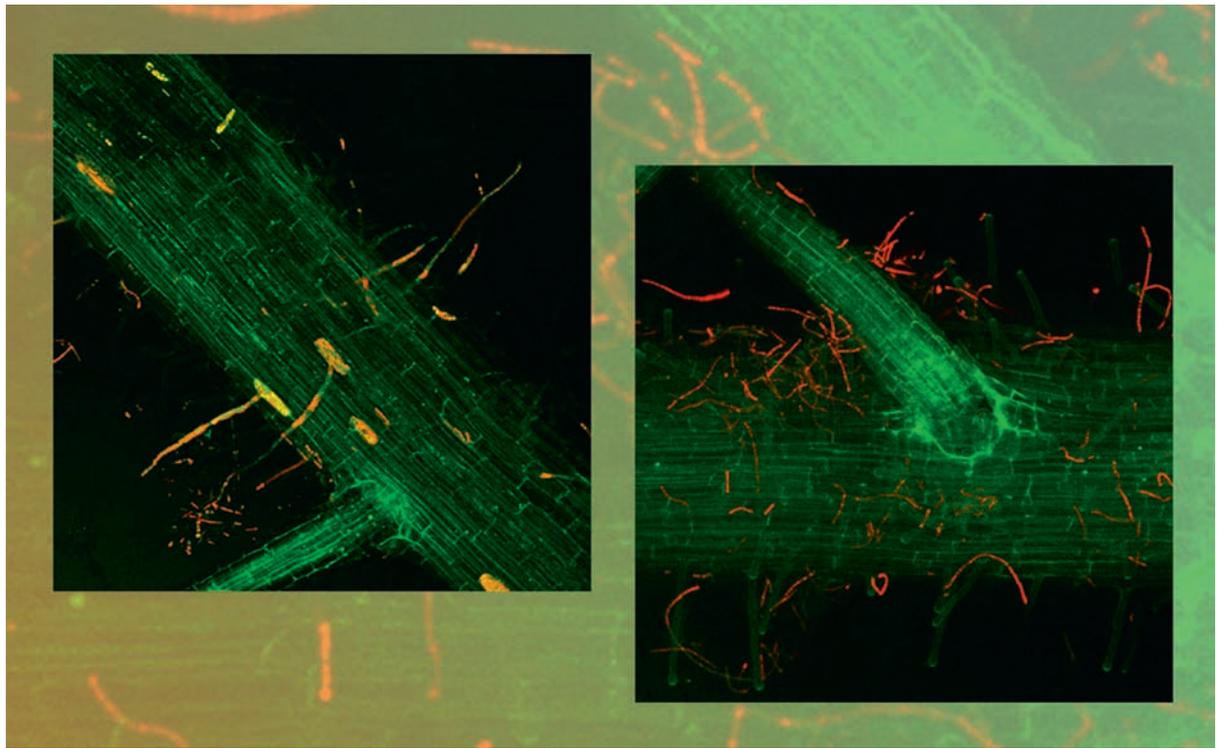


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SHORT COMMUNICATION

Endophytic Colonization of Rice (*Oryza sativa* L.) by the Symbiotic Strain *Nostoc punctiforme* PCC 73102

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Cyanobacteria are phototrophic microorganisms able to establish nitrogen-fixing symbiotic associations with representatives of all four of the major phylogenetic divisions of terrestrial plants. Despite increasing knowledge on the beneficial effects of cyanobacteria in rice fields, the information about the interaction between these microorganisms and rice at the molecular and structural levels is still limited. We have used the model nitrogen-fixing cyanobacterium *Nostoc punctiforme* to promote a long-term stable endophytic association with rice. Inoculation with this strain of hydroponic cultures of rice produces a fast adherence of the cyanobacterium to rice roots. At longer times, cyanobacterial growth in the proximity of the roots increased until reaching a plateau. This latter phase coincides with the intracellular colonization of the root epidermis and exodermis. Structural analysis of the roots revealed that the cyanobacterium use an apoplastic route to colonize the plant cells. Moreover, plant roots inoculated with *N. punctiforme* show both the presence of heterocysts and nitrogenase activity, resulting in the promotion of plant growth under nitrogen deficiency, thus providing benefits for the plant.

