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A Contribution to the Studies of Actinorhizal Symbioses of *Alnus* spp. in Slovenia

By

Tine GREBENC[1], Maja RUPNIK[2], Janez HACIN[3], Miha JANC[2] & Hojka KRAIGHER[1]

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Summary

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Alders (*Alnus* spp) can form multiple symbiosis with ectomycorrhizal fungi and nitrogen fixing bacteria from the genus *Frankia*. We have tried to isolate *Frankia* from root nodules of *Alnus glutinosa* and *A. incana*, to culture them in different culture media and inoculate the seedlings of *A. glutinosa, A. incana* and *A.viridis*. The root nodules were surface sterilized and cultured in a modified BAP medium (LI & al. 1996), a combination of BAP and nutrient medium (BAKER & al. 1979) and in a liquid medium. No growth was observed, although a reference strain of *Frankia* sp. from a culture collection in Oregon did grow in the combined medium. For inoculation, crushed surface sterilized nodules and sterile two-week old seedlings of alders, grown on the medium PNS (HACIN & al. 1997), were used. The nodulation was observed on *A. glutinosa* and *A. incana* in two growth conditions: in growth pouches and in vitro. PCR amplification with specific primers was performed to confirm the presence of a part of nitrogenase coding genes and to confirm isolates as *Frankia alni* species/group. We plan to continue with a screening for growth conditions and molecular characterization of *Frankia* strains from Slovenia and with combined inoculations with ectomycorrhizal fungi.

[1] Slovenian Forestry Institute, Večna pot 2, SI-1000 Ljubljana, Slovenia. E-mail: tinegrebenc@hotmail.com

[2] Biology Department, BF, Uni-Lj, Večna pot 111, Ljubljana, Slovenia.

[3] Dept. of Food Science & Technology, BF, Uni-Lj, Jamnikarjeva 101, Ljubljana, Slovenia.

Introduction

Alnus spp. can form tripartite symbiosis among roots, nitrogen-fixing actinomycetes and mycorrhizal fungi. Only a few ectomycorrhizal fungi are known to colonise roots of alders (MOLINA & al. 1994), while actinomycetes from the genus *Frankia* can induce root-nodules and provide nitrogen fixation in them. Fixed nitrogen is immediately incorporated into amino acids and transported to all symbiotic partners (ARNEBRANT & al. 1993). Inoculated alder seedlings have been found to grow better, showing no nitrogen deficiency.

In this project we have initialized studies of actinorhizal symbioses on alder in Slovenia by screening for actinomycetes from different alder species and sites, their molecular identifications, inoculum production and inoculation techniques on seedlings.

Material and Methods

Isolation of strains from root nodules

Nodules collected on *A. glutinosa* and *A. incana* from different locations in Slovenia were washed in a stream of water, surface sterilized in 2% sodium hypochlorite and rinsed four times in sterile distilled water. Lobes were crushed with a sterile mortal and pestle with a small amount of sterile distilled water (LI & al. 1996). Isolation by filtration was performed according to LI & al. 1996, using filters with mesh openings of 1.2µm, 0.45µm and 0.22µm. Filtrates were inoculated onto solid and liquid media. Isolation by sucrose-density fractionation was performed according to BAKER & al. 1979.

Frankia strain NAL010708 (source: USDA Forest Science, Corvallis, Oregon) was used as a reference. *Bradyrhizobium japonicum* strain TAL 102 (= USDA 110, Niftal collection) was used as a legume nitrogen fixing symbiont.

Media

Three different media were used to culture *Frankia* strains: modified BAP medium (LI & al. 1996), a combination of BAP and nutrient medium (BAKER & al. 1979) and a liquid medium with Tween. Inoculated media were incubated at room temperature for at least two months.

Nodulation test and nitrogenase activity

Seeds of *Alnus* spp. were surface sterilized for 15 minutes in 30% H_2O_2 with a drop of Tween 80, rinsed three times and germinated in sterile distilled water. Two week old seedlings were brought into nitrogen free PNS medium (HACIN & al. 1997) and grown at room temperature (20-25°C) with 16h day period in growth pouches and in vitro (in large test tubes). Three to five seedlings of each *Alnus* species were inoculated with 1ml of the crushed nodules suspension. Acetylene reduction test was performed on HP 5830A gas chromatograph on root system of two month old seedlings (HACIN & al. 1997). Results obtained were tested by t-test for biological, agricultural and medical research.

PCR amplification

DNA was extracted from nodules obtained during nodulation test and also directly from surface sterilized nodules collected on different locations. Extraction and purification was modified from KRAIGHER & al. 1995 as follows. Single lobe was crushed in 2% CTAB lysis buffer with 1% (wt/vol) polyvinylpyrrolidone and 20µl of 14M 2-mercaptoethanol. DNA was extracted with chloroform-isoamilalcohol (24:1) and 2-propanol, washed with 70% ethanol, dried and resuspended in sterile distilled water.

PCR amplifications were carried out as described by LUMINI & BOSCO 1996, modified in respect that glycerol was not used and the concentration of $MgCl_2$ was 2mM. V7 hypervariable region of the 16S ribosomal genes was amplified using primers specific for *Frankia alni* species/group (FGPS989ac-79) and for *Elaeagnus* group of *Frankia* strains (FGPS989e-80) in association with a reverse primer FGPL50'-73 (LUMINI & BOSCO 1996). The thermal profile was described by ROUVIER & al. 1996. To confirm the presence of nif region, the intergenic spacer (IGS) between nifD and nifK was amplified according to JAMANN & al. 1993.

