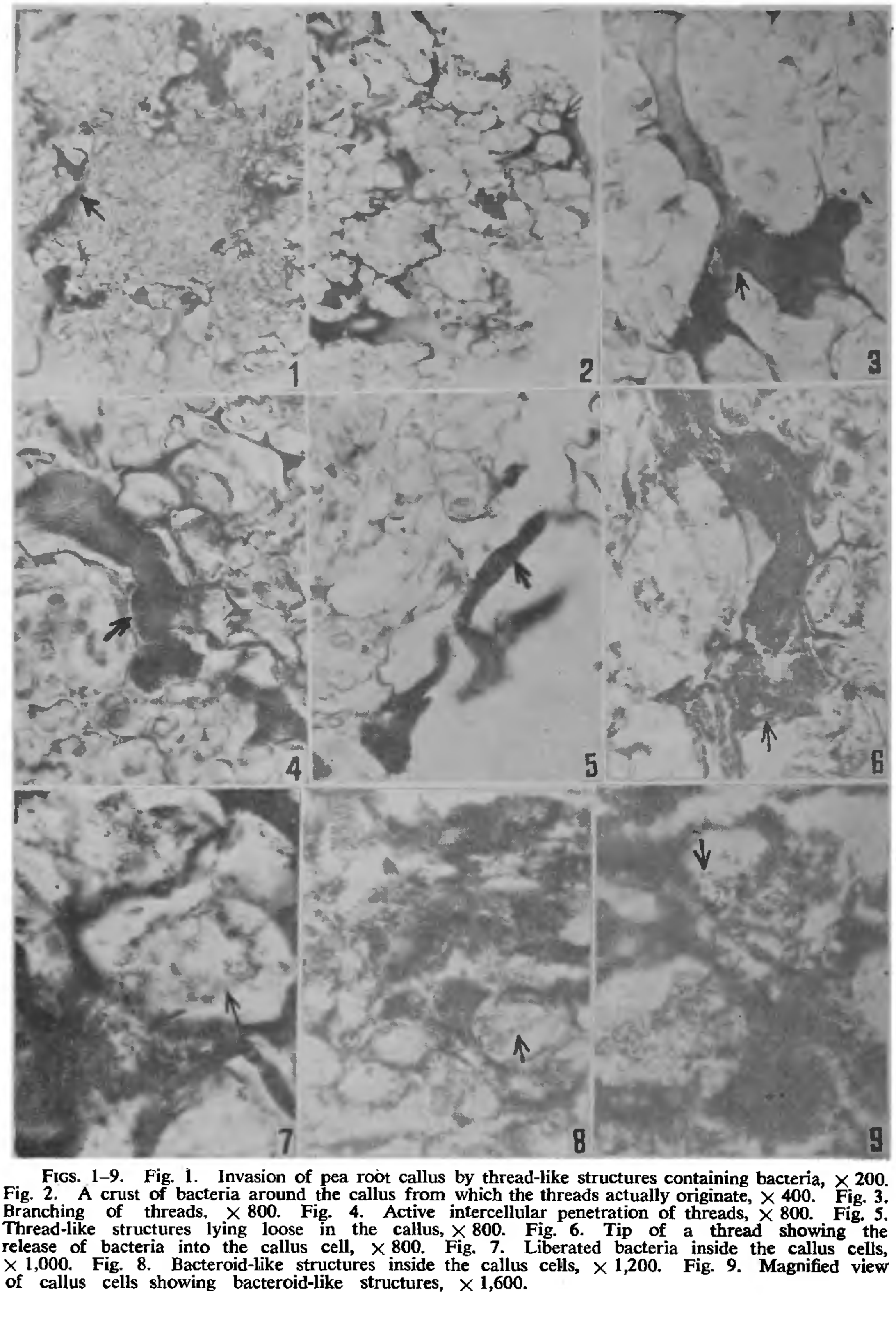
INTRODUCTION

THE formation of infection threads and nodules in legume-*Rhizobium* association has been studied in whole plants[1] and in isolated roots[2] under aseptic conditions. An unsuccessful attempt to establish symbiosis in isolated plant tissue (callus) was made by Veliky and La Rue[3] in 1967. This was followed by a successful attempt by Holsten *et al.*[4] in 1971, using soybean root callus infected with *R. japonicum*. Nitrogenase activity was detected in infected calli (by acetylene reduction technique) and the tissue contained infection thread-like structures and bacteroid-like cells. The results reported in this paper relate to our work with pea root callus infected with *R. leguminosarum*, using the method followed by Holsten *et al.*[4] with slight modifications.

