

Molecular Analysis of Phenotypic Diversity among Four *Frankia* Isolated from *Casuarina* Nodules in Egypt. 1- Someaclonal Variation among Four *Frankia* Isolates.

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Abstract: Four *Frankia* isolates namely Fc, Fe, Fg and Fh were isolated from nodules of four species of *Casuarina* plants grown on different Egyptian desert locations by using the double-layer technique. Isolates formed filamentous of *Frankia* on BAP-PCM medium lacking nitrogen. The growth pattern of four isolates was found to be growing exponentially and their metabolic activities in BAP-PCM liquid medium under stirred conditions. The Fe isolate was superior to the isolates in their growth, metabolic activity, effectiveness and ineffectiveness, compared with the others. Three somaclonal variation showed among 4 closely related *Frankia* isolates by using whole cells fatty acids analysis acetylene reduction activity. Among *Frankia* isolates were significant variations were detected qualitative and quantitative. Generally, all tested isolates contained saturated, unsaturated and branched fatty acids. Dominant fatty acids detected were saturated (15:0), branched (16:0 iso) and unsaturated (17:0 and 17:1B) which were 14.7, 29.9 and 23.5% respectively of the total acids content. N₂-ase activity was maximal at 5 to 7 days for isolates (271.72, 282.75, 250.15 and 240.70 n mol C₂H₄/ml/hr), respectively. Vesicles of isolates were formed with 25 hr on NH₄-free culture medium and reached peak numbers within 35 hr. The four tested isolates of *Frankia* were assessment of the effectiveness in plant their were different capable of inducing nodulation on the root of *C. glauca* as well as acetylene reduction activities.

Key words: *Frankia*, *Casuarina* spp., Fatty acids pattern, Growth curve, Acetylene reduction.

INTRODUCTION

In the tropics, the ability of *Casuarina* species to form symbiotic N₂ fixing association with *Frankia* is one attribute which makes these tree species potentially important for fuel-wood production, agroforestry and reclamation of infertile soils in the tropics, subtropics and arid zones (Girgis *et al.*, 2002). During the last decade hundreds of *Frankia* isolates have been obtained from Casuarinaceae nodules using different isolation techniques (Baket and Mullin, 1989). All isolates obtained from nodules were assigned to the genus *Frankia* on the basis of (i) morphological features, such as sporangium and vesicle formation in submerged liquid culture, (ii) chemical composition of certain cell constituents such as cell wall type III, phospholipids type PI and the presence of the diagnostic sugar 2-O-methyl-mannose and (iii) the ability to fix nitrogen and to nodulate plants (Lechevalier, 1986). Many isolates lacking some of these morphological and physiological characteristics of typical *Frankia* have been obtained from actinorhizal nodules (Mirza *et al.*, 1991).

Qualitative and quantitative analyses of total fatty acids composition have been shown to be useful for taxonomic investigations (Janse, 1991). Analysis of short chain fatty acids has been routinely used in the identification of anaerobic bacteria (Arellano *et al.*, 2000). Using this information along with physiological and genetical data, Lalonde *et al.*, (1988) proposed the recognition of species *Frankia elaeagni* and *Frankia alni*, with subspecies *pmmmerili* and *vandijkii*. However, all these investigations showed a characteristic fatty acid profile for all strains studied.

This study aims to characterize 4 *Frankia* isolates obtained from root nodules of *Casuarina* spp. on the basis of their cytochemical character using total fatty acids pattern.

MATERIALS AND METHODS

Source of Frankia Isolates:

Four trees of four *Casuarina* sp. were examined for nodulation at different Egyptian desert locations (Table 1). Nodules samples of root system developed under a depth of 30 cm were collected. The aerial parts

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were used to identify the species of *Casuarina* according to Johnson (1982). The nodules were air-dried over silica gel and stored at room temperature for *Frankia* isolation.

