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Light Requirement for Shoot Regeneration in Horseradish Hairy Roots[1]

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ABSTRACT

Hairy roots of horseradish (*Armoracia rusticana*) were induced by inoculation with *Agrobacterium rhizogenes* harboring Ri plasmid and cultured on phytohormone-free Murashige and Skoog medium after eliminating the bacteria. Hairy roots grew vigorously and sometimes formed yellowish calli under dark conditions. On the other hand, growth of hairy roots stopped after several weeks of culture with light, then shoots were regenerated. Frequency of shoot formation from hairy roots increased as the culture period in light lengthened and the light intensity increased. The shoot regeneration was induced by treatment with white or red light, but not with far-red light. Shoot regeneration by red light was inhibited by following treatment with far-red light. Red and far-red light reversibly affected shoot regeneration. Excised roots of nontransformed plants grew quite slowly on phytohormone-free Murashige and Skoog medium and occasionally formed shoots under white light conditions.

It is well known that dicotyledonous plants infected by *Agrobacterium rhizogenes* harboring Ri plasmid form numerous adventitious roots at the infected site. Concerning this phenomenon, several researchers clearly demonstrated that a portion of Ri plasmid (T-DNA) was integrated into plant genomic DNA and genes on the T-DNA caused both root formation and definite growth of the roots on phytohormone-free medium (1, 18, 21). The adventitious roots named as hairy roots grow vigorously in phytohormone-free medium and provide a useful material for studies on secondary metabolite production (9, 10). In a number of plant species, plant regeneration from hairy roots could be observed when they were cultured on medium with phytohormones (19). On the other hand, in some species, hairy roots cultured under light and/or dark conditions formed adventitious shoots on phytohormone-free medium (5, 12, 18).

It was previously reported that hairy roots of horseradish formed adventitious shoots when they were cultured in phytohormone-free medium under light conditions (11, 14). However, there have been no detailed analyses of effects of light on adventitious shoot formation. Therefore, we have conducted a series of experiments to clarify the effects of light on adventitious shoot formation in horseradish. In the present paper, we demonstrate and discuss the light requirement for shoot formation in both hairy roots and excised roots of nontransformed plants of horseradish.

MATERIALS AND METHODS

Induction and Maintenance of Hairy Roots

Hairy roots were obtained by inoculating sterilized leaves of horseradish (*Armoracia rusticana*) with *Agrobacterium rhizogenes* strain 15834 harboring Ri plasmid (pRi 15834). Several weeks after the inoculation, tip segments of hairy roots that appeared on the inoculated sites were cut off and cultured on hormone-free MS[2] semi-solidified (0.2% gerlite) medium containing an antibiotic (Claforan, 500 mg/L) at 25°C under dark conditions. Segments of growing hairy roots were transferred several times to the fresh MS medium to eliminate the bacteria and then transferred to hormone-free MS medium without antibiotics.

Culture of Hairy Roots

Hairy roots were cultured on hormone-free semi-solidified (0.2% gerlite) MS medium in Petri dishes. Culture conditions in darkness were 24 h dark at 25°C and those in light were either 16 h light/8 h dark or continuous light at 25°C. Light intensity of white light in Figures 1 and 2 and Tables I and II was measured with Toshiba Photocell Illuminometer SPI-5.

Red Light and Far-Red Light Treatment

Red light (0.6 W/m^2) was obtained by passing white light from a Toshiba lamp (FL4OSS. W/37) through a Mitsubishi No. 102 filter (3 mm) that cut off light shorter than 600 nm. Far-red light (0.8 W/m^2) was obtained from a Toshiba lamp (FL2OS-FR74) through an Asahikasei Deragurasu A filter (3 mm). Light intensity of white light, red light, and far-red light in Tables III through V was measured with KIPP & ZONEN Compensated thermopile CA 1.

RESULTS

Characterization of Hairy Roots

Two to four weeks after the bacterial inoculation, numerous adventitious roots appeared on the inoculated sites. Almost all of the adventitious roots grew fast on hormone-free MS medium after elimination of bacteria. These well-growing

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[2] Abbreviation: MS, Murashige and Skoog.

hairy roots exhibited frequent branching of lateral roots both in complete darkness and under light conditions (Table I). On the other hand, root tips isolated from nontransformed plants grew slowly without lateral root branching on hormone-free MS medium (Table I). Under dark conditions, both hairy roots and nontransformed roots rarely formed shoots (Table I). On the other hand, under light conditions (16 h light/8 h dark, about 17 μmol/m^2·s of light intensity), hairy roots produced many shoots with a high frequency, but nontransformed roots formed a few shoots with a low frequency (Table I).

