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Survival of *Frankia* Strains under Different Soil Conditions

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Abstract: Some factors affecting the establishment of *Casuarina-Frankia* symbioses were studied by following the survival of some *Frankia* strains exposed to different environmental soil conditions. This was accomplished by examining the effect of soil desiccation at 30°C temperature or to intermittent exposure of desiccated soil to 50°C (3h on three consecutive days) and different soil pHs on nodulation. Six *Frankia* strains were isolated from different *Casuarina* plantations and were used in this study. *Frankia* survival, was measured as the persistence of nodulation capacity using the plant infection technique. The survival of introduced *Frankia* fell markely in soils undergoing drying. The extent of decline varied with strain and soil. The decrease in numbers for all strains after desiccation was greater in reclaimed soil than other soils tested. The survival of all *Frankia* strains in response to desiccated soil was higher with intermittent exposure to 50°C than when desiccated at 30°C. The survival of introduced *Frankia* varied with adjusted soil pHs. Survival was often higher at pH 8, but usually was adversely affected by higher pH (pH 8.5). Recovery of introduced *Frankia* into autoclaved soils, exposed to different treatments, was significantly higher than non-autoclaved soil.

Key words: Actinorhizal plants, *Casuarina* species, *Frankia* survival, nodulation capacity, persistance, pH, temperature

Introduction

In Egypt, genus *Casuarina* is the only actinorhizal plant species capable of forming root nodules in symbioses with nitrogen -fixing filamentous soil bacteria (*Frankia*). Results obtained by many researchers (El-Lakany, 1983; Mansour and Baker, 1994; Safwat ***et al.***, 1994; Mansour ***et al.***, 1996) have encouraged the use of these actinorhizal plants in newly reclaimed soil as pioneer species for soil fertilization and as well as many other purposes. As the Egypt's population approaches 100 millions in the next five years, more lands will need to be reclaimed; therefore, expansion of agriculture is considered a major goal in our country, to meet the demand for food. *Casuarina* trees are in request in agroforestry systems that can be used for soil improvement.

In order to maximize the usefulness of *Casuarinas*, as actinorhizal nitrogen-fixing trees in agroforestry systems, for this purpose, it may necessary to introduce proper strains of *Frankia* into the soil. Introduction of *Frankia* strains to Egyptian soil has to be under intensive studies to select the best strains for best survival rate under our Egypt's extreme environmental conditions. Poor survival of introduced symbiotic N_2-fixing filamentous soil bacteria (*Frankia*) to soil leads to unsatisfactory nodulation of *Casuarina* plantations resulting in and reducing tree productivity and

survival rate. For example, a low % of nodulation and high mortality of seedlings in fourteen months old stands of *Casuarina* (*C.glauca* and *cunninghamiana*) was observed and to be caused by inadequate soil conditions for survival of introduced *Frankia* (Mansour, unpablished data). Poor and inter-seasonal soil survival of *Frankia* reduced *Casuarina* nodulation and subsequently reduced its effectivity.

The survival of *Frankia* is probably dependent on soil conditions especially in summer. Desiccation is the most likely the primary cause of decreasing the survival rate of *Frankia*. In addition, survival of *Frankia* may depend on soil texture as studies of *Rhizobium* species have shown (Bushby and Marshall, 1977). Subsequent survival in the dried soil may be decreased by high temperature in summer or alkalinity of soil.

The influence of soil type, on and/or the interaction with the effects of desiccation, high temperature and soil alkalinity on the survival of introduced *Frankia*, may account for *Frankia* survival differences and the variable growth responses of *Cauasrina* seedlings.

In the present studies, some conditions of importance for successful introduction of *Frankia* and for nodulation of Casuarinas in relation to soil type were studied.

