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PROSPECTS FOR EVALUATION OF *FRANKIA – CASUARINA* ASSOCIATION UNDER EGYPTIAN CONDITIONS I- BIOCHEMICAL AND MOLECULAR DIFFERENTIATION OF NATIVE *FRANKIA*

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ABSTRACT

Four native *Frankia* were isolated from nodules developed on the root of *C. glauca* and *C. cunninghamiana* grown on a range of Egyptian soils. Examinations of light microscopy revealed that all isolates exhibited structures characterizing *Frankia* i.e., hyphae, sporangia and vesicles (*in vitro*) Acetylene reduction activity using N-free BAP medium showed that N_2-fixing activity of those isolates ranged from 151.36 to 211.42 nmol C_2H_4/h/mg mycelial protein . The highest biomass yield were obtained from *Frankia* cultures in BAP liquid medium containing sodium propionate compared with other sources of carbon, i e , mannitol, fructose, mannose, sucrose and maltose. However, the growth pattern of all isolates under the above-mentioned conditions were similar to those of the 2 reference strains of *Frankia*. Prior to exponential growth, the isolates showed a lag phase of approximately 6-7 or 2-3 days under static and stirred conditions, respectively. On the other hand, they were sensitive to 7 antibiotics with different mechanisms of actions, i. e, streptomycin sulfate, neomycin sulfate, kanamycin sulfate, chloramphenicol, tetracycline, gentamycin and nalidixic acid. The similarities among the tested isolates which were examined by SDS-PAGE and agrose-gel electrophoresis techniques showed that they were varied in electrophoretic protein pattern as well as total chromosomal and plasmid DNA. Active hyphae and spores of all *Frankia* isolates were able to induce N_2-fixing nodules on the roots of *C. cunninghamiana* where endophytic colonization were confirmed by scanning electron microscopy.

Key words: Native *Frankia*, Biochemical characterization, Molecular differentiation, *Casuarina glauca*, *C. cunninghamiana*, Nodulation, N_2-fixation

INTRODUCTION

Frankia is an actinomycete able to fix atmospheric nitrogen either *in vitro* and *in planta* by infecting the root system and forming nodules on a number of non-legumes, termed actinorhizal plants (**Akkermans & Van Dijk, 1976; Callaham *et al* 1978, Diem and Dommergues, 1990 and Benson & Silvester, 1993**).

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Actinorhizal plants are economically and ecologically important for clearest land reclamation (**EL-Lakany, 1983**), regeneration of impoverished soils and sand dune stabilization (**National Research Council, 1984 and Dommergues, *et al* 1984**).

Since the first successful isolation and culture of *Frankia* from *Comptonia peregrina* (**Callaham *et al* 1978**) many isolates have been collected from the root nodules of actinorhizal host plants such as *Alnus* (**Baker *et al* 1979**) *Elaeagnus* (**Baker, *et al* 1980**), *Casuarina* (**Diem *et al* 1982; Diem & Dommergues, 1983; Girgis, 1993; Selim, 1999 and Zayed, 2001**); *Colletia*, (**Akkermans *et al* 1984**) and *Ceanothus* (**Lechevalier & Ruan, 1984**). These *Frankia* isolates have been characterized by their morphological structures such as hyphae, vesicles and polymorphic sporangia and N_2-fixation, carbon source utilization, restriction analysis of the genome and specific DNA or RNA, antibiotics sensitivity, determination of fatty acids composition, total *Frankia* protein patterns by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or the patterns of enzyme electrophoresis using non-denaturing ultra-low gelling point agarose polyacrylamide gel electrophoresis (ULGA-PAGE) (**Benson & Hanna, 1983; Benson *et al* 1984; Nazaret *et al* 1989, Benoist & Schwencke, 1990; Mirza *et al* 1991; Akkermans *et al* 1992; Carú, 1993; Girgis, 1993; Girgis & Schwencke 1993; Selim, 1995 and Zepp *et al* 1997**).

The present work report on biochemical and molecular characterization of 4 native *Frankia* isolates from root nodules of *Casuarina* grown on Egyptian soil. The nodulation and N_2-fixing effectivness of hyphae and spores produced by those isolates was also evaluated.

MATERIAL AND METHODS

Seeds and seedlings

Seeds and seedlings of *Casuarina cunninghamiana* were used in the present investigation. They were kindly provided by Desert Development Center (DDC), American University in Cairo, Egypt.

Reference strains

The designation and origin *Frankia* reference strains used in this study are indicated in Table (1).

Table 1. Origin of *Frankia* reference strains used in this study

Frankia	Original host	Geographical origin	Reference
UF 001	*C. glauca*	Ismailia	Selim 1999
UF 002	*C. glauca*	Zagazig	Selim 1999

Soil

A loamy sand soil with pH 8.3; E.C. 0.16 ds. m^{1} and 12; 1 and 100 ppm of N,P and K respectively was collected from El-Bostan location, Beheira Governorate. The samples were air dried, ground to pass into 2 mm sieve, mixed thoroughly prior to experimentation.

Experimental techniques

Isolation and characterization of native *Frankia*

Collection of nodules samples

Nodules samples were collected from the roots of *Casuarina* trees which were grown on soils of Sadat City, Nobaria City, Cairo-Zagazig road and Kafr El-Sheikh.

Isolation of *Frankia*

Frankia spp. were isolated from the collected *Casuarina* root nodules using the double layer method (Murry *et al.*, 1984). The selected nodules were cleaned from most soil and organic debris adhering to the surface by washing many times in water, followed by examination under the dissecting microscope to get rid of fine soil and organic particles still adhering to the nodule surface or inserted between nodule lobes. Separated lobes were surface sterilized in sodium hypochlorite (2.0%) and $HgCl_2$ (0.1% in 0.5% HCl) for 15 min then rinsed with sterile distilled water several times. Individual lobes were cultured for one week in nutrient broth at 29±2°C for testing the effectiveness of surface sterilization (Carrasco *et al* 1992).

The sterilized lobes were cut into small pieces by using a sterile scable. The small pieces of lobes were evenly distributed onto a bottom layer of 1.5% agar modified-Q-mod in petri dish. Three ml of the semi solid modified Q-mod medium was poured on the over layer, thus covering the pieces of nodule lobes (Caru, 1993), and then the plates were incubated at 29±2°C for 2-3 months. *Frankia* colonies grow out of nodule pieces and exhibited typical hyphae and sporangia were isolated on modified -Q-mod. medium (Lalonde & Calvert, 1979). *Frankia* isolates were subjected to purification trials (Diem & Dommergues 1983). The purified *Frankia* isolates were maintained on modified BAP liquid medium at 29±2°C and subculturing was usually done every week using the same medium.

