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The Response of a Free-Living and an Endophytic *Frankia* to Extreme Environmental Conditions

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Abstract

Growth of the free-living and N_2 fixation of the endophytic form of *Frankia* strain Ce4 were measured under various environmental conditions.

The maximum alkalinity for growth (protein production in a Tween, propionate, casamino acid-TPC nutrient broth) of the free-living form of *Frankia* was below pH 7.5. However, strain Ce4 was capable of fixing atmospheric nitrogen at pH 6.0–7.8 inside the root nodule of *Casuarina equisetifolia* Forst. raised on N deficient sand. The maximum electrical conductivity (EC) of TPC broth for the growth of the free-living form was less than 16 mS/cm. But, when living in symbiosis with the host, strain Ce4 was capable of fixing N_2 at soil water EC level of 18.4 mS/cm. The response to temperature of the free-living and the endophytic forms were essentially the same, the optimal temperature being 32° C.

It is concluded that *Frankia* is capable of fixing N_2 inside root nodules under conditions where the pH or salinity of the environment surrounding the root nodule is too high for the growth of the free-living *Frankia*. This indicates that the microsymbiont probably benefits from the symbiosis not only in a nutritional sense but also by receiving shelter against extreme soil conditions.

Keywords: *Casuarina*, *Frankia*, NaCl, pH, temperature

1. Introduction

Plant species which form nitrogen-fixing root nodules (actinorhizae) in symbiosis with the actinomycete *Frankia*, are called actinorhizal plants. The number of actinorhizal plant species exceeds 280. They are dicotyledons belonging to eight different plant families (Baker and Schwintzer, 1990).

Most actinorhizal plants are small shrubs. Only some tree species of the genera *Casuarina*, *Allocasuarina* and *Alnus* have significance in forestry. The family *Casuarina* consists of 4 plant genera and about 90 tree and shrub species. *Casuarina equisetifolia* Forst. (nomenclature follows Johnson, 1982) is one of the most widespread species among the family (Baker and Schwintzer, 1990).

All the *Frankia* strains isolated from actinorhizal plants so far are Gram positive, filamentous, spore-producing, branching actinomycetes. Sporangial size varies between 10–100 micrometers, and their shape is round, chain-like or very irregular. The hyphal diameter of *Frankia* is some 0.5–2 micrometers (Lechevalier and Lechevalier, 1990). Lignification of the host cell walls (Tjepkema et al., 1986; Berg and McDowell, 1988), which causes discontinuity of the intercellular air spaces inside the root nodule (Tjepkema et al., 1988; Zeng et al., 1989) is presumed to protect the nitrogen-fixing enzyme (nitrogenase) against oxygen, since root nodules of *Casuarina* lack the vesicles that are the sites of nitrogenase in other *Frankia* strains living in actinorhizae.

The carbon sources used by *Frankia* in symbiosis have not yet been identified, but short-chain fatty acids, intermediates of the tricarboxylic acid (TCA) cycle and pyruvate, are possible compounds (Tjepkema et al., 1986). In addition to carbon, the host also provides the microsymbiont with shelter against soil antagonists and removal of fixation products. The host plant also inhibits spore formation of the actinomycete in spore negative nodules with an unknown mechanism (Schwintzer, 1990).

The ammonium released by nitrogenase of the bacterium is assimilated in the plant through the action of glutamine synthetase or glutamine dehydrogenase to glutamine (Blom et al., 1981; Akkermans et al., 1983) and is transported in the vascular system to other plant compartments to serve as structural material for large amino acids and proteins. However, part of the amino acids accumulate in the nodule or are utilized by the actinomycete.

Bergersen (1982) has hypothesized that legume nodules provide not only nutrition to the microsymbiont but also provide shelter against extreme environmental conditions. The aim of this study has been to test this hypothesis for an actinorhizal plant by analyzing the response of a free-living and an endophytic form of *Frankia* to various environmental conditions.

2. Materials and Methods

Sources of seeds and Frankia

Seeds of *Casuarina equisetifolia* were obtained from Agam, Alexandria, Egypt. The selection of the *Frankia* strain Ce4 (Baobab Farm, Mombasa, Kenya) for the present study was based on earlier laboratory experiments (Miettinen and Smolander, 1989).