Materials and Methods

Soil chemistry and texture

Three soil types (sandy, loamy sand and sandy loam) collected during summer 2001, were used in this study (Table 1). All soils were characterized for pH, which was estimated in a 1:1 (soil: water) soil suspension. Organic carbon was determined by Walky-Black method (Nelson and Sommers, 1996). Available cations were estimated in 1:1 (Soil: water) suspension (Lanyon and Heald, 1982). Soil electrical conductivity (milli-Siemens m^{-1}; mS m^{-1}) was also determined in a 1:1 (soil:water) suspension. Inorganic nitrogen was determined using steam-distillation method (Mulvaney, 1996). Total nitrogen content was determined by Kjeldahl method (Bremner, 1996). Available phosphorus, extracted with buffered alkaline solution (Kuo, 1996), was also determined. Soil particle size composition was defined using the "sieve and sedimentation" procedure (Coventry and Fett, 1979). This procedure provided the percentages of gravel (> 2mm), sand (0.02-2 mm), silt (0.002-0.02 mm) and clay (<0.002 mm) (Table 1).

Plant materials

Seeds of *Casuarina cunninghamiana* were surface sterilized in 30% H_2O_2 for 15 min and thin washed with sterile distilled water. Sterilized seeds were germinated in flat containing acid -

Table 1: Soil characteristics

				Soluble Cations (meq L^{-1})					Texture (%)		
Soil	pH	EC*	O.C**	Ca^{2+}	Mg^{2+}	Available N (mg kg^{-1})	Total N (%)	Available P (mg kg^{-1})	clay	silt	sand
Sand	8.10	0.31	0.02	0.7	0.3	15.00	0.02	0.75	0.03	0.63	99.34
Loamy sand	7.80	0.84	0.32	5.5	0.6	52.50	0.07	9.40	8.90	3.50	87.60
Sandy loam	8.51	0.55	0.49	1.8	0.8	28.50	0.04	0.50	15.80	5.20	79.00

* Electrical conductivity (dSm^{-1}). **Organic Carbon (%).

Table 2: Maximum soil temperature (°C) reached at 5 cm soil depth at different soil types over the months of July 2001 to April 2002

			Month			
Soil type	location	Latitude/longitude	37073	37135	37257	37347
Sandy Soil	Ismailia	30° 40'/32° 15'	56.0	50.0	35.6	39.9
Loamy sand	Ismailia	30° 40'/32° 15'	50.0	39.2	35.0	39.0
Sandy loam	Zagazyg	30° 38/31° 30o	49.3	45.0	34.2	36.6

washed sterile sand and watered every day. After seed germination, two months old seedlings were used for determining the nodulation capacity of freshly collected and treated soil.

Frankia strains

Six *Frankia* strains were used in this study, which were isolated from root nodules collected from different *Casuarina* plantations (*C.cunninghamiana* and *C.glauca*). *C.cunninghamiana* was the host origin of *Frankia* strains HEG1, HEG 2, HEG 3 and HEG4 and *C.glauca* was the host of strains HEG5 and HEG6. Isolation was achieved following to the methods of Mansour ***et al.*** (1990). All strains were characterized for infectivity and effectivity of symbiotically fixing nitrogen.

Population of *Frankia* strains in soil samples

Populations of *Frankia* strains were indirectly estimated in soil samples by determination nodulation capacity. Nodulation capacity was determined by plant infection dilution method using MPN (most probable number) technique. Three replicate samples from each site were collected. A ten-fold serial dilution in sterile water was made for each soil after they have being mechanically agitated for 10 min. Five to six 8-week-old *Casuarina* seedlings, each growing in a tube containing sterile acid-washed, coarse sand, were inoculated with 1ml each of the appropriate dilutions.

Nodulation was scored after 12 to 16 weeks of seedlings inoculation. The MPN values were calculated according to Alexander (1982).

Experimental design

The conditions which affect survival of introduced *Frankia* to soil and for nodulation of *Casuarinas* were studied in two separate experiments.

Experiment 1. Survival of *Frankia* strains in response to temperature and soil type

In this experiment the effect of desiccation and storage, under two temperature regimes, of different soil types on the survival of different *Frankia* strains was investigated. The survival of *Frankia* strains was measured indirectly as the persistence of their nodulation capacity.

