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Growth requirements of Frankia strains isolated from Casuarina equisetifolia, and the influence of the isolates on the growth of the host plant

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SELOSTE: CASUARINA EQUISETIFOLIASTA ERISTETTYJEN FRANKIA-KANTOJEN KASVUVAATIMUKSET JA KANTOJEN VAIKUTUS ISÄNTÄKASVIN KASVUUN

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Frankia was isolated from the root nodules of *Casuarina equisetifolia* L. seedlings, grown in a growth chamber, after inoculation with soil originating from an old east Kenyan casuarina forest. Optimum pH for the growth of the two isolates ranged from 6.4 to 6.9. The optimum temperature for their growth was 32 °C. The growth of these cultures ceased at NaCl concentrations above 2 %. The influence of the isolates on the growth of the host plant was determined in a growth chamber experiment in which an American *Frankia* strain (HFPCcI3) was used as a reference. The biomass of the inoculated seedlings was 2.4 - 4.1 fold those of the non-inoculated control seedlings at the end of the 7-month experiment.

Tutkimuksessa eristettiin *Frankia*-viljelmä neljästä *Casuarina equisetifolian* juurinystyrästä. Kasvatuskaapissa kasvatetut isäntäkasvit oli siirrostettu itäafrikkalaisella kasuariinametsän pintamaalla. Eristetyt kannat kasvoivat parhaiten alustassa, jonka pH-arvoksi oli säädetty 6,4 - 6,9 ja kasvulämpötilaksi 32 °C. Yli 2 %:n NaCl-pitoisuuksissa viljelmien kasvu pysähtyi. Eristettyjen viljelmien vaikutus isäntäkasvin kasvuun määritettiin kasvatuskaappikokeella. Eristetyillä *Frankia*-kannoilla siirrostettujen taimien biomassa kehittyi 2,4 - 4,1 kertaiseksi kontrollitaimiin verrattuna.

Keywords: *Casuarina, Frankia*, growth response, nitrogen fixation.
ODC 181.351+176.1 *Casuarina*

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1. Introduction

The nitrogen-fixing actinomycete *Frankia* lives in symbiosis with more than 200 species of woody dicotyledonous plants (Lechevalier 1986). *Casuarina equisetifolia* L. is one of the most important of such actinorhizal species in tropical regions both ecologically and economically. It originates from Australia, but for centuries sailors have brought casuarinas to tropical coasts for ornamental purposes and to stabilize coastal sand dunes. Casuarina is also used as fuelwood and for construction work in the form of poles and beams (Advisory Committee on... 1984).

The first *Frankia* cultures were isolated in 1978 (Callaham et al. 1978). Owing to the economical importance of *Casuarina* species, *Casuarina - Frankia* symbiosis has been studied intensively (eg. Gauthier et al. 1981, 1985, Diem et al. 1982, Shipton and Burggraaf 1983, Zhang et al. 1984,1985, Reddell and Bowen 1985a,1985b,1986, Reddell et al. 1986, Ng 1987, Rosbrook and Bowen 1987, Sellstedt and Winship 1987, Reddell et al. 1988, Sellstedt 1988) and the growth requirements of *Frankia* isolates have been described (eg. Shipton and Burggraaf 1982, Zhang et al. 1985, 1986, Faure-Raynaud 1986). The first report of marked increases in the productivity of field-grown casuarina stands, resulting from inoculation with *Frankia*, has recently been published (Reddell et al. 1988).

The N_2-fixation capacity of actinorhizal root nodules is known to be dependent on the genotypes of both the host and endophyte (Fleming et al. 1987, Reddell and Bowen 1985b, Sougoufara et al. 1987), as well as on temperature (Reddell et al. 1985), moisture level (Sundström and Huss-Danell 1987), phosphorus content (Reddell et al. 1986), salinity (Ng 1987), and availability of micronutrients in the soil (Hewitt and Bond 1961, 1966).

