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Journal
Plant Signaling & Behavior, 12(1)

ISSN
1559-2316

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Publication Date
2017-01-02

DOI
10.1080/15592324.2016.1268313

Peer reviewed

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Plant Signaling & Behavior

ISSN: (Print) 1559-2324 (Online) Journal homepage: http://www.tandfonline.com/loi/kpsb20

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PLANT SIGNALING & BEHAVIOR
2017, VOL. 12, NO. 1, e1268313 (4 pages)
http://dx.doi.org/10.1080/15592324.2016.1268313

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SHORT COMMUNICATION

Blue light does not inhibit nodulation in *Sesbania rostrata*

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ABSTRACT
Earlier, we reported that root nodulation was inhibited by blue light irradiation of *Lotus japonicus*. Because some legumes do not establish nodules exclusively on underground roots, we investigated whether nodule formation in *Sesbania rostrata*, which forms both root and "stem" nodules following inoculation with *Azorhizobium caulinodans*, is inhibited by blue light as are *L. japonicus* nodules. We found that neither *S. rostrata* nodulation nor nitrogen fixation was inhibited by blue light exposure. Moreover, although *A. caulinodans* proliferation was not affected by blue light irradiation, bacterial survival was decreased. Therefore, blue light appears to impose different responses depending on the legume-rhizobial symbiosis.

ARTICLE HISTORY
Received 2 November 2016
Revised 27 November 2016
Accepted 29 November 2016

KEYWORDS
Blue light; *Sesbania rostrata*; *Azorhizobium caulinodans*; root nodulation; light exposure

Leguminous plants and rhizobia establish a symbiosis whereby root nodules containing bacteria that fix nitrogen gas into ammonia develop on a host root. Root nodulation is regulated by various environmental cues (drought stress,[1] salt stress,[2] and light stress[3]). We recently reported that root nodulation of *Lotus japonicus*, a model legume, was inhibited by white light irradiation, and that the inhibition of nodulation is caused by blue light perception by both the host plant roots and the rhizobia.[4] Moreover, blue light exposure to *L. japonicus* roots resulted in reduced nodule weight and decreased acetylene reduction activity (ARA). Higher plants are known to develop avoidance mechanisms, such as root negative phototropism[4,5] and the shade avoidance syndrome,[7] to survive under conditions of biotic or abiotic stress. We concluded that inhibition of nodulation by light is one of several avoidance responses that plants such as *L. japonicus* use to conserve energy especially under environmental stress because nitrogen fixation is an energy-intensive process.

Sesbania rostrata, a tropical legume, develops nodules on both root and stems in response to *Azorhizobium caulinodans* ORS571.[8,9] The crack entry infection process via adventitious root primordia on *S. rostrata* aerial stems, which leads to the initial formation of nodules, has been observed in the flooded roots of this species.[10,11] Although root nodulation of *L. japonicus* is inhibited by white light, stem nodulation of *S. rostrata* is not inhibited under high intensity sunlight, which includes the blue component of the spectrum. Thus, we hypothesized that stem nodulation would not be inhibited by blue light, but that the subterranean roots might be affected. Because inhibition of root nodulation in *L. japonicus* by white light is actually caused by blue light perception, we investigated the effect of blue light irradiation on nodulation on *S. rostrata* underground roots in response to inoculation with *A. caulinodans* ORS571.

To study the effect of light on root nodulation in *S. rostrata*, we irradiated roots with blue light supplied from above for 14 d and analyzed the nodulation process thereafter. Seeds were germinated on 0.8% (wt/vol) water agar for 3 d in the dark. The seedlings (with their roots shaded by black paper outside of the test tube) were grown on 1.5% (wt/vol) agar-solidified N-free, Broughton and Dilworth (B&D) medium[12] for 7 d under white light (70 μmol m^{-2} s^{-1}). The roots were then inoculated with *A. caulinodans* strain ORS571 (1.0×10^7 cells per plant), and the plants were grown for 14 d under blue light (80 μmol m^{-2} s^{-1}) in a vertical orientation with/without root shading in a 28°C growth chamber. For the unshaded plants, both the shoot and root were exposed to light, whereas when the root was shaded, only the shoot was exposed. Under these conditions, unshaded and shaded roots received approximately 60 μmol m^{-2} s^{-1} and approximately 5 μmol m^{-2} s^{-1} of light, respectively.