Frankia inocula were harvested from one liter of a 21 to 28-day-old culture grown in B medium (Lecheviler, 1984). Each inoculum was added to either autoclaved or non-autoclaved sieved soil. Autoclaving the soils was done by allowing the soils for 30 min at 123°C after they have been wetted with 15 to 20 ml of distilled water. After autoclaving, soil allowed to dry for 3-5 days

at 30°C. Ten ml of homogenized hyhae of each ***Frankia*** strains was applied evenly over the soil surface. The number of infective units (IU) in this inoculum is given in the tables of results (Tables 4 and 5).

After inoculum addition to soil surface, the soil was thorough mixed and allowed to dry for 5 days at 30°C. Three replicates of inoculated autoclaved or non-autoclaved soil were either maintained at previous temperature (LT treatment) or placed at 50°C for 3h, on each of 3 consecutive days (HT treatment) before returning to 30°C. These temperatures were realistic of those measured in the field (Table 2). After the temperature treatments the soil was subjected for determination of the nodulation capacity as mentioned above. The nodulation capacity was followed for 4 months.

Experiment 2. Survival of *Frankia* strains in neutral or alkaline soil

In this experiment only sandy soil was used, however reclaimed soil (loamy sand) and clay soil (sandy loam) were difficult for pH adjustment. Soil was treated with diluted (0.1N) H_2SO_4 to adjust pH to 7, 7.5, 8 and 8.5 after which ***Frankia*** inoculm was evenly applied over the soil surface and thoroughly mixed. The number of infective units (IU) in the introduced inoculum is given in the table of results (Table 6). The nodulation capacity was determined after 4 months.

Data treatment

Significant treatment effects in experiments 1 and 2 were identified using analyses of variance on log - transformed data. Systematic relationships between survival of each ***Frankia*** strain tested and soil properties were investigated using simple correlation analysis.

Results

Characterization of isolated *Frankia* strains

All isolated strains are demonstrated typically ***Frankia*** strains characteristics of intensive hyphal growth, production of vesicles in large quantities in media especially in those with N-free as well as production of sporangia with spore release in old cultures. ***Frankia*** strain HEG3 was characterized by high production of sporangia as well as high numbers of released spores.

All ***Frankia*** strains are capable to re-infect its host plant of ***Casuarina*** species (***C.cunninghamiana***, ***C.glauca*** and ***C.equestifolia***). A comparison among strains for high biomass production and the amount of atmospheric N_2 fixed in combination with ***C.cunninghamian*** seedlings is provided in Table 3. Evaluation of the data obtained, ***Frankia*** strain HEG3 recorded the highest followed by strain HEG6.

Population of *Frankia* strains in soil samples

In freshly or air-dried soils collected from different sites, no infective ***Frankia*** were observed, even in soils from rhizospheres of nodulated ***Casuarina*** trees.

Effect of soil type and temperature on survival of different *Frankia* strains

The fate of the introduced ***Frankia*** populations in different soils, determined as persistence of their nodulation capacity, is shown in Tables 4 and 5. Significantly higher numbers of ***Frankia***

Table 3: Comparison among different *Frankia* strains showing different effective responses with *Casuarina cunninghamiana* as host plant

Frankia strain	Date of first nodule observation (days)	Number of nodules plant^{-1}	Nodule dry dry weight ±SD (g plant^{-1})	Mean shoot height ±SD (cm)	Mean root length ±SD (cm)	Mean stem diameter ±SD (cm)
Control	0	0	0 0	11.60±1.34c	14.9± 4.80c	0.14±0.02d
HEG1	21	4	0.006±0.003b	20.10±1.67b	19.82±2.82c	0.72±0.04c
HEG2	15	10	0.007±0.003b	22.00±4.00ab	18.00±2.74c	0.42±0.05c
HEG3	18	9	0.006±0.002b	26.40±2.97a	30.60±11.37a	1.52±0.04a
HEG4	20	7	0.011±0.005a	19.60±1.14b	27.46± 5.12b	0.49±0.01c
HEG5	17	5	0.008±0.004b	20.00±2.12b	28.00± 6.71b	0.53±0.02c
HEG6	18	4	0.010±0.004b	23.60±2.41a	28.40± 3.05b	1.07±0.05b

