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Flowering time and concentration of secondary metabolites in floral organs of *Hypericum perforatum* are affected by spectral quality

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Abstract

Hypericin and pseudohypericin are the major bioactive constituents of floral parts of *Hypericum perforatum* L., mainly used for the treatment of neurological disorders and depression. The principle objective of the current study was to evaluate the effect of blue, blue and red mixed, and red light on flowering time and concentration of hypericin, pseudohypericin and hyperforin in the floral tissues of *H. perforatum* plants. The results revealed that red light promoted flowering and production of the three major medicinal components, indicating the influence of spectral characteristics of light on flowering of *H. perforatum* plants. Spectral quality of light was found to be an important factor in controlling the flowering of *H. perforatum* plants.

Key words: *Hypericum perforatum* L., artificial light, controlled environment, hyperforin, hypericin, long-day plant, St. John's wort

Introduction

Plants recognize changes in their light environment by sensing light quality using signal-transducing photoreceptors. Light signals detected by the photoreceptors directly or indirectly affect physiological, morphological, and anatomical features in plants (Goto, 2003). Light quality has been reported to influence the flowering time of many long-day plants (Runkle and Heins, 2006). Under different light-quality cues the proportion of total phytochrome in the active form (phytochrome photoequilibrium) has been reported to regulate floral morphogenesis (Weinig, 2002) and flowering time of several long-day plants (Downs and Thomas, 1990). In other studies, the ratio of R/FR has been shown to alter the flowering time of many plant species including *Antirrhinum majus, Campanula carpatica, Coreopsis grandiflora, Petunia x hybrida,* and *Hyoscyamus niger* (Runkle and Heins, 2006, and references therein).

Hypericum perforatum L. (St. John's wort) has been used medicinally for thousands of years. It is a long-day flowering plant as it flowers in Europe around St. John's Day (June 24) and its flowering was promoted under 16-18 h d^{-1} light period in controlled environments (S.M.A. Zobayed, unpublished data). The major medicinally important metabolites, hypericin, pseudohypericin and hyperforin, are located mainly in the floral tissues of this plant. The current experiments were conducted to evaluate the influence of spectral characteristics of light on flowering time and production of major metabolites in the floral tissues of *H. perforatum* plants.

Materials and methods

Plant material: Seeds of *H. perforatum* (cv. 'Standard', Murakami seed Co., Ltd., Yokohama, Japan) were sown in 128-cell plug seedling trays (Takii Seed Co., Ltd., Kyoto, Japan) filled with a commercial soil mixture (Yanmar Agricultural Equipment Co., Ltd., Osaka, Japan). Twenty-one days after sowing, plants were transplanted to individual pots (bore diameter, 7.5 cm; capacity, 250 mL), filled with the commercial soil mixture. Plants were supported with commercial wires and threads. The plants were cut at the eighth node of the main stem 31 days after sowing to produce lateral branches. Thirty-six days old plants (fresh weight, 498.2 ± 23.0 mg; dry weight, 97.0 ± 5.1 mg) were used as the plant materials. The plants were grown in a controlled environment room with air temperatures of 27/24°C (light/dark period), relative humidity of 60%, and a 16 h d^{-1} light period provided by a combination of blue fluorescent lamps (FLR40S-EB/M, Matsushita Electric Industrial Co., Ltd., Osaka, Japan) and red fluorescent lamps (FLR40S-ER/M, Matsushita Electric Industrial Co., Ltd.). The CO_2 concentration was 1000 μmol mol^{-1} and the photosynthetic photon flux (PPF) measured at the soil surface was 250 μmol m^{-2} s^{-1}.