Light Requirement for Shoot Formation from Hairy Roots

Root tips (1 cm) of hairy roots were excised and transferred to hormone-free MS medium in Petri dishes (9 cm). They were cultured under either complete darkness or light conditions (16 h light/8 h dark, about 17 μmol/m^2·s of light intensity). The growth rate of hairy roots in both the light and dark conditions were similar until the seventh week of culture, but the growth in the light stopped after 8 weeks of the culture (Fig. 1C). The longer the culture period in the light, the higher the frequency of shoot formation and the number of shoots per explant after 5 weeks of the culture (Fig. 1A). In the dark conditions, however, shoot formation was rarely observed throughout the culture period (Fig. 1B).

When hairy roots were cultured under light conditions for the first 4 weeks, no shoot formation was observed (Fig. 1A). However, the length of hairy roots increased from 1 to about 10 cm for the period. Therefore, hairy roots were cultured for 0 to 16 weeks under dark conditions and then transferred to light conditions (16 h light/8 h dark, 78 μmol/m^2·s of light intensity) to see the effects of the initial dark period. One week after the transfer to light conditions, morphological observations were carried out. When hairy roots were precultured for two weeks in darkness and transferred to the light, shoot formation was observed 1 week after the transfer (Fig. 2). Frequency of shoot formation and the number of shoots per explant tended to increase when the culture period in darkness was prolonged.

In the next experiment, the effects of duration of light irradiation were examined. Hairy roots that were precultured under dark conditions for 14 weeks were treated with light (78 μmol/m^2·s) during 1 to 168 h and then transferred to complete darkness. Frequency of shoot formation was measured one week after the start of light treatments. Frequency of shoot formation and the number of shoots increased in proportion to the duration of light treatment (Table II). When hairy roots were cultured for more than 24 h in light, hairy roots and regenerated shoots turned green. Similar results were also obtained in the case of the treatment with 7.8 μmol/m^2·s of light intensity (data not shown).

In another experiment, hairy roots were cultured for 24 h in continuous light where the light intensity was varied from 0.78 to 78 μmol/m^2·s, and then transferred to dark conditions. Frequency of shoot formation was determined one week after the start of light treatments. Both the frequency of shoot formation and the number of shoots increased in proportion to the degree of light treatment (Table II).

In the next experiment, both the intensity and the duration of light treatment were changed to give the same light fluence. At the same fluence (intensity × duration), the frequency of shoot formation and the number of shoots produced were variable. A short exposure (1.68 h) to a high light intensity (78 μmol/m^2·s) resulted in less shoot formation than a long exposure (168 h) to a lower light intensity (0.78 μmol/m^2·s). The duration of light exposure was more important than light intensity as far as shoot formation from hairy roots was concerned (Table II).

Effects of Red and Far-Red Light on Shoot Formation

In the above experiments, the treatment with white light induced shoot formation from hairy roots. Then effects of both red and far-red light on shoot formation were examined. Hairy roots that were precultured for 12 weeks in complete

Table I. *Characteristics of Hairy Roots and Nontransformed Roots*

Segments (1 cm) of root tips were cultured on hormone-free MS medium in complete darkness (D) or in 16 h light/8 h dark, with about 17 μmol/m^2·s light intensity (L). Length of roots (cm) was measured after 4 weeks of culture. Number of lateral roots (No. of lateral roots/cm main root) was counted after 4 weeks of culture. Frequency (%) of shoot formation ([No. of explants forming shoots/total explants] × 100) and number of shoots (No. of shoots/total explants) were recorded after 10 weeks of culture. Experiments of hairy roots were repeated three times with 20 replications and those of nontransformed roots were repeated three times with 10 to 15 replications. Values are expressed as the mean ± SE.