Keywords: cyanobacteria, heterocyst, *Nostoc punctiforme*, *Oryza sativa*, rice, symbiosis

Rice plants (*Oryza sativa* L.) require large amounts of mineral nutrients, including nitrogen and phosphorus, for their growth, development, and grain production (Sahrawat 2000). N

fertilizers, providing around 16 to 17 kg N per ton of rice, are being used to meet the crop demands (Choudhury and Kennedy 2004). These agricultural practices, heavily dependent on the application of synthetic fertilizers and pesticides, have raised environmental and health problems that include deterioration of soil fertility, overuse of land and water resources, polluted environment, and an increased cost of agricultural production. A big question today in agriculture is how to maintain crop production without deteriorating soil environmental quality (Abdalla et al. 2013; Singh et al. 2016). In this sense, promoting a stable symbiosis between agriculturally important crop plants and nitrogen-fixing microorganisms is a topic of special interest for reducing current environmental and health problems, derived from the use of chemical nitrogen fertilizers.

N₂-fixing cyanobacteria are naturally occurring prokaryotes that have a tremendous impact on C and N contents of wetland aquatic ecosystems because of their capability to photosynthetically fix CO₂ and anoxygenically fix atmospheric nitrogen. N₂ fixation takes place in differentiated cells called heterocysts by the activity of the nitrogenase enzyme complex (Kumar et al. 2010). Cyanobacteria, mainly *Nostocales*, are also known by their ability to establish symbiosis with plants, lichens, and algae, providing fixed nitrogen to the symbiotic partner. As a model organism of plant-cyanobacteria symbiosis, *Nostoc punctiforme* PCC 73102 (also known as ATCC 29133) has been extensively used (Bergman et al. 2007; Meeks and Elhai 2002). For plant colonization, these cyanobacteria differentiate short motile filaments called hormogonia that are attracted to the plant (Meeks and Elhai 2002). Differentiation of hormogonia is pivotal for plant colonization (Bergman et al. 2007). In terms of specificity, cyanobacteria can be considered promiscuous, since they are able to establish symbiosis with a wide range of plants from the division of bryophytes, pteridophytes, gymnosperms, and angiosperms (Adams et al. 2012; Bergman et al. 2007; Lobakova et al. 2003; Warshan et al. 2018). Taking advantage of this potential, attempts to establish a stable symbiosis with crop plants including tobacco (Gusev et al. 1986), wheat (Gantar et al. 1991), cotton (Prasanna et al. 2015), and sugar beet (Svircev et al. 1997) have been made to carry out associative N₂ fixation (Adams et al. 2012; Bergman et al. 2007).

In rice, epiphytic associations of N₂-fixing cyanobacteria to root plant surfaces have been noted (Freiberg 1999; Nilsson et al. 2002; Prasanna et al. 2009; Svircev et al. 1997; Whitton 2000). However, little is known about rice-cyanobacteria association at the molecular and structural levels or the mechanisms involved in cyanobacteria-plant recognition. In particular, previous short-term

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studies (4 days) on rice inoculation with cyanobacteria under laboratory conditions concluded that cyanobacteria reached a tight epiphytic association to the rice root epidermis, with occasional intercellular occupancy, providing fixed nitrogen to the plant exogenously without endophytic association (Nilsson et al. 2002). In this work we have studied the cyanobacteria-rice association under controlled laboratory conditions at longer incubation periods (up to 45 days) by means of laser scanning confocal microscopy (LSCM). Hormogonia-enriched cultures of the symbiotic model strain *Nostoc punctiforme* PCC 73102 (ATCC 29133) have been used for the first time to establish a long-term stable endophytic association with the model *Oryza sativa* Nipponbare rice variety. Ultrastructural analysis of this association shows that *N. punctiforme* intracellularly colonizes the root cells, reaching to the exodermis. This stable association stimulates plant growth in the absence of inorganic nitrogen.

Adherence of *Nostoc punctiforme* to rice roots. Coculture conditions for rice and cyanobacteria were first established under axenicity to maintain the coculture for more than 50 days. Seven days seedlings of the model *Oryza sativa* Nipponbare rice variety were grown hydroponically in closed containers with -N BG11₀ medium (without any combined nitrogen source). Plants were grown under 12/12 h light/dark cycles, meanwhile the roots were maintained in dark conditions. In this system, plants developed normally, with no apparent negative effects on the plants phenotype during the whole experimental period, including the ability to produce seeds (not shown). For the coculture experiments, *N. punctiforme* cultures enriched in fresh hormogonia were prepared and added to the hydroponic rice culture. A strong adherence of cyanobacteria filaments to the rice roots, preferentially to embryonic and postembryonic root crowns, was observed (Fig. 1). At 48 h postinoculation,