Germination of the seeds

The influence of soil water hydrogen ion concentration on the germination of the seeds of *C. equisetifolia* was studied at pH levels from 2.1 to 11.1. The pH of the water solutions was adjusted with KOH and H_2SO_4. Each solution (200 ml) was poured into Erlenmeyer flasks and about one hundred seeds were placed on the solution in each flask. The floating seeds were kept on the solutions for a period of 5 days at a temperature of 30° C. The solutions were replaced with fresh solutions daily. The number of germinated seeds were counted at the end of the experiment. The influence of environmental NaCl concentration on the germination of the seeds of *C. equisetifolia* was studied at electrical conductivity (EC) levels from 0.03 to 22.5 mS/cm. The EC of the water solutions was adjusted with NaCl. Two hundred ml of each solution were poured into Erlenmeyer flasks and about one hundred seeds placed on the solutions. The floating seeds were kept on the solutions for a period of 1 week (EC levels of 0.03 to 10.0) to 3 weeks (EC levels of 12.0 to 22.5) at a temperature of 30° C. The flasks were sealed with plastic caps to prevent evaporation. The number of germinated seeds were counted at the end of the experiment.

Surface sterilization of seeds and inoculation of seedlings

Seeds were immersed in a solution of 30% H_2O_2 for 20 min, and rinsed with sterile water (Sellstedt and Winship, 1987). The *Frankia* inocula were grown in a Tween, propionate, Casamino acid medium (TPC) (Weber et al., 1988) at 32° C for 1 to 5 months. The cells were harvested by centrifugation (500×g, 1–10 min), washed and resuspended in sterile water. The suspension was subsequently homogenized by repeated flushing through an injection needle, and then dispensed at 1 ml doses to each seedling at the age of 2–6 weeks. Uninoculated seedlings were left as controls.

Growth substrate and raising conditions

White, plastic containers were filled with *Frankia*-free quartz sand (particle diameter 0.5–1.2 mm) and watered with nutrient solution (Huss-Danell, 1978) supplemented with starter nitrogen at the beginning. The seedlings were subsequently watered with nitrogen-free nutrient solution. In the greenhouse of Hyytiälä Forestry Field Station the length of the day varied from 12–19 hr, and the maximal solar radiation within a single day ranged from 150–800 $\mu molm^{-2}s^{-1}$. The relative humidity of the air (RH) varied between 40 and 100%, and the air temperature (T) fluctuated between 20 and 35° C during the experiments. Both RH and T were measured continuously using a Lambrecht hydrothermograph (type 252). In the laboratory, the metal halide lamps (Osram HQI-T, 400 W) were adjusted to a day length of 19 hr. Air relative humidity was ca. 100%.

Adjustment of soil temperature, pH and EC

The seedlings for the temperature experiment were raised in containers immersed in water baths under laboratory conditions. Temperature levels of 15 and 20° C were maintained using Lauda (K2RD, 1 kW) refrigerator. The other temperature levels were maintained by means of thermostatic heaters (Jäger, 300 W, accuracy ±0.7° C). The temperature inside the seedling containers was measured continuously with a copper-constantan thermocouple (Honeywell). The seedlings for the pH and EC experiments were raised in greenhouse. Diluted (1:4), nitrogen-free nutrient solutions was adjusted to the desired pH levels (Table 1) by adding suitable proportions of K_2HPO_4 and NaH_2PO_4. The combined concentration of the phosphates at each pH level was 1.67 g/l.

Table 1. The pH of the nutrient solutions used as treatments for *C. equisetifolia*, and the pH of the soil water at the end of the six month experiment. The standard error is given in parenthesis (the number of replicates was 10 at each treatment).

pH of the nutrient solution	pH of the soil water
5.5	6.0 (0.07)
6.2	6.3 (0.05)
6.5	6.5 (0.04)
6.9	6.9 (0.04)
7.4	7.3 (0.04)
7.7	7.5 (0.04)
8.0	7.7 (0.09)
8.3	7.8 (0.11)

Table 2. Sodium chloride content and electrical conductivity (EC) of the nutrient solutions used as treatments for *C. equisetifolia*. The standard error is given in parenthesis (the number of replicates was 10 at each treatment).