Characteristic		Hairy Roots	Nontransformed Roots
Length of roots	D	8.6 ± 2.9	6.1 ± 1.6
	L	8.7 ± 2.8	4.7 ± 1.3
Number of lateral roots	D	1.12 ± 0.84	0.08 ± 0.25
	L	0.76 ± 0.84	0.07 ± 0.17
Frequency of shoot formation (%)	D	7 ± 6	2 ± 3
	L	86 ± 9	23 ± 16
Number of shoots	D	0.16 ± 0.19	0.02 ± 0.03
	L	4.96 ± 0.19	0.74 ± 0.50

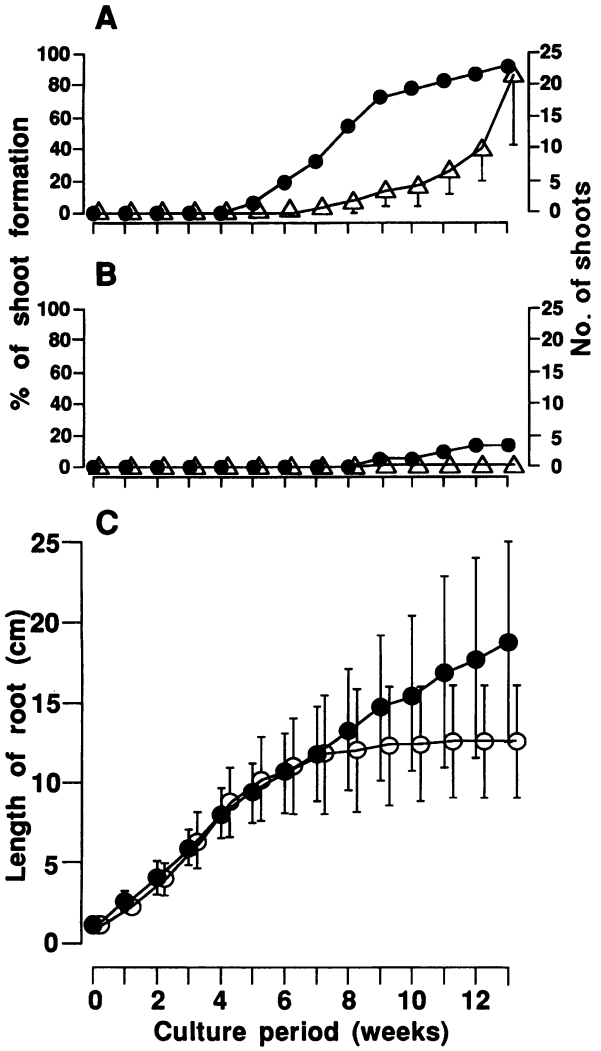

Figure 1. Shoot formation (A and B) and hairy root lengthening (C). Root tip segments (1 cm) of hairy roots were cultured in light (16 h light/8 h dark, about 17 μmol/m^2·s of light intensity) or in dark with 22 or 21 replications, respectively. A and B, Frequency (%) of shoot formation ([No. of explants forming shoots/total explants] × 100, ●) and number of shoots (No. of shoots/total explants, △). A, Light; B, dark; C, growth of hairy roots cultured in light (○) or in dark (●). Flags indicate SE.

darkness were cultured for 1 week under various light conditions (16 h light/8 h dark). Neither dark control nor those treated with far-red (0.8 W/m^2) light formed shoots (Table III). On the other hand, treatments with both white light (3.0 W/m^2) and red light (0.6 W/m^2) similarly induced shoot formation at a high frequency (Table III).

In the next experiment, hairy roots were cultured with red or far-red light or red light followed by far-red light for 5 d and then transferred to darkness. With the red light treatment, many shoots were formed from hairy roots, but only a few shoots were formed with far-red light treatment (Table IV). Shoot formation induced by 7-min red light was partially inhibited by the following 7-min far-red light irradiation (Table IV). In the case where the duration of both red and far-red light irradiation was altered to 20 min or 1 h each, the shoot formation induced by red light was also inhibited by the following far-red light irradiation (data not shown). In those cases, duration of red light:duration of far-red light was 1:1. This ratio was changed to 1:1, 1:3, and 1:7 under the condition that duration of red light was 5 min and that one cycle of red light followed by far-red light was 40 min. In the case where the ratio of red light:far-red light was 1:7, the frequency of shoot formation was almost the same as that of the dark control (data not shown).

In the next experiment, hairy roots were cultured under repeated irradiation with red and far-red light (Table V). Induction of shoot formation was determined by the kind of light that was radiated at the end of the 2-h cycles. If red light was the last one, shoot formation was induced, and if far-red light was the last one, shoot formation was inhibited (Table V).

