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短 報（Short communication）

Nodulation of *Alnus japonica* and *Casuarina equisetifolia* in liquid culture after inoculation with *Frankia*

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Abstract

Evaluation of infectivity and effectivity of different *Frankia* strains inoculated to *Alnus japonica* and *Casuarina equisetifoila*, in liquid culture system, was carried out. Plant materials of *A. japonica* and *C. equisetifolia* were grown in liquid culture (1/4-strength Hoagland's nutrient solution; pH 6.8; 100-ml glass bottles), and their roots were inoculated with strains of *Frankia*. Seedlings of *A. japonica* developed abundant root hairs and formed red swellings on lateral roots 1 week after inoculation with a *Frankia* strain isolated from a root nodule of *A. japonica*. The red swellings developed into root nodules. Rooted cuttings of *C. equisetifolia* formed root nodules 3 weeks after inoculation with *Frankia* strains isolated from root nodules of *Casuarina*. Nodule roots of *C. equisetifolia* grew upwards in the nutrient medium, exhibiting negative geotropism. These results demonstrated that liquid culture is a suitable method to evaluate nodule development over time by non-destructive observations. This method will be useful for further research on actinorhizal symbioses.

Key words : nodulation, water culture, *Alnus, Frankia*, *Casuarina*, negative geotropism

1. Introduction

Members of the genus *Frankia*, a soil-inhabiting actinomycete, infect roots of some woody dicotyledonous plants and induce the formation of nodules, which are specialized symbiotic organs that fix atmospheric nitrogen. These plants, known as actinorhizal plants (Baker and Schwintzer 1990, Yamanaka and Okabe 2008), are important for forestry, land reclamation, and natural ecosystems, and as a model for genetic engineering of plant–microbe symbioses. Therefore, much attention has been paid to actinorhizal symbioses (Torrey 1990, Benson and Dawson 2007).

Root-nodule microorganisms were first isolated and cultured from actinorhizal plants in 1978 (Callaham et al. 1978). Since then, much research has been focused on the biology of the actinomycete *Frankia* isolated from different habitats. *Frankia* isolates have been characterized based on their host specificity (the range of plants they infect), their mode of infection, and their efficacy in promoting growth of the host plant. Many studies have evaluated the effects of inoculating *Frankia* strains onto plant roots. In those studies, inoculations have been conducted using various procedures depending on the size and traits of plants and the facilities available.

Gibson (1987) described various systems for evaluating nodulation and nitrogen fixation by legumes. Based on the systems used to study legumes, actinorhizal plants have been cultivated using bottles or tubes (Smolander and Sundman 1987, Hilger et al. 1991), plastic growth pouches (Valverde and Wall 1999) and an aeroponics system in which plants were grown with roots bathed in nutrient mist (Zobel et al. 1976). Plants cultivated in pots or containers have also been used in studies on *Frankia* (Benoit and Berry 1990). Although root nodules develop well on plants grown in sand or other substrates (Zhang and Torrey 1985, Torrey 1990), it is difficult to observe nodule development directly in such systems. In contrast, liquid cultures allow visualization of nodule development over time. For liquid cultures, various vessels with different sizes have been used (Torrey 1990, Myrold 1994). Smolander and Sundman (1987) and Hilger et al. (1991) used small bottles (120- and 50-ml) for liquid cultures of *Alnus* to evaluate its nodulation capacity in soil. The advantage of using small bottles is that they are economical in terms of space, allowing many replicates for experiments. In spite of this, there have been no reports of nodulation trials using pure cultures of *Frankia* and plant

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materials in small bottles.

In this study, we evaluated nodulation and nitrogen fixation of some actinorhizal plants (*Alnus* and *Casuarina*) after inoculation with *Frankia* isolates. The plant materials were grown in liquid cultures in 100-ml glass bottles.

