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IDENTIFICATION OF SOME LOCAL *FRANKIA* STRAINS BASED ON PHYSIOLOGICAL AND MOLECULAR VARIATION

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ABSTRACT

In this investigation, set of five *Frankia* strains were isolated from root nodules of *Casuarina* trees from five different regions in Egypt and were identified based on their physiological and molecular genetic variations. Results showed that growth parameters of *C. glauca* inoculated with the five *Frankia* strains grown in loamy sandy soil were both significantly higher than those of plants grown in clay soil. The effect of the *Frankia* strains under study on number of nodules, percentages of seedling that formed root nodules (nodulation frequency) and activity of acetylene reduction in clay soil as well as loamy sandy soil was addressed. The numerical analysis of the investigated parameters was found to be a useful tool for differentiation between the *Frankia* strains. We have analyzed 9 randomly amplified polymorphic DNA (RAPD) primers against five *Frankia* strains. Results showed that the number of amplified fragments differed between the strains. Some primers were useful in identifying unique DNA polymorphisms of all strains tested. Some fragments were found to be polymorphic (not common). These unique fragments could be recommended as markers for distinguishing between the applied strains of *Frankia*. Statistical analysis of RAPD-PCR polymorphisms showed similarities between *Frankia* strains ranged from 67.3 to 85%. The phylogenetic tree confirmed the genetic diversity between the *Frankia* strains under investigation. Genomic fingerprinting assay using RAPD-PCR was excellent methodology for differentiating between the *Frankia* strains. The correlation between the phylogenetic and the phylophenetic trees of the five *Frankia* strains were also discussed.

INTRODUCTION

Frankia is an actinomycete able to fix atmospheric nitrogen either *in vitro* or in plants by infecting root system and forming root-nodules on a number of non-legumes termed actinorhizal plants (Diem and Dommergues, 1990 and Benson and Silvester, 1993). Numerous studies have shown that inoculating different pro-venances or clones of acti-norhizal plants with *Frankia* increased the nitrogen fixation (acetylene reduction activity) and different growth parameter of actinorhizal plants used (Bulloch, 1994, Selim and Schwencke, 1995, Selim *et al*., 2000 and Selim *et al*., 2003).

Genomic fingerprinting assays using randomly amplified polymorphic DNA (RAPD) are excellent method-logies and were originally developed to identify genetic polymorphisms in plant **(**More *et al*., 1994; Mehling *et al*., 1995 and Harn *et al*., 1997), fungal **(**Fritsch *et al*., 1993**),** and prokaryotic genomes **(**Grajal-Martin *et al*., 1993**)** and are fast and sensitive means for identifying small differences between similar complex genomes.

RAPD methodology has been used for differentiation and tracking of specific strains within the actionmycetes, including *Corynebacterium* **(**Kutchma *et al*., 1998**),** *Mycobacterium* **(**Heath *et al*., 1986; Hadrys *et al*.**,** 1992 and Mahadevan, 1992**),** *Nocardia* spp. **(**Liesack *et al*., 1991**),** *Renibacterium* **(**Goodfellow, 1989**)** and *Streptomyces* spp. (Mohamed *et al*., 2001; Mahfouz and Mohamed, 2002; El-Domyati and Mohamed, 2004; Mohamed and Galal, 2005 and Mohamed *et al*., 2006).

The application of molecular tools to questions related to the genetics, ecology and evolution of actinorhizal symbiotic systems has been especially fruitful during the past two years. Host plant phylogenies based on molecular data have revealed markedly different relationships among host plants than have previously been suspected and have contributed to the development of new hypotheses on the origin and evolution of actinorhizal symbiotic systems (Beth and Dobritsa, 1996). Genetic diversity among *Frankia* strains nodulating members of the family *Casuarinaceae* was revealed by different molecular PCR tools (Mirza *et al*., 1994; Rouvier *et al*., 1996; Guetsky *el al*., 2005; Huguet *et al*., 2005 and Chavez and Margarita, 2006) and by two dimensional poly acrylamide gel electrophoresis (Benson *et al*., 1984).

The aim of this study was to determine activities of five *Frankia* strains infecting *Casuarina glauca* plants. In addition, determination of the genetic diversity of the *Frankia* strains under investigation was also aimed by RAPD-PCR.

MATERIALS AND METHODS

Soils: Two representative samples of loamy sandy and clay soils were collected from El-Bostan location, Behera Governorate and King Mariout City, Alexandria Governorate, Egypt, respectively.

Seeds: Seeds of *C. glauca* were kindly provided by Desert Development Center (DDC), American University in Cairo, Egypt.

