Competition among symbiotic cyanobacterial Nostoc strains forming artificial associations with rice (Oryza sativa)

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Abstract

Competition among four symbiotically competent Nostoc strains, colonizing rice roots, was examined using hetR-DGGE (denaturing gradient gel electrophoresis) as strain identification. Although mixed in various combinations, only one strain at a time associated with the rice roots. Nostoc strain 8964:3 was the most competitive and our data suggest that its competitive fitness was dependent on rapid hormogonial spreading as displayed on agar plates. Furthermore, rice roots induced hormogonia in all tested Nostoc strains, but only Nostoc strain 9104 showed positive chemotaxis towards the root. Inhibition of growth of competing cyanobacterial strains was not apparent.

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1. Introduction

Creation of artificial associations between nitrogen-fixing micro-organisms and plants of great agricultural importance could potentially reduce the demand for chemically produced nitrogen-fertilizers. Cyanobacteria might have great potential in such artificial associations as they (in particular the genus Nostoc) naturally have a broad host range and can infect all major plant organs [1]. In addition, Nostoc possesses specialized cells, the heterocysts, preventing destruction of nitrogenase by oxygen [2], and the genus is one of the most common soil cyanobacteria [3].

Attempts have been made to form artificial associations between free-living, nitrogen-fixing cyanobacteria (such as, Chlorogloeopsis, Anabaena and Synechococcus), and cell/callus cultures of for example Panax, Medicago, and Nicotiana [4–6]. In addition, associations between intact plants such as, wheat, corn, bean, and sugar beet and soil cyanobacteria of the genus Nostoc, Anabaena and Cylindrospermum have been established with varying success [7–14].

Through screening of Nostoc strains collected from natural plant symbioses, such as with Gunnera, Anthoceros and cycads, a selection of rice root associating strains were identified [15]. The association was tight and the cyanobacteria could not be removed by washing or by sonication. When associated with rice roots, the Nostoc strains increased their nitrogen fixation and their presence appeared to improve the growth of the rice plants [15]. Only individual cyanobacterial strains were tested for association in the previous study. However, under natural conditions, cyanobacteria are likely to exist in mixed populations and may compete for suitable substrates or hosts to
colonize. To our knowledge, the competitive capacity of mixed populations of cyanobacterial strains forming symbiotic or artificial associations with plants is unknown.

The aim of the present study was to examine if competition may occur among selected *Nostoc* strains with known associative competence [15]. A prerequisite for our analyses was that the selected *Nostoc* strains belonged to genetically different groups as verified by short tandemly repeated repetitive (STRR)-PCR fingerprinting [16] and hetR-DGGE (denaturing gradient gel electrophoresis) patterns [17]. Secondly, the strains were examined depending on hormogonial (motile cell stage acting as infection unit [1]) spreading, chemotactic behavior towards rice, hormogonia induction by rice, and depending on the capability of the individual tested cyanobacterial strains to inhibit growth of each other.

2. Material and methods

2.1. Organisms

Four *Nostoc* strains (8901:1, 8964:3, 8981, 9104) originally isolated from *Gunnera* spp. plants (Dr. E. Söderbäck, Stockholm University, Sweden) were used. All strains were previously proven to successfully associate with rice roots as shown in artificial association assays [15]. They were cultivated in liquid BG110 media [18] and grown under the conditions described for rice cultivation. The liverwort *Blasia*, used for chemotactic experiments was grown as described by Knight and Adams [19].

2.2. Competition

Competition experiments were performed on rice roots under the conditions described for rice cultivation. The roots of 10 days old rice plants were challenged with 1 ml of a mixture of 2 or 3 strains, added in equal amounts (OD 3.0 at 720 nm). After 7 days co-culture, the roots were rinsed and sonicated for three minutes to remove loosely attached cyanobacteria. The roots from each competition setup were crushed separately in 200 µl TE buffer, and washed in 500 µl of the same buffer 4 times before direct use in hetR-PCR.

To investigate the hormogonial spreading of the four *Nostoc* strains, they were allowed to grow in equal amounts (OD 3.0 at 720 nm) on agar plates (1.5%) containing BG110. All four strains (5 µl of each) were added to the same plate and the spreading and possible dominance of one strain was measured during a period of 9 days. Hormogonial spreading were measured as radial growth from original spot in mm.