Table 1: *Casuarina* species grown in 4 locality of North Sinai Governorate.

<i>Casuarina</i> species	Age (year)	Locality	Soil texture ¹	pH ²	EC ³
<i>C. equestifolia</i>	10	Arish (1)	Sandy	7.58	0.56
<i>C. glauca</i>	15	Arish (2)	Sandy	7.99	0.59
<i>C. cunninghamiana</i>	7	El-Nobaria (1)	Sandy clay loam	8.19	2.05
⁴ <i>C. hybrid</i>	5	El- Nobaria (2)	Sandy clay loam	8.15	1.95 ¹

Soil texture was determined by the pipette method of particle size analyses; ²pH value was determined in soil water suspension 1:2.5 ; ³EC (Electrical conductivity in mmohs/cm² at 25°C was determined) ⁴*C. hybrid* (*C. cunninghamiana* x *C. glauca*).

Isolation of *Frankia* Isolates:

Frankia were isolated by using the double-layer technique described by Diem and Dommergues (1983). Fresh nodules formed on *Casuarina* roots, the isolates were propagated by washing the culture twice with sterile saline solution, disruption through a sterile needle syringe (0.6 X 30 mm). The cultures were inoculated on BAP-PCM medium (Girgis and Schwencke, 1993) and incubated at 28 °C with magnetic stirring at 150 rpm. The isolates were compared with identified Egyptian *Frankia* strain Sa 1 (Catalogue no. UBF 020803) previously isolated from *C. glauca* by Girgis *et al.* (2002).

Total Protein:

Total protein of *Frankia* isolates was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard proteion.

Vesicles Formation:

Vesicles counts were made by sonicating the mycelia of isolates of each vial for 30 S. at 60 W. The number of vesicles was counted using a hemocytometer as described by Girgis *et al.* (1992).

Growth Patterns of *Frankia* Isolates:

The isolates of *Frankia* were inoculated at a rate of 1µg protein/ml into BAP-PCM medium and incubated at 28 ± 2°C at 2 weeks under stirred conditions. After 3, 6, 9 and 12 day post incubation the developed mycelia were harvested by centrifugation at 5000 rpm.

Data were plotted against time and the exponential growth phase of each isolate was used to calculate:

- Specific growth rate (μ) according to the equation: $\mu = \frac{\ln A_1 - \ln A_0}{t_1 - t_0}$. Where (Painter and Marr, 1963): μ = specific growth rate; A_1 = mycelial protein at t_1 time; A_0 = mycelial protein at t_0 time; t_1 = time after a particular period of exponential growth phase; t_0 = time at the beginning of the exponential growth phase.
- Doubling time (td) according to the equation: $td = \frac{\ln 2}{\mu}$. Where: td= doubling time; μ = specific growth rate; $\ln 2$ = natural logarithm of 2. The level of growth in term of µg protein/ml medium was also recorded at the end of the exponential phase (6 days from inoculation).
- Viability of *Frankia* isolates was determined using INT (2-(ρ -iodophenyl)-3-(ρ -iodophenyl)-5-phenyl-tetrazolium chloride) reduction activity technique as described by Prin *et al.*, (1990).
- Nitrogenase activities of *Frankia* isolates were determined in 3 days old culture in BAP-PCM medium without ammonia by the acetylene reduction assay as described by Hardy *et al.* (1968). Ethylene production from acetylene was expressed as n mols C₂H₄. h⁻¹, ml⁻¹ per culture.

Efficiency of *Frankia* Isolates:

The infectivity of *Frankia* isolates was tested on *C. glauca* seedlings using modified hydroponic Gibson system (Vincent, 1970). Seedlings were irrigated with 1/4 strength Hoagland complete nutrient solution up to the 5th week of growth (about 3-5 cm in plant height). The nutrient solution was replaced by the same solution without nitrogen. Each seedling was inoculated with 20 µg protein of homogenized hyphae of 6 day-old *Frankia* culture Girgis *et al.* (1990). Six weeks after inoculation, seedlings were harvested and the root systems were examined for the number and dry weight of nodules. The nitrogenase activity was determined in their nodules according to Hardy *et al.*, (1968).