Following the root nodulation tests, no significant differences in shoot and root lengths were observed when unshaded and shaded plants were compared (Fig. 1a and b). However, although the root nodule number of *L. japonicus* unshaded roots was drastically decreased compared with that of the shaded roots (shaded; 4.11 ± 0.20 vs. unshaded; 1.76 ± 0.21)[4], the number of nodules developed on *S. rostrata* unshaded roots was slightly, but significantly increased, compared with the shaded roots (Fig. 1c). However, the nodule weight/nodule of the unshaded roots decreased (Fig. 1d). In addition, both white and pale pink nodules were evident on the roots of the shaded plants, but green nodules were common on the unshaded plants (Fig. 1f and g). Nevertheless, no significant difference in acetylene reduction activity (ARA) per plant was observed between the shaded and unshaded conditions (Fig. 1e). By

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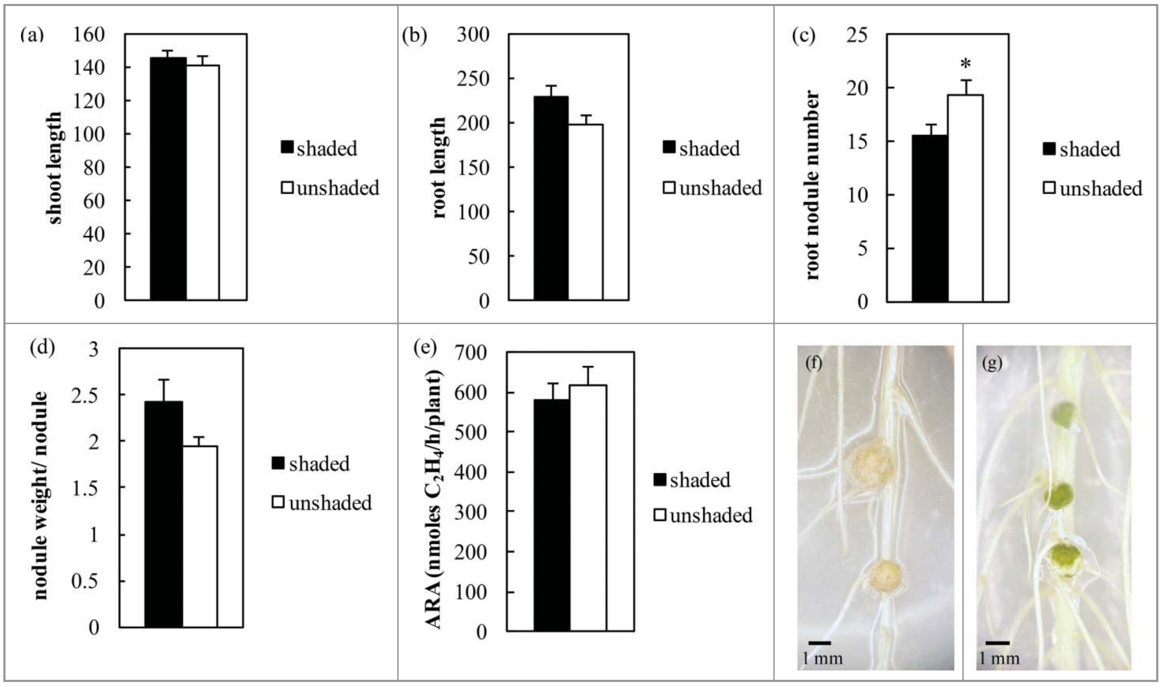

Figure 1. Effect of blue light on growth and nodulation in *Sesbania rostrata* inoculated with *Azorhizobium caulinodans* ORS571. (a-e) Plants were grown under blue light (80 μmol m^{-2} s^{-1} from above) for 14 d with or without root shading. (a) Shoot length, (b) root length, (c) nodule number per plant, (d) nodule weight per nodule, and (e) acetylene reduction activity (ARA) per plant were measured. Values are means $\pm$ SE (24 plants per treatment). *Statistically significant at $P<0.05$ (Student's *t*-test). (f-g) Photograph of root nodules 14 d after inoculation under (f) shaded or (g) unshaded condition. This experiment was performed four times with similar results.

