

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Studies on the Growth, Culture Behavior and Infectivity of two *Frankia* Strains UFECeI5 and LLR43 Grown in Medium Containing Antibiotic Substances

Samira R. Mansour

Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt

Abstract: Antibiotics Penicillin G and Rifamycin were screened for their effect on morphology, growth and infectivity of two *Frankia* strains CeI5 and R43. Rifamycin at concentration $10\mu\text{g ml}^{-1}$ was found to produce morphological changes and alternation in infectivity of *Frankia* strain R43. Penicillin G showed no effect at the same concentration and had inhibitory effect at higher concentrations as Rifamycin.

Key words : *Frankia*, Actinorhizal plants, Antibiotic, Infectivity

Introduction

Frankia is the generic classification of a group of actinomycetes, which symbiotically associate with a variety of woody, dicotyledonous plants. Plants infected by *Frankia* are known collectively as actinorhizal. *Frankia* infects the roots of these plants, which respond by forming specialized structures known as nodules. In pure culture, *Frankia* is characterized by extensive branches hyphae bearing terminal swelling termed vesicles and sporangia which contain spores.

Within the past three decades ideas concerning the relationship of *Frankia* to its host have changed substantially. Becking (1970) proposed that nodule forming actinomycetes are obligate symbionts, but then, in 1978, the first confirmed isolation and culturing of *Frankia* occurred (Callaham *et al.*, 1978). These achievements have sparked new interest in understanding the natural relationships of *Frankia* with its host. In an elegant study done by Baker (1987), it concluded that pure *Frankia* strains have a broad host range but fall under four well-defined host specificity groups. Among these group is *Casuarina* host specificity group, which includes *Frankia* strains that have capability to infect different *Casuarina* species as a host. However, pure *Frankia* cultures isolated from root nodules of *Casuarina* species are falling under two different host specificity groups. The first group includes isolates that have capabilities to re-infect *Casuarina* as their original host (Group 2). However, the second group are the isolates which failed to re-infect their host (Group 3) (*Casuarina*) but effectively nodulate members of another host specificity group, Eleagenaceae. This phenomenon can be explained by whether in the course of isolation process or the way in which a culture of *Frankia* is grown modifies the infective capacity of *Frankia* isolates. It also possible to state that under soil conditions *Frankia*

strains, with association of natural existence of different microbial population, may behave differently. Soil conditions were found to have a significant role in survival and establishment of *Frankia* strain with its host (Reddell *et al.*, 1986; Smolander, *et al.*, 1988). However, there is little understanding of the behavior, in relation to host specificity, of *Frankia* in soil. Existence of antibiotic producing organisms among microbial population in soil is taken in an account that antibiotic substances produced by these organisms may affect the behave of *Frankia* strains in soil.

In the light of present state of knowledge it seems more appropriate to gain indirect indications of its behave in medium containing antibiotic substances so that sensible experimental schemes might be generated. Hence, in this work, investigation of growth response, effectivity and infectivity of some *Casuarina* isolates grown in medium containing antibiotics was carried out.

Materials and Methods

Source of isolates: Two *Frankia* strains were used in this study. The source, designation, catalog number and their host specificity were shown in Table 1. Both of these strains were isolated from *Casuarina* root nodules and failed to re-infect their host. These *Frankia* strains can infect other actinorhizal host plants, therefore they belong to host specificity group 3 (Table 1).

Inoculum preparation, growth conditions and morphological characterizations: The *Frankia* strains were propagated in defined B medium (Murry *et al.*, 1984) supplemented with 2 mM NH_4Cl and subcultured for three successive growth incubation time periods, each one was 6-10 days. Short incubation period in this medium was applied to obtain rapidly intensive hyphal growth devoid of differentiated structures. Filamentous samples were

examined under Nomarski interference contrast optics and found to be free of vesicles, sporangia, or spores. The hyphae were harvested by centrifugation at 4,000 rpm with washing in sterile distilled water, homogenized with Potter-Elvehjem homogenizer, and inoculated into B medium with different concentration of antibiotics. Two different types of antibiotics, Penicillin G (*benzylpenicillin*) and Rifamycin were added to the B medium in which their concentrations will reach 1, 10, 50, 100 $\mu\text{g ml}^{-1}$ of the medium. Five replicate flasks containing 50 ml of liquid medium for each treatment were inoculated with 0.05 ml Packed Cell Volume (PCV), with 3.8 $\mu\text{gm protein ml}^{-1}$, of hyphal homogenate and incubated at 28°C.