Treatments and growing conditions: The experiment was designed to evaluate the effects of different light qualities: blue light (B), mixture of blue and red light (BR), and red light (R). Blue and red fluorescent lamps were used as light sources. The spectral characteristics of each light source are listed in Table 1. There was a notable difference in the red/far-red ratio (R/FR) for the three light sources; however the phytochrome photoequilibrium (Pfr/P) value, which is an indicator of phytochrome response to R/FR, was almost the same. The experiment was conducted in a closed transplant production system (Nae terasu, Taiyo-Kogyo Corp., Tokyo, Japan). Environmental conditions common to all treatments were 27/24°C air temperatures (light/dark period), a 16 h d^{-1} light period, 60% relative humidity, and 1000 μmol mol^{-1} CO_2 concentration. As the seedlings grew, the distance between the lamps and the growing points of the plants was adjusted to maintain a constant PPF (250 μmol m^{-2} s^{-1}). The positions of the seedlings within the treatments were rearranged to minimize the variation of PPF. Subirrigation with a nutrient solution (Otsuka hydoponic composition adjusted to EC 1.2 dS/m and pH6.0, Otsuka Chemical Co., Ltd., Osaka, Japan; 4.2 mmol L^{-1} NO_3^-, 1.3 mmol L^{-1} $H_2PO_4^-$, 1.0 mmol L^{-1} Ca^{2+}, 0.38 mmol L^{-1} Mg^{2+}, 2.2 mmol L^{-1} K^+, 0.4 mmol L^{-1} NH_4^+) was applied once a day after germination.

Fig. 1. Developmental stages of *H. perforatum* flowers. At stage 1, flower buds were entirely green and about 1-3 mm in length. Stage 2 buds had the first visible yellow petals, 3-4 mm. Stage 3 buds had exposed yellow petals with small dark glands on the exposed surface, 4–6 mm. Stage 4 buds were slightly larger with yellow petals and dark glands clearly visible, 6-8 mm. Stage 5 buds were the most mature closed buds with dark glands visible on petals and anthers, over 8 mm. Stage 6 buds consisted of newly open flowers. Stage 7 flowers withered.

Table 1. Spectral characteristics of each fluorescent lamp

	B	BR	R
Photon flux (μmol m^{-2} s^{-1})			
300-400 nm (UV)	1.4	1.4	1.3
400-500 nm (B)	198.0	102.1	17.4
500-600 nm (G)	41.8	46.2	50.2
600-700 nm (R)	10.2	101.7	182.4
700-800 nm (FR)	2.1	12.5	22.4
B/R (400-500 nm/600-700 nm)	19.5	1.0	0.1
R/FR (600-700 nm/700-800 nm)	4.9	8.1	8.1
Phytochrome photoequilibrium (Pfr/P)*	0.8	0.8	0.8

*The value of phytochrome photoequilibrium was calculated using the equation proposed by Hanyu *et al.* (1996).

Table 2. The number of flower buds of *H. perforatum* plants grown under different light quality for 33 days (69 days after sowing)

Light quality	Flower developmental stages						
	1	2	3	4	5	6	7
B	4.6 a	3.1 a	3.0 b	1.5 a	0.8 b	0.3 a	1.0 b
BR	6.3 a	4.0 a	5.1 a	2.3 a	2.1 a	0.8 a	2.1 b
R	6.1 a	1.4 b	2.9 b	2.2 a	1.9 ab	0.8 a	5.1 a

The data are means from 29-31 plants and different letters indicate significant difference at $P<0.05$ level according to Tukey-Kramer test.

Flowering: The definition of floral developmental stages was modified from Murch *et al.* (2002) (Fig. 1). The time courses of the percentage of plants with flower buds (stage 1) and the percentage of plants with open flowers (stage 6) 69 days after sowing were observed. The days from bearing of the flower bud (stage 1) to flowering (defined as bud opening) (stage 6) were measured. The number of the flower buds, and the fresh and dry weights of flower buds were measured 69 days after sowing.

Extraction and determination of hypericin, pseudohypericin, and hyperforin concentrations: Samples representing seven distinct stages of flower development in BR treatment and stages 3 and 6 in B and R treatments were collected 69 days after sowing and stored at -85°C until used for the analysis of hypericin, pseudohypericin, and hyperforin as described by Mosaleeyanon *et al.* (2005). The concentrations of the medicinal components in the sampled flower buds were expressed as mg g^{-1} flower bud DW, and the total medicinal content of each flower bud (g/flower bud) was calculated by multiplying the concentrations of the medicinal components by bud dry weight. The medicinal content of total flower buds of each plant (g/plant) was calculated by multiplying the concentrations of medicinal components in the sampled flower buds in BR treatment by the number of flower buds per plant in each treatment, assuming that the total medicinal contents of each flower bud (g/flower bud) during each treatment were same.

Statistical analysis: The experiment was conducted twice with 18 replications. Means and standard errors obtained from replicate experiments were subjected to an analysis of variance (ANOVA). The means were compared using the Tukey-Kramer test at a 5% level of significance. The means of the percentage of plants with flowers were compared using Fisher's exact test with Bonferroni correction.