Infectivity test

For this purpose, *Casuarina* seedlings were fed for at least 2 weeks with N-free ¼ Hoagland solution. Each plant was inoculated with 20 µg protein of 7 day old culture homogenized hyphae (Selim & Schwencke, 1995). Three replicates were used for each treatment. Three months later, the root systems were examined for the presence of nodules, number of nodules and their nitrogenase activity.

Scanning electron microscopy (SEM) of *Casuarina* nodules

A representative samples of nodules were subjected to ultra sectioning and then thin fixed in 2.5% glutaraldehyde for 24 h at 4°C, then post-fixed in 1% osmium tetroxide for 1h at room tem-

perature **(Harley and Fergusen, 1990)**. The specimens were then dehydrated with ascending concentrations of acetone, critical point dried, and finally sputter coated with gold. The examination, measurements and photographing were done through a Jeol Scanning Electron Microscope (JSM-T 330 A) equipped with image recording and processing system (SemAfore).

Nitrogenase activity

Nitrogen fixation was estimated by the acetylene reduction assay (ARA) method according to **Hardy *et al* (1968)**. Assay was done both "*in vitro*" for pure *Frankia* cultures and "*in planta*" for *Frankia* present in nodules. A measurable and reproducible chromatographic signal both with *Frankia* cultures and with whole nodulated plants was obtained after 3 & 24 hours of incubation with acetylene at 28°C. Assays were performed using whole nodulated plants under near-identical condition to avoid unwanted variables, as discussed by **Huss-Danell (1990) and Vessey (1994).**

Effect of carbon source on the growth of *Frankia*.

The growth of *Frankia* isolates on different carbon sources was tested in BAP-liquid medium, supplemented with either sodium propionate, galactose, lactose, mannose, maltose, mannitol, sucrose, sodium citrate, fructose or sodium pyruvate at a concentration of 0.18 gram carbon/L. All carbon sources were sterilized by Millipore filtration (pore size 0.22 μm), and *Frankia* isolates were grown on different carbon sources for 15 days **(Carú, 1993)**. Total protein of representative samples was determined according to **Bradford (1976).**

Effect of antibiotics

The antibiotic resistance of *Frankia* isolates was determined in liquid BAP medium containing 7 different antibiotics (kanamycin sulfate, nalidixic acid, gentamycin sulfate, tetracycline, chloramphenicol, neomycin sulfate, and streptomycin sulfate) at a concentration of 10 & 20 and 40 μg ml^{-1} medium. Stock solutions of the antibiotics in distilled water were sterilized by Millipore filter (0.22 μm). The media were inoculated and incubated at 29±2°C. Representation samples were collected after 15 days and the total protein was determined as shown by **(Bradford 1976).**

Electrophoretic pattern of *Frankia* protein

Analysis of *Frankia* protein was carried out for differentiation between reference strains and isolates by using the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, the tested *Frankia* were grown on BAP liquid medium for 5 days at 29+2 °C. The cells were harvested by centrifugation at 5000 rpm for 15 min and washed twice with 0.085% NaCl. Fifty μl of 10 mg/ml Lysozyme were added and pipette four times to mix. Samples were incubated at 37°C for one hour to digest the cell wall. The samples were then cooled to room temperature. A volume of 40 μl lysis buffer was added to the samples. Samples were incubated in a boiling water bath for 2 min and centrifuged for 5 min at 15000 rpm.

A volume of 10 µl mercaptoethanol (10% v/v) was added to 100 µl containing 100 µg protein extract of each sample. Samples were boiled for 10 min and 100 µl of each sample were loaded on gel after adding one drop of the loading buffer (bromophenol blue and glycerin).

Total cell DNA of *Frankia*

Isolation of chromosomal DNA of 4 *Frankia* isolates (UF010, UF011, UF012 and UF013) and two reference strains (UF001 and UF002) were carried out using Promega cot No = A 1120 kit called wizerd ® genomic DNA purification kit. The method of **Normand *et al* (1983)** was used for plasmid DNA isolation.

Effect of *Frankia* inoculum form on nodulation and N_2-fixation of *C. cunninghamiana*

Preparation of *Frankia* inoculum

Frankia cells stored in modified Q-mod medium, need two or three passages in BAP liquid medium to obtain sporangia-free exponential cells **(Selim and Schwencke, 1995)**. For all tested *Frankia,* culture was propagated by inoculating 1 µg protein per ml **(Bradford, 1976)** from exponentially growing syringe disrupted (5-6 timer using a 0.6 mm sterile needle) *Frankia* cells. *Frankia* grown in modified-Q-mod medium produce hyphae, sporangia (or spores) and vesicles **(Selim, 1999)**. Cultures enriched in one of these different morphological variant were obtained as follows:

Sporangia free exponentially growing hyphae were obtained from cells stored in modified-Q-mod medium and cultured three times in BAP liquid medium supplemented with phosphatidyl – choline and incubated at 29 ±2°C for one week with agitation at 200 rpm with a cross-type magnetic bar to be used as a starter. Spore-enriched cultures were collected after 24 days of growing *Frankia* isolates in BAP liquid medium without N-sources **(Selim & Schwencke 1995)**.

All those morphologically different cultures of *Frankia* were washed and centrifuged once at 5000 rpm for 15 min in NH_4^+ free BAP liquid medium and once in ¼ Hoagland solution without N-source **(Hoagland & Arnon 1938)** and resuspended in ¼ Hoagland solution without N-source. Finally they were syringe-homogenized (using syringe a needle 0.6 mm diameter), their protein concentration were determined according to **Bradford (1976)** to be used as inocula (20 µg/plant).

Raising of *Casuarina* seedling

Seeds of *Casuarina cunninghamiana* were sterilized by immersing for 2 min in concentrated H_2SO_4 , then washed with sterile distilled water until they reached neutral pH **(Selim & Schwencke, 1995)**. Two month old seedlings were transferred into 20 cm diameter pot (two plants), irrigated for at least 2 weeks (twice a week) with ¼ Hoagland solution with (NH_4^+) and then 3 weeks with NH_4^- free ¼ Hoagland solution under greenhouse condition **(Selim & Schwencke, 1995)**.