Fatty Acids Extraction, Preparation and Analysis:

Mycelia at 9 days old incubation by centrifugation at 5000 rpm for 10 min and washed twice with sterile distilled water. The fatty acids were saponified, mehtylated and extracted as described by Miller and Berger (1985). The samples were analyzed using MIDI Microbial Identification System (USA) which consists of a Hewlett Packard HP 5890 A gas chromatograph with a 25 m X 0.2 mm 5% methylphenyle silicone fused silica capillary column, H2 as carrier gas and a flame-ionization detector, an automatic sampler, an integrator and

a computer identifies the fatty acids, using data of a fatty acid library and a calibration mix of known fatty acids (Microbial ID) as a reference.

RESULTS AND DISCUSSION

Existence of Nodulated Casuarina in Tested Sites:

Data in table (1) showed that, soil textures of the tested sites were sandy with pH levels ranged from 7.2 to 8.2. Soil salinity showed considerable variations among tested sites. While the site examined in Arish was non-saline (0.56 and 0.59 EC), other sites were characterized by moderated or high level of salinity. The latter find were observed in El-Nobaria (1.95 and 2.05). Four isolates of *Frankia* isolated from nodulated trees of *Casuarina sp.*, they were namely Fq, Fg, Fc and Fh according species and detected to the system described by Lechevalier (1986). The cellular and endophytic capabilities of those isolates were also compared with those of the reference strain of *Frankia* Sa1 (Catalogue no.UBF 020803).

Somaclonal Variations:

Morphological Characteristics of Frankia Isolates:

Under optimal conditions the growth of the isolates formed microclonies showing a loose internal net of hyphae non-pigmented and without mucilage which are called "open mesh" (Schwencke, 1991). The 5 days-old microclonies were all isolates about 750 to 900 μm diameter. Those microclonies are not morphological and physiological homogeneous. Activity growing hyphae were evenly distributed in the colonies, no lysing hyphae and few sporangia were observed (Fig. 1). Light microscpic examination revealed branched, sepatate hyphae and sporangia of different sizes, number and shapes. The isolates formed rounded are AriCe (3) poor growth 22.13 $\mu\text{g/ml}$ for isolates. This shows that, the nutritional requirements of strain Fc and Fe differed from those of the others.

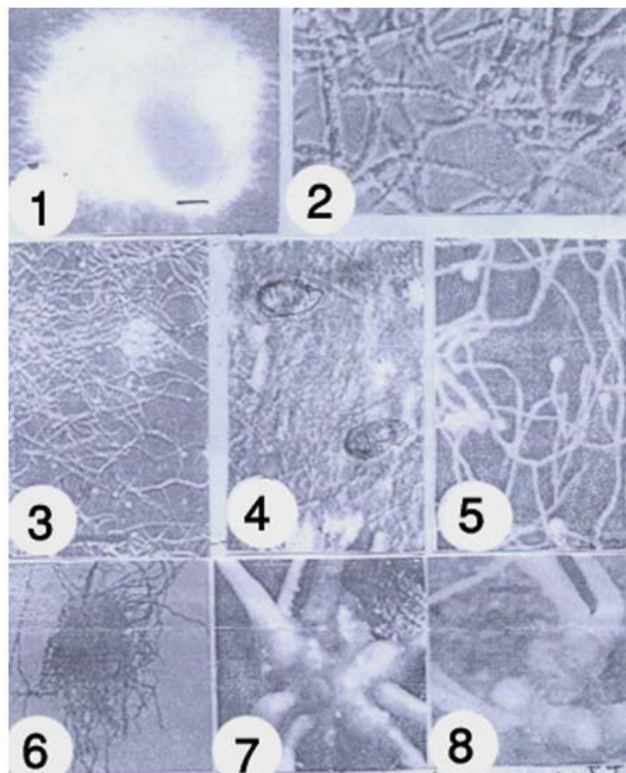


Fig. 1: Morphological charactes of *Frankia* (Fe isolate) grown on BAP-PCM medium (*in vivo*).