contrast, in *L. japonicus*, shoot growth was inhibited because both nodule number and ARA of the unshaded roots were significantly smaller compared with the shaded roots. Because, in *S. rostrata*, ARA did not differ between shaded and unshaded treatments (Fig. 1e), neither stem nor root growth was affected (Fig. 1 a,b). Taken together, in the *S. rostrata-A. caulinodans* symbiosis, a significant inhibitory effect by blue light irradiation on root nodule formation was not observed.

Next, we analyzed the expression of *NIN*, a nodulation gene marker. Two-days after inoculation, *S. rostrata* roots were quickly frozen in liquid N_2 and stored at -80°C. Total RNA was prepared with an RNeasy Plant Mini Kit (Qiagen). DNase I treatment was performed using DNase RT-Grade (Wako). A one-step SYBR Primescript RT-PCR Kit (Takara) was employed. Transcript levels were normalized against the *SrGAPDH* (glyceraldehyde 3-phosphate dehydrogenase) transcript. The nucleotide sequences of the primers used are: *SrNIN* gene, 5′- GGGAATAGTTGGCACAGCCTTCAC −3′ and 5′- AGAGGATATTCCGCTTTGCT −3′); *SrGAPDH* gene (5′- CATTTGAAGGGTGGTGCCAAG −3′ and 5′- CATT GACTCCAACAACAAACATGG −3′).

The expression of the *NIN* gene in *S. rostrata* was significantly increased by symbiont inoculation whereas very little expression occurred in the uninoculated roots as previously reported in various species (Fig 2a).[13-15] As expected, based on the results in Fig. 1, *NIN* expression levels did not differ between the shaded and unshaded roots (Fig. 2b). This result was related to the slight, but significant increase in nodule numbers on the unshaded roots compared with the shaded roots (Fig. 1c), thus further demonstrating that *NIN* expression levels are correlated with nodule number.

Because *S. rostrata* develops stem nodules under high intensity sunlight and root nodulation was not inhibited by irradiating the roots with blue light, we hypothesized that the sensitivity of *A. caulinodans* to blue light might be different from that exhibited by *M. loti*.[4] To investigate whether blue light exposure influences rhizobial growth, *A. caulinodans* (1.0×10^6 cells per ml) were cultured under blue light. We cultured the bacteria without shaking at 28°C in either blue light (90 μmol m^{-2} s^{-1}) or in the dark because it was difficult to make certain that the cells in each tube would be exposed to sufficient light (90 μmol m^{-2} s^{-1}) under shaking condition. To estimate bacterial growth, the absorbance 610 (A_{610}) of the bacterial culture was measured every 24 h from 24 to 144 h. As shown in Fig. 3, *A. caulinodans* growth under blue light as measured by absorbance was not inhibited during the logarithmic growth phase but a slight, but significant, absorbance difference was observed between the blue light and dark treatments at 96, 120 and 144 h. Cell numbers were checked at the end of logarithmic phase (72 hours) and stationary phase (144 hours) using dilution plate methods. However, no difference in viability was observed at 72 h based on cell number counts between dark ($6.69 \pm 1.26 \times 10^8$ cell/ml) and blue light ($7.77 \pm 0.39 \times 10^8$ cell/ml) treatments. In contrast, cell numbers under blue light-illuminated conditions ($5.12 \pm 0.03 \times 10^7$ cell/ml) after 144 h were drastically decreased compared with the dark treatment ($6.63 \pm 1.36 \times 10^8$ cell/ml). A gene encoding deoxyribodipyrimidine photolyase, which is homologous to the *M. loti*, *Cryptochrome* gene, is present in the *A. caulinodans* genome and may be involved in blue light perception. From these data, we conclude that blue light does not affect rhizobial growth