Soil inoculum, collected from the top soil of an old casuarina stand in Gede, Kenya, has been used successfully for a number of years to improve the post-planting survival of casuarina at the Baobab Farm, South-eastern Kenya. These promising field trials encouraged us to study the *Frankia* population present in this inoculum.

The aim of the present study was (i) to isolate *Frankia* strains from nodules induced by soil from the Baobab Farm, (ii) to compare the growth response of casuarina seedlings inoculated with the isolates and a reference strain (HFPCcI3) isolated from *C. cunninghamiana*, U.S.A. (Zhang et al. 1984), and (iii) to investigate the influence of different pH, temperature and salinity levels on the growth of the *Frankia* isolates.

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Roles of the authors: PM carried out the field and laboratory work according to detailed advice provided by AS, and wrote the manuscript, which was then finalized jointly by the two authors.

2. Materials and methods

21. Selection of the host plants

A soil sample (about 500 g) was collected on 17 August 1986 at Northern Nursery, Baobab Farm, Mombasa, Kenya. The air-dry soil sample was stored for five months at room temperature (20 - 35 °C) sealed in a plastic bag before further studies in Finland.

The sample was mixed (1:2) with sand (particle diameter 0.5 - 1.2 mm) and vermiculite . The mixture was divided into two 2 - 1 jars (250 g of inoculum in each) in which the pre-germinated *Casuarina equisetifolia* seeds were sown. The absence of *Frankia* contamination on the seed was checked in a simultanous growth chamber experiment. The seed was of local Mombasa origin (earlier introduced from Gede).

The seedlings were grown in a growth chamber (PAR 160 - 200 $\mu molm^{-2}s^{-1}$, day length 12 h, relative humidity 10-30 %, day temperature 28 - 30 °C, night temperature 15 - 17 °C).

Unidentified plant pathogens caused the death of several seedlings during the following weeks.

The first root nodule was found 14 weeks after sowing. One week later, the seedlings were removed to individual 100 ml test tubes containing the sand-vermiculite mixture. After four weeks the tubes were stoppered and the nitrogenase activity of the seedlings estimated as acetylene reduction (Hardy et al. 1968) at an acetylene concentration of 10 % using 150 and 60 min incubation periods. The ethylene produced was measured using an Aerograph 200 gas cromatograph.

Seedlings no. 23 and 26 were selected for the isolation procedure owing to their high momentary nitrogenase activity, whereas seedlings no. 13, 19 and 22 were selected due to their vigorous overall appearance (Table 1).

Table 1. Nitrogenase activity of the inoculated *Casuarina equisetifolia* seedlings at the age of 20 weeks expressed as the amount of reduced acetylene (mean, n=2).

Taulukko 1. Casuarina equisetifolian *nitrogenaasiaktiivisuus 20 viikon iässä ilmaistuna juurinystyröiden pelkistämän asetyleenin määränä (keskiarvo, n=2).*

Seedling, no.	Acetylene reduction ($nmolC_2H_4xh^{-1}$)	Isolated culture(s)
1	310	
2	415	
3	95	
4	443	
5	189	
6	166	
7	167	
8	89	
9	106	
10	228	
11	294	
12	179	
13	244	Ce1, Ce2
15	222	
16	156	
17	489	
18	361	
19	528	
20	533	
21	180	
22	244	
23	604	Ce3
24	334	
25	472	
26	734	Ce4

22. Isolation of *Frankia*

Excised root nodules of the selected host plants were surface sterilized by soaking in 1 % OsO_4 for 1 - 6 minutes (Lalonde et al. 1981) and washed five times in sterile water before being chopped up using tweezers. Pieces of root nodule were aseptically transferred to PAC and TPC (supplemented

with glucose) culture medium (Weber et al. 1988).