Results and Discusion

We have tried different methods for separation and isolation of *Frankia* strains from root nodules. None of the methods showed any growth of *Frankia* sp. on any of the selected media either due to contamination or no growth was observed at all. The reference strain NAL010708 however yielded a typical growth on the combined medium. All seedlings grown in growth pouches (*A. glutinosa* only) developed root nodules within one month after inoculation with the crushed nodules suspension. Only a few isolates nodulated *A. glutinosa, A. incana* and *A. viridis* in test tubes and the overall growth of seedlings in vitro was slower.

All nodulated seedlings tested for nitrogenase activity showed a reduction of acetylene but no significant difference per gram of dried nodules or whole seedlings was found between different isolates. The average nitrogenase activity was equivalent to 2.8 μmoles ethylene formed from acetylene per gram of dried whole seedling per hour.

PCR amplification of V7 hypervariable region of 16S ribosomal genes was performed on five isolates. Using primers specific for *Frankia alni* from three of the five isolates a single fragment slightly shorter than 1000bp was amplified (Fig. 1, lines 2-7). As expected no specific amplification was observed using primers specific for *Elaeagnus* infective group of *Frankia*, since all isolates were collected on *Alnus* spp. as a host plant. No specific amplification was observed using the same primers on *Bradyrhizobium japonicum* strain TAL 102.

In a separate test seven more isolates were used for PCR amplification with primers specific for IGS in nifDK. In all twelve isolates including *Bradyrhizobium japonicum* strain TAL102 the amplification was successfull (Fig. 1, lines 9-15, data not shown for *B. japonicum*) yielding an app. 1400bp long fragment which could correspond to IGS according to LUMINI & BOSCO 1996.

The amplification of the ribosomal genes has been tested in order to confirm the genus *Frankia*, which in combination with nifDK, used in order to confirm the nif region typical for nitrogen fixing bacteria, can be used to identify the genus *Frankia* and to exclude all unspecific amplifications of mitohondrial or plant DNA. Amplified nifDK IGS region can also be used for further characterization of isolates by restriction fragment lenght polymorphism and nif-HaeIII fingerprint (JAMANN & al. 1993).

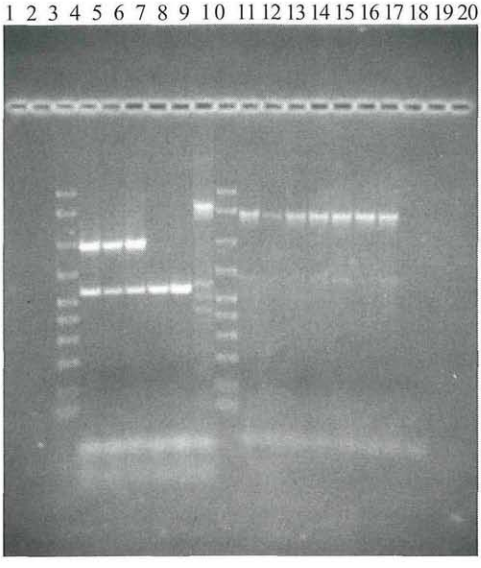

Fig. 1. PCR amplification of V7 hypervariable region in 16S ribosomal genes and intergenic spacer (IGS) in nifDK. Lines 3 and 10: molecular marker (bands at 2000, 1500, 1000 700, 500, 400, 300, 200, 100, 50 bp). Lines 4-9: amplification of V7 hypervariable region with primers specific for *Frankia alni* species/group. Lines 11-17: amplification of intergenic spacer in nifDK with primers FGPD685-85 and FGPK700'-92.

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References

ARNEBRANT K., EK H., FINLAY R.D. & SÖDERSTROM B. 1993. Translocation of nitrogen between *Alnus glutinosa* seedlings inoculated with *Frankia* sp. and *Pinus contorta* seedlings connected by a common ectomycorrhizal mycelium. - New Phytol. 124: 231-242.

BAKER D., TORREY J.G. & KIDD G.H. 1979. Isolation by sucrose-density fractionation and cultivation in vitro of actinomycetes from nitrogen-fixing root nodules. - Nature 281: 76-78.

HACIN J.I., BOHLOOL B.B. & SINGLETON P.W. 1997. Partitioning of ^{14}C-labelled photosynthate to developing nodules and roots to soybean (*Glycine max*). - New Phytol. 137: 257-265.

JAMANN S., FERNANDEZ M.P. & NORMAND P. 1993. Typing method for N_2-fixing bacteria based on PCR-RFLP - application to the characterization of *Frankia* strains. - Molecular Ecology 2: 17-26.

KRAIGHER H., AGERER R. & JAVORNIK B. 1995. Ectomycorrhizae of *Lactarius lignyotus* on Norway spruce, characterised by anatomical and molecular tools. - Mycorrhiza 5: 175-180.

LI C.Y., STRZELCZYK E. & POKOJSKA A. 1996. Nitrogen-fixing endophyte *Frankia* in Polish *Alnus glutinosa* (L.) Gartn. - Microbiol. Res. 151: 371-374.

LUMINI E. & BOSCO M. 1996. PCR-restriction fragment lenght polymorphism identification and host range of single-spore isolates of the flexible *Frankia* sp. strain UFI 132715. - Appl. Envir. Microbiol. 62 (8): 3026-3029.

MOLINA R., MYROLD D. & LI C.Y. 1994. Root symbiosis of red alder: technological opportunities for enhanced regeneration and soil improvement. - In: HIBBS D.E. & al. (Eds.), The biology and management of red alder, pp.23-46. - Oregon State University Press, Corvallis, Oregon.

ROUVIER C., PRIN Y., REDDELL P., NORMAND P. & SIMONET P. 1996. Genetic diversity among *Frankia* strains nodulating members of the family *Casuarinaceae* in Australia revealed by PCR and restriction fragment lenght polymorphysm analysis with crushed root nodules. - Appl. Envir. Microbiol. 62 (3): 979-985.