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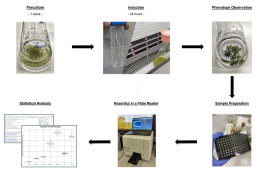
Quantitative Assessment of Hormogonia Induction in Nostoc punctiforme by a Fluorescent Reporter Strain (Neubauer et al. 2025)



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We use this protocol and it's working

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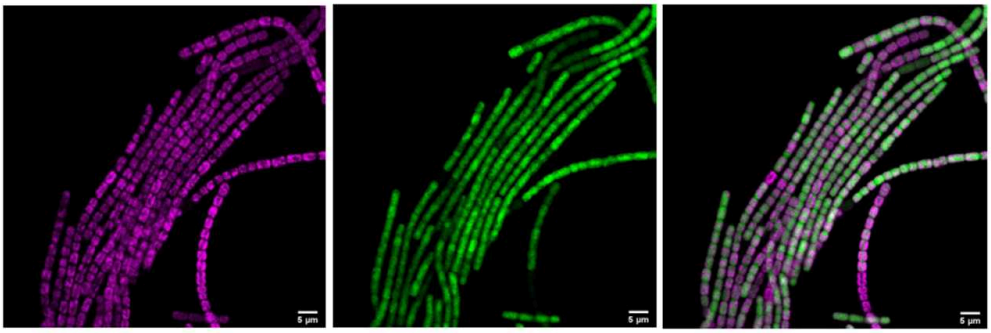
Abstract

While symbiotic plant-cyanobacteria interactions hold significant potential for revolutionizing agricultural practices by reducing the application of artificial nitrogen fertilizers, the genetic underpinnings of the symbiotic interaction between the plant host and the cyanobiont remain poorly understood. In particular, the molecular mechanisms through which host plants induce the formation of motile cyanobacterial filaments (hormogonia), essential for colonization and initiation of symbiosis, are not well characterized. In this protocol, we present a novel yet objective method for quantifying hormogonia induction. We have developed a reporter strain of Nostoc punctiforme PCC 73102 capable of quantifying hormogonia induction in response to diverse biotic and abiotic stimuli. Our innovative approach, using a cyanobacterial hormogonia reporter strain, allows high-throughput screening of the hormogonia-inducing effect of a wide array of environmental and plant signals. This method is expected to greatly advance our understanding of the genetic determinants underpinning plant-cyanobacteria symbioses.



Confocal images of the pilA reporter strain after exposure to *Anthoceros* exudates for 24 hours. Left panel: chlorophyll autofluorescence; Middle panel: GFP fluorescence; Right panel: left and middle panels merged. Scale bar: 5 µm.

Materials

Far red light

We applied three panels of a far-red LED module; 50-60 µmol m-2 s-1 (Green Power LED module HF far red, IONC 9290004645, Koninklijke Philips N.V., NL).

Induction by HIF

For an optimal hormogonium induction process, use *Anthoceros agrestis* exudate by collecting its $BG11_0$-MES medium after one month of HIF production. This HIF-containing medium can be used in a 1:1 volume ratio.

Cyanobacterial culture conditions

The laboratory model strain *Nostoc punctiforme* PCC 73102 (also known as ATCC 29133 and UCO 154) was obtained from the culture service of the Institute of Plant Biochemistry and Photosynthesis, Sevilla, Spain. It was routinely maintained in solid BG11 (Rippka et al., 1979) medium containing 1% weight/volume (w/v) Bacto Agar (Becton, Dickinson and Company, France). Solid cultures were kept on a shelf at 25°C under reduced illumination (20-25 µmol m-2 s-1) in a growth chamber.

Liquid cultures were prepared in BG11 from solid cultures and incubated at 20-25°C, with a continuous light exposure of 45-55 µmol m-2 s-1 on an orbital shaker (100-120 r.p.m.). Mutant strains derived from *N. punctiforme* were maintained under the same conditions, but growth media were supplemented with 25 µg/ml Neomycin to preserve the genetic modification.

***Nostoc* culture grown in BG11.**

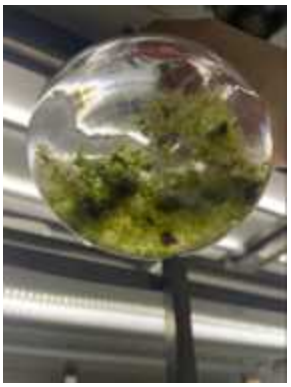
Plant culture

Thallus tissue of the hornwort *Anthoceros agrestis* Bonn isolate (Szövényi et al., 2015) was cultivated in liquid BCD medium (Cove et al., 1996) under the same conditions as described above. To induce the secretion of the hormogonia-inducing factor (HIF), 1 g (fresh weight) of thallus tissue was transferred to $BG11_0$ ($BG11_0$ without nitrogen, (Rippka et al., 1979)) and supplemented with 5 mM 4-Morpholineethanesulfonic acid (MES, Merck KGaA, Germany) to stabilize the pH at 7.4. The cultures were grown in 500 mL Erlenmeyer flasks containing 200 mL $BG11_0$ sealed with a sponge plug for one week.

Gunnera tinctoria plants were cultivated in a greenhouse in pots. *Gunnera* mucilage containing the HIF was obtained from the emerging leaves, using a sterile spoon.

Rice (*Oryza sativa*) and wheat (*Triticum aestivum*) plants were germinated axenically and grown hydroponically in BG11. To obtain HIF, they were maintained for 1 week in $BG11_0$ under illumination. This $BG11_0$ medium was used in the experiments to induce hormogonia.