NaCl content of the nutrient solution, %	EC of the nutrient solution, mS/cm	EC of the leached nutrient solution, mS/cm
0	0.5	0.6 (0.04)
0.05	1.2	1.7 (0.14)
0.13	2.5	3.8 (0.33)
0.25	3.9	5.6 (0.25)
0.35	5.6	6.9 (0.20)
0.50	7.5	7.2 (0.13)
0.75	8.2	9.5 (0.14)
1.00	10.0	11.7 (0.16)
1.25	11.6	14.1 (0.23)
2.00	18.0	18.4 (0.17)

The EC level of the nutrient solution was adjusted with NaCl (Table 2). The treatments were repeated twice during the first two months, and thereafter fortnightly. In addition, the seedlings were given pure water every day with mist sprinklers to compensate for evapotranspiration. The pH of the soil water running out of the bottom holes of the seedling containers was measured using a Metrohm 632 pH meter. The EC of the soil water was measured using a Beckman Conductivity Bridge (constant 0.01 cm^{-1}).

Measurements of nitrogen fixation of endophytic Frankia

After the treatment period, the total dry mass of the seedlings (65° C, 48 hr) was measured. The mean nitrogen concentration (mg/g) of the inoculated (5–7 plants) and uninoculated seedlings (5–7 plants) was determined by the Kjeldahl method. The amount of symbiotically fixed nitrogen (mg/plant) was estimated as the difference between the mean nitrogen content (dry mass multiplied by the nitrogen concentration) of the inoculated seedlings and the mean nitrogen content of unnodulated control seedlings (Bergersen, 1980).

Measurements of growth of the free-living Frankia

The inoculum of *Frankia* was grown for one month in TPC medium at 28° C. The cells were harvested by centrifugation (5000 rpm/12 min), washed three times and resuspended in sterile water. The suspension was subsequently homogenized by repeated flushing through an injection needle (0.08×60 mm),

with a final flushing through a 0.5×16 mm needle, and then dispensed at 0.5 ml doses into test tubes containing 8 ml TPC medium (Weber et al., 1988). Four replicates were incubated at each pH, temperature and NaCl treatment. The pH levels were modified by altering the phosphate buffer, and the combined concentration of K_2HPO_4 and NaH_2PO_4 was 1.67 g/l. The phosphates were added to the medium after separate sterilization. The pH of the medium did not vary by more than 0.2 pH units during the incubation. The growth of *Frankia* was assessed according to Smolander et al., (1988). The cells were washed three times with distilled water and stored at −18° C. Frozen cells were suspended in 2 ml 0.1 N HCl, kept in boiling water for 30 min, and then sonicated for 30 sec. The sonicated suspension was centrifuged (300×g, 12 min), and the protein content of the supernatant measured by the Coomassie brilliant blue G 250 method (Bradford, 1976) using bovine serum albumin as standard.

Analysis of data

The data were analyzed using a SAS nonlinear regression procedure. The program produces least-squares estimates of the parameters of a nonlinear model. It also lists nonlinear least squares summary statistics including regression (SSr), residual and total (SSt) sum of squares. In addition, the program calculates and asymptotic 95% confidence interval for the estimate of the parameter (Gallant, 1987). Calculation of the standard error (SE) for nitrogen fixation is based on the standard errors of the nodulated and nonnodulated seedlings.

3. Results

Growth requirements of the Frankia *isolate*

Frankia isolate Ce4 grew (produced protein) at temperatures of 19.7–32.0° C (Fig. 2a), and at pH 6.4 and 6.9 (Fig. 1a). Maximal growth of the isolate occurred at 32.0° C. At an EC level of 9 mS/cm the growth was clearly inhibited (Fig. 3a). No growth was observed under conditions, where the temperature of the growth medium was 14.4° C and 37.4° C, or at pH 4.2, 5.3 and 7.5, or at EC above 16 mS/cm.

Influence of soil conditions on the endophytic nitrogen fixation

All inoculated seedlings grown at different pH levels were nodulated by the end of the experiment. The hydrogen ion concentration had no clear effect on dry mass production (Fig. 1d), or on actinorhizal nitrogen fixation (Fig. 1c) of *C. equisetifolia* within the examined range.