2. Materials and Methods

2.1 Preparation of plant materials

Two actinorhizal plants, *Alnus japonica* (Thunb.) Steud. and *Casuarina equisetifolia*, L., were used in these experiments. Seeds of *A. japonica*, from seed stock kept at the Forestry and Forest Products Research Institute, were soaked in running water for several days. The seeds were immersed in 0.5% (w/v) benomyl for 1 h, and then placed on a sheet of Kimwipes (Nippon Paper Crecia, Tokyo) to remove excess solution. The sterilized seeds were transferred aseptically to sterile Petri dishes containing 0.9% water agar medium. The Petri dishes were sealed with parafilm and incubated in a growth chamber at 28°C under continuous light, supplied by a fluorescent lamp (photosynthetic photon flux density = 124 µmoles/m^2/s). After germination, seedlings were transplanted onto 150 ml sterilized perlite in a 300-ml conical flask. To each flask, 90 ml sterile 1/4- strength complete Hoagland's nutrient solution, pH 6.7 (Hoagland and Arnon 1938) was added. Seedlings in the conical flask were grown in the growth chamber until they reached the two- or three-leaf growth stage.

For *C. equisetifolia*, shoot cuttings were prepared as described by Lundquist and Torrey (1984). The cuttings (6-cm long) were cut from shoot tips. The shoots were initially surface-sterilized by immersion in 95% ethanol for 1 min, then soaked in 1% sodium-hypochlorite solution for 5 min and washed five times with sterilized water. The sterilized shoots were planted in 500 ml sterilized pumice (particle size 2–3 mm in diameter: commercial name: Hyugatsuchi) moistened with 200 ml sterile distilled water in a lidded cylindrical glass bottle (14.5 cm deep, 10 cm inner diameter). The bottles were sealed with parafilm, and the lower part of the bottle was covered with aluminum foil to block the light. The bottles were placed in a growth chamber under the same conditions described above for *A. japonica*. Root development from the cuttings was evaluated by observing the materials from the side and the base of the bottle once a week.

2.2 Liquid cultures

The plant materials were cultured in 100-ml glass vials with polypropylene screw caps (SV-100, Nichiden-Rika Glass, Tokyo, Japan, Fig. 1). The vials were filled with sterile 1/4-strength complete Hoagland's nutrient solution (pH 6.8). A hole (12.5 mm diameter) was made in the lid of each vial, and the hole was closed with a silicone plug. To add plant materials to this system, the plug was cut in half and the plant material was sandwiched between the two

Fig. 1. Growth and nodulation of plant materials in liquid cultures. (a) *Alnus japonica*. Arrows show red swellings that developed after inoculation with *Frankia* strain AJ01 isolated from a root nodule of *A. japonica*. Red swellings developed into root nodules (see Fig. 3a). Photo was taken 8 days after *Frankia* inoculation. (b) *Casuarina equisetifolia*. Arrows show root nodules that formed after inoculation with *Frankia* strain CaE03 isolated from a root nodule of *C. equisetifolia*. Note upward growth of lateral roots and nodule roots (negative geotropism). Photo was taken 34 days after *Frankia* inoculation.

Table 1. Names and origins of *Frankia* strains used in this study.

Strain	Host plant	Location
AJ01	*Alnus japonica*	Tsukuba, Ibaraki, Japan
CaE01	*Casuarina equisetifolia*	Taketomi, Okinawa, Japan
CaE02	*C. equisetifolia*	Taketomi, Okinawa, Japan
CaE03	*C. equisetifolia*	Nago, Okinawa, Japan
T7	*C. cunninghamiana*	Ismailia, Egypt

halves. The plug was inserted into the hole in the cap, with the plant roots immersed in the nutrient solution. The vials were covered with a black plastic sheet to block the light and placed in the growth chamber. One week before inoculation with *Frankia*, the plant materials were transferred to vials containing sterile 1/4-strength nitrogen-free Hoagland's solution (Hoagland and Arnon 1938).