MATERIALS AND METHODS

Seeds of *Pisum sativum* var. *baunville* were surface sterilised with cetavlon (1%) and germinated on Murashige-Skoog's[5] (MS) basal medium. The roots were cut off when 2–3 cm long and transferred to MS medium supplemented with 2, 4 - D and kinetin. Callus formation was observed after 1 month. The callus was transferred to MS basal medium and allowed to remain there for 5 days. Pure cultures of *R. leguminosarum* were isolated from pea root nodules by conventional procedure[6] and maintained on yeast extract mannitol agar (YEMA) slants.

One ml of a YEM broth culture containing actively growing bacteria was transferred to each test-tube containing MS basal liquid medium with actively growing callus and incubated in darkness at 25 ± 2° C. Uninoculated calli served as controls. After seven days incubation, the callus mass was washed twice in MS basal liquid medium under aseptic conditions. The solution containing the callus mass was filtered through sterile cheese cloth and calli transferred from the cheese cloth to MS basal solid medium. They were incubated for



FIGS. 1–9. Fig. 1. Invasion of pea root callus by thread-like structures containing bacteria, × 200. Fig. 2. A crust of bacteria around the callus from which the threads actually originate, × 400. Fig. 3. Branching of threads, × 800. Fig. 4. Active intercellular penetration of threads, × 800. Fig. 5. Thread-like structures lying loose in the callus, × 800. Fig. 6. Tip of a thread showing the release of bacteria into the callus cell, × 800. Fig. 7. Liberated bacteria inside the callus cells, × 1,000. Fig. 8. Bacteroid-like structures inside the callus cells, × 1,200. Fig. 9. Magnified view of callus cells showing bacteroid-like structures, × 1,600.

another period of 14 days in dark. Similar treatment was also given to calli which were kept as control.

The calli were fixed in FAA for 24 hours, passed through tertiary butyl alcohol series and embedded in paraffin wax. Microtome sections were cut to 10 μ and slides were prepared by staining with Safranin-Fast green combination using Canada balsam as the mounting medium. The slides were observed under a light microscope.

Nitrogenase activity of infected calli was analysed by the acetylene reduction technique[4] as follows: The samples were purged with air, stoppered and injected with 0·5 ml of acetylene. The acetylene-ethylene conversion was analysed by gas chromatographic method.

Results

Microtome sections of infected calli showed infection thread-like structures (hereafter referred to as threads) ramifying the callus tissue (Fig. 1). The threads originated from the bacterial mass which formed a crust around the callus (Fig. 2). Some threads branched repeatedly (Fig. 3). A definite wall could be seen around the thread as in Fig. 4. The figure also indicates intercellular penetration of the thread. Often, threads were observed lying loose near the peripheral cells of the callus (Fig. 5). At certian points, the threads were ruptured at the tip and the bacteria were being liberated as in Fig. 6. Some of the cells of the callus contained bacteria (Fig. 7). At least 5–10% of the peripheral cells of the infected calli were seen filled with bacteroid-like structures (Fig. 8) which were different from those shown in Fig. 7. When magnified, the bacteroid-like structures (Fig. 9) resembled bacteroids in the cells of nodules of intact plants. The data regarding nitrogenase activity as revealed by the reduction of acetylene to ethylene are presented in Table I.

Table I

Nitrogenase activity in the infected and control samples of pea root calli

Serial No. of infected calli	Period of incubation: 1 hour — μ moles of $C_2H_2 \rightarrow C_2H_4$ per tube	Period of incubation: 3 hours — μ moles of $C_2H_2 \rightarrow C_2H_4$ per tube
1	0·000942	0·001262
2	0·000442	0·000875
3	0·002075	0·002775
4	0·001008	0·001388
5	0·000504	0·000625
Controls (Uninfected)	No activity	No activity

Discussion

In general, the infection thread-like structures in pea callus were bigger than those observed in *in vivo* in nodulated pea plants and in infected root cell cultures of soybean[4]. The bacteroid-filled cells in pea callus were also usually restricted to the peripheral portion of the callus as observed by Holsten and associates in cell cultures of soybean[4]. In a personal communication to one of us (after seeing our photographs of infected calli), Dr. R. W. F. Hardy of Du Pont Laboratory, U.S.A., comments that our pea callus threads are more abundant and larger than the infection thread-like structures (Pseudo-infection threads) which were observed by Holsten and associates[4] in soybean root callus. He also mentions that the presence of bacteroid-filled cells appears to be a promising feature. Whether or not the thread-like structures observed in these studies could be regarded as a feature arising out of true symbiosis can only be understood by future investigations. However, some of the infected callus cultures were analysed for nitrogenase activity in Dr. Hardy's laboratory. Measurable enzyme activity was detectable. La Rue and Goodchild, in their work on soybean, claim to have successfully established symbiosis using a different methodology. They found continued growth of infected callus which was regarded as a positive sign of symbiosis (La Rue, personal communication). We have also observed continued growth of infected pea calli. Further work on the fine structure and nitrogenase activity of *Rhizobium*-infected calli of different leguminous plants is in progress.

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