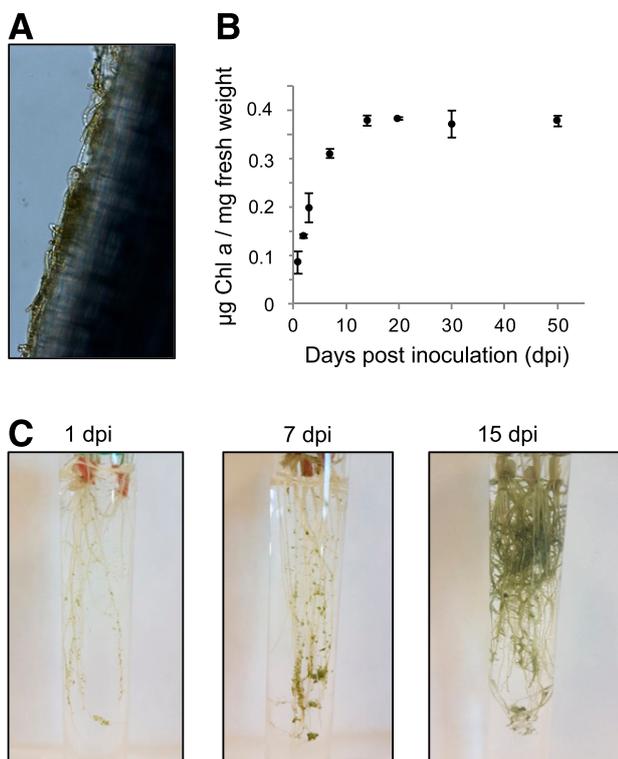


Fig. 1. Association of *Nostoc punctiforme* to rice roots. **A**, Bright field microscopy of a rice root incubated for 48 h in the presence of *N. punctiforme*. **B**, Cyanobacterial association to rice roots, quantified as micrograms of chlorophyll a (Chl a)-per milligram of root weight. The values are the means \pm standard error. **C**, Appearance of rice roots inoculated with *N. punctiforme* at 1, 7, and 15 days postinoculation (dpi).

filaments were found clearly attached to the root surface (Fig. 1A). Similar cyanobacterial adherence to rice roots at short times of coculture (4 days) has been reported previously (Nilsson et al. 2002). However, in these studies the process of adherence was monitored just for the first 6 h of cocultivation, in which a rapid phase of adherence was found that lasted less than 1 h, followed by a second phase after a lag period. We have studied instead the adherence of *N. punctiforme* to rice roots up to 50 days postinoculation (dpi). The adherence of this cyanobacterium to the rice roots increased with the time of cocultivation until reach a plateau at 15 dpi (Fig. 1B). In the roots of seedlings between 1 to 7 days-old, cyanobacteria filaments appeared mainly as microcolonies, whereas in samples from 15 to 45 days-old plants they formed dense layers and clumps (Fig. 1C). Cyanobacterial filaments remained attached to the roots after washing with running tap water, indicating a strong adherence of the cyanobiont to the plant tissues. As a control, the cyanobacterium *Nostoc* sp. strain PCC 7120, neither symbiotic nor hormogonia-forming, was used in similar experiments. In this latter case only a faint epiphytic association, easily washed by tap water, was found (Supplementary Fig. S1). It is important to note that even though the roots were incubated in long-term dark conditions, in the presence of *N. punctiforme* they appeared completely green (Fig. 1), thus suggesting that photosynthetic pigments of the cyanobiont were still present.

Long-term colonization trials. In order to gain insights into the type of cyanobacteria-plant association and the ability of *N. punctiforme* to colonize root cells, the progression of this association was followed by means of LSCM. Roots samples were taken at 7, 20, 30 and 45 dpi, washed extensively with running tap water and visualized (Fig. 2). Since *N. punctiforme* cells retained photosynthetic pigments, the presence of this cyanobacterium was followed by monitoring cyanobacterial chlorophyll (Chl) autofluorescence. At 7 dpi, a strong epiphytic association of *N. punctiforme* filaments to the epidermis was already detected (Fig. 2A). Filaments also clumped in the proximity of the emergence of secondary roots, that could act as