Results

Effects of light quality on flowering, concentrations of medicinal components and medicinal content of flower buds: The percentages of plants with flower buds in all light quality treatments rapidly increased from 3 days after observation of the first bud and were saturated within 9 days (Fig. 2). Plants grown in R light produced buds earlier than those grown in B or BR, while plants in the two latter treatments produced their first flower buds on the same day. Fifty percent of plants grown in B, BR, and R had buds by 56, 54, and 52 days after sowing, respectively. The time between appearance of the first flower bud and flowering in B, BR, and R treatments was 13, 12, and 12 days, respectively. The percentage of plants with flowers, 69 days after sowing was highest in R treatment, 1.2 and 2.0 times that of plants grown in BR and B (Fig. 3). The total number of flower buds per plant was almost the same under different light conditions (Table 2). At 69 days after sowing, the number of flower buds at stages 1, 4, and 6 was similar among the treatments; however the largest number of buds at stages 2, 3, and 5 was found in plants with BR treatment, and stage 7 buds were most abundant in R treatment.

Hypericin concentrations in stage 3 and 6 flower buds were similar under different light treatments (Fig. 4). Pseudohypericin concentration in stage 3 flower buds was significantly higher in the B treatment than in BR and R treatments, but was similar among the treatments in stage 6 buds. Hyperforin concentration in stage 3 flower buds was higher in R treatment than B and BR treatments, but like pseudohypericin was almost the same among the treatments by stage 6.

Dry weight of stage 3 flower buds in R treatment was greater than that in B treatment, and almost the same as that in BR treatment

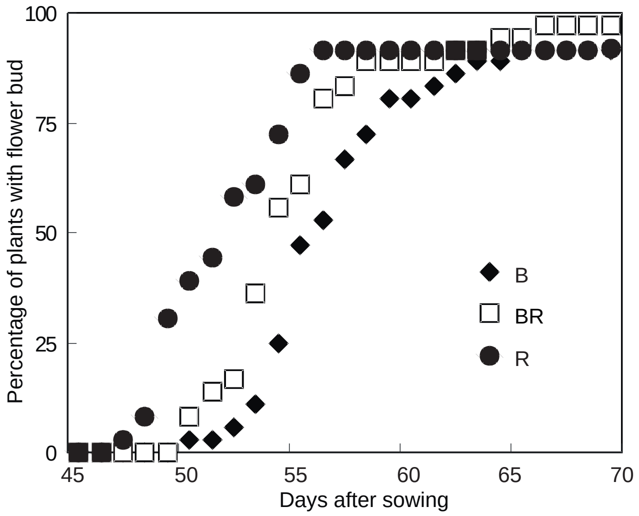

Fig. 2. Time courses of the percentage of plants with stage 1 flower buds, n=36.

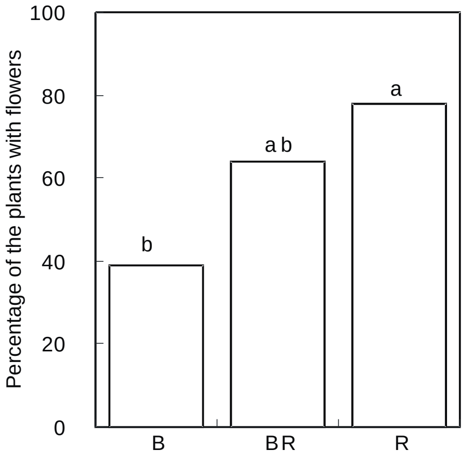

Fig. 3. Percentage of plants with flowers grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P \leq 0.05$, determined by the Fisher's Exact test with Bonferroni correction. Each bar represents mean ± S.E. of 36 replicates.

(Fig. 5). Dry weight of stage 6 flower buds was highest in R treatment. Total content of hypericin and pseudohypericin in stage 3 flower buds was almost the same among the treatments (Fig. 6). The hyperforin content in R treatment was higher than that in B treatment, and was almost the same in BR treatment. The contents of the three medicinal components in stage 6 flower buds in R treatment were significantly higher than those in B and BR treatments.

The total content of hypericin, pseudohypericin, and hyperforin in flower buds of plants 69 days after sowing (µg/plant) was highest in R treatment (Fig. 7).