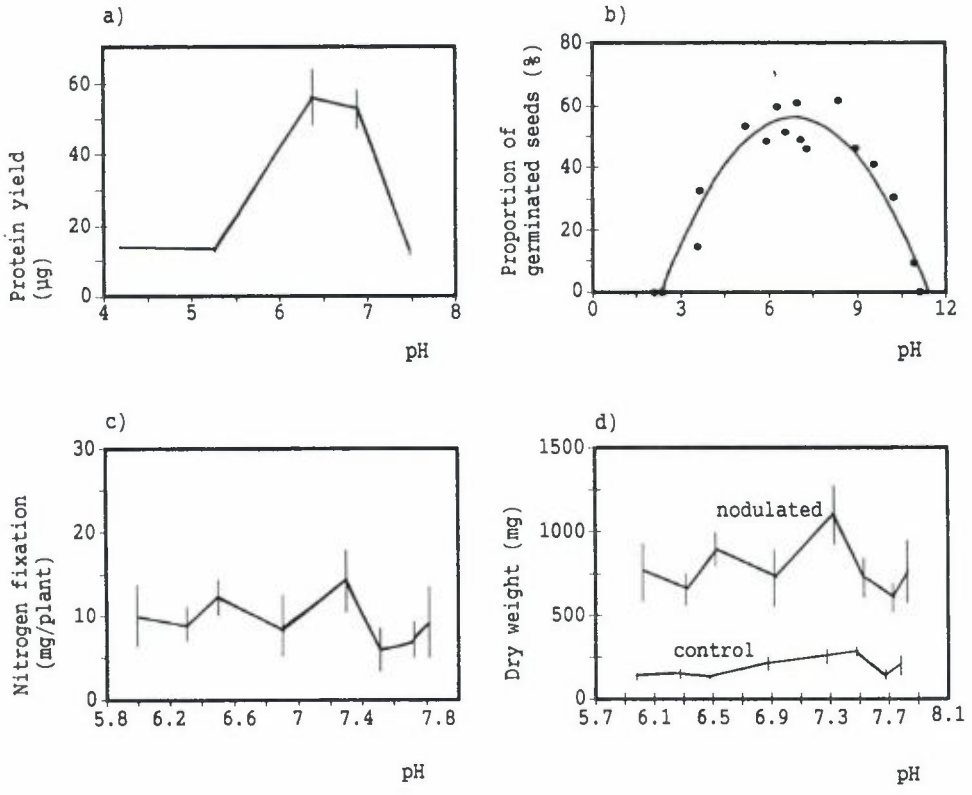

Figure 1. Effect of pH on *Frankia* and *C. equisetifolia*

a. Growth of *Frankia* isolate Ce4 on TPC medium, buffered with K_2HPO_4-NaH_2PO_4, incubated at 28° C for 23 days. The arrow indicates the size of the inoculum. Values are means ±SE, n = 4.

b. Proportion of germinated seeds of *C. equisetifolia*. The seeds were allowed to float on solutions adjusted to different pH values for a period of 5 days.

c. Nitrogen fixation of endophytic *Frankia* isolate Ce4 estimated by the difference between the mean nitrogen content of the nodulated plants (5 replicates) and the mean nitrogen content of the control plants (5 replicates). Values are means ±SE. Treatment period was 187 days.

d. The average dry weight of *C. equisetifolia*. Values are means ±SE, n = 5.