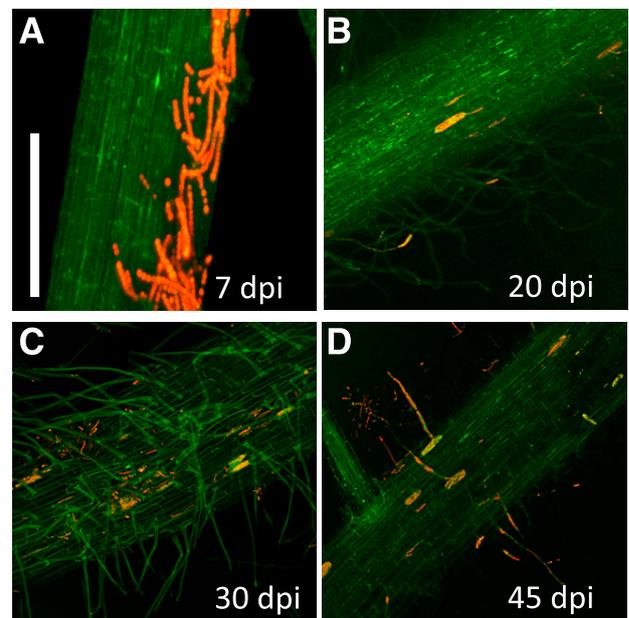


Fig. 2. Colonization of *Nostoc punctiforme* in rice roots. **A** to **D**, Rice roots inoculated with *N. punctiforme* at 7, 20, 30, and 45 days postinoculation (dpi), respectively, were visualized by a confocal microscope. Stacks generated from 90 to 130 frames are shown. In green, suberin autofluorescence from the plant cell walls; in red, cyanobacterial chlorophyll autofluorescence. Scale bar = 100 μ m.

the primary site of colonization. This epiphytic association reinforces previous findings describing a tight external association of cyanobacteria to rice roots after 4 days of coculture (Nilsson et al. 2002). At 20 dpi, however, *N. punctiforme* was also observed colonizing primary root surfaces (Fig. 2B). Longer times of incubation increased colonization, being cyanobacterial cells more abundant in the root epidermis (Fig. 2C). Remarkably, in samples from 45 days-old plants, the cells of the plant epidermis were completely colonized intracellularly by *N. punctiforme* (Fig. 2D). These results provide evidences of a long-term, stable endophytic colonization of rice roots by *N. punctiforme*. As a control, the nonsymbiotic cyanobacterium *Nostoc* sp. strain PCC 7120 was used. Filaments of this strain were never found inside the plant tissues, being some of them trapped in the vicinity of the root hairs after washing with running tap water (Supp. Figure 1B and C).

The colonized root structures were further analyzed. Thus, transverse sections of large radical roots were prepared and visualized (Fig. 3). According to the images of root slices, cyanobacteria were able to colonize cells of both the epidermis and exodermis, but did not transverse the sclerenchyma layer (Fig. 3A). By contrast, in a previous study on cyanobacteria-rice association with different cyanobacterial and rice varieties (Nilsson et al. 2002), *Nostoc* cells followed the contours of the outer surface of the root epidermis, and only occasionally occupied intercellular spaces in the epidermal root layer, which was interpreted as the first events in the colonization process (Nilsson et al. 2002). However, a cyanobacterial strain isolated from soil, *Nostoc* 2S9B, used to explore wheat plant colonization in liquid culture, penetrated the root epidermis and cortex and was present in intercellular spaces forming packages, and sometimes within wheat plant cells (Gantar et al. 1991). In our system, *N. punctiforme* was only found intracellularly but not within the symplast (Fig. 3B), thus indicating an apoplastic colonization route.