Microscopic examination was made at one, two, three and four weeks after inoculation then the cell transferred to 1ml of 3% glutaraldehyde for 3h to which 1 ml of 50% glycerol was added. Examination of samples transferred to glass microscope slides after 12 h was made with the microscope using phase contrast optics. Five microscopic fields per slide were examined for each flask per each treatment. Five slide replicates were carried for each flask. Growth yield was assessed by estimating total protein of washed hyphae at 7, 15 and 30 days after inoculation using the Bio-Rad protein assay following the method described by Murry *et al.* (1984).

Infectivity and effectivity studies: *Frankia* strains CeI5 and R43 propagated in B medium with either Penicillin G or Rifamycin at concentration 1 and 10 $\mu\text{g ml}^{-1}$ of medium were tested for their capacities to infect different *Casuarina* species. Higher concentrations of both Penicillin G and Rifamycin were not tested for unhealthy hyphal growth of both *Frankia* strains.

Seedlings preparation: Seeds of different *Casuarina* species (*C.cunninghamiana*, *C.glauca* and *C.equisetifolia*) were propagated following the method of Mansour and Torrey (1991). *Csuarina* seeds were courtesy obtained from Dr. El-Lakany, Dessert Development Center of the American University in Cairo. When plants were about 6 cm tall (1-3 months depending on species), young plants were transferred to water-culture jars containing 1/4-strength Hoagland's solution (Hoagland and Arnon, 1950) lacking nitrogen. Five jars were used for each species with three plants per jar.

Inoculum preparation and inoculation: Hyphal growth of *Frankia* strains CeI5 and R43 propagated in B medium, containing antibiotic substances, were harvested at the end of third week of inoculation. Harvested hyphae

were washed in distilled water with centrifugation and homogenized. The inoculum was applied drop wise along the roots of each seedling using 0.01 ml PCV of culture plant⁻¹. Five inoculated jars with each *Frankia* strains CeI5 and R43 grown in B medium served as controls. Also, five uninoculated jars served as negative controls. Plants were grown in the green house without aeration. Observations of nodule formation were made at regular intervals and nodule number and percentage of nodulation recorded.

Results and Discussion

Morphological characterization: Morphological responses of two *Frankia* strains, CeI5 and R43, to the different concentrations of antibiotics used in this study are illustrated in Table 2 (a and b). High concentrations of Penicillin G and Rifamycin greatly inhibited vesicle and sporangium formations for both *Frankia* strains CeI5 and R43. *Frankia* strain R43 was much sensitive to different concentrations of Penicillin G than CeI5, as a result less frequency in vesicle and sporangium formations was recorded. However, with Rifamycin *Frankia* strain R43 showed high frequency of vesicle formations. Abnormal vesicle morphology, double spherical swellings, of *Frankia* strain R43 was also observed in B medium containing 10 $\mu\text{gm ml}^{-1}$ of Rifamycin (Fig.1). The potential usefulness of the unusual form of vesicles as a genetic marker depends on whether this structure is due to a stable genetic mutation or not, more studies are in need. However, the effect of this unusual form has on other traits such as infectivity, was investigated in this study.

Growth of *Frankia* strains: Growth of CeI5 and R43, expressed as $\mu\text{gm protein}^{-1}\text{ml}$ in the four different concentrations of antibiotics tested, was highly suppressed by high concentrations of these antibiotics (Fig.2. a, b, c and d). This result agrees with those of *Rhizobium* species recorded by Davey and Papavizas (1961) and Pattison and Skinner (1973). Greater affects were recorded at 30 days after inoculation. Decreases were attributable to autolysis late in cultured period. These results are in confirmation with results obtained by Mansour *et al.* (1991). In the same time, the higher concentrations of antibiotics used enhanced and increased the cells autolysis. *Frankia* strain R43 showed significantly lower ($P < 0.05$) protein content compared with *Frankia* strain CeI5 when propagated in B medium containing different concentration of Penicillin G (Fig. 2.a. and b). At low concentrations, Rifamycin recorded less effect on growth for both *Frankia* strains (Fig. 2.c and d).