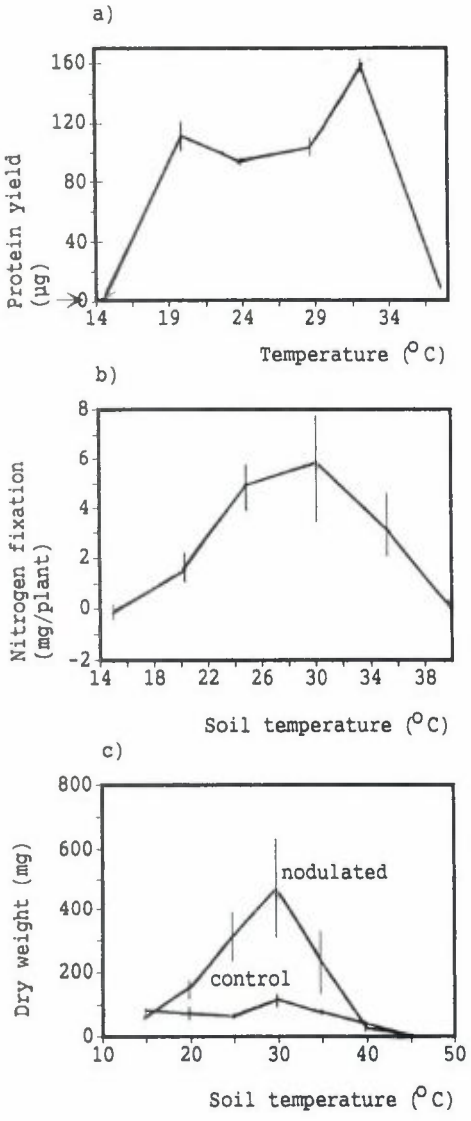

Figure 2. Effect of temperature on *Frankia* and *C. equisetifolia*.

a. Growth of the *Frankia* isolate Ce4 (30 day incubation period) on TPC medium at pH 6.7. The arrow indicates the size of inoculum. Values are means ±SE, n = 4.

b. Nitrogen fixation of endophytic *Frankia* isolate Ce4 estimated by the difference between the mean nitrogen content of the nodulated plants (7 replicates) and the mean nitrogen content of the control plants (7 replicates). Values are means ±SE. Treatment period was 137 days.

c. The average dry weight of *C. equisetifolia*. Values are means ±SE, n = 7.

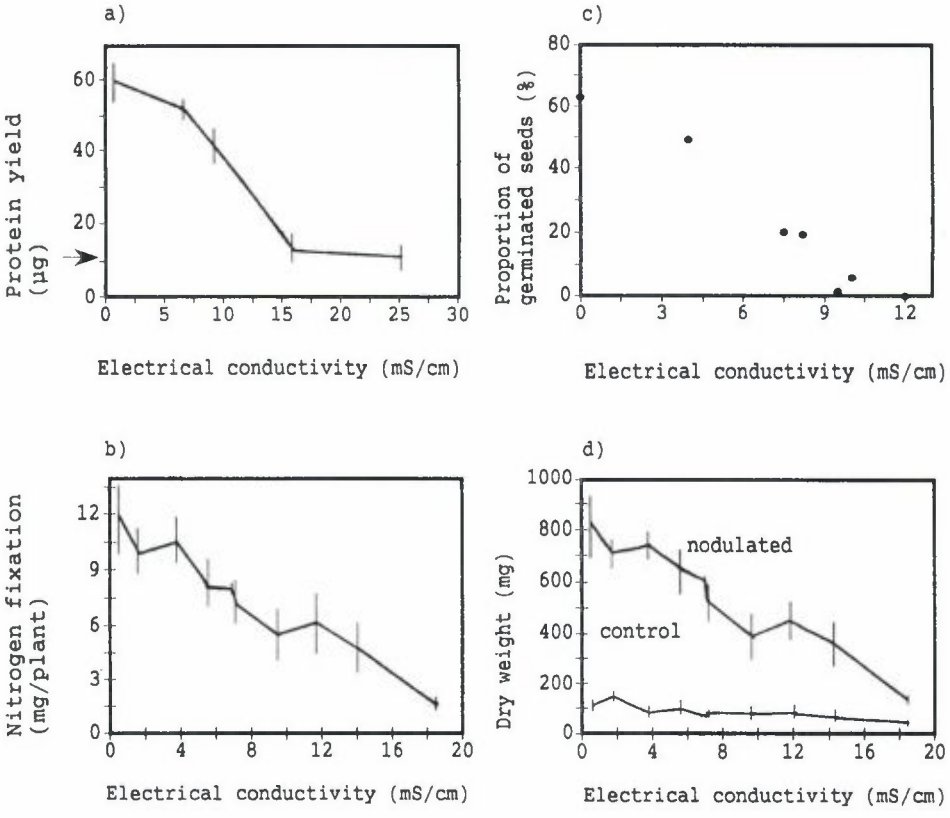

Figure 3. Effect of EC on *Frankia* and *C. equisetifolia*.