Plant inoculation

Development *Casuarina* plants were inoculated with *Frankia* isolates applied into a 3-cm deep hole, prepared by a

6-mm diameter wood stick with a conical end introduced at about 1-cm from the top root. This ensure a uniform distribution of the inoculum around the roots (**Selim & Schwencke, 1995**). Inocula were always prepared to contain 20 µg of *Frankia* protein per ml within the same final volume (4 ml) of ¼ Hoagland solution without N source (**Selim & Schwencke, 1995**).

RESULTS AND DISCUSSION

Frankia colonizing *C. glauca and C. cunninghamiana*

Cultural and morphological features

Four *Frankia* isolates were obtained from healthy nodules collected from the roots of *C. glauca* and *C. cunninghamiana* trees grown on different localities in Egypt, using the method described by **Murry *et al* (1984) and Carrasco *et al* (1992)**. After 2-3 months inoculated modified Q-mod solid media gave white globoid colonies grew out of nodules with a maximum of about 0.3 mm diameter. *Frankia* isolates obtained were transferred into BAP liquid medium. The growth patterns of all isolates in BAP liquid medium were similar to those of 2 reference *Frankia* UF001 and UF002 in the same medium.

When the microorganisms were subcultured several times, the filamentous culture developed. Under light microscope, The morphological features of typical *Frankia* structure were very similar to the reference *Frankia* strains .In liquid BAP medium, all isolates formed branched septate hyphae (from 0.1 to 0.6 µm in diameter). Spores and sporangia were in a lateral or terminal position on the hyphae with sizes from 1.0-1.5 and 4.0-15.0 µm respectively. (Figs. 1A & B). In the same above mentioned medium without N source a spherical vesicles ranging from 1.0-1.5 µm in diameter were formed in abundance, (Fig. 1 C). However, *Frankia* isolates were differed in their growth rate.

Nodulation and N_2-fixation

Under defined greenhouse conditions, all *Frankia* isolates were able to nodulate *Casuarina* seedlings (Fig. 2). The effective nodules formed by all *Frankia* isolates showed normal structure and typical internal organization with endophyte developing vesicles within the cortical cells of the host tissue (Fig. 3).

Data in Table (2) show the nitrogenase activity *"in vitro"* of the *Frankia* isolates, measured as acetylene reduction. The values of nitrogenase activity of *Frankia* isolates and strains grown in nitrogen free BAP liquid medium ranged from 151.36 to 211.42 n mol C_2H_4/h/mg *Frankia* protein.

Growth responses to different carbon sources

The ability of the *Frankia* isolates to utilize various carbon sources (galactose, lactose, mannose, maltose, mannitol, fructose, sucrose, sodium citrate and sodium pyruvate were compered with sodium propionate as control when present singly in BAP liquid medium as a sole carbon and energy source under static conditions (see Fig. 4).

The recorded data in Fig. (4) obviously show that, sodium propionate was the best carbon source with the isolates the UF011, UF012, UF013 and strains

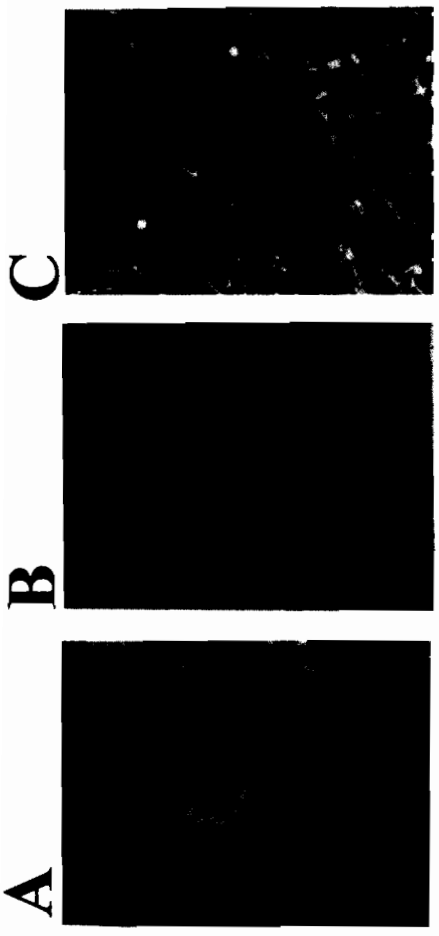

Fig. 1. Photographs of light microscopic examination of *Frankia* isolates grown in BAP liquid medium as visualized by epifluorescence after acridin orange vital staining.
A) Hyphae, B) Sporangia, C) Vesicles.

Fig. 2. A photograph illustrating root nodules of *Casuarina* bearing many nodules lobes with various size

Table 2. I*n vitro* specific nitrogenase activity of four *Frankia* isolates and two reference strains grown in N free BAP medium

Frankia isolates	UF010	UF011	UF012	UF013	UF001	UF002
Nitrogenase activity (nmol C_2H/h/mg protein)	191.69	182.47	151.36	202.89	172.01	211.42

UF001 and UF002 which gave biomass yield of 11.5, 12.5, 12.7, 14.3 and 16.1 µg protein/ml medium, respectively. On the other hand, higher biomass yield was obtained from *Frankia* isolate UF010 when grown with mannose, maltose, mannitol, sucrose or fructose as carbon sources giving, 17.6; 16.3; 16.3; 16.1 and 15.9 µg protein/ml, respectively. However all *Frankia* isolates grew well in BAP liquid medium containing sodium propionate as a sole carbon source, perhaps, because this compound is a standard carbon source in the medium used for isolation of *Frankia* **(Carú, 1993)**.