1) Compact *Frankia* colony, 2) Reproductive torulose hypha, 3) Mature sporangia showing sporangium disruption and spore release, 4) Globose sporangia liberating mature sporangiospores, 5) Vesicles produced by *Frankia* grown on N-free medium, 6) Nodule (6 month old), 7 and 8) Root nodules dichotomously branched and the apex of each nodule lobe gave rise a negative geotropic root.

N₂-ase activity was maximal at 4 to 5 days for the isolates Fc, Fe, Fg, and Fh (271.75, 221.55, 267.2 and 240.70 n mol C₂H₄/ml/hr), respectively. Vesicles of isolate Fc were formed within 25 hr on NH₄-free culture medium and reached peak numbers within 30 hr. They were about 3.0 μ in diameter, phase bright and reached their greatest frequency after 30 hr at which time the N₂-ase activity peaked. A high increase in vesicles number was detected and maximum (86±7 X 10⁴ and 69±9 X 10⁴ vesicles/ml, respectively) was obtained at 5 and 6 days for the latter isolates, there were positive correlations between C₂H₄-reducing activity and vesicles formation (Tjepkema *et al.*, 1981).

The metabolic activities i.e., IRA and N₂-ase activities were generally in line with the growth. Using total protein content and IRA together provide complementary methods for accurate study of the changes of during its different growth phases. However, higher activities were detected in strain Fc and isolate Fe being 285.12, 312.50 μ mol/INTF/ml and 281.71 and 260.85 n mol C₂H₄/ml/hr, respectively. Assessment of cell viability in *Frankia* culture is of particular importance in studies designed to improve cell culture methods, or after treatments that attempt to transform genetically the organisms (e.g., electroporation) (McEwan and Wheeler, 1997). IRA technique is more effective than a previously used method of acridine orange as a vital stain reported by Schwencke (1991). The IRA decline observed in *Frankia* hyphae at the end of the growth phase indicates that many hyphae were probably subjected to senescence and death during the so-called stabilization phase.

Frankia Isolates Affectivity:

Measurements *in planta* showed that the three tested isolates of *Frankia* were capable of inducing nodulation on the roots of *C. glauca*. The Fe isolate produced number of nodules with dry weight nearly similar to those obtained from the reference strain (Fig. 1). Vesicles in nitrogen-free media of different size, number and shapes of the isolates related to reference *Frankia* strain Sa1 (Table 1). *Frankia* isolates from *Casuarina* are known to be relatively difficult to be isolated where as are shown growing in culture compared to other *Frankia* strains (Nazaret *et al.*, 1989). The double layer agar technique is advantageous in that case of contaminants does not necessarily overgrow the *Frankia* colonies and that microaerophilic *Frankia* colonies are not directly exposed to the atmosphere. In the current study, the double layer agar technique with slight modifications described by Diem *et al.*, (1982) was used. Looped tips of newly developed nodules were used for the isolation of the endophytes and the outer cortical layers of the nodule lobes were peeled off to eliminate most of contaminants (Diem and Dommergues, 1983). In the current experiments, *Frankia* colonies were formed inside the agar medium and hyphae never grew out of the agar.

Growth Patterns of Frankia Isolates:

The growth rates of *Frankia* isolates were measured by increases in protein (μg/ml). It is obvious that *Frankia* isolates recorded the highest and lowest growth rates (Fig. 2) and hence the longest doubling times (Table 2). However, the isolates were shown by reference strain in terms of the amount of protein produced ml⁻¹ medium after 6 days of incubation.