DISCUSSION

Adventitious shoot formation *in vitro* is under the control of various factors, including the genetic (4) and physiological (16) states of the explants, and chemical and physical factors of the culture conditions (16). Among these factors, effects of phytohormones, especially auxin and cytokinin, have been examined in detail in a number of plant species (3, 8, 17). Concerning the physical factors, the effects of temperature during culture have frequently been investigated. Even though the light condition is an important factor for shoot regeneration, only a limited number of studies have been reported. In a few species, shoots were spontaneously regenerated from hairy roots on hormone-free medium under light conditions (5, 18). In horseradish hairy roots, light treatment induced shoot formation on hormone-free medium (11, 14).

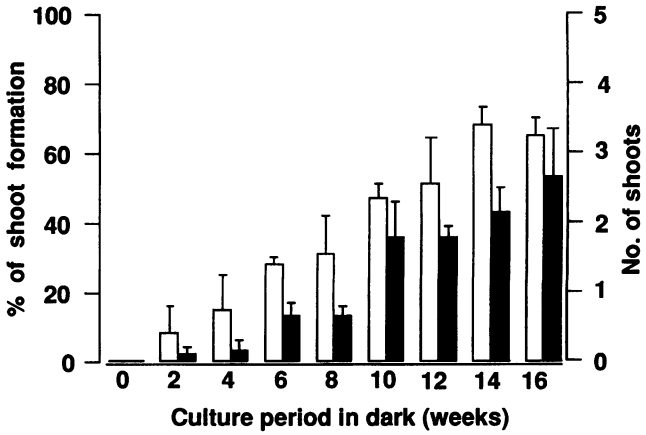

Figure 2. Effects of culture periods in dark on shoot formation in hairy roots. Root tip segments (1 cm) of hairy roots were cultured in the dark for 0 to 16 weeks, then transferred to light conditions (16 h light/8 h dark, 78 μmol/m^2·s of light intensity). One week after transfer, the frequency (%) of shoot formation ([No. of explants forming shoots/total explants] × 100, □) and number of shoots (No. of shoots/total explants, ■) were recorded. Experiments were repeated three times with 18 to 32 replications. Flags indicate SE.

Table II. *Effects of the Duration of Light Treatment and/or Light Intensity on Shoot Formation in Hairy Roots*

Root tip segments (1 cm) of hairy roots were cultured in complete darkness for 12 to 14 weeks, then cultured at various light intensities (0-78 μmol/m^2·s) for 0 to 168 h. Except for hairy roots cultured for 168 h in light, hairy roots were transferred to complete darkness after the light treatments indicated. One week after the start of the light treatments, the frequency (%) of shoot formation ([No. of explants forming shoots/total explants] × 100), number of shoots (No. of shoots/total explants), and frequency (%) of greening explants ([No. of greening explants/total explants] × 100) were recorded. All experiments were repeated two to four times with more than 30 replications. Values are expressed as the mean ± SE.

Intensity (μmol/m^2·s) × Duration (h)	Shoot Formation	No. of Shoots	Greening
	%		%
78 × 0	7 ± 1	0.12 ± 0.02	0
78 × 1	17 ± 6	0.28 ± 0.10	5 ± 2
78 × 24	35 ± 6	0.75 ± 0.33	38 ± 6
78 × 168	58 ± 4	1.34 ± 0.18	88 ± 7
0 × 24	7 ± 1	0.14 ± 0.02	0
0.78 × 24	20 ± 3	0.29 ± 0.02	29 ± 1
7.8 × 24	27 ± 7	0.55 ± 0.15	38 ± 8
78 × 24	35 ± 6	0.75 ± 0.33	38 ± 6
0 × 168	13 ± 6	0.20 ± 0.10	0
78 × 1.68	19 ± 6	0.36 ± 0.12	8 ± 5
7.8 × 16.8	29 ± 6	0.48 ± 0.10	26 ± 11
2.6 × 50.4	32 ± 3	0.70 ± 0.08	36 ± 6
0.78 × 168	54 ± 5	1.83 ± 0.47	59 ± 5

As we have shown, both hairy roots and the roots of nontransformed horseradish plants formed shoots on hormone-free medium under light conditions (Table I). However, the frequency of shoot formation in the roots of nontransformed plants was lower than that in hairy roots. This might be partially related to the differences in the rate of cell division and cell elongation between hairy roots and normal roots. In hairy roots, a certain period of growth before light irradiation was necessary for the shoot formation (Figs. 1 and 2). Physiological changes that happened during hairy root growth might be important for the formation of adventitious shoots.