Effect of antibiotics on *Frankia* growth

The sensitivity of four isolates of *Frankia*, and two strains towards variety of antibiotics with different mechanisms of action (kanamycin sulfate, nalidixic acid, gentamycin sulfate, tetracycline, chloramphenicol, neomycin sulfate and streptomycin sulfate) with three concentrations (10, 20 and 40 µg/ml medium)

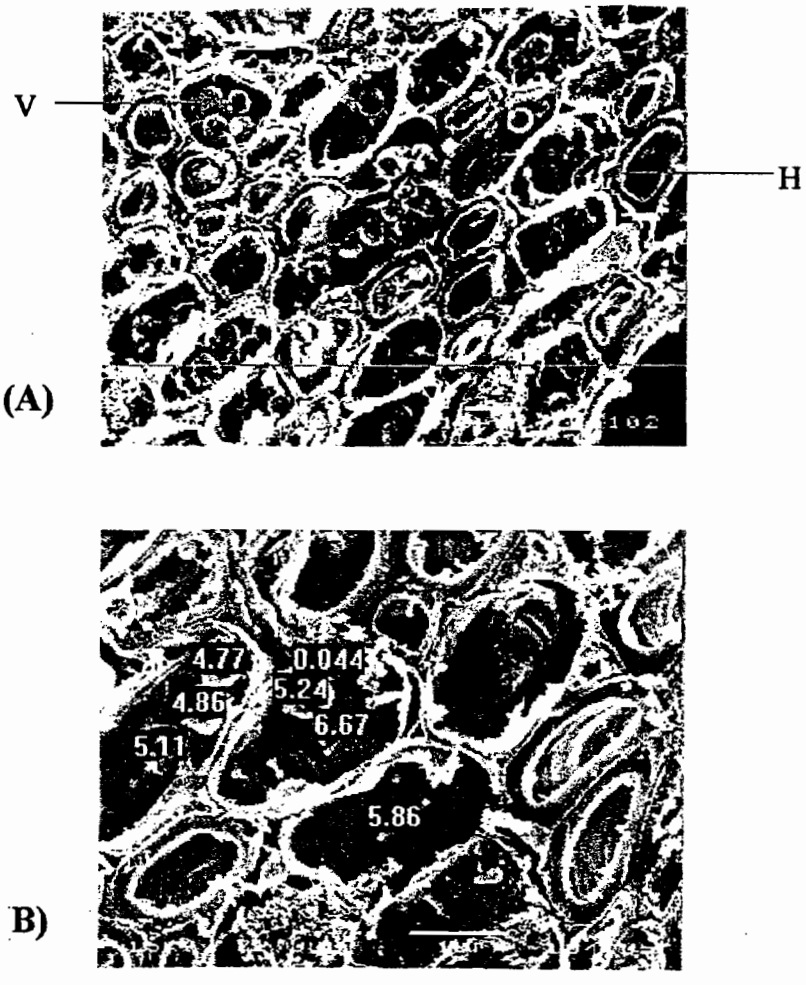

Fig. 3. Electron micrograph of effective nodule from *Casuarina* induced by *Frankia* isolate, showing *Frankia* vesicles (V) and hyphae (H) embedded in the cytoplasm of cortical cells (24 weeks after inoculation)
* Bar scale = 10μm (A) 1000 X (B) 2000 X

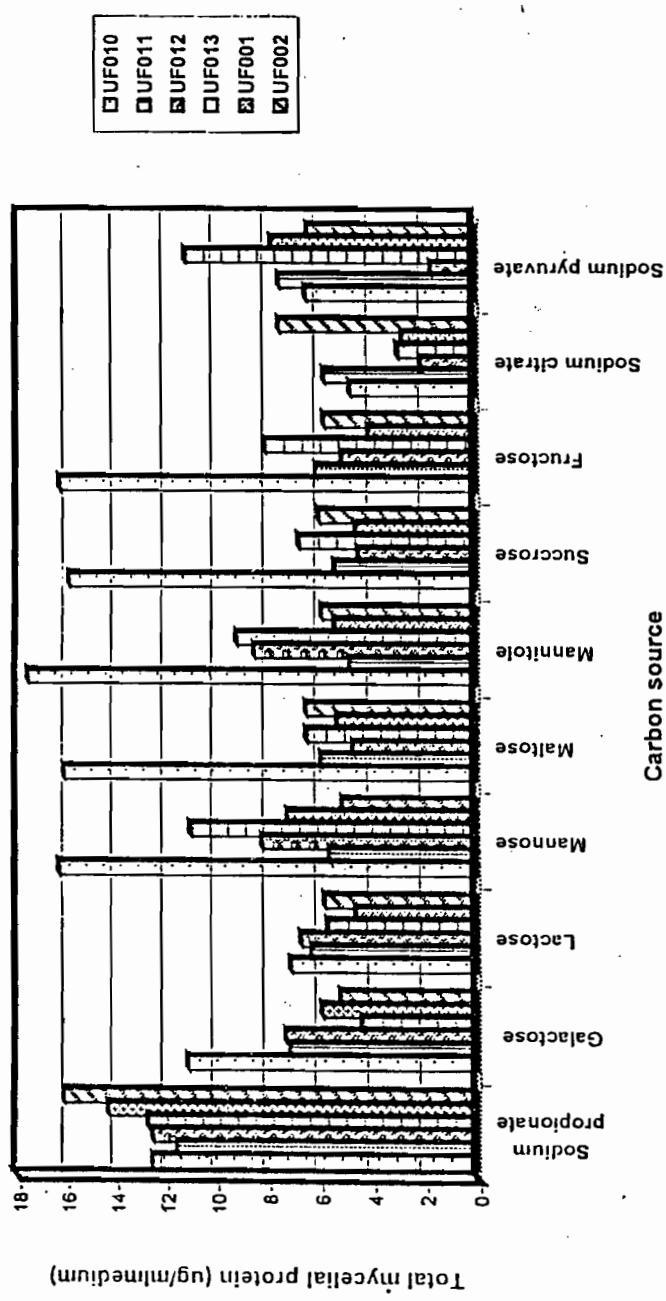

Fig. 4. Protein concentrations of four isolates and two reference strains of *Frankia* grown in BAP liquid medium with different carbon sources

was determined in BAP liquid medium after 15 days of incubation at 29±2°C (see Table, 3).

Generally, *Frankia* biomass yield decreased with increasing the antibiotic concentration used. However, the isolates UF012 & UF013 grown in media supplemented with streptomycin sulfate and isolates UF012 and strain UF002 in chloramphenicol (Table, 3) showed a very high sensitivity even at the lowest concentration of antibiotic used (10 µg/ml). In these cases, the initial *Frankia* protein used as inoculum (1 µg protein/ml) was either unchanged indicating complete growth inhibition or even cell lysis may occurred since the amount of protein determined at the end of the incubation period was either unchanged or decreased less than the amount of *Frankia* protein as inoculum.

Few studies on antibiotic sensitivity of *Frankia* have been published, praticularly on strains isolated from the genera *Alnus* and *Elaeagnus* **(Normand and Lalonde, 1986).** A spontaneous out ineffective neomycin-resistant *Frankia* from an *Alnus*-compatible strain has also been obtained **(Faure, Raynaud *et al* 1990).** The resistance of *Frankia* strains to various antibiotics may be useful in studies of survival and competitiveness in soil, differentiation between the closely related *Frankia* strains and identification of their presence in nodules on plants inoculated with a mixtures of strain **(Carú, 1993; Girgis, 1993; Selim, 1995 and Selim *et al* 2000).** The antibiotics assay can also become a useful tool as a specific genetic markers **(Carú, 1993).** Resistance to antibiotic is the most useful since it is not only a stable marker for strains, but also enables one to use positive selection in genetics work specifically in DNA cloning.