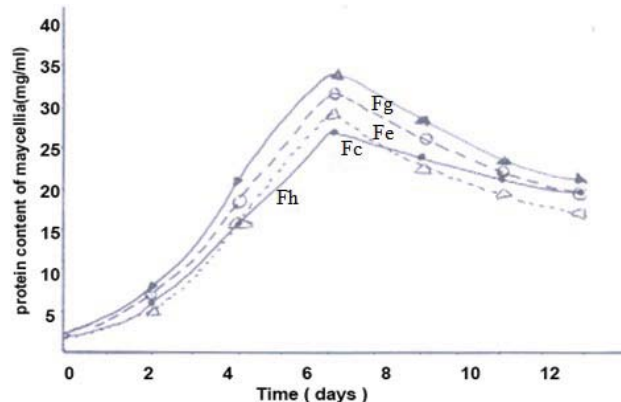


Fig. 1: Growth curves of 4 *Frankia* isolates under stirred conditions in BAC-BCM medium.

According to the growth in BAP-CM medium as shown in Table (2) the isolates could be classified into (1) good growth: the maximum amount of protein after 6 days from incubation was 34.75 μg/ml for isolate Fe, while the reference strain Sa1 was 36.15 μg protein/ml; (2) intermediated growth 29.25 and 25.42 μg/ml for isolates Fh and Fc, respectively.

Table 2: Growth and nodulation characters of 4 *Frankia* isolates.

<i>Frankia</i> isolates					

<i>Frankia</i> properties	Fc	Fe	Fg	Fh	* Sa1
Growth characters					
- Specific growth rate (μ)	0.6	0.7	0.6	0.6	0.8
- Doubling time (day)	1.50	1.26	1.42	1.40	1.09
- Growth (μ g protein/ml)	29.25	32.45	34.75	25.42	36.15
- Viability (μ mol. INTF/ml)	285.12	312.50	325.42	182.25	375.25
- N_2 ase activity (n mol C_2H_4 /mg/h)	205.21	260.18	250.25	185.10	270.25
- Number of vesicle ($\times 10^5/ml^1$)	55.00	65.45	75.35	76.21	90.89
Nodulation characters					
- Number of nodules plant ⁻¹	10.5	10.2	14.2	9.4	12.5
- Dry weight of nodule (mg plant ⁻¹)	17.15	19.25	27.25	16.24	20.25
- N_2 ase activity (n mol C_2H_4 /mg/h)	550.21	601.15	695.75	475.25	702.15

*Reference *Frankia* strain, each value represents an average of five replicates.

The recorded figures were 10.5, 10.2, 14.2, 9.4 and 12.5 for nodule number plant⁻¹ and 17.15, 19.25, 27.25, 16.24 and 20.25 (mg/ plant⁻¹) for nodule dry weight of Fc, Fe, Fg and Fh compared with Sa (as standard control) respectively. The above-mentioned finding was also parallel to the records of the activity and specific activity of acetylene reduction. The Fg isolate gave lower levels of nodulation and acetylene reduction activities.

Data in Table (2) clearly showed that the maximum nodulation and N₂-fixation, measured by acetylene reduction activity (ARA) of seedlings, was obtained by isolate Fg followed by the reference strain Sa1. Furthermore, mean nodules weight was higher in Fg isolate than the other isolates. The superiority of these isolate as far as their effectiveness is concerned could be related to the high ability to fix more N₂-fixation *in vitro* than the others. However, this type of relationship should be considered with caution.

Total Cellular Fatty Acids:

The four *Frankia* isolates were varied in quantitative and qualitative of total cellular fatty acid composition as shown in Table (3). The fatty acid composition is qualitatively similar for several *Frankia* strains reported previously (Mirza *et al.*, 1991 and Thnlid *et al.*, 1989). However, comparatively lower amounts of 10:20H and 18:20H carbon fatty acids and higher amounts of 17:1B; 15:0 iso and 17:1 were detected in all the isolates used. While non of the fatty acids was identified as signature molecule for the genus *Frankia*, a profile of five fatty acids appears to be characteristic for this genus (Mirza *et al.*, 1991). These fatty acids (15:0; 16:0 iso; 17:0; 17:1 and 18:1 iso F) constituted nearly 80% or more of the total fatty acids of all *Frankia* isolates.