Frankia strain	Plant dry weight (g plant^{-1}) ±SD		Nitrogen yield (g plant^{-1}) ±SD	
	Root	Shoot	Root	Shoot
Control	16.4±5.54c	15.2±2.89d	0.12±0.01d	0.16±0.03f
HEG1	18.0±5.45c	39.3±10.1cd	0.47±0.04b	0.74±0.05e
HEG2	16.8±2.68c	65.1±8.74cd	0.27±0.03c	1.86±0.12d
HEG3	49.8±16.23a	210±89.79a	0.69±0.03a	6.04± 0.11a
HEG4	16.1± 3.79c	54± 9.03cd	0.21±0.02c	1.72± 0.10d
HEG5	15.3± 4.10c	85.2±13.26c	0.12±0.02d	2.17± 0.01c
HEG6	39.4± 9.38b	156±47.2b	0.45±0.03b	5.48± 0.02b

Mean values for growth measurment (n = 9), for *Casuarina*/*Frankia* combinations, per colume followed by the same letters are not significantly different at 0.05 level of probability (Duncan's Multiple Range test)

Table 4: Log_{10} numbers of different *Frankia* strains surviving in different autoclaved soils under low (LT) and high (HT) temperature treatments
Values are the mean of nodulation capacity of soil samples (n = 3).
Initial inoculum: Log I U (Infective Unit) g^{-1} soil = 2.73.

	Soil types					
	Sandy soil treatment		Loamy sand treatment		Sandy loam soil treatment	
Frankia strains	LT	HT	LT	HT	LT	HT
HEG1	1.51	2.34	1.04	0.97	1.59	2.34
HEG2	1.51	2.32	0.89	1.18	1.52	2.34
HEG3	1.90	2.34	1.15	1.42	2.04	2.34
HEG4	1.23	1.30	0.65	1.11	1.30	1.38
HEG5	1.08	1.30	0.74	1.00	1.15	1.32
HEG6	1.30	1.32	0.89	1.18	1.36	1.56

cells were recovered from dried soils after storage at high temperature compared to the lower temperature storage. However, both the magnitude of this effect and comparative survival of ***Frankia*** cells for each strain between soils within a temperature treatment depended on the soil type and ***Frankia*** strains. Greater survival occurred in soils with low values of available nitrogen and Ca, which were inter-correlated ($r = 0.989$, $P < 0.004$). The survival of ***Frankia*** strains was more

Table 5: Effect of temperature (LT low temperature; HT temperature) on the survival of different *Frankia* strains in different non-autoclved soil types , determined as persistance of their nodulation capacity (infective unit, IU g^{-1} soil)
Values are the mean of nodulation capacity of soil samples (n = 3).
Initiat inoculum: Log I U g-1 soil = 2.73.

	Soil types					
	Sandy soil treatment		Loamy sand treatment		Sandy loam soil treatment	
Frankia strains	LT	HT	LT	HT	LT	HT
HEG1	1.42	2.04	0.60	0.30	1.34	1.59
HEG2	1.52	1.41	0.30	0.60	1.34	1.51
HEG3	1.41	2.11	0.65	0.89	1.30	2.23
HEG4	1.15	1.30	0.65	0.26	1.15	0.26
HEG5	1.08	1.30	0.56	0.69	1.04	1.08
HEG6	1.15	0.89	0.74	0.83	1.15	1.23

Table 6: Log $_{10}$ number of different *Frankia* cells surviving in different soils under amended pHs
Values are in means for three determinations of nodulation capacity. Initiat inoculum: Log I U g^{-1} soil = 2.73.