2.3. Denaturing gradient gel electrophoresis (DGGE)

PCR was performed on crushed rice roots with associated cyanobacteria, using primers toward the cyanobacterial hetR gene, designed to amplify a 270 bp region [17]. The forward primer was attached to a GC-clamp in the 5’-end. Primers sequences were synthesized at Cybergene, Novum, Sweden:

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Forward: 5’-CGCCCGCCGCCGCGCCGGCGCCGGCGCCGCCGCCCCCGCCCCAAGTGTGCAATA-
TACATGAC-3’
Revers: 5’-TCAATTGTCTTTTTTCTTC-3’
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Two µl template of cyanobacterial suspension (individual strains; free-living or from crushed root) was added. The conditions of the PCR were: one cycle of 95 °C for 6 min, 30 cycles of 93 °C for 1 min, 52 °C for 1 min, 70 °C for 1 min, one cycle of 70 °C for 10 min and final step at 4 °C. The PCR reaction (total 50 µl) contained 100 pmol of each primer, 1U of DNA polymerase (Dynazyme, Oy, Espoo, Finland), 10 µM of dNTP, and the buffer supplied with the enzyme was used according to instructions of the manufacturer. The PCR reactions were performed in Perkin-Elmer GeneAmp PCR system 2400 (Shelton, Conneticut, USA). The generated PCR products were separated through DGGE on 8% (wt/vol) polyacrylamide gradient gels in 0.5 mM TAE buffer (20 mM Tris-acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM di-sodium EDTA). The denaturing gradient ranged from 20–45%. The gradients were formed with 8% (wt/vol) acrylamide stock solutions through mixing of one stock with no denaturant and one with 100% denaturant (7 M urea, 40% [vol/vol] formamide). The gels were electrophorized for 3–4 h at 60 °C and 200 V in BioRad DCode™ System (Hercules, California, USA). The gels were stained in 5 µg ethidium bromide/ml. As control, DGGE was performed on PCR products from cultured cyanobacteria either one by one, or in combinations of four. To exclude that the plant roots influenced the result, DGGE patterns of cyanobacteria associated individually with rice...
roots were compared to those of cultured isolates. The experiments were repeated three times.

2.4. Inhibition experiments

Investigations were made to see if the four cyanobacterial strains could inhibit the growth of the others. The cyanobacterial strains were grown individually in 15 ml Falcon tubes on semisolid (0.5%) agar slopes containing BG110, and in liquid cultures of the same media for three weeks. After three weeks, the four strains from the liquid cultures were spread over individual agar plates (1.5%) containing BG110 and were allowed to dry. Semisolid agar (0.5%), also containing BG110, was poured onto the same plates. The new agar layer was left to set, after which small pieces of growing cyanobacteria from the Falcon tubes were placed on the agar plates. The pieces, containing the putative inhibiting strains, were put one by one, or two at a time on the plate. The plates were incubated in conditions described for cyanobacterial cultivation for three weeks. Any inhibition would be detected as a clear zone in the basal growing mat surrounding the inhibitory Nostoc strain. The experiment was repeated two times.

2.5. Chemotaxis

Chemotaxis was investigated by the capillary method developed by Knight and Adams [19]. Exceptions to the method were that hormogonia was induced by red light for 18 h and that 5 ml of hormogonial suspension were used in each Petri dish compartment. Putative attractants were: crushed rice root or stem from plants grown with or without nitrogen, Blasia exudates from plants with or without nitrogen, and as control cyanobacterial media BG110. After 24 h incubation under the same conditions as described for rice growth, as well as in the conditions described for cyanobacterial cultivation, the number of hormogonia in the microslides was counted using a light microscope. The experiment was repeated six times.

2.6. Hormogonia induction

To investigate the capacity of rice to induce hormogonia, the method described by Rasmussen et al. [20] was used. Cyanobacteria (1 g FW, OD 3.0 at 720 nm) were placed in Eppendorf tubes and the putative hormogonia inductant were added: i.e., crushed rice roots from plants grown with or without nitrogen. As control the cyanobacterial media BG110 ± nitrogen were used. After 18 h incubation in 24 °C and 18 μmol photons m⁻² s⁻¹, the number of hormogonia was counted in a light microscope. The experiment was repeated three times with three replicates in each trial.

3. Results

3.1. Cyanobacterial competition

During competition assays, only one Nostoc strain at a time associated with the rice roots. The hetR-DGGE analyses demonstrated a single band per competition experiment, corresponding to a single strain (Fig. 1). One strain, Nostoc strain 8964:3, was dominating on the rice roots as its hetR-DGGE fingerprint was the only to appear when combined with the other strains (Fig. 1). When strain 8964:3 was not included, the second most successful strain was Nostoc strain 9104 (Fig. 1). However, when using non-associated, cultured isolates, each

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) patterns of cyanobacteria competing on rice roots, using a PCR amplified region of the hetR gene. (a) Competition of two cyanobacterial strains in the combinations described above each lane. The first four lanes represent the hetR products obtained from cultured Nostoc strains 8901:1, 8964:3, 8981 and 9104. (b) Competition of three strains in the combinations described above each lane. The last four lanes, represent (as in (a)) the individual cyanobacterial patterns from the strains when in culture. To the far right are hetR-DGGE patterns from all four strains when mixed from pure cultures.
Nostoc strain was amplified with the same PCR efficiency, as all bands of the four strains appeared with the same intensity using hetR-DGGE (Fig. 1(b)). Moreover, the bands from cultured cyanobacteria migrated to the same position on the DGGE gels as bands from cyanobacteria co-cultured individually with rice, indicating that the plant material did not interfere with the cyanobacterial DGGE patterns.