Growth kinetics of *Frankia* isolates

A comparison between the growth kinetics of the four *Frankia* isolates and two reference strains grown in BAP liquid medium under stirring and static conditions during 10 and 20 day respectively at 29±2°C are presented in Figs (5&6). Growth curve of *Frankia* isolates and strains cultures can be depicted graphically by plotting the total mycelial protein µg/ml against time in. Figs (5 A&B) show the growth curve of *Frankia* isolates when grown in BAP liquid medium under stirred conditions. It could be noticed that growth patterns of the *Frankia* isolates were generally similar, and it grew rapidly in stirred culture conditions with maximal biomass yield after 7 or 8 days of growth being -19.6, 23.5, 22.1, 24.5, 24.8 and 20.3 µg protein/ml medium for *Frankia* isolates UF010, UF011, UF012, UF013, and the reference strains UF001, UF002 respectively.

On the other hand, *Frankia* isolates grown in BAP liquid medium under static conditions (Figs. 6 A& B) showed comparatively slower growth rates with low biomass yield. The maximum yield was ranged from 16 to 17 µg protein/ml medium after 16 days from inoculation, except for isolate UF010 which had a maximal growth yield of 15.4 µg/ml after 18 days of incubation. It could be concluded that *Frankia* isolates and strains used in this study grew faster and gave a maximum mycelia growth (µg protein/ml) under stirred growth conditions in comparison with the static conditions.

Table 3. Effect of different antibiotics with three concentrations (µg) on growth of four isolates and two referance strains of *Frankia* grown in BAP liquid medium after 15 days

	Kanamycin sulfate			Nalidexic acid			Gentamycin sulfate			Tetracycline			Neomycin sulfate			Chloramphenecol			Streptomycin sulfate		
	10	20	40	10	20	40	10	20	40	10	20	40	10	20	40	10	20	40	10	20	40
UF010	5.8	3.6	3.2	11	9.1	8.9	4.0	3.8	1.5	7.4	3.7	3.6	3.5	3.5	2.4	4.3	2.9	2.6	4.6	3.5	3.3
UF011	4.5	3.8	2.1	4.8	3.5	1.9	5.3	3.1	1.2	4.6	2.9	2.1	5.1	3.9	2.3	4.0	1.8	0.5	3.4	2.3	0.5
UF012	7.2	6.8	1.8	5.8	4.5	3.2	4.6	1.3	1.1	2.9	2.4	1.8	3.0	1.9	0.5	0.5	0.5	0.5	0.5	0.5	0.5
UF013	11.9	8.9	1.2	8.9	5.6	4.0	2.5	1.5	1.1	4.1	2.5	1.2	1.5	1.3	0.5	6.5	4.3	1.4	0.5	0.5	0.5
UF001	6.9	6.7	2.5	6.7	5.4	2.5	5.0	3.5	0.5	3.1	2.8	2.6	5.6	2.0	1.4	3.5	2.4	1.4	2.1	2.0	1.4
UF002	7.8	6.3	4.3	3.7	4.6	3.4	2.5	2.0	1.6	4.6	3.2	1.4	1.1	0.5	0.5	0.5	0.5	0.5	1.5	0.5	0.5

Measured by the amount of mycelia protein in µg/ml medium produced under different treatment

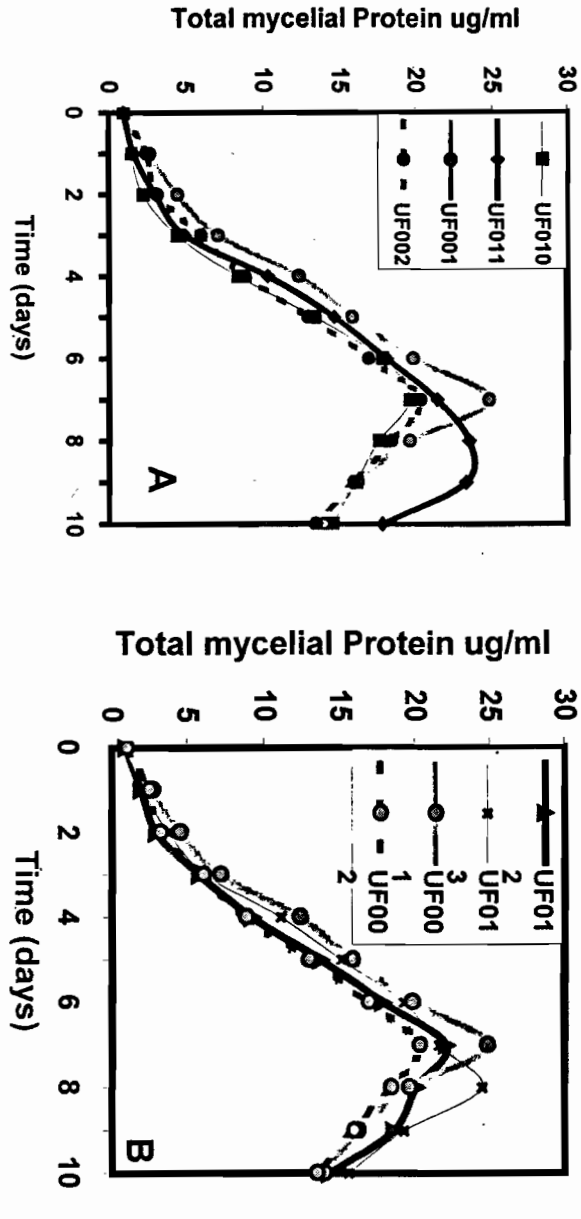

Fig. 5. Growth curves of four isolates and two reference strains of *Frankia* inoculated in BAP liquid medium under stirred conditions

