FEMS Microbiology Letters 15 (1982) 181–184
Published by Elsevier Biomedical Press

Extranodular growth of *Frankia* on *Casuarina equisetifolia*

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Received 19 January 1982
Accepted 3 May 1982

1. INTRODUCTION

17 genera of non-leguminous angiosperms bear N_2-fixing root nodules initiated by an actinomycete known as *Frankia* [1,11]. The expanding volume of current literature attests to the significance vested in this symbiosis. However, to the best of our knowledge, no publications up to this point have dealt with the occurrence and behaviour of *Frankia* outside its specific niche, the nodule. Reported here are observations indicating that *Frankia* is able to grow outside the nodule of *Casuarina equisetifolia* and to proliferate in its vicinity. Our study is focused on *Casuarina* because these tropical and subtropical non-legumes play a major role by virtue of their ability to thrive on nitrogen-deficient soils.

2. MATERIAL AND METHODS

2.1. Cultivation and inoculation of the Casuarina *seedlings*

Seeds of *Casuarina equisetifolia* were washed and surface-sterilized with concentrated H_2SO_4 for 2 min and rinsed several times with sterile distilled water. They were then germinated in sterile, moist sand for 10 days at 30°C. 6-week-old seedlings were transferred into empty petri dishes (9 cm diam.); the stems were maintained upright with a plastic clip fitted inside the rim and the roots were positioned lying horizontally on the bottom. The petri dishes were filled with 20 ml of a sterile quarter strength Hoagland solution [5]. The lids of the petri dishes had a radial slot near the rim in which the seedling stems were inserted. The lid and the rim of the petri dishes were painted black to prevent the proliferation of algae. The seedlings were placed in a controlled environment chamber (day/night: 16/8 h; day temperature/night temperature: 25/20°C). Each week, for 4 weeks, the Hoagland solution was renewed; when necessary, sterile water was added to compensate for evaporation. When the seedlings were ca. 10 weeks old, the Hoagland solution was replaced by a sterile N-free Hoagland solution. One set of the seedlings was inoculated by adding into each petri dish 1 ml of a water suspension of crushed nodules (ca. 10 mg fresh wt.). These nodules had been freshly collected on nodulated *Casuarina* grown in tubes or pots and had been surface-sterilized before being crushed. In some petri dishes, sterile distilled water was used instead of the N-free Hoagland solution. Another set of the seedlings remained uninoculated to serve as a control or to be used later in infectivity tests described in Section 3.

2.2. Microscopic observations of the root systems

To observe the root systems of the seedlings without disturbing them, we removed the lid of each petri dish and placed the whole device with the seedling under a dissecting microscope (×40). Such observations were made every week after