When the four strains were grown together and competed on BG110-agar plates, the same result as from the hetR-DGGE investigation was obtained. The strain that appeared dominating and displayed the largest hormogonial spreading on the plate was Nostoc strain 8964:3, expanding its radius almost 28 mm (Fig. 2). Again, the second most dominating strain was Nostoc strain 9104, spreading 22 mm during the same time period (Fig. 2).

3.2. Chemotaxis

Rice was found to chemotactically attract Nostoc strain 9104 (Fig. 3), but the attraction varied depending on temperature and whether the plant had been grown with or without nitrogen. The highest attraction (approximately 550 hormogonia/microslide) was seen using stem extract from rice plants grown with nitrogen and at 24°C (Fig. 3(b)). Contrary, at the same temperature, using rice grown in absence of nitrogen, the root extract attracted most hormogonia (approximately 250 hormogonia/microslide, Fig. 3(a)). At 30°C, fewer total hormogonia were detected, approximately 80 hormogonia/microslide (Fig. 3(c) and (d)).

In order to evaluate the chemotactic method, the investigations performed by Knight and Adams [19] were repeated using Nostoc strain LBG1. We obtained similar attraction towards Blasia exudates, although
were used as control. The values are means ± SE of 9 experiments.

with fewer hormogonia (data not shown). Blasia exudates did not attract the Nostoc strains used in this study. However, Nostoc strain LBG1 was attracted to rice roots, mainly towards extracts from rice grown with nitrogen at 24 °C (data not shown).

3.3. Hormogonia induction

Rice root extract induced hormogonia in all four Nostoc strains (Fig. 4). The highest induction (approximately 220 hormogonia) was found in Nostoc strain 9104. Most hormogonia were generally induced by rice root extract from plants grown without nitrogen. The exception was Nostoc strain 9104, which formed most hormogonia in the presence of rice root extract from plants grown on nitrogen.

3.4. Inhibition

Finally, the ability of individual cyanobacteria to inhibit growth and spread of neighboring cyanobacterial strains was examined. However, none of the four Nostoc strains prevented the growth of the other three strains (data not shown).

4. Discussion

To our knowledge, this is the first time competition at the strain level has been identified among Nostoc strains forming artificial associations with rice roots. Of the four tested strains, Nostoc strain 8964:3 was the most competitive, even though all strains were selected for having a high rice root associating capacity and all being symbiotically competent [15,16].

Hormogonia are known prerequisites for symbiotic competence and their importance can be understood as natural hosts have been shown to release a hormogonia inducing factor (HIF) to aid establishment of cyanobacterial symbioses [19–24]. Our data show that not only natural host plants [19] but also non-host plants can induce hormogonia, supporting earlier investigations [10,13]. Interestingly, Nostoc 8964:3 did not form the highest number of hormogonia, indicating that competitive fitness is not directly related to the number of hormogonia formed initially. However, competitive fitness may require a continuous high production of hormogonia, or perhaps more importantly, a high speed of hormogonial motility and spreading, as indicated by our results. Our results therefore suggest rapid hormogonial spreading, a prominent feature in the highly competitive Nostoc strain 8964:3, to be more important for competitive success during artificial association than chemotaxis, inhibition of competing strains, and initial hormogonia formation.

Nostoc strain 8964:3 displayed a low positive chemotaxis towards rice compared to Nostoc strain 9104. However, cyanobacteria were in the competition assay added to rice roots in comparatively large quantities, and co-cultivation occurred in a restricted space where long-distance attraction may not have been needed for association to occur. Chemotaxis may, however, be important in establishment of natural cyanobacterial symbioses [19,24].

Nostoc strain 9104 was attracted to different plant organs depending on whether the plant was grown with or without nitrogen (N). Potentially roots, the natural site for N-uptake in plants, excrete some attracting signal under N-deplete conditions aimed at nitrogen-fixing organisms. The reason for the high chemo-attraction observed towards rice stem under N-replete conditions can at present stage only be speculative and call for further experiments. However, also during single strain co-cultivation with rice roots [15], Nostoc strain 9104 displayed the highest association to rice plants grown with nitrogen.

In conclusion, the data show that Nostoc strains compete when associating with rice roots and that one strain can out-compete other strains. Of the four tested Nostoc strains, strain 8964:3 was the most competitive. We propose this capacity to be ascribed to a highly efficient spreading of hormogonia. Neither inhibition of competing cyanobacterial strains or ability to respond to chemotactic attraction seemed to be crucial for associative success, although they may be of importance under natural conditions. As in natural cyanobacterial symbioses where only a specific range of cyanobacteria is allowed entry into a specific host [25], selectivity at the strain level also is apparent in artificial associations. Furthermore, the specificity seen in natural cyanobacterial symbioses [25] may be a consequence of the competitive fitness of individual cyanobacterial strains, rather than a physical or chemical barrier established by the host or the environment.
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