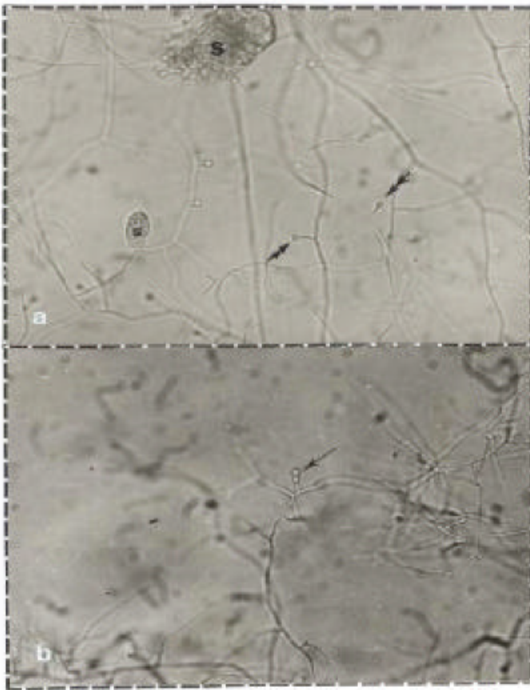


Fig. 1: Light photomicrograph of *Frankia* strain LLR43 showing the unusual double spherical form (arrow) of vesicle which is occasionally found when R43 grown in B medium supplemented with 10 $\mu\text{g ml}^{-1}$ Rifamycin. Magnification= 1000X

Fig. 2c

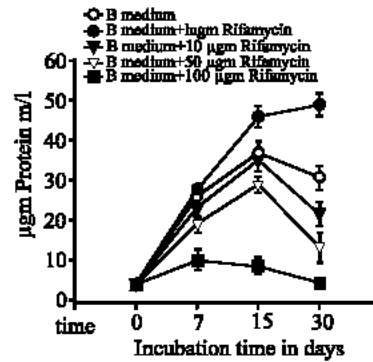


Fig. 2d

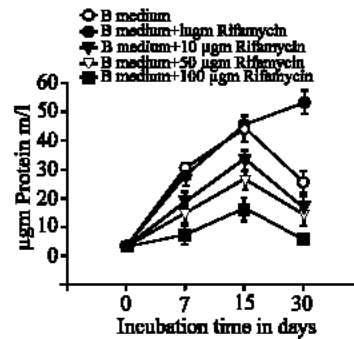


Fig. 2: Growth responses of *Frankia* strains UFGCeI5 and LLR43 to different concentrations of Penicillin G (a and b respectively) and Rifamycin (c for CeI5 and d for R43) assessed as $\mu\text{g protein ml}^{-1}$ after 7, 15 and 30 days of inoculation. Vertical lines represent standard deviations

Fig. 2a

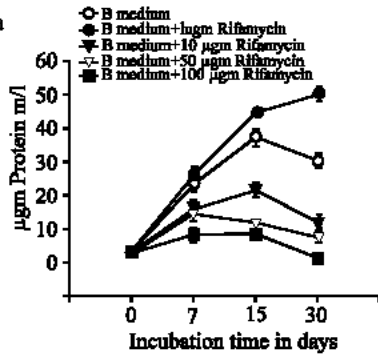
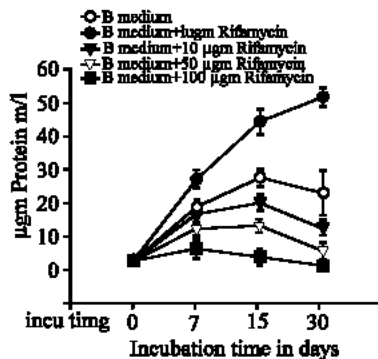