TPC medium contained

K_2HPO_4	300 mg
$NaH_2PO_4 \times 2H_2O$	260 mg
$MgSO_4 \times 7H_2O$	200 mg
$CaCl \times 2H_2O$	10 mg
NH_4Cl	100 mg
Na-FeEDTA	10 mg
biotin	2 mg
Tween 80	1 ml
casaminoacids	500 mg
Na-propionate	500 mg
glucose	1000 mg
H_3BO_3	1.5 mg
$MnSO_4 \times 7H_2O$	0.8 mg
$ZnSO_4 \times 7H_2O$	0.6 mg
$CuSO_4 \times 7H_2O$	0.1 mg
$(NH_4)_6Mo_7O_{24} \times 4H_2O$	0.2 mg
$CoSO_4 \times 7H_2O$	0.01 mg
distilled water	1000 ml

The PAC medium was similar to TPC except that it contained Na-acetate 2500 mg and glucose 1000 mg instead of Tween 80. Both media were adjusted to a pH level of 6.7 - 7.00.

Over 100 isolation tubes were prepared. Seemingly pure cultures of actinomycetes were obtained in four tubes.

23. Influence of temperature, pH and NaCl on the growth of the isolates

Two of the *Frankia* isolates, Ce1 and Ce4, were 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 (0.8 x 60 mm), the final flushing through a 0.5 x 16 mm needle, and then dispensed at 0.5 ml doses into test tubes containing 8 ml TPC medium. Four replicates were incubated at each pH, temperature and NaCl treatment.

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 s. The sonicated suspension was centrifuged (3000 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.

The incubation temperatures investigated were 14.4, 19,7, 23.8, 28.6, 32.0 and 37.4 °C. The temperature did not change by more than 1.1 °C during the incubation period (18 days for Ce1, 30 days for Ce4).

The pH levels investigated were 4.2, 5.3, 6.4, 6.9 and 7.5. The desired pH levels were adjusted before autoclaving the TPC medium (without phosphates) by adding either HCl or NaOH to the medium. The sterile medium was buffered with suitable proportions of autoclaved K_2HPO_4 or $NaH_2PO_4 \times 2H_2O$. The combined concentration of the phosphates at each pH level was 1.67 g/l. The pH of the medium did not change by more than 0.2 pH-units during the experiment. The inoculated tubes were incubated for 23 days at 28 °C.

The NaCl concentrations investigated were 0 , 0.5 , 1 , 2 , 4 and 7 %. The test tubes, containing 10 ml of TPC, were incubated for one month at 28 °C. The cultures were aerated three times per week by whirling.

24. Influence of the isolates on the growth of the host plant

Casuarina equisetifolia seeds were surface-sterilized by soaking in 30 %-H_2O_2 for 20 minutes (Sellstedt and Winship 1987), and then sown in 450 ml plastic jars filled with a mixture of sand (particle size 0.5 - 1.2 mm) and vermiculite (sand/vermiculite = 6/1). The seedlings were fertilized once a week with full-strength nutrient solution supplemented with 0.36 mM NH_4NO_3 (Huss-Danell 1978). The plants were kept under controlled condition in a growth chamber with a photoperiod of 17/7 h, day/night temperature 25/15 °C; relative air humidity 75 %, PAR 200 $\mu molm^{-2}s^{-1}$ as Sellstedt and Winship (1987). At the age of 17 days eight replicate seedlings (one/jar) were inoculated with the isolates Ce1, Ce2, Ce3, Ce4 and the reference strain HFPCcI3 using a completely randomized lay-out . The inocula had been grown in 10 ml TPC medium at 28 °C for one month, washed, and then suspended in the nutrient solution and dispensed at 1.25 ml doses to each experimental seedling. In addition, eight seedlings were inoculated with the sterile nutrient solution to serve as controls. The first root nodule appeared five weeks later (inoculum Ce3). The height of the seedlings and their dry biomass (24 h/80 °C), biomass division ratio, root nodule mass and root nodule number was determined after 28 weeks.