Source of *Frankia* strains: Five *Frankia* strains named UF010, UF015, UF020, UF023 and UF024 isolated from root nodules of *Casuarina* trees at five different regions in Egypt were kindly provided by Biofertilizers Unit, Fac. Agric., Ain Shams University.

Experimental technique: A greenhouse pot experiment was conducted at the Unit of Biofertilizers, Faculty of Agriculture, Ain Shams University, to evaluate the effects of inoculation with different *Frankia* strains on the performance of *C. glauca* in two types of soil. For this purpose, polyethylene bags (20X30 cm) with 5 Kg capacities were packed with either of loamy sandy or clay soils. Two months old seedlings of *C. glauca* were transplanted into the polyethylene bags contained either of the 2 tested soils. Seedlings were fed with Hoagland solution containing NH_4^+ for 2 weeks and 3 weeks with the same solution without NH_4^+ (Selim and Schwencke, 1995). *Casuarina* plants were then inoculated with either of the tested *Frankia* strains by adding 20µg of the mycelial protein/plant. Developed plants were fed with Hoagland without nitrogen source for 6 months.

At the end of the experiment period *Casuarina* plants grown under different treatment were harvested to record shoot height (cm/plant), plant dry weight (g/plant), nodulation frequency (%), number of nodules per plant, dry weight of nodules (mg/plant) and acetylene reduction assay (Hardy *et al*., 1968) expressed in nmols C_2H_4/h/plant. The

results were analyzed according to Snedecor and Cochran (1967).

DNA extraction and purification: *Frankia* strains were grown on BAP-modified medium (Fontaine *et al*., 1986). Strains were grown in 50 ml portion of the medium in 250 ml conical flasks. Each flask was inoculated with 5 ml of stock culture and incubated at 28°C for 3-4 weeks. On centrifugation of the growth cultures at 14000 rpm for 20 min at 4°C, the pellets were collected and used for DNA extraction. The DNA extracts of the five *Frankia* strains was prepared and purified as described by Mohamed *et al*. (2001) (a modified method of Marmur, (1961**)**. The final DNA pellets were resuspended in 50µl TE buffer, pH 8.0 and its concentrations were adjusted (100 ng/µl) using the spectrophotometer as described by Sambrook *et al*. (1989).

RAPD-PCR analysis: Based on the methods of Williams *et al*. (1990) and Mohamed *et al*. (2001), the RAPD-PCR analysis of the five *Frankia* strains under investigation was carried out using 9 random primers (Table 1). The PCR mixture was conducted in a volume of 50 µl as reported by Mohamed *et al*. (2001) using 100 ng DNA**.** The PCR program started with one cycle for denaturation at 95°C for 4 min followed by 40 cycles, each consists of 94°C for 1 min; 37°C for 1 min and 72°C for 2 min. The final segment was extended for 7 min. The PCR products were electrophoresed on 1.2% agarose gel for 2.5 h at 80 V followed by staining with 0.5% ethidium bromide as described by Sambrook *et al*. (1989). The DNA bands were visualized under UV transilluminator. For analysis, the fragments of the DNA polymorphisms for each isolate were scored as 1 for present and 0 for absence. The similarity coefficient (F) between the five *Frankia* strains was defined by the formula of Nei and Li (1979)**.** A phylogenetic tree was derived from the distance by un-weighted paired-group method (Sneath and Sokal, 1973).

Table-1: Nucleotide sequences of 9 random primers used for RAPD-PCR analysis.

Primers name	Sequences (5′---------3′)
OPA-02	TGCCGAGCTG
OPA-07	GAAACGGGTG
OPA-16	AGCCAGCGAA
OPA-18	AGGTGACCGT
OPB-11	GTAGACCCGT
OPB-14	TCCGCTCTGG
OPB-15	GGAGGGTGTT
OPC-07	GTCCCGACGA
OPC-08	TGGACCGGTG

RESULTS AND DISCUSSION

In Egypt, genus *Casuarina* is the only actinorhizal plant species capable of forming root nodules in symbioses with nitrogen-fixing filamentous soil bacteria (*Frankia*) (El-Lakany, 1983; Mansour and Baker, 1994 and Mansour *et al*., 1996). In addition, 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 (Mansour 2003).