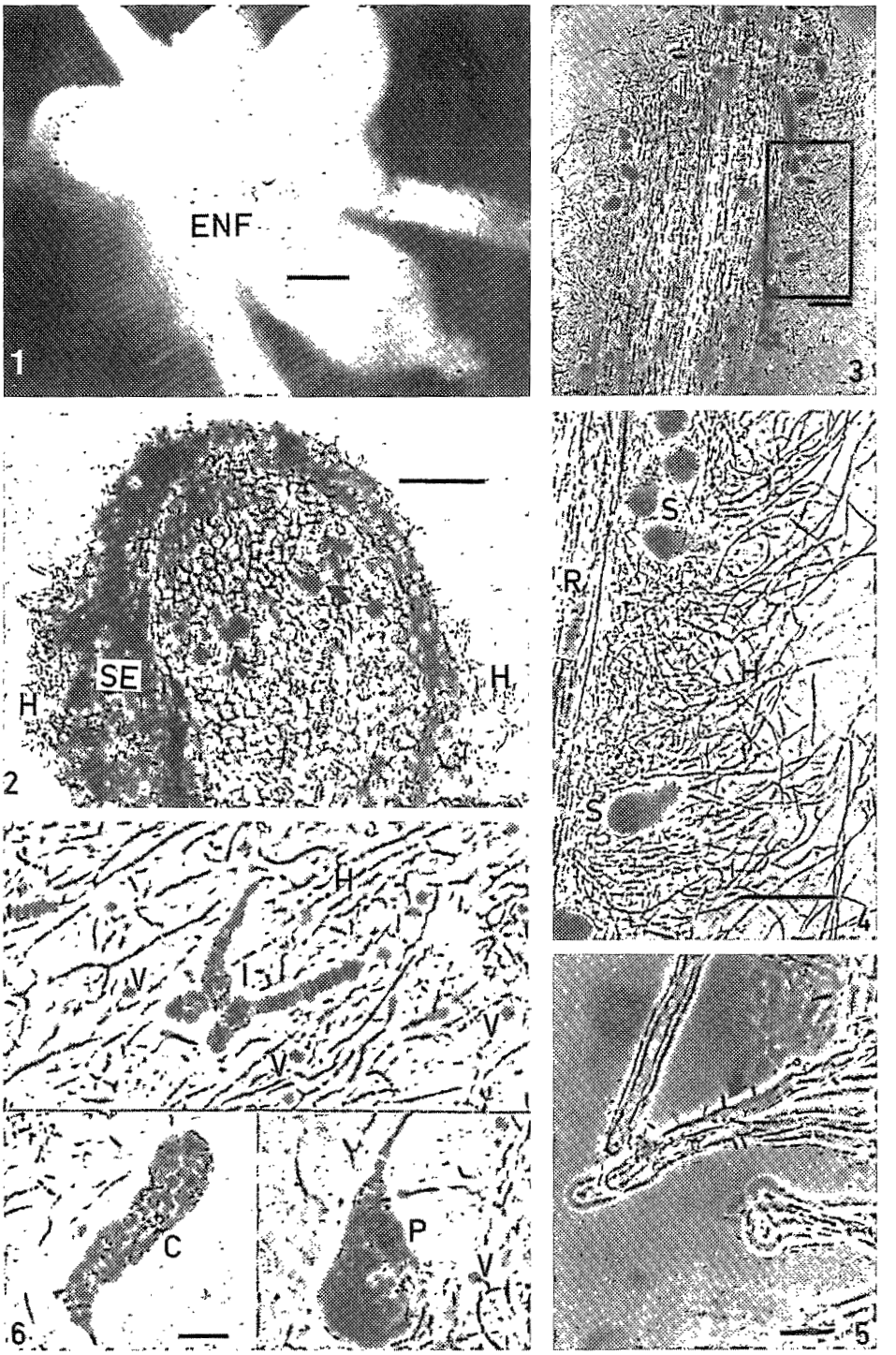

Fig. 1. Extranodular colony of *Frankia* (ENF) growing in the vicinity of a nodule of *Casuarina equisetifolia* as observed under dissecting microscope. Note the fluffy whitish aspect of the colony. Bar=200 μm.

inoculation for the presence of nodules. After the initiation of nodulation, the seedlings were cultivated for 3–4 additional weeks. Adjacent roots or microbial colonies growing around the newly formed nodules were then excised and stained with trypan blue in lactophenol (0.1%, w/v) for microscopical observation (×250). Thick sections of nodule lobes were cut with a freezing rotary microtome and stained with trypan blue.