3. Results and discussion

31. Growth requirements of the *Frankia* isolates Ce1 and Ce4

Maximal growth of the isolates occurred at 32.0 °C (Fig. 1). At this temperature isolate Ce4 raised the pH of the culture medium up to 8.1 during incubation. This probably inhibited growth. The effect of temperature on the growth of the isolates was in agreement with observations reported earlier (Zhang et al. 1986, Shipton and Burggraaf 1983).

pH levels of 6.4 and 6.9 were most favourable for the growth of *Frankia* (Fig. 2.). Zhang et al.(1986) have determined a pH curve for the growth of HFPCcI3, which is rather similar to that for isolates Ce1 and Ce4.

NaCl concentrations as low as 0.5 % clearly inhibited the growth of the *Frankia*

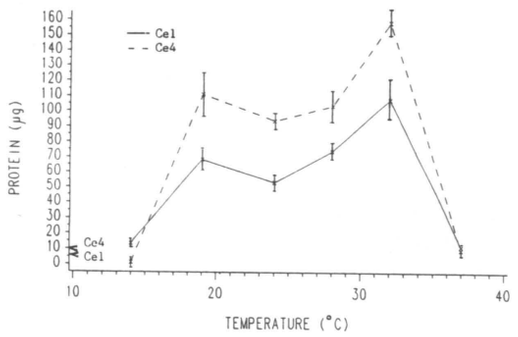

Figure 1. Effect of temperature on the growth of the *Frankia* isolates Ce1 (18 day incubation period) and Ce4 (30 day incubation period). The isolates were grown in TPC medium at pH 6.7. Arrows indicate the size of the inoculum, columns show standard error.

Kuva 1. Lämpötilan vaikutus Frankia - *kantojen Ce1 (18 vrk:n kasvatusaika) ja Ce4 (30 vrk:n kasvatusaika) kasvuun. Kannat kasvatettiin TPC-ravintoliemessä pH-arvossa 6,7. Nuolet osoittavat siirroksen koon, pylväät osoittavat keskiarvon keskivirheen.*

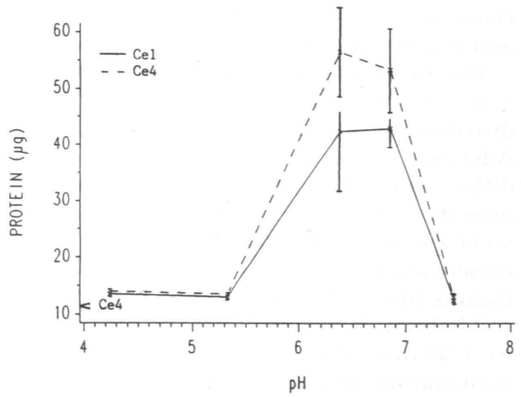

Figure 2. Growth of the *Frankia* isolates Ce1 and Ce4 at different pH levels (TPC medium, buffered with K_2HPO_4-NaH_2PO_4, incubated at 28 °C for 23 days). Arrow indicates the size of the Ce4 inoculum (Ce1-inoculum size was 6.0 µg), columns show standard error.

Kuva 2. Frankia *kantojen Ce1 ja Ce4 kasvu eri pH tasoilla (TPC-ravintoliemi, K_2HPO_4-NaH_2PO_4-puskuri, kasvatettu 28 °C:ssa 23 vrk). Nuoli osoittaa Ce4-kannan siirroksen koon (Ce1-kannan siirroksen koko oli 6 µg), pylväät osoittavat keskiarvon keskivirheen.*

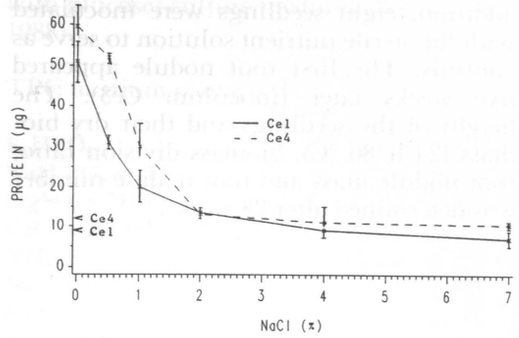

Figure 3. Growth of the *Frankia* isolates Ce1 and Ce4 at different NaCl concentrations (TPC medium, pH 6.7, incubated at 28 °C for one month). Inoculum size is indicated with an arrow, columns show standard error.