In this study, five *Frankia* strains **(**Figure 1) were used and their activities on growth parameters and nitrogen fixation in loamy sandy and clay soils were determined. Data presented in the growth parameters and number of nodules of *C. glauca* inoculated with different *Frankia* strains grown in loamy sandy soil (Table 2) was significantly higher than those of plants grown in clay soil (Table 4). The highest number of nodules was obtained from *C. glauca* grown on the

clay soil and loamy sandy inoculated by *Frankia* strains UF015 and UF020 being 8.7 and 6.0 nodule/plant, respectively. The percentages of seedlings that formed root nodules (nodulation frequency) were generally higher in clay soil than loamy sandy soil except for *Frankia* strain UF020. *C. glauca* grown on clay and loamy sandy soil gave high record of nodules dry weight than that grown in clay soil.

The highest activity of acetylene reductase was obtained from *C. glauca* plants grown on clay soil inoculated with *Frankia* strains UF024 and UF015 being 2490.7 and 3912.3 nmol/C_2H_4/h/plant, respectively. Numerous studies have shown that inoculation with *Frankia* strains increase the growth parameters (shoot and root length, shoot and root dry weight), number nodulation and N_2-fixation of the host plants **(**Diem and Dommergues, 1990; Benson and Silvester 1993; Bulloch, 1994; Selim 1995; Selim and Schwencke 1995; Selim *et al*., 2000; Zayed 2001 and Selim *et al*., 2003)**.**

Arbitrary numerical scoring for the effect of the five *Frankia* strains on growth parameters as well as nitrogen fixation was suggested (Data not shown). Clustering of all scoring units was determined as mentioned by Sneath and Sokal (1973) and the results phylo-phenetic trees are given in Figures-2 and 3 for loamy sandy and clay soils, respectively. The data reveal the presence of two major related clusters, one includes, UF020 and the second include UF010, UF015, UF023 and UF024. It was also found that the later cluster contained two subclusters. UF023 *Frankia* strain was in subcluster, while UF010, UF015 and UF024 *Frankia* strains were fell in the other subcluster. As interestingly, no difference between the clusters of loamy sandy and clay soils was noted. The differences were in the similarities (%) between the clusters, as it was higher in case of loamy sandy soil than clay soil. The similarities ranged between 76.6 and 97.3% in case of loamy sandy soil (Table 3) and from 66.7 to 87.2% for clay soil (Table 5). This is compatible with their effect of growth parameters as discussed above.

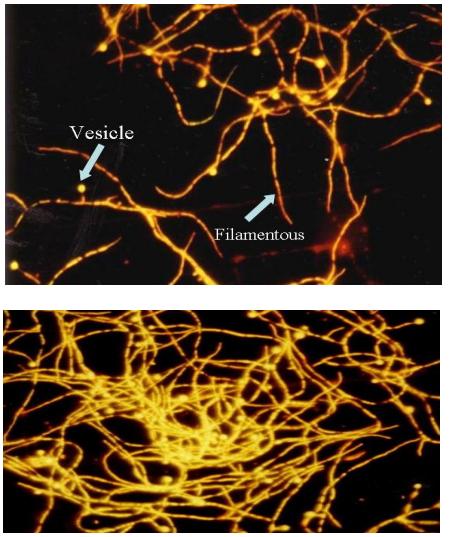

Figure-1: Micrographs show the morphology of *Frankia* (nitrogen-fixing filamentous soil bacterium) strains used in this study.

Table-2: Characteristics of five *Frankia* strains inoculated *C. glauca* grown in loamy sandy soil for 6 months.

Parameters	*Frankia* strains				
	UF010	UF015	UF020	UF023	UF024
Shoot height (cm/plant)	64.5^{d}	53.0^{b}	62.8^{e}	97.5^{c}	62.0^{e}
Shoot dry weight (g/plant)	3.1^{b}	1.8^{c}	1.9^{c}	8.8^{d}	2.3^{c}
Root length	39^{a}	35^{a}	31.5^{ac}	52.5^{d}	35.8^{a}

Root dry weight (mg/plant)	1.85[e]	1.4[f]	0.81[a]	1.96[e]	1.35[f]
No. of nodules/plant	1.0[d]	1.7[fi]	6.0[a]	2.0[df]	1.3[d]
Nodulation frequency (%)	20	30	100	30	30
ARA nmols C_2H_4/h/plant	841.41	1006.4[f]	656.1[i]	1512.5[e]	1068[f]

Means not followed by the same letter are significantly different by Ducan's LSD test (P< 0.05).

Table-3: Similarity between five *Frankia* strains based on their effect on *C. glauca* growth parameters grown in loamy sandy soil.