Quantity and concentration of medicinal components in flower buds at different stages: The concentrations of major metabolites in floral tissues were explored in plants grown under BR. In this treatment, the dry weight of flower buds increased from stage 1-6, and then decreased (Fig. 8). The concentrations of the medicinal components in flower buds of plants 69 days after sowing are shown in Fig. 9 (A). Hypericin and pseudohypericin concentrations were highest in stage 2 flower buds, and subsequently declined. Hyperforin concentration was highest in stage 2 buds and subsequently decreased, was constant during

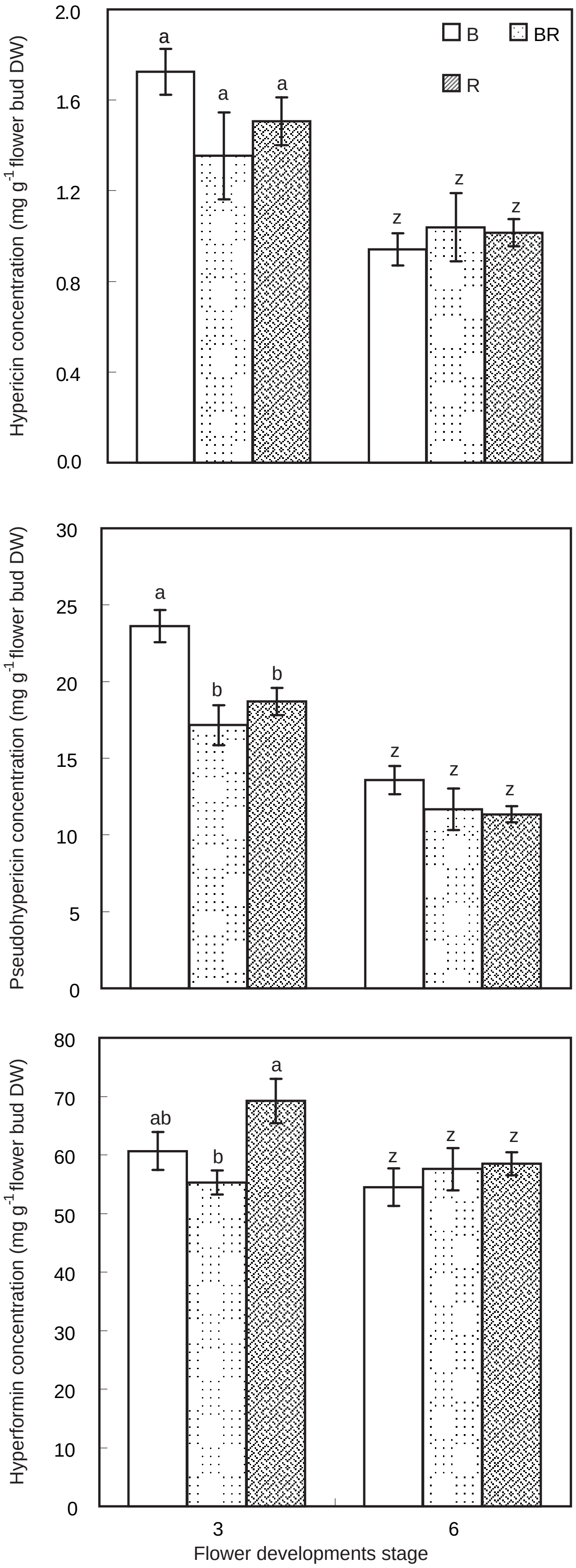

Fig. 4. The hypericin, pseudohypericin, and hyperforin concentrations of stage 3 and 6 flower buds of plants grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S.E. of 11-12 (stage 3) and 6-14 (stage 6) replicates.

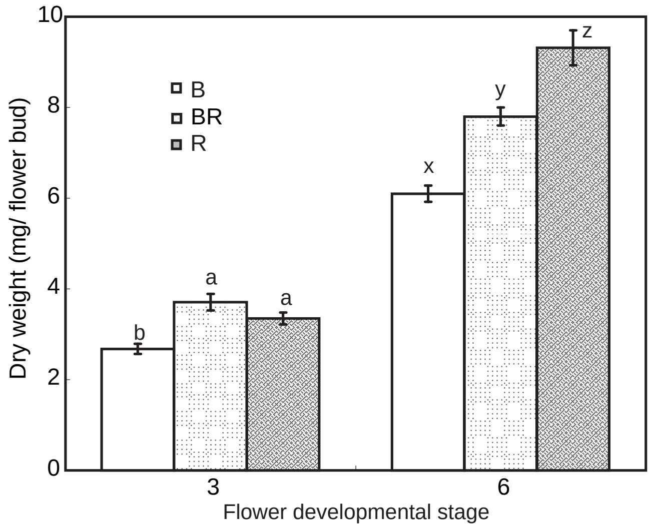

Fig. 5. Dry weights of stage 3 and 6 flower buds of plants grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S. E. of 24-27 (stage 3) and 8-19 (stage 6) replicates, respectively.