Fig. 2b



Infectivity and effectivity: The results of infectivity trials with CeI5 and R43 were tabulated for three *Casuarina* species (Table 3). Only *Frankia* strain R43 incubated in B medium with 10 $\mu\text{g ml}^{-1}$ Rifamycin was infective with all *Casuarina* species. *Casuarina cunninghamiana* and *C. glauca* seedlings showed nodule initiation within four weeks, and *C. equisetifolia* seedlings showed delayness of nodulation, 6-7 weeks after inoculation. Significant differences ($P < 0.01$) in total nitrogen content in comparison with uninoculated controls were recorded among different *Casuarina* species. It was interesting that *C. cunninghamiana* showed the highest nodulation percent and total nitrogen content. Alteration of host specificity at low concentration of rifamycin for *Frankia* strain R43, than was recorded Baker (1987), was surprising and it can be used as new tool for *Frankia* classification. This needs to explore the other *Frankia* strains that failed to nodulate *Casuarina* host specificity group. This new finding, of the effect of specific antibiotics on some

Table 1: Host origin, trivial designation, and host-specificity group (HSG) of *Frankia* strains Cel5 and R43 used in this study. Cross-inoculation studies done by Torrey (1989) including seedlings from three families Casuarinaceae, Elaeagnaceae and Myricaceae. Scores: + =all plants nodulated, ± = less than 50% nodulated, - = no nodulation, space = not tested

Trivial designation	Catalog Number	host origin	Nodulation results							HSG	References
			<i>C.cumm.</i>	<i>C.equis.</i>	<i>C.glauca</i>	<i>E.ang.</i>	<i>Hip.rh.</i>	<i>M.cerf.</i>	<i>M.gale</i>		
Cel5	UFG 026605	<i>Casuarina equisetifolia</i>	-	-	±	+		+	+	3	Berg, unpublish
R43	LLR 02022	<i>Casuarina cunninghamiana</i>	-	-	-	+	+	+		3	Lechevalier <i>et al.</i> , 1987

Table 2: Morphological characteristics of *Frankia* strains Cel5 and R43 grown in B medium with different concentration of Penicillin G

Morphological expression in culture* (Time in weeks)																
Antibiotic concentration ($\mu\text{g ml}^{-1}$)	Cel5								R43							
	Vesicles				Sporangia				Vesicles				Sporangia			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Control	++	+++	+++	+++	±	++	++	+++	++	+++	+++	++	++	+++	+++	++
1	++	+++	+++	++	++	+++	+++	++	+	±	±	-	++	+	±	-
10	+	++	++	±	++	+++	++	+	+	±	-	-	+	±	-	-
50	+	++	+	±	++	+++	+	±	±	±	-	-	±	±	-	-
100	+	+	-	-	+	±	-	-	±	±	-	-	±	±	-	-

(b) Morphological characteristics of *Frankia* strains Cel5 and R43 grown in B medium with different concentration of Rifampicin

Morphological expression in culture* (Time in weeks)																
Antibiotic concentration ($\mu\text{g ml}^{-1}$)	Cel5								R43							
	Vesicles				Sporangia				Vesicles				Sporangia			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Control	+++	+++	+++	+++	±	++	+++	+++	++	+++	+++	+++	++	+++	+++	++
1	++	++	++	++	++	++	++	+	++	++	+++	++	+	+	+	+
10	++	++	++	+	++	+++	+	±	±	++	++	+	+	+	±	±
50	++	++	++	+	++	++	+	-	±	++	±	±	+	±	±	-
100	++	+	±	-	+++	±	-	-	±	±	±	-	++	±	-	-

* Frequency per field: - = 0; ± = 1-5; + = 6-10; ++ = 11-20; +++ =>20

Table 3: Infectivity and effectivity of the *Frankia* strains Cel5 and R43 propagated in B medium containing Rifampicin at concentrations 1 and 10 $\mu\text{g ml}^{-1}$ medium