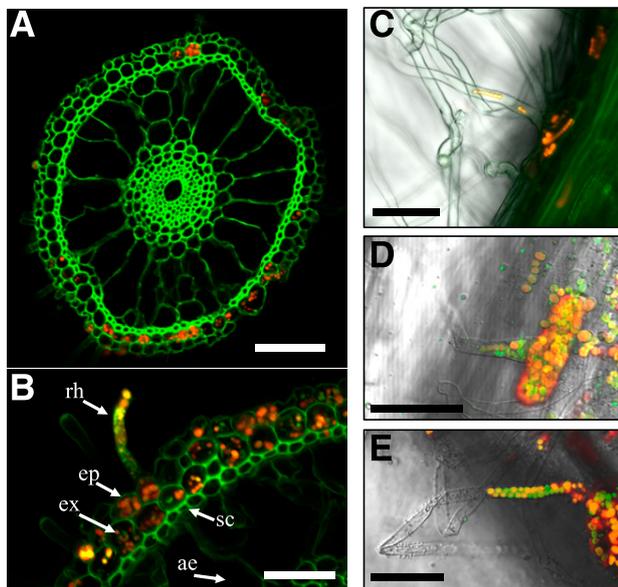


Fig. 3. Ultrastructural analysis of colonization of rice roots by *Nostoc punctiforme*. **A** and **B**, Root cross-sections generated from rice plants 45 days after inoculation with *N. punctiforme*. ep = epidermis, ex = exodermis, sc = sclerenchyma layer, ae = aerenchyma lacunae, rh = root hair. **C** to **E**, Root hairs being colonized. Stacks were generated from 90 to 130 Z sections. In green, suberin autofluorescence from the plant cell walls; in red, cyanobacterial chlorophyll autofluorescence. Scale bars in **A** = 100 μ m and **B** to **E** = 50 μ m.

We noticed that, at longer times, root hairs also showed some degree of colonization (Fig. 3B). In this case, the infection started in the plant body and then extended through the root hairs (Fig. 3C to E). In most cases, short filaments resembling hormogonia were also found inside the root hair (Fig. 3C). Root hairs might offer a facilitated point of entry for endophytes, as it has been found in many cases that intracellular plant-microbe interactions begin with intracellular access of the microbe through root hairs. This is the case in the very well-studied *Rhizobium* or *Frankia* symbioses (Charpentier and Oldroyd 2010). In these systems, root hair curling, induced by the production of nodulation factors (Nod), is the primary event in the symbiotic process (Santi et al. 2013). This was not observed in our system, in which root tips were not affected. In this sense, it is possible that penetration is facilitated by production of hydrolytic enzymes. Cell-wall degrading enzymes are important for endophytes to break plant cell walls and colonize the cells. Genes encoding cell-wall degrading enzymes widely exist in the genomes of endophytic bacteria (Liu et al. 2017). It has been shown that symbiotic *Nostoc* strains show enriched functions, including motility toward the plant, uptake of phosphate, branched-chain amino acids and ammonium, production of nostopeptolides and the ability to breakdown parts of the plant cell wall hemicelluloses, such as xylan (Liaimer et al. 2015; Warshan et al. 2018). The latter may be a key feature in the intracellular colonization of plants by symbiotic *Nostoc* strains. An endo-1,4- β -xylanase for xylan degradation was identified as highly upregulated in *Nostoc* sp. Moss2 during physical contact with the moss *Pleurozium schreberi* (Warshan et al. 2017). Further studies on the expression and function of hydrolytic enzymes during cyanobacteria plant invasion and colonization are required to a better understanding of the biological processes involved in the rice-*Nostoc* association.

Effect of *Nostoc* inoculation on plant growth. Endophyte bacteria promote plant growth through nitrogen fixation, phytohormone production, nutrient acquisition, and by conferring tolerance to abiotic and biotic stresses (Kandel et al. 2017). For this, the effect of *N. punctiforme* endophytic association on the rice growth in the absence of combined nitrogen has been evaluated (Fig. 4). Although a small positive effect on the aerial part of the plant was observed, significant increases in both the root length and fresh weight were measured in plants grown in the presence of *N. punctiforme*, being these roots ca. 2 times longer and weightier than the respective controls (Fig. 4). Positive effects on plant growth and grain production have been observed previously after addition of other cyanobacteria strains in field trials (Singh et al. 2016) and under laboratory conditions (Nilsson et al. 2002; Prasanna et al. 2009; Svircev et al. 1997), although no evidence of a functional endophytic colonization of rice roots has been described.

Our results are in agreement with the hypothesis that N_2 fixed by the cyanobiont might be transferred to the plant, promoting growth in nitrogen-limited conditions. In order to corroborate this hypothesis, we first explored for the presence of heterocysts in the colonized structures, which are the terminally differentiated cyanobacterial nitrogen-fixing cells. Heterocysts can be detected from the comparison of phase-contrast and fluorescence images of a filament as a lack of autofluorescence from photosynthetic pigments, an indication of complete heterocyst maturation (Santamaría-Gómez et al. 2018). Interestingly, we were able to detect mature heterocysts in all the colonized tissues of the plant (Fig. 5). In some cases, contiguous heterocysts were also found (Fig. 5D). The presence of contiguous heterocysts has been noted in other cyanobacteria-plant symbioses (Bergman et al. 2007). In order to ascertain whether these heterocysts are functionally active inside the plant cell, nitrogenase activity was measured in rice roots that

had been inoculated with *N. punctiforme*. As shown in Table 1, a high nitrogenase activity was measured in inoculated roots in the absence of combined nitrogen. Since heterocysts differentiation is only activated in the absence of combined nitrogen (Kumar et al. 2010), a negative control of plants inoculated in the presence of nitrogen was added. A faint epiphytic association to plant roots of filaments containing vegetative cells was observed (not shown). As expected, only residual nitrogenase activity was detected in these conditions. As a control of specific endophytic colonization, rice roots inoculated with the nonsymbiotic strain *Nostoc* sp. strain PCC 7120 only showed a residual nitrogenase activity in the absence of combined nitrogen (Table 1).