stages 3-6, and then increased again. The medicinal contents 69 days after sowing in BR treatment are shown in Fig. 9 (B). Hypericin and pseudohypericin contents in stage 1-4 flower buds increased during flower development, and remained high in stages 4-7. Hyperforin content increased throughout flower development.

Discussion

The present experiments were undertaken to evaluate the influence of spectral characteristics of light on flowering of *H. perforatum* plants without altering the phytochrome photoequilibrium values, or the PPF at the growing points of plants. We found that R light promoted flowering in *H. perforatum*: plants grown in R light developed flower buds earlier than those grown in B or BR, and the percentage of plants with flowers in R treatment was higher than that in B and BR treatments. The number of days from appearance of the first flower bud to flowering in B, BR, and R treatments were almost the same; therefore we concluded that the pace of flower development was not influenced by light quality.

The floral transition in *Arabidopsis*, a quantitative long-day plant, is regulated by at least four flowering pathways: the photoperiod response, the vernalization response, the autonomous pathway, and the gibberellin (GA)-dependent pathway (Mouradov *et al.*, 2002). *H. perforatum* may be a long-day plant because it flowers around St. John's Day (June 24) in Europe, and its flowering was promoted under a 16-18 h d^{-1} light period in a controlled environment (S.M.A. Zobayed, unpublished data). Furthermore, flowering of *H. perforatum* plants does not require vernalization. Therefore, phytochrome may have a role in the flowering time of *H. perforatum* plants. Many studies on the control of flowering by light quality focus on promotion of flowering by a decrease in R/FR, which in turn decreases the value of Pfr/P. In the current study, although R/FR was different among the three light quality treatments, the value of Pfr/P was the same; therefore the results of the current study cannot be explained by any flowering pathway known to date. Therefore we deduced that proportions of blue