	Autoclaved sandy soil				Non-autoclaved sandy soil			
	pH treatment				pH treatment			
Frankia strains	7	7.5	8	8.5	7	7.5	8	8.5
HEG1	1.15	0.97	1.59	0.89	0.89	0.83	1.32	0.74
HEG2	1.15	1.08	1.52	0.74	1.23	1.04	2.04	1.88
HEG3	2.04	1.32	2.34	1.15	1.42	1.23	1.59	1.15
HEG4	2.04	2.32	2.04	2.04	2.34	2.34	1.41	1.36
HEG5	2.23	1.90	2.11	0.96	2.04	1.30	2.11	1.51
HEG6	2.15	2.04	2.34	0.92	0.89	0.83	1.30	1.32

strongly correlated negatively with amount of nitrogen available in the soil (r ranged from -0.993 to -0.998 with $P<0.002$). Meanwhile, the rest of other soil properties did not influence survival of different ***Frankia*** strains, no correlations among them were recorded.

The nodulation capacity for all ***Frankia*** strains was significantly lowered in non-autoclaved soils than autoclaved soils, except for ***Frankia*** strains HEG4 and HEG5 desiccated at high temperature, in sandy soil, where no significant differences recorded.

Frankia strain HEG3 recorded highest survival rate among ***Frankia*** strains tested with different temperature treatments in different autoclaved soils, with one exception for sandy and sandy loam soils in which no significant difference with both strains HEG1 and HEG2 at high temperature treatment (Table 4). In non-autoclaved soil types, HEG3 was the highest survival rate only at elevated temperature (Table 5).

Effect of soil pH on nodulation capacity of different *Frankia* strains

Alkaline sandy soil with pH 8, achieved by treating soil with diluted H_2SO_4, showed higher nodulation capacity of ***Frankia*** strains. However, the magnitude of this effect depended on the

strain of ***Frankia*** cultures (Table 6). In autoclaved soil, ***Frankia*** strains HEG3 and HEG6 demonstrated the highest nodulation capacity, however at higher pH 8.5 ***Frankia*** strain HEG4 was significantly the highest value. In non-autoclaved soil, ***Frankia*** strain HEG5 was more resistant to alkaline soil (pH 8.5) than the other ***Frankia*** strains.

Discussion

Soil type affected the response of ***Frankia*** strains to both desiccation and storage at elevated temperatures. Under these conditions, soil with less available N, was more favorable to survival of different ***Frankia*** strains. However low nodulation capacity was observed for all soils tested. In comparison to induced inoculum, this effect could also be related to soil deficiencies of Molybdenum or Cobalt or both since these have significant effects on nodulation (Hertogh ***et al.***, 1964; Low and Evans, 1962). This reason may also explain the poorer survival of ***Frankia*** strains in Egyptian soil as well as the absence of spore-containing sporangia in nodules of ***Casuarina*** species, that considered the more resistant unit under drying conditions (Burleigh and Torrey, 1990). Interstingly, significant differences in susceptibility to drying were found among strains.

Recovery of low numbers, calculated as presistance of nodulation capacity, of tested ***Frankia*** strains in nonsterile soils in comparison with sterile ones could be possible. This can be explained by a: competition of ***Frankia*** for nutrients with other organisms b: autoclaving the soil will permit the ***Frankia*** to grow without interference from other soil microorganisms which ***Frankia*** is characterized by its slow growth rate. In addition, autoclaving usually releases nutrients which may have provided ***Frankia*** with an opportunity to grow more (Baker, personal communication).

Desiccation and storage at elevated high temperature showed an increase in infective units for different ***Frankia*** strains. Significant greater recovery at high temperature in compared to low temperature (30^{0}C) may be that effect of high temperature on stimulation of spores to be germinated.

The recorded low survival of ***Frankia*** strains in response to high soil pH (pH 8.5, Table 6) is consistent with the observed effect of pH on the viability of ***Frankia*** strains (Burggraaf and Shipton, 1982) or it could also be resulted of loss of this adaptation (Smolander ***et al.***, 1988). However, significant increase in persistence capacity of ***Frankia*** strains in sandy soil were limited to relatively neutral pHs (pH 7 and pH 8) level found to be suitable for the growth of ***Frankia*** strains in pure cultures or soil conditions from which nodules were collected.

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