Taken together, the results presented here provide clear indications of a stable colonization involving the model organisms *Nostoc punctiforme* and *Oryza sativa* Nipponbare, and showing a N_2 fixation activity associated to the colonized plant roots. Thus, the use of these organisms to establish plant symbiosis opens future research aimed at the identification of the signals and metabolic changes involved in such interaction.

Organisms and growth conditions. Rice seedlings (*Oryza sativa* L.) cultivar Nipponbare (NPB, *japonica*) were surface-sterilized by washing first with distilled water and then with 0.5% (wt/vol) calcium hypochlorite for 20 min. Later, the seeds were thoroughly washed with sterilized tap water and were kept for germination on a wet filter paper in a container. Rice plants were grown hydroponically in tanks containing BG11₀ (-N) medium (free of combined nitrogen) (Rippka et al. 1979). Experiments were carried out in a growth chamber at 28°C,

75% relative humidity, 12-h light and 12-h dark cycles, and light intensity of $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Nostoc punctiforme PCC 73102 (ATCC 29133) and *Nostoc* sp. strain PCC 7120 were obtained from the Biological Cultures Service of the Institute of Plant Biochemistry and Photosynthesis. These strains were grown either in BG11₀ medium (-N) or BG11₀ supplemented with 2.5 mM NH_4Cl and 5 mM TES-NaOH buffer (pH 7.5) (+N), at 30°C in continuous light ($50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in shaken (100 rpm) liquid cultures or on medium solidified with 1% (wt/vol) Difco agar. In order to avoid hormogonia differentiation, filaments of *N. punctiforme* were grown in BG11₀ medium supplemented with 2.5 mM NH_4Cl , 5 mM TES-NaOH buffer (pH 7.5) and 4 mM sucralose, to a concentration of 2 to 3 μg of Chl per milliliter (Splitt and Risser 2016). Then, filaments were washed and incubated in BG11₀ (without sucralose) for 16 h for production of hormogonia.

Cocultivation of *Nostoc* strains and rice plants. Seedlings of rice grown for 7 days were suspended in 4-liter closed tanks containing at least 20 plants, in +N or -N BG11₀ medium. After 4 to 5 days of acclimation, *N. punctiforme* or *Nostoc* sp. strain PCC 7120 inoculants containing hormogonia were added to the solutions at a final concentration of 0.5 μg of Chl per milliliter. Cocultivation was carried out in a growth chamber for up to 45 days, as described above for rice plants.

To determine association to plant roots, Chl *a* content was measured according to Arnon (1949). Root samples were directly taken at different times of coculture. Chl values referred to the root fresh weight.