Frankia strains	UF010	UF015	UF020	UF023	UF024
UF010	100.0				
UF015	91.4	100.0			
UF020	81.8	78.0	100.0		
UF023	92.7	84.2	76.6	100.0	
UF024	97.3	94.1	83.7	90.0	100.0

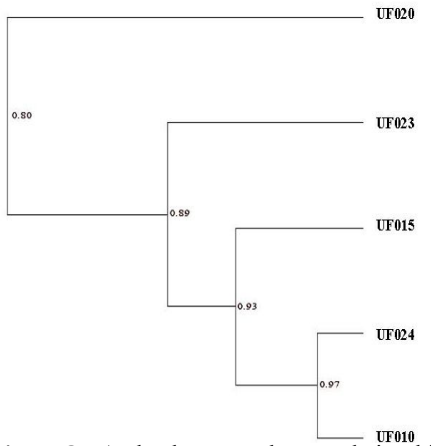

Figure-2: A dendrogram shows relationship between five *Frankia* strains based on *C. glauca* growth parameters grown in loamy sandy soil.

Table-4: Characteristics of five *Frankia* strains inoculated *C. glauca* grown in clay soil for 6 months.

Parameters	*Frankia* strains UF010	UF015	UF020	UF023	UF024
Shoot height (cm/plant)	50[b]	70.3[a]	52.5[b]	52.5[b]	56[b]
Shoot dry weight (g/plant)	1.5[c]	6.0[a]	1.7[c]	1.7[c]	1.6[c]
Root length	33[a]	36.3[a]	20.8[c]	20.8[c]	22.5[b]
Root dry weight (mg/plant)	0.43[d]	0.52[c]	0.65[b]	0.65[b]	0.46[d]
No. of nodules/plant	4.3[b]	8.7[a]	2.7[c]	2.7[c]	4.7[b]
Nodulation frequency (%)	80	80	50	50	100
ARA nmols C_2H_4/h/plant	2171.8[b]	3912.3[a]	1762.2[c]	1762.2[c]	2490.7[d]

Means not followed by the same letter are significantly different by Ducan's LSD test (P< 0.05).

Table-5: Similarity between five *Frankia* strains based on their effect on *C. glauca* growth parameters grown in clay soil.

Frankia strains	UF010	UF015	UF020	UF023	UF024
UF010	100.0				
UF015	78.3	100.0			
UF020	78.3	66.7	100.0		
UF023	81.3	66.7	70.6	100.0	
UF024	87.2	77.6	58.5	74.3	100.0

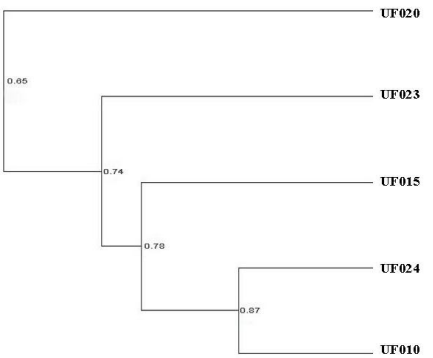

Figure-3: A dendrogram shows relationship between five *Frankia* strains based on *C. glauca* growth parameters grown in clay soil.

RAPD-PCR analysis: Molecular genetic markers have been developed into powerful tools to analyse genetic relationships and genetic diversity. As an extension to the variety of existing techniques using polymorphic DNA markers, the random amplified polymorphic DNA (RAPD) technique may be used in molecular ecology to determine taxonomic identity, assess kinship relationships, analyze mixed genome samples, and create specific probes. Main advantages of the RAPD technology include (i) suitability for work on anonymous genomes, (ii) applicability to problems where only limited quantities of DNA are available, (iii) efficiency and low expense (Hadrys *et al*., 1992).

In this study, highly purified DNA extracts of the five *Frankia* strains were used as templates for RAPD-PCR. Data revealed that no amplified fragments were observed in any of the negative controls (PCR mixture without any DNA templates). Data in Tables -6 and 7 showed that the number of amplified fragments differed with different primers, which is expected. On the other hand, the number and sizes of amplified fragments differed from one strain to another for the same primer. Data also showed that a total number of 88 amplified fragments were obtained, out of which 27 unique fragments were distributed as follows: 8, 7, 4, 1 and 7 for strains UF010, UF024, UF023, UF015, and UF020, respectively. In addition, 75, 67.1, 80.7 and 72.7% out of the 88 fragments were amplified from the DNA of the UF010, UF024, UF023, UF015, and UF020 strains. Results in Table-8 revealed that the similarity between the DNA of the five *Frankia* strains in this study ranged from 67.3 to 85%. Results in Figure-8 showed the phylogenetic tree of the five *Frankia* strains (Figure-8) that the first cluster included strains UF020 with similarity of 71%, while, strain UF023 lied in subcluster with similarity of 75%, UF024 in another subcluster with similarity of 77% and the last two *Frankia* strains lied in one subcluster with similarty of 85%.

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