2.3 Inoculum preparation and inoculation technique

We used one *Frankia* strain to inoculate *A. japonica* seedlings and four strains of *Frankia* to inoculate *C. equisetifolia* (Table 1). All strains were cultured in N-free BAP liquid medium (Murry et al. 1984) in dark at 24°C for 4–6 weeks (AJ01), 2 weeks (CaE02, CaE03), 2–10 weeks (CaE01), or 60 weeks (T7). The 60-week-old *Frankia* culture was used to examine the nodulation capacity of old mycelia with spores, which are thought to be responsible for nodulation (Lalonde and Calvert 1979, Mansour and Torrey 1991). For inoculation, the cultures were homogenized at 10000 rpm (10 s) with an Ultra-Turrax mixer (TP 18/10S4, IKA, Staufen, Germany), and then poured into a 50-mL tube and centrifuged at 2320 *g* for 20 min to collect the hyphal fragments. The fragments were mixed with sterilized distilled water, centrifuged at 2320 *g* for 20 min, and the supernatant discarded. The procedure was repeated twice to wash the hyphal fragments. The plant materials sandwiched in the silicone cap were raised to remove the roots from the nutrient solution, and then 1 ml *Frankia* suspension (equivalent to 10 µl packed cell volume after centrifugation at 2320 *g* for 20 min), was applied to the roots.

2.4 Measurements

Observations of nodule formation were made regularly from 1 week after *Frankia* inoculation, and plants were harvested at 4 weeks after inoculation. Plant dry weight, nodulation, and acetylene reduction activity (ARA) were measured as described by Yamanaka et al. (2005). The t-test was used to examine the effect of *Frankia* inoculation on growth and ARA of *A. japonica* seedlings. One way of analysis of variance was to examine the effects of *Frankia* strains on the growth, nodulation, and ARA of *C. equisetifolia* cuttings.

3. Results

3.1 *Alnus japonica* nodulation

Roots of *A. japonica* seedlings formed abundant root hairs in liquid culture (Fig. 2a). One week after *Frankia* inoculation, red swellings (Fig. 1a) were observed along the roots of 9 out of 16 inoculated seedlings. These swellings developed into root nodules (Fig. 3a). All inoculated seedlings formed root nodules during the course of the experiment (Table 2). The average dry weight and ARA of nodulated seedlings were significantly higher than those of the control.

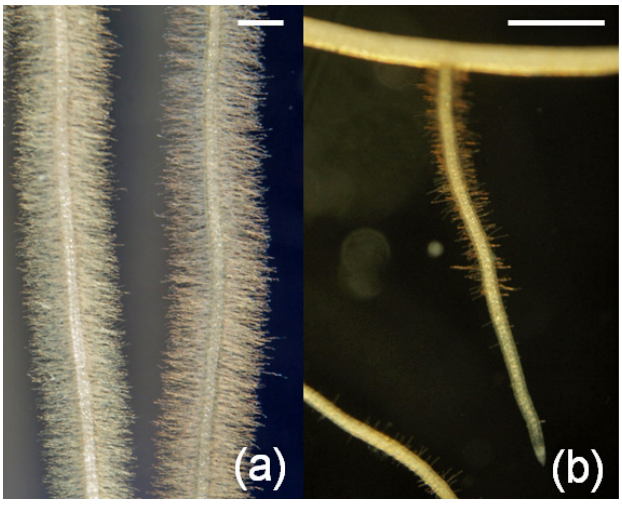

Fig. 2. Abundant root hairs on roots of *Alnus japonica* (a) and sparse root hairs on roots of *Casuarina equisetifolia* (b) in liquid culture. Scale bars = 1 mm.

Table 2. Nodulation and acetylene reduction activity (ARA) in *Alnus japonica* and *Casuarina equisetifolia* in liquid culture after inoculation with *Frankia*. Values are means (standard errors in parentheses). Means followed by different letters are significantly different at $P < 0.05$ (*t*-test).