Kuva 3. Frankia *kantojen Ce1 ja Ce4 kasvu eri NaCl-pitoisuuksissa (TPC-ravintoliemi, pH 6.7, kasvatusaika 1 kk, kasvatuslämpötila 28 °C). Nuoli osoittaa siirroksen koon. Keskiarvon keskikivirhe on osoitettu pylväin.*

isolates investigated (Fig. 3). No growth was observed at NaCl concentrations above 2 %. This supports the results reported by Dawson and Gibson 1987 (cf. also Shipton and Burggraaf 1982).

The prevailing pH, salinity and temperature of the soil along the geographical distribution of *Casuarina* (Sillanpää 1982, Advisory Committee on... 1984) often differs from the optimal range for the growth of *Frankia* in pure culture. The metabolism of *Frankia* cells may be more closely adapted to the environmental conditions inside the root nodule than to the soil habitat. However, it may be misleading to conclude that the pH, salinity and temperature requirements of *Frankia* are the same in the soil or inside the root nodule as they are in artifical medium.

32. Effect of the *Frankia* isolates on the growth of *Casuarina equisetifolia*

The host plant growth responses achieved in a growth chamber experiment were promising. All the inoculated seedlings formed root nodules and differed significantly ($P<0.05$, df = 49, Tukey) from the non-inoculated control seedlings as regards their biomass and height (Table 2). The biomass increment achieved through inoculation in our experimental setup was 2.4 to 4.1 fold, which is slightly less than expected (Rosbrook and Bowen 1987, Reddell and Bowen 1985b). In addition, the biomass of the seedlings inoculated with the reference isolate HFPCcl3 was significantly higher than that of the seedlings inoculated with Ce3. Biomass and height of seedlings inoculated with the various Ce isolates did not differ significantly from each other.

Inoculation with isolate Ce1 caused the highest growth increase per root nodule mass (A/B-ratio; Table 2).

The average number of root nodules induced by the *Frankia* isolates ranged from 8.3 to 34.3 per plant, their average biomass varying between 56.3 and 110,8 mg (Table 2). These values are rather similar to those reported by Reddell and Bowen (1985b) and Rosbrook and Bowen (1987). The ratios between the biomass of the seedlings and the nodule biomass obtained in our study were to some extent higher than those reported by Sellstedt (1988).

The non-nodulated control seedlings allocated a relatively high proportion (42 %) of the carbohydrates to increasing their root biomass, whereas the inoculated seedlings allocated most of the carbohydrates to the leaves (57 % - 66 %, Table 2) - which are in accordance to observations reported by Sellstedt (1988).

The present study showed that the soil inoculum used at Baobab Farm contains efficient *Frankia* strains which can significantly improve the growth of *Casuarina equisetifolia* under growth chamber conditions. The *Frankia* population in the soil inoculum appears to be homogeneous as regards nitrogen fixation because different *Frankia* isolates induced similar growth responses to the host plants.

Table 2. Effect of a *Frankia* inoculum on the growth of *Casuarina equisetifolia* in a 7 months growth chamber experiment. Biomasses are given as dry weights, standard deviation is shown in parentheses, n=8.