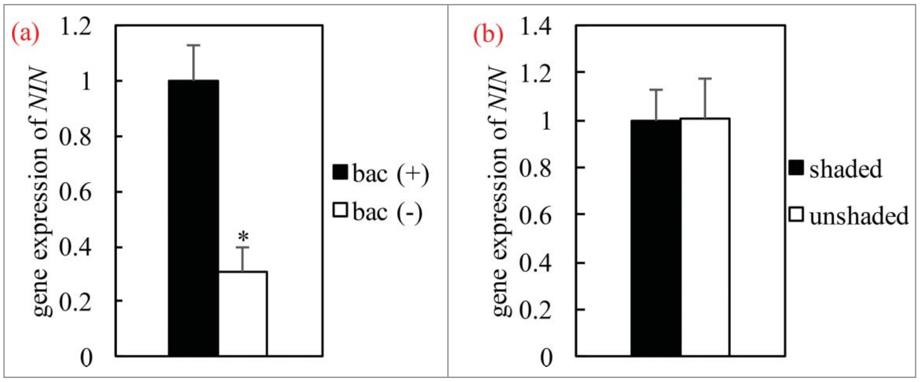

Figure 2. Relative *NIN* expression from the shaded or unshaded root in *Sesbania rostrata* with or without *Azorhizobium caulinodans* ORS571 inoculation. (a-b) Plants were grown with or without root shading under light from above (80 μmol m^{-2} s^{-1} from above) for 2 d with inoculation or not. Difference of *NIN* expression of (a) inoculation or not, and (b) root shading or not were investigated. The *NIN* transcript levels were normalized to that of *GAPDH* as an internal control. Values are means ± SE (9 plants per treatment). *Statistically significant at $P<0.05$ (Student's *t*-test). This experiment was performed three times with similar results. The identity of nucleotide sequences between *S. rostrata* and *L. japonicus* (Lj2g3v3373100.1) was 76% (672 amino acids in length), and this nucleotide sequences were deposited at DDBJ as accession number: LC194191.

based on absorbance measurements, but rhizobial survival is reduced by long-term blue light exposure.

Under the experimental conditions used in the nodulation tests in our research, nodules developed on *S. rostrata* roots grown in agar-solidified N-free B&D medium following inoculation with *A. caulinodans*. On the other hand, if blue light exposure had caused inhibition in this plant, neither stem nor root nodules would have developed. Therefore, the mechanism of blue light inhibition described for *L. japonicus*[4] is not conserved in the symbiosis between *S. rostrata* and *A. caulinodans*. Moreover, based on genome sequence comparisons, *A. caulinodans* is phylogenetically related to prototypes of rhizobia and not to *M. loti*.[16,17] In the symbiosis between *L. japonicus* and *M. loti*, the influence of blue light on nodule development is profound. Analyzing the steps between light perception and the initiation of nodule development, however, requires further study.

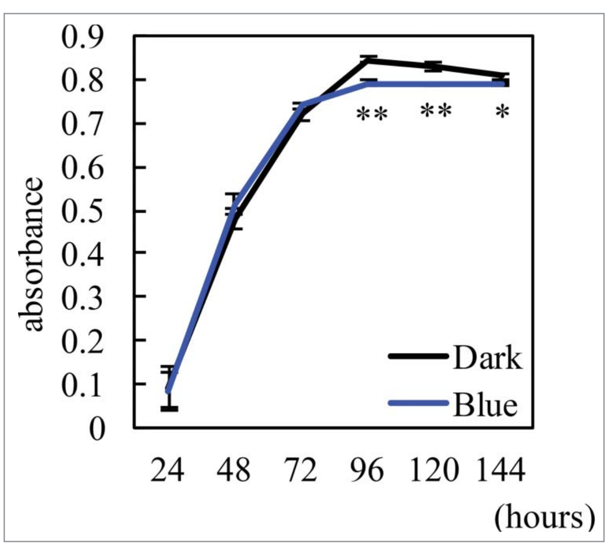

Figure 3. Proliferation of Azorhizobium caulinodans ORS571. *A. caulinodans* was cultured under blue light (90 μmol m^{-2} s^{-1}) or kept in the dark. Values are means ± SE (10 samples) and statistically significant at $P<0.05$ (Student's *t*-test). This experiment was performed three times with similar results.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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