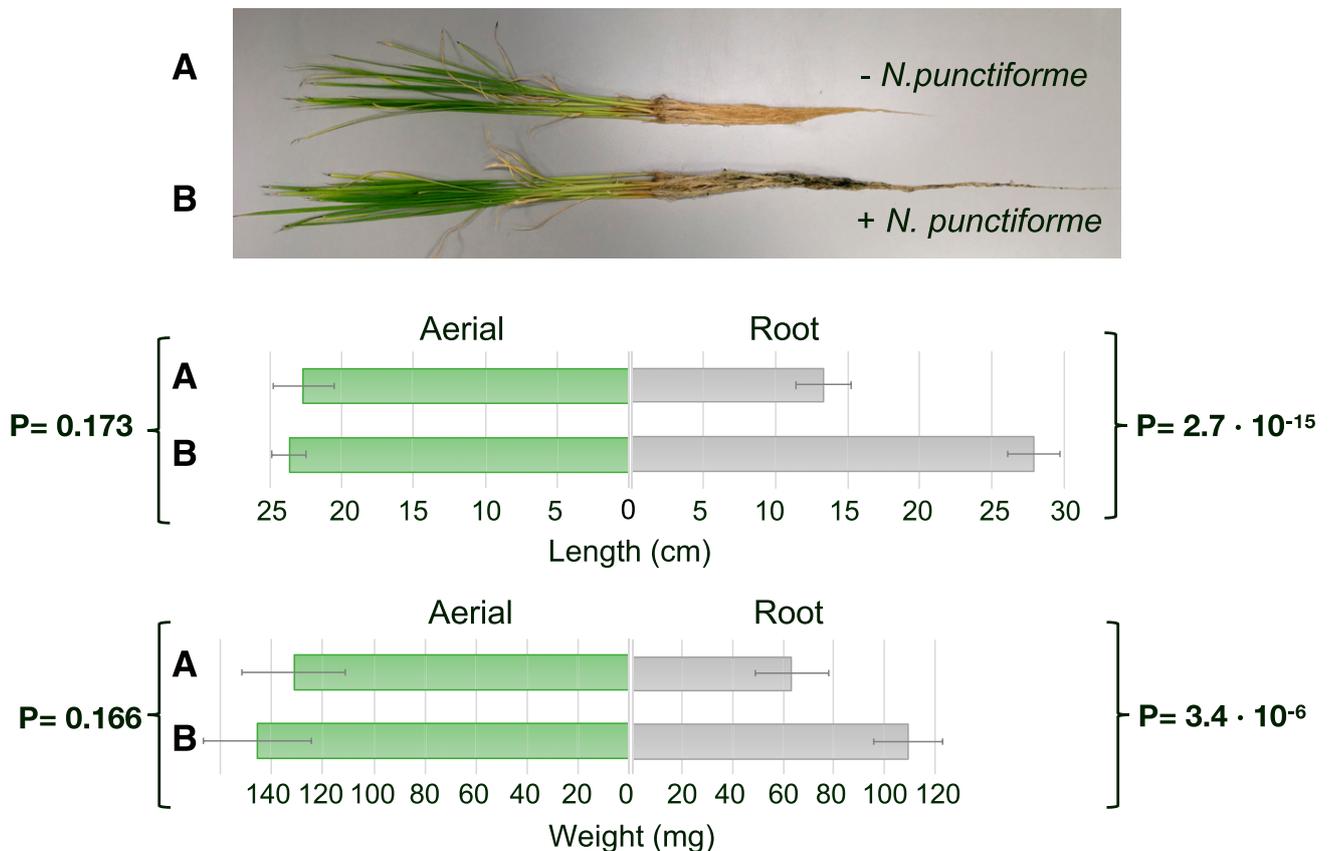


Fig. 4. Effect of *Nostoc punctiforme* inoculation on plant growth. **A**, Rice plants grown in media free of combined nitrogen in the absence or **B**, presence of *N. punctiforme* were photographed and measured at 20 days postinoculation. The values are the means \pm standard error. P indicates *P* value from the Student's *t*-test.

To determine *N. punctiforme* inoculation on plant growth, rice plants were grown in media free of combined nitrogen in the absence or presence of *N. punctiforme*. At day 20 after inoculation, 20 plants were excised, were thoroughly washed with running tap water, and weight and length were measured.

Sample preparation and confocal microscopy. For confocal microscopy, fresh rice roots were cut and washed intensively with tap water. Alternatively, root slices were obtained by excision with a blade. Samples were mounted in a coverslip and were examined with a Leica TCS SP2 confocal microscope, using HC PL-APO CS $\times 20$ or HCX PLAM-APO $\times 63$ 1.4 NA oil immersion objectives. Cyanobacterial autofluorescence was excited with 488 nm light irradiation from an Argon laser, and fluorescence emission was monitored across windows of 650 to 700 nm. Root lignin and suberin autofluorescence was similarly excited with 476 and 488 nm laser irradiation, and fluorescence emission was monitored across windows of 510 to 533 nm. Z series containing from 90 to 130 frames were stacked and processed with the Image J program (version 1.41).

Nitrogenase activity in plant roots. Determination of nitrogenase activity was carried out by a method based on that described by Stewart et al. (1967). Rice roots (1 g) extensively

washed with tap water were incubated in 2 ml of BG11₀ in small flasks. Rubber flask caps were placed to block gas exchange. After incubating the cultures for 30 min, 2 ml of acetylene were injected into the flasks, using a syringe with a needle, 1-ml gas samples were taken every 30 min for 3 h, and the ethylene produced was determined by gas chromatography. Nitrogenase activity was calculated as nanomoles of ethylene per gram of roots per hour.