Table 3: Fatty acids composition of 4 *Frankia* isolates.

<i>Frankia</i> isolates					

Number of carbon atoms in fatty acid	Fc	Fe	Fg	Fh	* Sa1
Saturated					
13	-	1.05	1.25	0.95	1.05
14	-	0.72	1.10	0.75	0.92
15	9.25	7.35	25.15	12.50	31.55
16	2.19	2.50	4.37	3.10	1.75
17	6.05	1.95	12.95	4.95	13.15
18	-	-	1.75	2.05	0.45
Unsaturated					
15:1 A	-	-	0.75	-	-
15:1 B	5.75	-	2.10	0.95	3.00
16:1 A	4.25	4.75	-	4.13	0.57
16:1 B	27.05	25.15	22.75	25.10	18.12
17:1 B	-	-	0.95	-	0.75
18:1 C	-	-	-	-	0.67
Branched					
12:0 iso	-	0.52	-	-	-
14:0 iso	3.10	2.75	2.85	2.18	7.05
15:0 iso	1.75	-	7.05	5.12	1.75
16:0 iso	1.82	1.75	1.45	0.72	0.95
17:0 iso	3.15	-	1.72	0.55	-
18:0 iso	5.25	1.02	2.95	12.01	18.75
Hydoxy					
11:0 20H	-	0.75	-	-	-
11:0 20H	2.88	2.01	-	-	3.12
11:0 20H	-	-	-	0.95	-
Unknown	-	-	2.00	-	-
Unknown	-	1.09	1.01	-	1.01
Unknown	-	-	0.85	1.01	0.71

* Sa1 = Reference *Frankia* strain, each value recorded an average from two replicates.

Generally, all tested isolates contained saturated, unsaturated and branched fatty acids. Dominant fatty acids detected were saturated (i.e, 15:0), branched (i.e, 16:0 iso) and unsaturated (17: 1B) which were 14.7; 29.9 and 23.5% respectively of the total acids content. *Casuarina-Frankia* isolates showed quantitatively and qualitatively significant differences. Isolate namely Fg could be characteristic by unsaturated fatty acids 18:C, 12, 14 and 18:1 iso. moreover, these strain showed higher amount of 15:0 compared with all the other strains tested. Isolate Fc was contained branched fatty acid 15:0 iso. Moreover, variation in fatty acid quantity was detected in *Casuarina-Frankia* isolates, although high quantity was detected in some strain like isolate Fg and Fh which was contained 18 and 16 fatty acids, other strain like Fc showed low quantity (12 fatty acids) indicating that there were significant differences between the isolates qualitatively and also quantitatively.

The results indicated that there were significant variations among *Casuarina-Frankia* isolates from *Casuarina* tested not only qualitative but also quantitative. However, the analyses of total fatty acid composition have been shown to be useful for taxonomic investigations (Janse, 1991 and Mirza *et al.*, 1991).

As analyzed by MIDI Microbial Identification System (Microbial I D, New York, Dem USA)

Advances in gas chromatography technology now give new power to the analysis of fatty acids, on its application to microbial classification. Moreover, the profile of fatty acids appears to be quite conserved at the genus-level (Mirza *et al.*, 1991 and Girgis, 1993). Culturing of *Frankia* under standardized conditions followed by extraction of their fatty acids and gas chromatographic analysis provides data for identification and characterization between *Frankia* strains. However, it could be concluded that the analysis of total cellular fatty acids can be useful and rapid technique in the characterization among closely related *Frankia* strains.

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