<i>Frankia</i> strain	Treatment	Nodulation (+, -)									Plant total nitrogen content±SE (mb)			Estimated mg N fixed per nodulated plant		
		species			species			species			species		species		species	
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Cel5	Control*	-	-	-	0	0	0	0	0	0	4.17±0.33	3.99±0.39	4.66±0.20	-	-	-
	1 $\mu\text{g ml}^{-1}$	-	-	-	0	0	0	0	0	0	4.15±0.42	4.06±0.31	4.60±0.25	-	-	-
	10 $\mu\text{g ml}^{-1}$	-	-	-	0	0	0	0	0	0	3.83±0.46	3.94±0.34	4.64±0.22	-	-	-
R43	Control*	-	-	-	0	0	0	0	0	0	4.01±0.33	3.95±0.32	4.20±0.24	-	-	-
	1 $\mu\text{g ml}^{-1}$	-	-	-	0	0	0	0	0	0	4.16±0.35	3.88±0.34	4.33±0.21	-	-	-
	10 $\mu\text{g ml}^{-1}$	+	+	+	70	66.7	50.05	2	1	1	59.34±3.81	35.20±1.62	18.80±0.47	55.43	31.24	14.38
Uninoculated	-	-	-	0	0	0	0	0	0	3.88±0.31	3.96±0.32	4.42±0.21	-	-	-	
Control																

Fifteen seedlings were tested for each *Casuarina* species for each treatment. Species 1 = *C. cunninghamiana*, species 2 = *C. glauca*, specie 3 = *C. equisetifolia*
 * Plants inoculated with either Cel5 or R43 propagated in B medium

Frankia strains, can be a clue to explain the phenomena of non-infective *Casuarina* isolates to their original host which discussed by Torrey (1989). Therefore, depending on the results obtained, association of different microbial flora under certain environmental conditions may have a

role for establishing of such associations. Reddell *et al.* (1986) and Smolander *et al.* (1988) showed that soil conditions play a major role in surviving and establishing the symbiotic relationship between *Frankia* and its host. Knowlton, *et al.* (1980), proved the role of associated

bacteria, *Pseudomonas*, in establishing and enhancement the infection of actinorhizal plants by *Frankia* under sterile conditions.

References

- Baker, D.D., 1987. Relationships among pure cultured strains of *Frankia* based on host pecificity. *Physiol. Plantarum*, 70: 245-248.
- Becking, J.H., 1970. Plant-endophyte symbiosis in non-leguminous plants. *Plant Soil*, 32: 11-654.
- Callaham, D., P. Del Tredici, J.G. Torrey, 1978. Isolation and cultivation *in vitro* of the actinomycetes causing root nodulation in *Comptonia*. *Sci.*, 199: 899-902.
- Davey, C.B., G.C. Papavizas, 1961. Translocation of streptomycin from coleus leaves and its effects on rhizosphere bacteria. *Sci.*, 134: 1368-1369.
- Hoagland, D.R., D.I. Arnon, 1950. The water-culture method for growing plants without soil. *California Agr. Exp. Sta. Circ*, 347-401.
- Knowlton, S., A. Berry, J.G. Torrey, 1980. Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. *Can. J. Microbiol.*, 26: 971-977.
- Lechevalier, M.P., D.P. Labeda, J.S. Ruan, 1987. Studies on *Frankia* sp. LLR02022 from *Casuarina cunninghamiana* and its mutant LLR02023. *Physiol. Plant*, 70: 249-254 .
- Mansour, S.R. and J.G. Torrey, 1991. *Frankia* spores of strain HFPCgI4 as inoculum for seedlings of *Casuarina glauca*. *Can. J. Bot.*, 69: 1251-1256.
- Murry, M.A., M.S. Fontain and J.G. Torrey, 1984. Growth kinetics and nitrogenase, induction in *Frankia* sp. HFPArI3 grown in batch culture. *Plant Soil*, 61-78.
- Pattison, A.C. and F.A. Skinner, 1973. The effects of antimicrobial substances on *Rhizobium* spp. and their use in selective medium. *Appl. Bact.*, 37: 239-250.
- Reddell, P., G.C. Bowen and A.D. Robson, 1986. Nodulation of *Casuarinaceae* in relation to host species and soil properties. *Aust. J. Bot.*, 34: 435-444.
- Smolander, A., C. Van Dijk and V. Sundman, 1988. Survival of *Frankia* strains introduced into soil. *Plant and Soil*, 106: 65-72.
- Torrey, G.J., 1989. Specificity among *Casuarinaceae* in root nodulation by *Frankia*. *Plant and Soil*, 118: 157-164.