Taulukko 2. Frankia-*siirroksen vaikutus* Casuarina equisetifolian *kasvuun 7 kk kestäneessä kasvatuskaappikokeessa. Biomassat on annettu kuivapainoina, keskihajonta on merkitty suluissa, n=8.*

Characteristic	Inoculum					
	Ce1	Ce2	Ce3	Ce4	HFPCcl3	control
Shoot height, cm	30.7 (6.1)	27.4 (5.0)	24.6 (6.0)	28.2 (4.2)	31.0 (4.8)	16.4 (2.4)
Biomass of the seedling, g	3.60 (1.0)	3.03 (0.4)	2.65 (0.7)	3.42 (0.8)	4.36 (1.1)	1.09 (0.1)
A: Increase in biomass as compared to control seedlings, g	2.5	1.9	1.6	2.3	3.3	0
Biomass division ratio of root, %	25.7 (6.9)	26.8 (4.5)	32.0 (11.4)	30.9 (10.1)	23.3 (4.6)	42.0 (4.2)
Biomass division ratio of stem, %	12.3 (5.1)	10.5 (3.2)	12.8 (6.7)	10.1 (3.1)	11.0 (3.7)	11.4 (3.2)
Biomass division ratio of leaves, %	62.0 (17.1)	62.8 (10.5)	56.7 (8.1)	59.1 (16.2)	65.7 (20.6)	46.6 (3.6)
Number of root nodules	34.3 (7.6)	30.0 (9.1)	12.6 (8.6)	8.3 (4.2)	18.8 (13.4)	0 (0)
B: Biomass of the root nodules, mg	65.3 (47.0)	56.3 (26.0)	92.5 (47.0)	106.4 (49.3)	110.8 (42.1)	0 (0)
Growth increase (A) / Root nodule biomass (B) - ratio	38.3	33.7	17.3	21.6	29.5	0

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Total of 35 references

Seloste

Casuarina equisetifoliasta eristettyjen Frankia-kantojen kasvuvaatimukset ja kantojen vaikutus isäntäkasvin kasvuun

Työssä tutkittiin mikrobiologisin menetelmin itäkenialaisella Baobab-maatilalla käytetyn maasiirroksen *Frankia*-populaatiota. Pieni erä (n. 500 g) maasiirrosta, jota on jo vuosia käytetty varmistamaan kasuariinaviljelmien symbioottisen typensidonnan toimivuus, tuotiin Suomeen syksyllä 1986. Maanäytteeseen istutettiin *Casuarina equisetifolia*n taimia, jotka kasvatettiin kontrolloiduissa kasvatuskaappiolosuhteissa. Neljästä taimien juuriin muodostuneesta juurinystyrästä eristettiin *Frankia*-viljelmä. Eristystyöhön otetut taimet valikoitiin hyvän ulkonäön ja korkean hetkellisen nitrogenaasiaktiivisuuden perusteella (taulukko 1).

Frankia-viljelmien symbioottinen tehokkuus määritettiin kasvatuskaappikokeella, jossa kullakin viljelmällä siirrostettiin 8 *Casuarina equisetifolia*n tainta. Lisäksi kokeessa verrattiin kenialaisten *Frankia*-viljelmien tuottamaa kasvuvastetta vertailukannan HFPCcI3 (isäntäkasvi *C. cunninghamiana*) tuottamaan kasvuvasteeseen sekä siirrostamattomiin kontrollitaimiin. Taimet kasvatettiin typpiköyhässä hiekka-vermikuliittiseoksessa.

Seitsemän kuukautta kestäneen kasvatuksen jälkeen kontrollitaimet olivat jääneet merkitsevästi siirrostettuja taimia pienemmiksi. Kenialaisten *Frankia*-viljelmien tuottamat kasvuvasteet eivät poikenneet merkitsevästi toisistaan, vertailukanta HFPCcI3 osoittautui kuitenkin heikointa kenialaista kantaa merkitsevästi tehokkaammaksi (taulukko 2).

Kahden *Frankia*-viljelmän kasvun lämpötilaoptimiksi saatiin 32 °C (kuva 1). pH-optimi vaihteli 6.4 ja 6.9:n välillä (kuva 2) Yli kahden prosentin natriumkloridipitoisuudessa tutkittujen kantojen kasvu pysähtyi (kuva 3).