Plant	Number of plants		Nodulation	No. of lobes/	DW of nodulated	ARA
Frankia strain	tested	nodulated	rate (%)	nodulated plant	plant (g)	(μmolC_2H_4/plant/day)
Alnus japonica						
AJ01	16	16	100	19.7 (2.5)	0.017a (0.002)	0.04a (0.01)
Uninoculated	13	0	0	—	0.010b (0.001)	0.00b (0.00)
Casuarina equisetifolia						
CaE01	20	16	80.0	11.7 (1.5)	0.039 (0.002)	0.19 (0.06)
CaE02	7	4	57.1	3.5 (1.0)	0.038 (0.002)	0.04 (0.04)
CaE03	13	9	69.2	9.9 (1.8)	0.043 (0.002)	0.34 (0.22)
T7	10	9	90.0	13.7 (2.4)	0.046 (0.003)	0.32 (0.15)
Uninoculated	14	0	0	—	0.038 (0.003)	0.00 (0.00)

3.2 *Casuarina equisetifolia* nodulation

Cuttings of *C. equisetifolia* developed roots with a few root hairs (Fig. 2b) in liquid culture. Some lateral roots on these cuttings grew upwards. Eight days after inoculation with T7, red nodules were observed on roots of *Casuarina* cuttings. Root nodules were observed 13 days after inoculation with CaE01 or CaE03, or 19 days after inoculation with CaE02 (Fig. 1b). The number of root lobes on nodulated *Casuarina* was not significantly different among the four *Frankia* strains (Table 2).

Casuarina nodules were typical *Myrica*-type nodules from which roots developed at the tip (Fig. 1b, 3b). These nodule roots grew upwards (Fig. 1b).

Only the nodulated cuttings showed ARA (Table 2); however, some had no ARA. There was no significant difference in dry weight between *Frankia*-inoculated and uninoculated *Casuarina* cuttings (Table 2).

4. Discussion

In these experiments, we used a simple and effective method to evaluate nodule formation and development in different host plants inoculated with various *Frankia* strains. This method did not require aeration, unlike that described by Torrey (1990), who reported that aeration of nutrient solutions in culture might be essential or desirable for seedling growth. In the present study, aeration of the solution appeared to be unnecessary, since *A. japonica* seedlings developed roots with abundant root hairs (Fig. 2a), which are required for successful root infection and nodule development (Zobel et al. 1976). Development of root hairs may be improved under anaerobic conditions because of accumulation of ethylene, which stimulates root hair development (Jackson 1985, Abeles et al. 1992).

In the present study, a 60-week-old culture of T7 effectively induced nodules on roots of *C. equisetifolia* (Table 2). The efficacy of this old inoculum in inducing nodulation may be related to the presence of spores, a characteristic feature of *Frankia* cultures (Mansour et al. 1990, Dewedar and Mansour 1992). Old cultures developed spores that germinated and formed hyphae when transferred to fresh medium (Tzean and Torrey 1989), thereby facilitating infection and nodule formation (Mansour and Torrey 1991). Burleigh and Torrey (1990) reported that *Frankia* spores were three orders of magnitude more infective than hyphae. That is, because the hypha is the infective tool, the fresh hyphae that germinated from spores were more infective than older hyphae.

In *Casuarina* cuttings inoculated with *Frankia*, root nodules formed and nodule roots grew upwards (Fig. 1b and 3b), exhibiting negative geotropism. The lateral roots also grew upwards (Fig. 1b). Upward growth of nodule roots, but not lateral roots, was reported previously (Bond 1956, 1957). One possible explanation for the upward growth of roots is that the gravitropism of the root could be disturbed by ethylene (Abeles et al. 1992), which accumulates in roots under