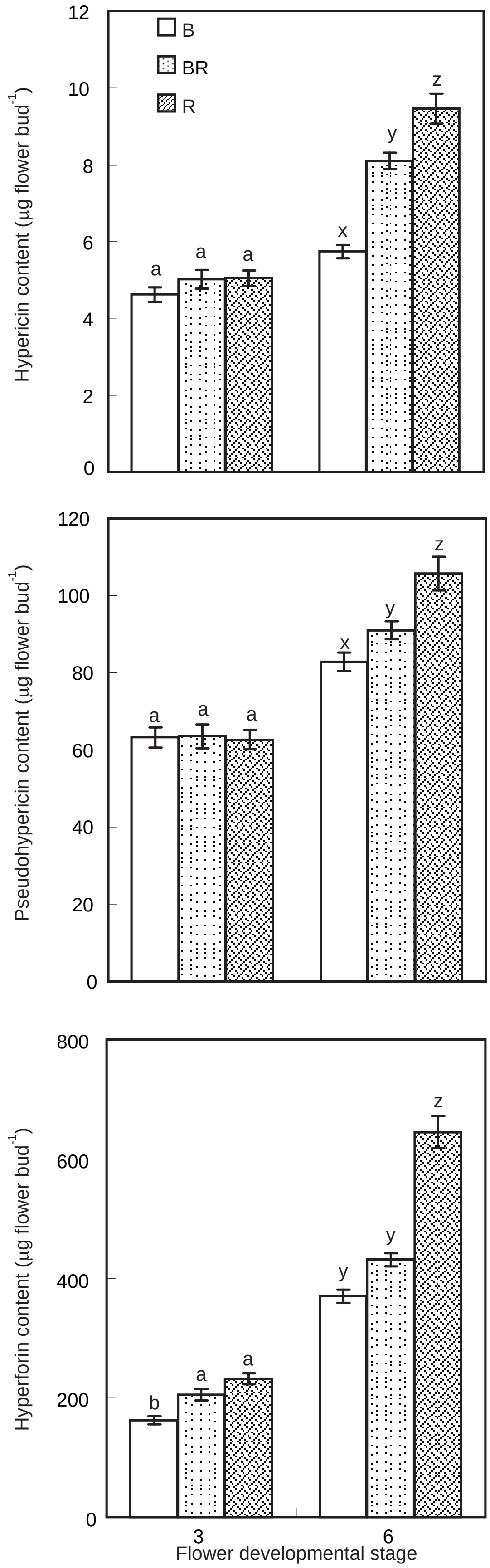

Fig. 6. The hypericin, pseudohypericin, and hyperforin contents of stage 3 and 6 flower buds of plants grown under different light conditions for 33 days (69 days after sowing). Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S.E. of 24-27 (stage 3) and 8-19 (stage 6) replicates, respectively.

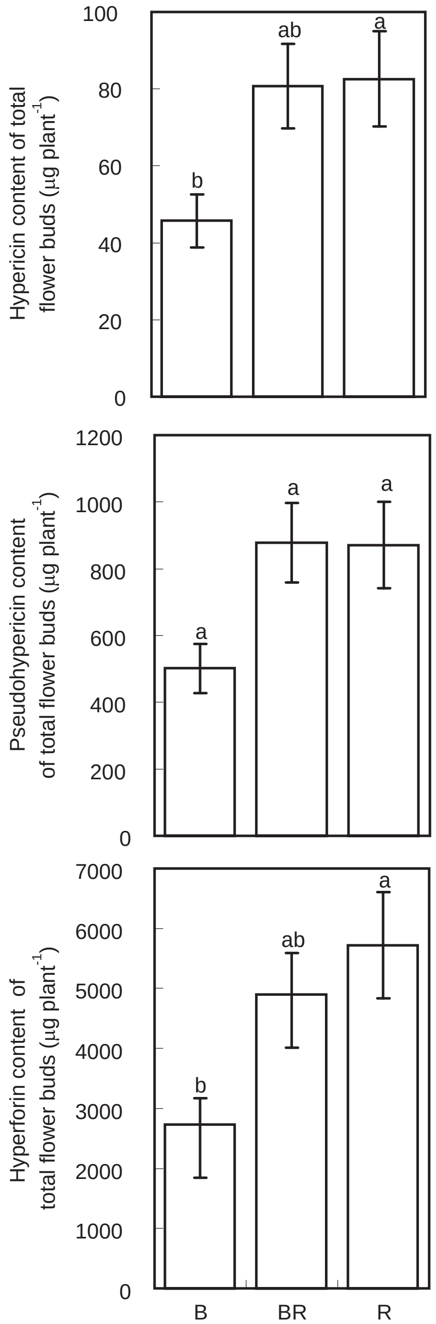

Fig. 7. The hypericin, pseudohypericin, and hyperforin contents of total flower buds of plants grown under different light conditions for 33 days (69 days after sowing). The contents of total flower buds were calculated by multiplying the medicinal content of each flower bud in BR treatment by the number of flower buds per plant in each treatment. Plants without flower buds were not included in the calculation. Different letters indicate significant differences between the treatments at $P < 0.05$, determined by the Tukey-Kramer test. Each bar represents mean ± S.E. of 29-31 replicates.

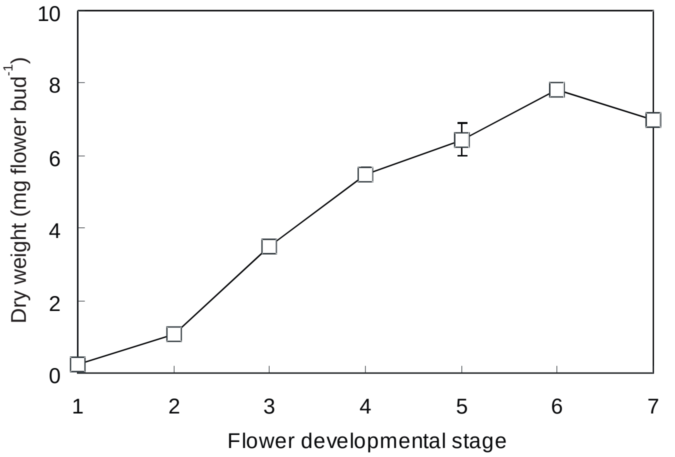

Fig. 8. Dry weight of flower buds of plants grown in BR treatment for 33 days (69 days after sowing). Error bars represents ± S.E. of 13–27 replicates.