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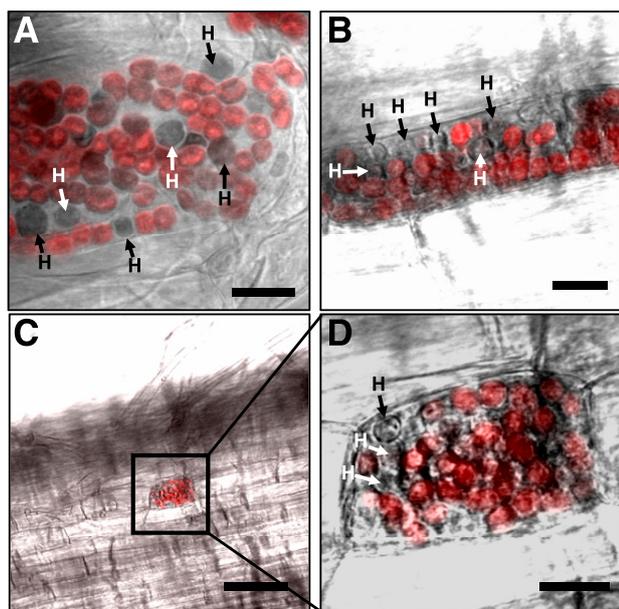


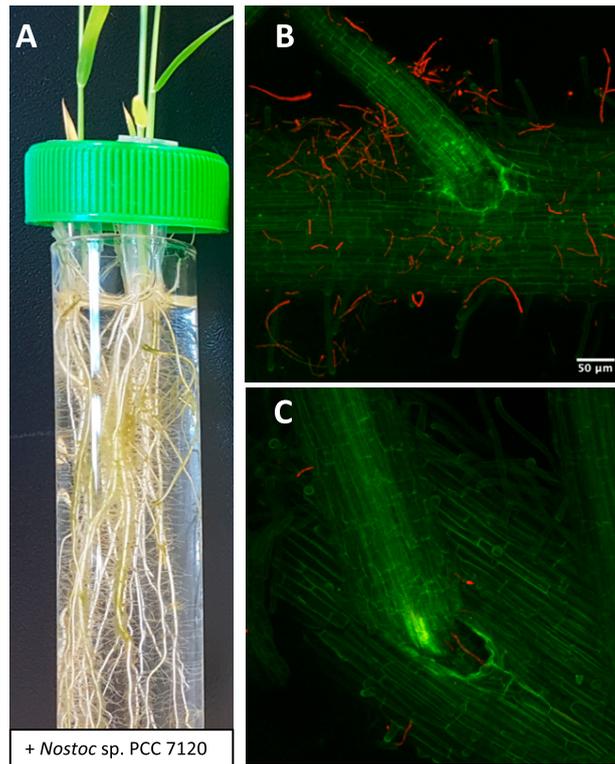
Fig. 5. Analysis of the presence of mature heterocysts in colonized tissues. The presence of mature heterocysts (H; indicated by arrows) was detected by the loss of autofluorescence of photosynthetic pigments. Merged images of red autofluorescence and bright field are shown. Scale bars in **A**, **B**, and **D**, indicate 50 μ m and **C**, 10 μ m.

Table 1. Nitrogenase activity measured in rice roots inoculated with *Nostoc punctiforme* and *Nostoc* sp. strain PCC 7120^a

Growth media	<i>N. punctiforme</i>	<i>Nostoc</i> sp. strain PCC 7120
BG11 ₀ (-N)	174.58 \pm 18.22	3.7 \pm 4.08
BG11 (+N)	0.73 \pm 0.57	0.0 \pm 0.00

^a Rice plants inoculated with the symbiotic strain *Nostoc punctiforme* or the nonsymbiotic *Nostoc* sp. strain PCC 7120 were grown for 20 days, in the absence (-N) or presence (+N) of a source of combined nitrogen. Roots were excised, were washed extensively, and were prepared for nitrogenase activity. Mean and standard deviation of three independent measurements are shown in nanomoles per gram per hour.

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Supplementary Figure S1. Association of *Nostoc* sp. PCC 7120 to rice roots. A) Appearance of rice roots inoculated with *Nostoc* sp. PCC 7120 20 days post inoculation. B) Rice roots inoculated with *Nostoc* sp. PCC 7120 were directly visualized by a confocal microscope. C) Rice roots inoculated with *Nostoc* sp. PCC 7120 after washing with running tap water. Stacks generated from 100 z frames are shown. In Green, suberin autofluorescence from the plant cell walls; in red, autofluorescence of photosynthetic pigments.