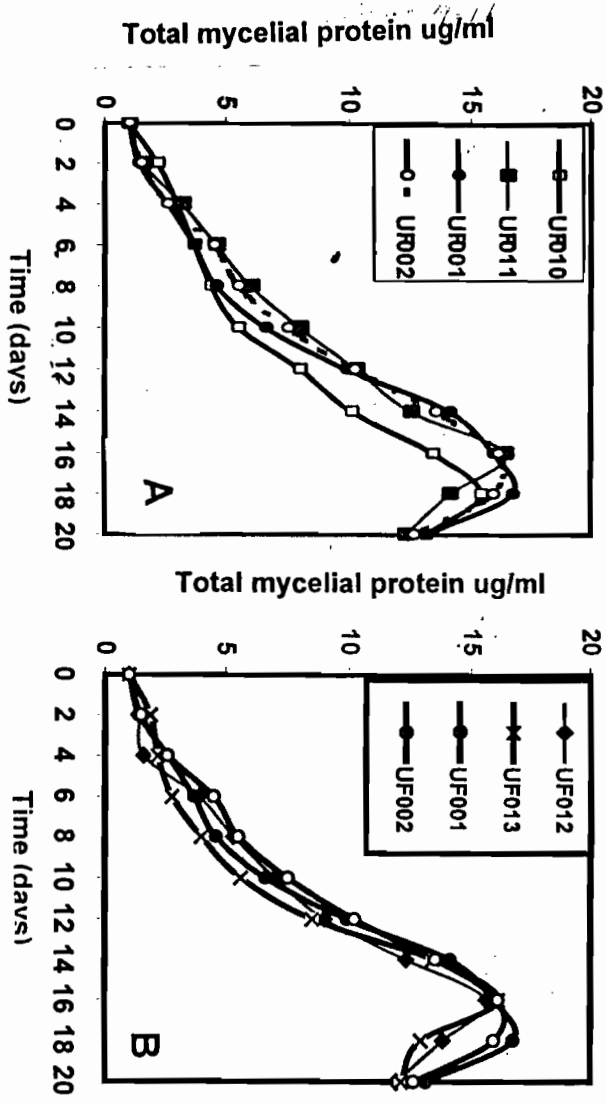

Fig. 6. Growth curves of four isolates and two reference strains of *Frankia* inoculated in BAP liquid medium under static conditions

Analyses of total protein

Protein analysis by SDS polyacrylamide gel electrophoresis (SDS-PAGE) has been used to characterize differences between *Frankia* strains **(Benson & Hanna, 1983; Nazaret *et al* 1989; Baker & Mullin, 1992 and Selim *et al* 2000).**

The present results of the SDS-PAGE analyses electrophoretic pattern of total protein extracted from the four *Frankia* isolates are given in Figs. (7) & (8). According to the computer analyses of the data. Twelve bands were common in all *Frankia* tested i.e., 82.67; 79.33; 75.50; 63.75; 57.62; 54.59; 51.18; 49.44; 42.20; 37.79; 23.19 and 16.63 KDa. *Frankia* isolate UF010 showed 21 bands ranged from 82.67 to 14.90 KDa, while 6 bands were absent with molecular weight of 81.15; 61.54; 32.96; 29.46; 25.97 and 20.34 compared with other tested *Frankia*. Moreover, *Frankia* isolate UF011 obviously showed 30 bands observed at 82.67 to 12.29 KDa. There are 11 novel bands with molecular weights of 81.15; 61.54; 44.86; 36.35; 34.49; 32.96; 31.52; 29.46; 25.97; 20.34; 12.29 KDa compared with the isolate UF010. *Frankia* isolate UF012 showed 30 bands present at 82.67 to 12.29 KDa. There were also 4 bands differentiating between UF012 and UF011 which were found in molecular weights 69.48; 40.24; 34.49 and 18.62 KDa, but, there were 11 different bands observed at 81.15; 69.48; 61.54; 44.86; 36.35; 32.96; 31.52; 29.46; 25.97; 20.34 and 12.29 KDa, compared with the isolate UF010. Electrophoretic profiles of isolate UF013 showed a 5 novel bands at molecular weight 72.56; 67.94; 66.16; 52.60 and 43.80 KDa while two bands were absent at molecular weights 67.10 and 27.64 KDa compared with the isolates UF010, UF011, UF012. On the other hand, *Frankia* strain UF001 gave 30 bands ranged from 82.67 to 12.29 KDa, but there were two absent bands at 71.03; 14.90 KDa compared with the *Frankia* isolate above. Protein pattern of UF002 showed a two novel bands at molecular weights 77.23 and 56.50KDa, while two bands were absent at 73.82 and 46.32 KDa compared with the other *Frankia* isolates.

A dendrogram of the relationships among *Frankia* isolates and reference strains, based on the computer analysis of the total protein is given in, Fig. (8). It could be indicated that the highest percentage of similarity index was detected between isolate UF011 and UF012 (group 1) being 90%. In case of isolate UF013 and strain UF001 (group 2) it was 89%. The similarity between group 1 and group 2 was 83%, but it was 77% between the last two groups and strain UF002. Isolate UF010 gave 69% similarity to the other tested *Frankia*.

From the results of total protein analysis of *Frankia* isolates and reference strains it could be concluded that the isolates UF010, UF011, UF012, UF013 belong to the genus *Frankia*.

Analyses of total chromosomal and plasmid DNA

The isolated chromosomal and plasmid DNA were subjected to agarose gel electrophoresis and stained with ethidium promeide, the banding pattern visualized in ultraviolet light. Figure (9) shows a pulsed field gel electrophoresis of chromosomal DNA from isolates UF010, UF011, UF012 and UF013 and reference strains UF001 and UF002 of *Frankia* using λ/ Hind III marker. It is obvious

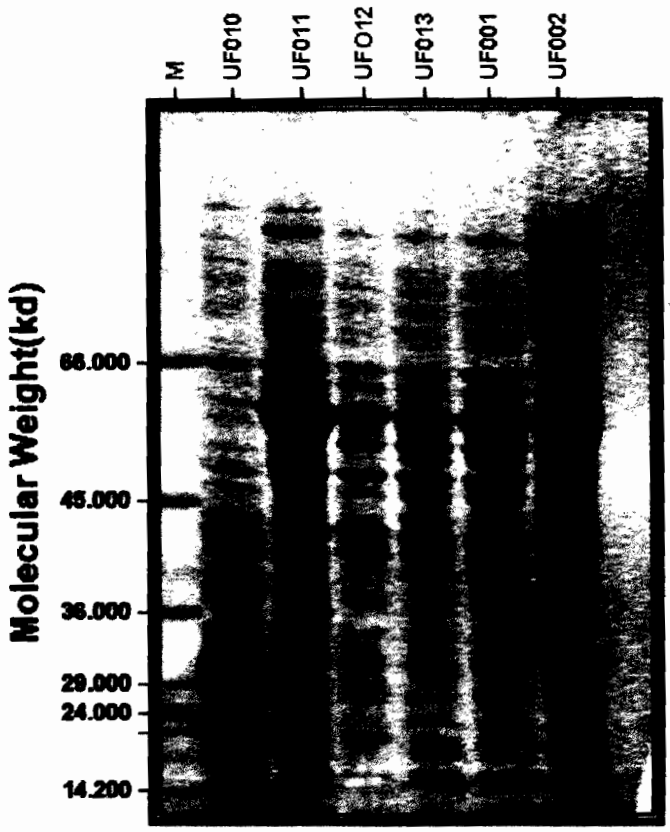

Fig. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of total protein of four *Frankia* isolates and two reference strains. The protein was extracted from exponentially growing *Frankia* cells in BAP liquid medium under stirrid condition