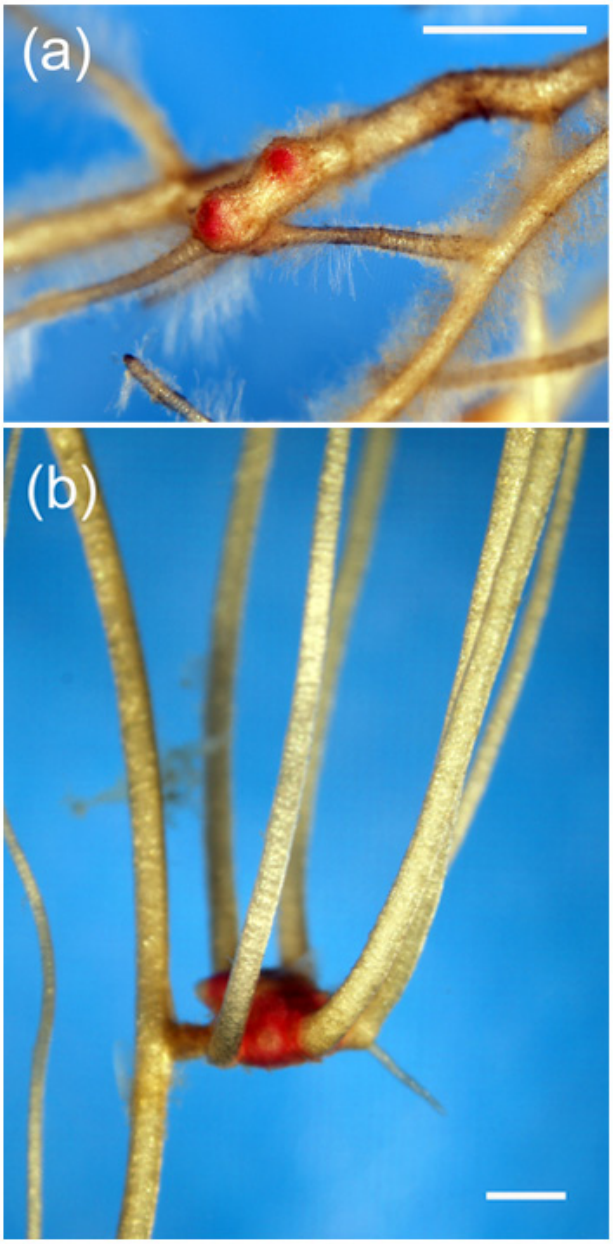

Fig. 3. (a) Root nodule in *Alnus japonica* that developed after inoculation with *Frankia* isolate AJ01. Photos were taken 28 days after inoculation. (b) Root nodule on roots of *Casuarina equisetifolia* showing nodule roots growing upwards. Nodules formed after inoculation with *Frankia* isolate CaE02. Photos were taken 34 days after inoculation. Scale bars = 1 mm.

anaerobic conditions (Jackson 1985).

The strong growth and nodulation shown by *A. japonica* in liquid cultures might be related to the fact that this species commonly grows in water-logged conditions (Kitamura and Murata 1979). Similarly, some alder species growing in wet soil (Farrar 1995) have been used as plant materials in liquid cultures because of their strong growth performance (Smolander and Sundman 1987, Smolander 1990). This characteristic of plants, that is, their ability to tolerate flooding stress, is an important attribute in selecting species for liquid cultures. As long as appropriate species are selected, liquid cultures would be useful for further studies on actinorhizal symbioses (Torrey 1990).

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水耕栽培でのハンノキおよびトキワギョリュウへの根粒菌フランキア接種による根粒形成

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要 旨

ハンノキおよびトキワギョリュウの根粒形成を水耕栽培にて観察した。ハンノキおよびトキワギョリュウの無菌苗を、100 mL のねじ口瓶に入れたホーグランド氏液（4 倍希釈、pH 6.8）にて育てた。ハンノキ苗の根系は根毛を豊富に形成し、フランキア根粒菌の接種後 1 週間目には赤色の肥大部が認められ、その後、根粒に発達した。一方、トキワギョリュウ苗は、菌の接種後 3 週間目に根粒を形成した。トキワギョリュウの根粒先端から伸長した根は養液中を上方に伸長し負の重力屈性を示した。このように水耕栽培は、根粒の形成を非破壊的に観察することが可能であり、様々な菌を用いた接種試験や栄養条件の根粒形成への影響などの実験に用いることができる。

キーワード：根粒形成、水耕栽培、ハンノキ、フランキア、モクマオウ、負の重力屈性

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