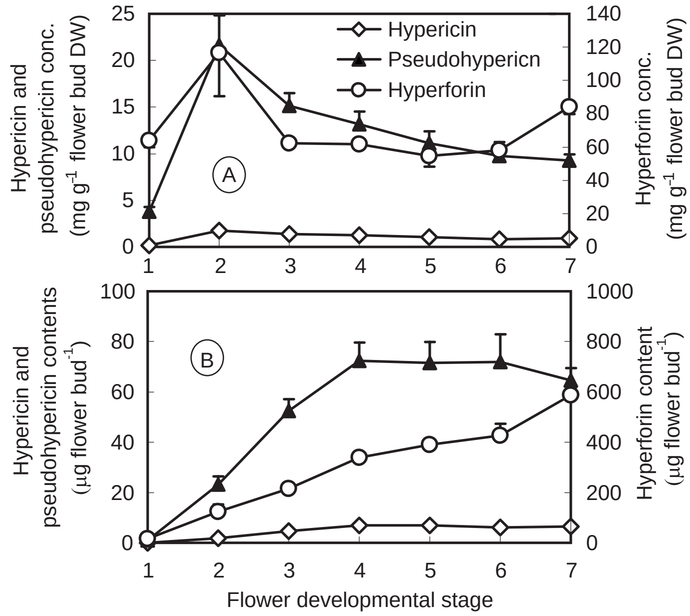

Fig. 9. Hypericin, pseudohypericin, and hyperforin concentrations (A) and contents (B) in the flower buds of plants grown in BR treatment for 33 days (69 days after sowing). Each bar represents mean ± S.E. of 7-13 replicates.

or red light to PPF and blue/red ratio (B/R) may affect several points in the flowering pathway.

The hypericin, pseudohypericin and hyperforin concentrations of *H. perforatum* grown in a greenhouse were previously reported to be highest in yellow flower buds about 3-4 mm (flower developmental stage 2) with a subsequent decline (Murch *et al.*, 2002). Our findings (Fig. 9) were in agreement with the previous results. Hypericin and pseudohypericin accumulate in significant quantities in dark glands. Stamens have higher concentrations of hypericin and pseudohypericin than any other organ of *H. perforatum* plants and also contain a significant number of dark glands (Zobayed *et al.*, 2006). The stamen anthers are formed during floral stages 1 to 2. The concentrations of hypericin and pseudohypericin in stage 2 flower buds were relatively high because the dry weight of other floral organs than stamens may be smaller at stage 2 than at stages 3 to 7. Therefore, decreasing hypericin and pseudohypericin concentrations from stages 2 to 7 is attributable to the increase in dry weight of the flower organs other than the stamens through flower development.

The stamens of flower buds develop during stage 1 to 4, and then stop developing (Zobayed *et al.*, 2006). Accordingly, the hypericin and pseudohypericin contents of flower buds increased during stages 1 to 4, and then were saturated. In contrast, the hyperforin content of flower buds continued to increase throughout flower development. The mechanism of hyperforin synthesis is little known, and differs from that of hypericin and pseudohypericin. Hyperforin may not accumulate in the dark glands; and hyperforin content of flower buds does not appear to be associated with stamen development.

Although the concentration of medicinal components in stage 6 flowers were almost the same under different light quality treatments; at stage 3, the hypericin and pseudohypericin concentrations were higher in B treatment, while the hyperforin concentration was higher in R treatment. The dry weight of stage 3 flower buds was higher in R treatment than in B treatment. The amount of anther tissue which accumulated hypericin and pseudohypericin in significant quantities may be relatively low, and the amount of any other floral organs which accumulated hyperforin may be relatively high. Calculating the medicinal contents in flower buds (g/flower bud) by multiplying the concentrations of medicinal components by flower bud dry weight, we demonstrated that the medicinal contents were highest in R treatment compared to those in B and BR treatments (Fig. 6).

The hypericin, pseudohypericin, and hyperforin contents of total flower buds of plants 69 days after sowing were higher in R treatment than those in B and BR treatments. Therefore, red light promoted the development of flower buds and produced flower buds with a higher content of secondary metabolites compared to blue light under constant Pfr/P values. We also conclude that spectral quality of light is an important factor in controlling the flowering of *H. perforatum* plants.

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