Anthoceros agrestis **liquid cultures.**



Closer look at liquid culture of ***Anthoceros agrestis*****.**

Troubleshooting

Before start

Nostoc & Plant Culture and Induction Methods

See Materials section.

Preculture

For the induction of hormogonia, liquid cultures of the cyanobacterial strains were prepared in BG11. Six days prior to hormogonia induction, the culture was transferred to antibiotic-free $BG11_0$ that was supplemented with 4 mM sucralose (Merck KGaA, Germany) to repress the formation of hormogonia (Splitt and Risser, 2016). Additionally, the culture was homogenized by passing through a needle (0.8 × 50 mm), and the density was adjusted to a chlorophyll a concentration of 0.5 µg/ml. The concentration of chlorophyll a was assessed by a 1:10 dilution of *N. punctiforme* in methanol (Mackinney, 1941).

Replicates

We recommend starting with 4 replicates for each treatment, using 250 mL Erlenmeyer flasks filled with 100 mL medium.

Controls

Hormogonia formation can be induced by exposing the reporter strains physical or chemical stimuli such as far red light or different plant exudates.
As controls, we recommend a negative control from cultures in $BG11_0$ without any additional treatment and a positive control which is either induced by far red light or by HIF from *Anthoceros agrestis*.

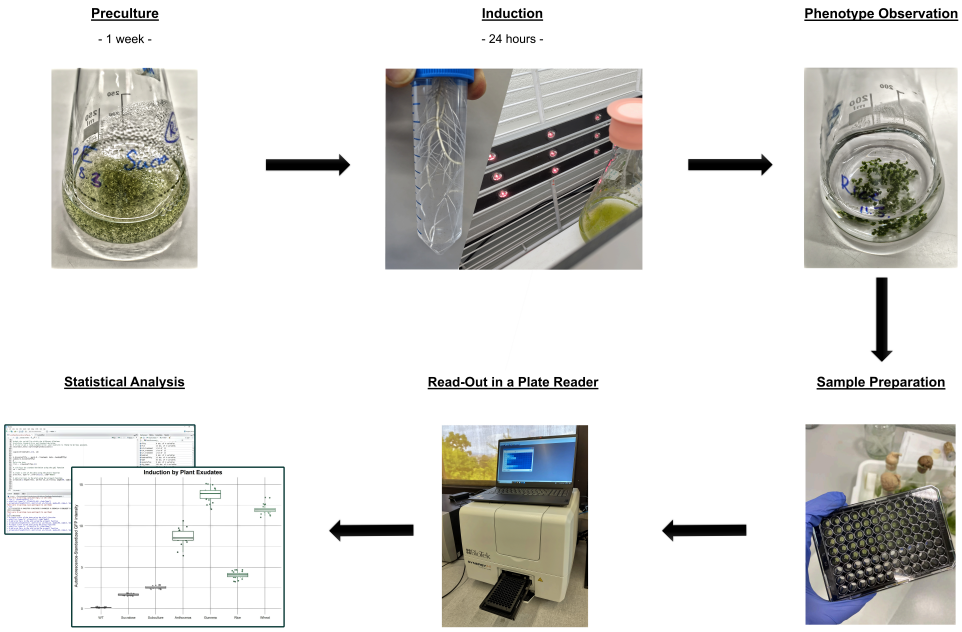
***Nostoc* pilA reporter strain after exposure to *Anthoceros* exudates.** Single flask on the left: control culture (pilA strain grown without exposure to *Anthoceros* exudates); Four flasks on the right: pilA reporter strain exposed to *Anthoceros* exudates in four replicates.

Moreover, two more controls can be carried out: a wild type strain of N. punctiforme as well as a reporter sample that is kept in the hormogonia repressing medium ($BG11_0$ + 4 mM sucralose).

Wild type *Nostoc* after exposure to *Anthoceros* exudates. Flask on the left: *Nostoc* culture after exposure to *Anthoceros* exudates for 24h; Flask on the right: Control *Nostoc* culture (*Nostoc* grown with sucralose).

Overview of the Protocol Steps

1



Hormogonia Induction

2 Prior to hormogonia induction, wash the cultures twice with $BG11_0$: Centrifuge 100 mL of the *Nostoc* reporter culture at 3000 ×*g* for 5 min. Resuspend the culture in 100 mL of $BG11_0$.

Repeat centrifugation and resuspension. Centrifuge again and resuspend in the final volume of $BG11_0$ (and additional supplements).

3 Induce hormogonia formation by exposing the reporter strains to a physical or chemical stimulus such as far red light or different plant exudates.

Quantification of Hormogonia

4 Spin down the *Nostoc* reporter cultures and quickly homogenize 100 mL of each culture by passing through a needle (0.8 × 50 mm).

5 A blank sample with $BG11_0$ for the correction of autofluorescence and turbidity was used in each measurement (measurement was made in at least eight wells).

6 Add 200 µL aliquots of the samples into the wells of a black 96-well microplate (Nunc, Denmark).

7 We recommend to carry out at least 12 measurements (12 wells, technical replicates) for each biological replicate.

8 Measure GFP fluorescence, turbidity, and Chlorophyll a autofluorescence using a VARIOSKAN LUX microplate reader.

Turbidity was measured at 730 nm. Chlorophyll a autofluorescence and GFP were measured at 650-700 nm and 485-520 nm, after excitation at 650 nm and 485 nm, respectively.

VARIOSKAN LUX microplate reader and 96 well plates used.

Statistical Analysis

9 To test the effect of abiotic and biotic factors on GFP fluorescence, a one-way ANOVA in combination with the Tukey Honest post hoc test was used in R (Core, 2008).

Protocol references

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