Frankia	UF010	UF011	UF012	UF013	UF001	UF002
UF010	1	0.72	0.75	0.64	0.69	0.63
UF011		1	0.9	0.81	0.82	0.77
UF012			1	0.84	0.85	0.77
UF013				1	0.89	0.8
UF001					1	0.71
UF002						1

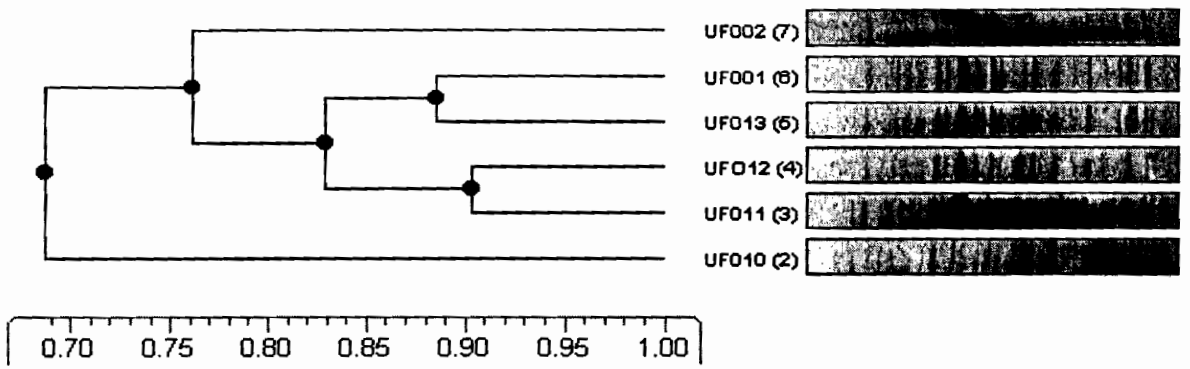

Fig. 8. Dendrogram showing relationships between four isolates and two reference strains of *Frankia*

that banding pattern of chromosomal DNA of the four above mentioned isolates (lanes 1, 2, 3 and 4) and the two reference strains of *Frankia* (lanes 5 and 6) had the same position on the agaros gel with a molecular weight more than the marker used (lane 7). These results, therefore, provide a simple alternative to DNA association studies for allocation of *Frankia* isolates to the reference strains.

Figure (10) shows the results of a trial to isolate plasmid DNA from the four *Frankia* isolates (lanes 2, 3, 4 and 5), the two reference strains (lanes 6 and 7) using λ/Hind III marker (lane 1). It is clear that *Frankia* isolates UF012 and UF013 and the reference strains harbor plasmid on the same band posit-ion on the gel and the molecular weight of the bands tend to be a large plasmid (It has a molecular weight more than the first band of the marker used which has a molecular weight of 23, 13 Kb. In case of *Frankia* isolates UF010 and UF011, the amount of isolated plasmid DNA probably was not sufficient to be visible under UV light or it has not plasmid at all.

The mean genome size of an organism gives an idea of the complexity of its metabolism as expressed by **Watson, (1976)** that for each protein there is at least one gene. The genome size of *Frankia* is twice that of *E. coli* and like that of *Streptomyces* that measures 10.000 Kb. In some strains of *Frankia*. plasmids ranging from 8 to 190 Kb **(Normand and Lalond, 1986).**

Effect of inoculum form of *Frankia* on nodulation and N_2-fixation of *C. cunninghamiana*.

The effect of inoculation with different morphological structures namely, exponential growing hyphae and spores of the four isolates UF010, UF011, UF012 and UF013 and the two reference strains UF001 and UF002 on nodule formation and nitrogenase activity of *Casuarina cunninghamiana* plants grown on loamy sand soil, was determined after 6 months Table (4). In control treatment (uninoculated plants) no nodules was noticed on plant roots which indicate the absence of effective indigenous *Frankia* in the soil used. Thus, it becomes evident that nodule formation, when occurred, is exclusively due to the introduced test organisms

Generally, higher records of nodulation and nitrogenase activity were obtained from the inoculated plants with exponentially growing hyphae of all tested *Frankia* compared to *Frankia* spores. The nodulation frequency were 80, 90, 90, 100, 90 and 100% for plants inoculated with the growing hyphae of isolates UF010, UF011, UF012 and UF013 of *Frankia* and the reference strains UF001& UF002 respectively. The corresponding nodulation frequency of *C. cunninghamiana* inoculated with *Frankia* spores were compared with the results obtained with hyphae being 30, 30, 30, 70, 70 and 70% in the same above mentioned respective order.

The same trend of results was obtained for ARA expressed as n moles C_2H_4/h/plant root which reflects nitrogenase activity and demonstrates that *Casuarina* nodules in intact plants were capable to fixing nitrogen, and the rate of nodulation was almost associated with nitrogenase activity. A biotic factors in the soil as well as the physiological conditions of the root hairs and *Frankia* must all be favorable. Therefore, a low nodulation frequency in some cases does not