a. Growth of *Frankia* isolate Ce4 on TPC medium, pH 6.7, incubated at 28° C for one month. The arrow indicates the size of inoculum. Values are means ±SE, n = 4.

b. Nitrogen fixation of endophytic *Frankia* isolate Ce4 estimated by the difference between the mean nitrogen content of the nodulated plants (5 replicates) and the mean nitrogen content of the control plants (5 replicates). Values are means ±SE. Treatment period was 187 days.

c. Proportion of the germinated seeds of *C. equisetifolia*. The seeds were allowed to float on solutions adjusted to different EC values for a period of 5 days.

d. The average dry weight of *C. equisetifolia*. Values are means ±SE, n = 5.

All inoculated seedlings raised at 20–30° C formed root nodules during the experiment, whereas no nodulation occurred at 40° C or 45° C. At 15° C only 43% of the inoculated seedlings were nodulated by the end of the experiment. The maximal dry mass production of the inoculated seedlings occurred at 30° C (Fig. 2c). The average dry weight of the inoculated seedlings at this temperature was 0.47 g (SE=0.16), which was about five times greater than that of the uninoculated seedlings.

The amount of actinorhizal nitrogen fixation showed a bell-shaped regression with soil temperature (Fig. 2b). The soil temperature explained 98.6% of the variation in the actinorhizal nitrogen fixation ($(SSr/SSt)^2 = 0.9862$, df = 3. Maximal actinorhizal nitrogen fixation occurred between 25° C and 35° C and no nitrogenase activity was detected at 15° C and 40° C. According to the statistical model, the actinorhizal nitrogen fixation reached its maximum at 28.7° C.

The seeds of *C. equisetifolia* were able to produce primary roots between pH 3.6 to 10.9. A parabolic regression emerged between the pH of the medium and germinination of the seeds (Fig. 1b). pH explained 99.4% of the variation in seed germination $(SSr/SSt)^2 = 0.9940$, df = 14). The optimal pH levels for seed germination occurred between 4.5 and 8.0

All the inoculated seedlings grown at different electrical conductivity (EC) levels were nodulated by the end of the experiment. The unnodulated control seedlings did not show any notable response to soil salinity after 186 days, but dry mass production (Fig. 3d), and actinorhizal nitrogen fixation (Fig. 3b) of the inoculated *C. equisetifolia* seedlings decreased with increasing soil salinity.

Electrical conductivity of the soil water explained 94% ($r^2 = -94$, df = 8 of the variation in actinorhizal nitrogen fixation. The average amount of fixed nitrogen ranged from 1.6 (EC level 18.4 $mScm^{-1}$) to 11.9 (EC level 0.9 $mScm^{-1}$) mg per plant.

Germination of the tde seeds was also strongly affected by the NaCl concentration of the environment. The electrical conductivity of the environment explained 96% ($r^2 = 0.96$) of the variation in the germination rate of the seeds (Fig. 3c) at EC 0 to 12 $mScm^{-1}$. The maximal germination percentage (63%) of the seeds occurred at the lowest EC investigated, 0.03 $mScm^{-1}$. The seeds were unable to germinate above an EC of 11.2 $mScm^{-1}$.

4. Discussion

The ability of *Frankia* to live inside the root nodule is probably an important benefit for the bacterium in the competition among soil micro-organisms, especially under extreme soil conditions. In the present study, free-living *Frankia*

was unable to grow if the pH of the nutrient solution was above 7.5, which supports the report by Zhang et al. (1986). However, actinorhizal nitrogen fixation occurred and *C. equisetifolia* grew well at soil water pH levels of 6.0–7.8, and Mwanza (1990) has reported actinorhial nitrogen fixation by *C. equisetifolia* at a pH of 8.7 in a nursery experiment in Kenya. In addition, the pH range for the germination of the seeds of *C. equisetifolia* varied from 3.6 to as high as 10.9 in the present study. The pH of the sap of many plant species is almost completely independent of the soil pH, varying within a range of about 5–5.5 (Mengel and Kirkby, 1979). Thus, the detection of symbiotic nitrogen fixation in actinorhizae when soil alkalinity exceeds the pH tolerance range of *Frankia* could be explained by the protective effect of the root nodule.