Adventitious shoot formation in the hairy roots depended on both the duration and intensity of light (Table II). The duration of light clearly affected the rate of shoot formation, whereas the intensity of light showed only slight effect (Table II), especially in the case where the hairy roots were cultured for 1 week (data not shown). Adventitious shoot formation in horseradish required continuous or intermittent light irradiation (Table V). These results indicated that the adventitious shoot formation in horseradish by light involved several essential processes induced by light. The greening of plants was also apparently regulated by light in a complex and not yet fully understood way. As indicated in this paper, such adventitious shoot formation is coordinated with the processes of greening of hairy roots (Tables II–V). These results also suggested indirectly that shoot formation was regulated by light, and that other changes induced by light might affect shoot formation.

Shoot formation in horseradish hairy roots can be induced by white light and red light, but not by far-red light (Table III). Far-red light irradiation given just after red light irradiation partially inhibited the shoot-inducing effects of red light (Table IV), and shoot formation was reversibly regulated by red and far-red light (Table V). These results indicate that phytochrome must be involved in the shoot formation from horseradish hairy roots.

The effect of light on adventitious shoot formation varies depending on the plant species. Adventitious shoot formation in tobacco calli was reported to be stimulated by blue light and inhibited by red light (15, 20). On the other hand, red light stimulated shoot formation in *Pseudotsuga menziesii* (7), petunia (2), and apple (13). To clarify the underlying mechanisms for light involvement in this process, we are currently attempting to elucidate the involvement of phytochrome in light-induced shoot formation of horseradish.

Table III. *Effects of Red or Far-Red Light on Shoot Formation in Hairy Roots*

Hairy roots were cultured for 1 week under various light conditions; complete darkness (Dark), 16 h light/8 h dark treatment with white light (W, 3.0 W/m^2), red light (R, 0.6 W/m^2), or far-red light (FR, 0.8 W/m^2). Other treatments were the same as in Table II. Experiments were repeated twice with more than 38 replications. Values are expressed as the mean ± SE.

Treatment	Shoot Formation	No. of Shoots	Greening
	%		%
Dark	4 ± 1	0.06 ± 0.03	0
W	49 ± 5	1.48 ± 0.19	71 ± 6
R	53 ± 1	1.36 ± 0.01	57 ± 1
FR	10 ± 3	0.20 ± 0.04	0

Table IV. *Inhibition of Shoot Formation in Hairy Roots by Far-Red Light*

Hairy roots were cultured for 5 d under various light conditions. In red light (R) treatment, hairy roots were cultured first in red light for 7 min and next in darkness for 7 min. These treatments were repeated for 8 h/d and continued for 5 d. In far-red light (FR) treatment, red light treatments were replaced by far-red light and other conditions were the same as those of R treatment. In red and far-red light (R/FR) treatment, dark periods after red light irradiation in R treatment were replaced by far-red light and other conditions were the same as those in R treatments. Other treatments were the same as in Table II and more than 54 replications were performed.

Treatment	Shoot Formation	No. of Shoots	Greening
	%		%
R	41	1.08	41
R/FR	20	0.43	29
FR	2	0.02	0

Table V. *Red and Far-Red Light Photoreversibility of Shoot Formation in Hairy Roots*

Hairy roots were cultured for 5 d under various light conditions or in darkness (Dark). In R treatment, hairy roots were cultured first in red light (R, 5 min) and next in darkness for 115 min. These treatments were repeated continuously 5 times/d and continued for 5 d. In R/FR treatment, hairy roots were cultured first in R and second in far-red light (FR, 35 min), and then in darkness for 80 min. In R/FR/R, R/FR/R/FR, or R/FR/R/FR/R treatments, R and FR treatments were repeated and dark periods were shortened to 75, 40, or 35 min, respectively. Other conditions in R/FR, R/FR/R, R/FR/R/FR, and R/FR/R/FR/R were the same as those of R treatment. Other treatments were the same as in Table II. Experiments were repeated three times with more than 30 replications. Values are expressed as the mean ± SE.

Treatment	Shoot formation	No. of Shoots	Greening
	%		%
Dark	8 ± 4	0.10 ± 0.03	0
R	33 ± 5	0.54 ± 0.06	41 ± 5
R/FR	19 ± 1	0.28 ± 0.05	15 ± 4
R/FR/R	39 ± 1	0.69 ± 0.06	43 ± 3
R/FR/R/FR	14 ± 3	0.20 ± 0.04	16 ± 5
R/FR/R/FR/R	37 ± 1	0.52 ± 0.06	43 ± 1

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