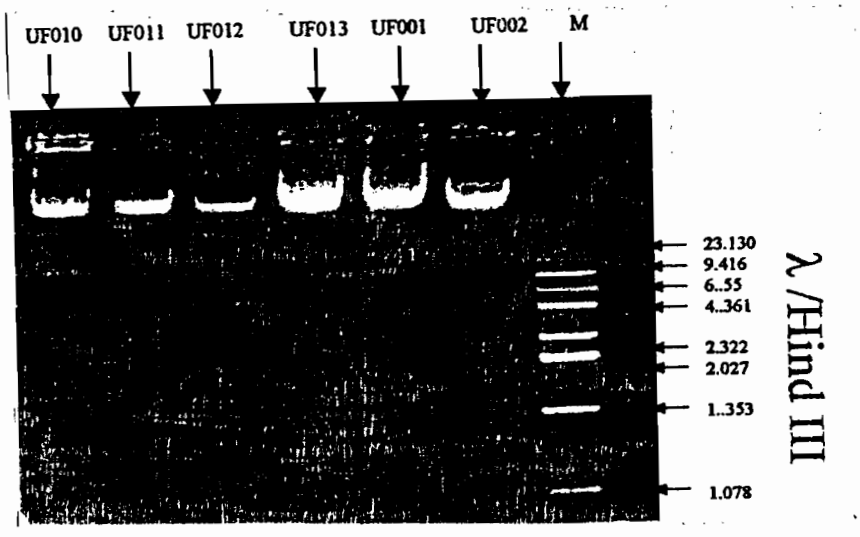

Fig. 9. Pulsed field gel electrphoresis of chromosomal DNA profile of four isolates and two reference strains of *Frankia*

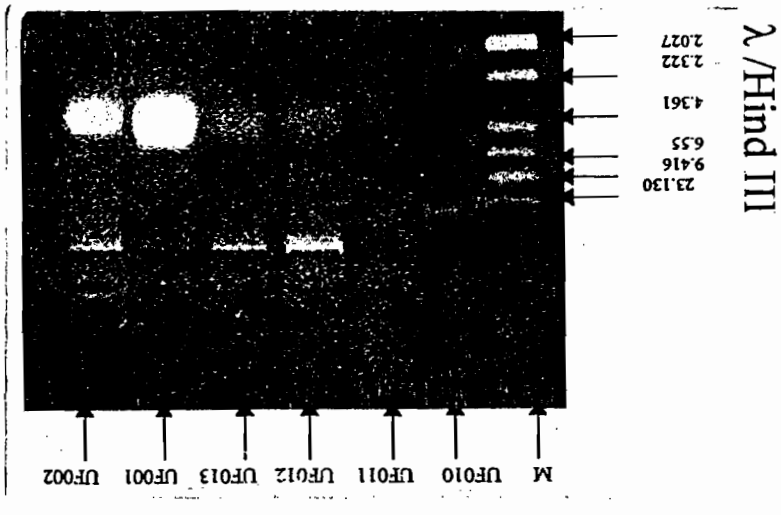

Fig. 10. Gel electrophoresis of plasmid profile of four isolates and two reference strains of *Frankia*

Table 4. Effect of inoculum form of *Frankia* on nodulation and N_2 fixing activity of *C. cunninghamiana* grown on loamy sand soil for 6 months under green house condition

Inoculum / Parameter	Exponential hyphae						Spores					
	UF010	UF011	UF012	UF013	UF001	UF002	UF010	UF011	UF012	UF013	UF001	UF002
Nodulation frequency (%)	80	90	90	100	90	100	30	30	30	70	70	70
Number of nodules/plant	19	6	6.5	11	13	13.4	2	4	2	4.33	10	7.33
Dry weight of nodule (mg) / plant	486.2	523	389	310	762	621	36	481.3	266	219.7	414.2	187
ARA	4138	2131	2690	3120	3963	4650	890	1870	778	2119	2896	3167

* ARA: Acetylene reduction activity n mol C_2H_4/h/plant root

necessarily reflect a scarcity of *Frankia*, but it can also be due to soil and plant factors regulating, infection and nodule.

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مجلة اتحاد الجامعات العربية للدراسات والبحوث الزراعية ، جامعة عين شمس ، القاهرة ، ١١(١) ، ٩٧ - ١٢٠ ، ٢٠٠٣

اتجاهات تقييم تعايش الفرانكيا والكازوارينا تحت الظروف المصرية
١- التفرقة البيوكيماوية والجزئية للفرانكيا المتوطنة

[٩]

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تم في هذه الدراسة الحصول علي ٤ عزلات للفرانكيا UF010,UF011,UF012, and UF013 من العقد المتكونة علي جذور *C. cunninghamiana, C. glauca* النامية في أماكن مختلفة بالأراضي المصرية ، وأظهرت الفحوصات المعملية بالميكروسكوب الضوئي ان كل العزلات ذات تراكيب مميزة لأفراد هذا الجنس (الهيفات , الأكياس الجرثومية , الحويصلات) وكذلك تحفيز إنتاج عقد جذرية علي جذور *C. cunninghamiana* تحت ظروف الصوبة وظهرت حويصلات للفرانكيا في القطاعات الرقيقة المأخوذة للعقد بعد تجهيزها وفحصها بالميكروسكوب الإلكتروني الماسح وتراوحت كفاءة اختزال الأستلين بواسطة هذه العزلات من ١٥١,٣٦ - ٢١١,٤٢ نانومول ايثيلين/ساعة/كجم بروتين مسيليومي كما أمكن الحصول علي أعلي إنتاجية للنمو الحيوي عند تنمية هذه العزلات في بيئة B A P السائلة المحتوية علي بربيونات الصوديوم مقارنة بمصادر الكربون الأخرى (المانيتول ، الفركتوز ، المانوز ، السكروز ، والمالتوز) وفي كل الأحوال كان نمط نمو جميع العزلات مماثلا السلالتين المرجعيتين UF001,UF002 حيث أظهرت العزلات طور سكون لمدة من ٦ -٧ أيام تحت ظروف المزارع ومن ٢ - ٣ أيام في حالة المزارع المقلبة كما كانت العزلات جميعها حساسة لسبعة أنواع من المضادات الحيوية ذات ميكانيكيات تأثير مختلفة وهي كبريتات الاستربتوميسين ، كبريتات النيوميسين ، كبريتات الكاناميسين، الكلورامنفيكول ،التتراسيلكين ، جنتاميسين ، حمض الناليدكسك وبتقدير دارجات التشابه بين العزلات من خلال تقنيات - SDS Plyacrylamide Gel - Electrophoresis (SDS-PAGE) ظهر ان هذه العزلات تتباين من حيث نمط هجرة البروتين وكذلك كمية DNA الكروموسومي والبلازميدي وعلي الرغم من قدرة الهيفات والجراثيم المأخوذة من كل من العزلات مجال الدراسة علي تحضير إنتاج العقد الجذرية علي جذور بادرات *C. cunninghamiana* .

تحكيم: أ.د السيد أحمد صالح أ.د ربيع الشهاوى