EC levels as low as 9 mS/cm clearly inhibited the growth of *Frankia* stain Ce4, and no growth was observed at EC of 16 mS/cm. This supports the results reported by Dawson and Gibson (1987, cf. also Shipton and Burggraaf, 1982). In spite of its sensitivity to salinity, *Frankia* strain Ce4 was able to infect the host at a nutrient solution EC of 18.4 $mScm^{-1}$ and to form nitrogen-fixing actinorhizae. Ng (1987) has reported actinorhizal nitrogen fixation by *C. equisetifolia* at a level of as high as 500 mM NaCl (corresponding to 2.9% NaCl, or EC value 21 $mScm^{-1}$), and El-Lakany and Luard (1982) have observed survival of *C. equisetifolia* at the same level of soil salinity.

These results seem to indicate that the presence of the host enables *Frankia* to fix N_2 inside root nodules under conditions where the pH or salinity of the soil water are too high for the growth of free-living *Frankia*. The microsymbiont thus seems to benefit from the symbiosis not only in a nutritional sense but also by receiving shelter against unsuitable environmental conditions. This viewpoint has earlier been emphasize for leguminous symbiosis by Bergersen (1982).

In the present study, nitrogen fixation of an endophytic *Frankia* was determined by measuring the amount of nitrogen accumulated in the tissues of *C. equisetifolia*. The accuracy of the measured values is unclear because an unknown amount of fixed nitrogen is lost, due to senescence of plant organs before the measurements. The uninoculated seedlings may have also utilized more nitrogen from the growth substrate than the inoculated seedlings (Witty, 1983), which would result in underestimation of the amount of symbiotically fixed nitrogen. According to Gauthier et al. (1985), the relative fertilizer uptake of nitrogen-fixing seedlings of *C. equisetifolia* is 4.1% and that of non-nitrogen-fixing seedlings is 8.8%.

The nitrogen concentration of the inoculated seedlings varied from 11.3 to 16.3 mg/g in the present study. The nitrogen concentration of the control

seedlings ranged between 3.9 and 10.9 mg/g. Nitrogen concentrations of *C. equisetifolia* determined by Gauthier et al. (1981), Reddell and Bowen (1985), Rosbrook and Bowen (1987) and Sellstedt (1988), fit in the presented range. The amount of symbiotic N_2 fixation was less than 15 mg/plant (Figs. 1c, 2b, 3b), which is slightly less than the amounts reported by Reddell and Bowen (1985) and Rosbrook and Bowen (1987) for slightly larger seedlings of *C. equisetifolia.*

A temperature of 37° C was too high for the growth of *Frankia* strain Ce4 (Fig. 2a). This is in agreement with observations reported earlier by Zhang et al. (1986) and Shipton and Burggraaf (1983). *C. equisetifolia* ceased to grow (Fig. 2c) and no actinorhizal nitrogen fixation was detected (Fig. 2b) at a slightly higher temperature. A temperature of 14.4° C was too low for the growth of *Frankia* strain Ce4 (Fig. 2a). This was about the same temperature as the minimum for actinorhizal nitrogen fixation. *C. equisetifolia* also suffered from chilling at a temperature of 15.0° C, which was observed as a marked decline in growth. These observations support the results reported by Reddell et al. (1985) concerning the influence of soil temperature on the growth of *Casuarina cunninghamiana.* The absence of nitrogen fixation at 15° C would be expected to limit the natural distribution of *C. equisetifolia* to areas where soil temperatures are below 15° C for the major part of the potential growing season.

The optimal temperature for the growth of the free-living *Frankia* strain Ce4 (Fig. 2a), the dry mass production of *C. equisetifolia* (Fig. 2c), and the functioning of actinorhizal nitrogenase (Fig. 2b), was close to 30° C. This indicates that the host, the microsymbiont, and the actinorhizal structure have almost the same thermal optimum.

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