3. RESULTS AND DISCUSSION

Up to 7 weeks after inoculation, uninoculated seedlings bore no nodule at all. By contrast, all the seedlings that had been inoculated were nodulated as early as the fourth week. Direct examination of the root systems under the dissecting microscope showed that 7 weeks after inoculation, 30–70% of the nodules in all the inoculated root systems were partially covered with fluffy whitish colonies composed of very thin hyphal filaments (0.9 μm), which appeared to be different from fungal hyphae (Fig. 1). Under higher magnification, the morphology of the filaments appeared similar to that of an actinomycete. Moreover, in the hyphal network, there were many spore-containing sporangia and vesicles similar to those formed by *Frankia* in vitro (Figs. 4 and 6). Sections of nodules suggested that the growing hyphae originated from the inner tissue of the nodule lobe (Fig. 2). The sites of emergence of the hyphae had the form of round protruding spots (ca. 200 μm diam.) at the surface of the nodules. In later stages, the hyphae proliferated around these spots, resulting in the typical fluffy colonies, which we designated as extranodular *Frankia* colonies (ENF). In some cases, ENF extended along the rootlets adjacent to the nodules, covering them up to 2–3 mm but apparently without penetrating the rootlet (Figs. 3 and 4). Sporangia in the ENF colonies exhibited a wide range of sizes and shapes (Fig. 6), much like those observed in pure cultures of *Frankia* from *Casuarina* [3] or other host plants (e.g. [9]). ENF vesicles were spherical (2.5–3.0 μm) and resembled typical vesicles obtained in vitro (Fig. 6). It is interesting to note that up to now typical spherical vesicles have never been observed inside the *Casuarina* nodules [11,12]. Fig. 5 shows that in the region invaded by ENF, rod-shaped particles were attached perpendicularly to the root hairs. We do not know the nature and the role of these particles. They could be bacteria-like contaminants or undetermined structures of *Frankia.* They were strikingly reminiscent of the 'exo-encapsulation threads' involved in the infection process of *Alnus* [7].

Some ENF colonies were carefully detached from the mother nodule and used to inoculate 10-week-old seedlings grown in N-free Hoagland solution. After 3–4 weeks, all the seedlings were heavily nodulated, indicating that *Frankia* from ENF colonies was infective.

4. CONCLUSION

Light microscope observations presented here suggest that, under the conditions of the experiments, *Frankia* was able to escape from some nodules as actively growing hyphae and to subse-

Fig. 2. Section through a nodule lobe showing the site of emergence (SE) of extranodular hyphae of *Frankia* (H). Heavily infected cells inside the nodule (arrow). Bar = 100 μm.

Fig. 3. ENF growing along roots adjacent to the nodules. Bar = 100 μm.

Fig. 4. Higher magnification of outlined area in Fig. 3 showing hyphae (H), spores-containing sporangia (S) on the root surface (R) of *C. equisetifolia.* Bar = 100 μm.

Fig. 5. Bacteria-like rods (arrow) perpendicularly attached to the surface of root hairs in an area invaded by ENF (out of focus in the picture). Bar = 10 μm.

Fig. 6. Micrograph montage showing hyphae (H), vesicles (V) and different types of sporangia observed in an ENF colony. Club-shaped (C), pear-shaped (P) and irregular-shaped (I) sporangia. Bar = 10 μm.

quently proliferate in the vicinity of the nodule and on the adjacent rootlets where it produced vesicles and spore-containing sporangia, structures considered to be specific to *Frankia*. Since no significant growth of *Frankia* was detected in other sites of the root system of *Casuarina equisetifolia*, this extranodular growth occurring spontaneously was thought to be distinct from the growth of *Frankia* in the rhizosphere, which is a consequence of the well-known rhizosphere effect. The latter type of growth has been recently reported by Lalonde et al. [8] after spot inoculation of the root systems of *Alnus*. The ability of *Frankia* to grow and sporulate outside the nodule probably contributes actively to the dissemination of this actinomycete in the soils, a problem already encountered by Houwers and Akkermans [6]. Since vesicles were abundant in the ENF colonies, it was inferred that free N_2 fixation could occur in the extranodular zone. Further experiments are planned to verify this hypothesis.

Interestingly enough, it was possible to harvest the *Frankia* structures that grew outside the nodule and to use them as an infective inoculum for *Casuarina equisetifolia*; this finding indicates that the *Frankia* from ENF colonies retained their infectivity, in contrast with the non-infective strains we have hitherto isolated from nodules of *Casuarina* using the serial dilution technique and synthetic media [3,4]. This last result raises the question as to the origin of deviating non-infective strains already posed by the Dutch group [2,10].

It is unknown at this point whether the extranodular growth of *Frankia* occurs in natural conditions or in host species other than *Casuarina equisetifolia*.

ACKNOWLEDGEMENTS

Additional financial support was provided by Délégation Générale à la Recherche Scientifique et Technique, Paris (Contrat LAT OG2). We thank M. Boureau for the photographs.

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