Adventitious shoot regeneration from *Begonia × erythrophylla* petiole sections is developmentally sensitive to light quality

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The influence of light quality on organogenesis in vitro was investigated using *Begonia × erythrophylla* petiole explants. Pre-treatment of in vitro donor plants by growth in the dark or under far-red or blue light reduced their competence for shoot formation when compared with those grown under red or white light. Culture of competent petiole explants under far-red, blue light or in the dark reduced the number of shoots produced per explant compared to those cultured under red or white light. Explants were found to be developmentally sensitive to both far-red and blue light, because meristem, but not primordia development was inhibited. In addition, blue light inhibition of shoot formation is not mediated directly through phytochrome, as few shoots formed on explants cultured under a mixture of red and blue light which resulted in a high \( P_{fr}/P_{tot} \) (0.82) and would allow shoot formation in the absence of blue light. Unlike the inhibitory influence of far-red light, which is reversible, exposure to blue light permanently reduces an explant's competence for shoot formation. Our results suggest that phytochrome and an independent blue light photoreceptor, possibly a cryptochrome, can regulate shoot production from *B. erythrophylla* petiole explants.

**Introduction**

Plants monitor both the magnitude (‘quantity’) and wavelength distribution (‘quality’) of their radiation environment via a number of photo-sensory pigments, called photoreceptors. These include the photoreversible phytochromes, blue light/UV-A absorbing cryptochromes and phototropins (Quail 2002), and UV-B receptors (Wade et al. 2001). These groups of photoreceptors co-operate to control many aspects of plant growth and development. Although a great deal of information is available regarding the influence of light quality on whole plants in situ, very little is known about the influence of light quality on plant regeneration and development in vitro. Both blue and red light have been implicated in the control of morphogenesis in vitro, with blue light promoting regeneration in some tissue culture systems (Weis and Jaffe 1969, Seibert *et al.* 1975) and red light in others (Kadkade and Seibert 1977, Kadkade and Jopson 1978). These results suggest that both phytochromes and cryptochromes could be important for controlling morphogenesis in vitro (Lercari *et al.* 1986).

Kadkade and Seibert (1977) showed that shoot formation from lettuce cotyledon explants was regulated by phytochrome, with the classic induction-reversion response observed. More recently, two studies have shown that red and blue light can interact to control development in vitro. Using tomato phytochrome mutants, Bertram and Lercari (2000) demonstrated that competence for shoot regeneration, from hypocotyl explants, is regulated by phytochrome B1 and that blue light, acting through a blue light receptor, is antagonistic. Muleo *et al.* (2001) showed that plum shoot proliferation involves two distinct processes, axillary bud

**Abbreviations** – B, blue; FR, far-red; MS, Murashige and Skoog; NAA, napthaleneacetic acid; \( P_{fr}/P_{tot} \), phytochrome photoequilibria (the amount of the active form of phytochrome over the total amount); R, red; SIM, shoot-inducing medium; W, white.
differentiation and axillary bud development. They suggested that red light regulates apical dominance via phytochrome, whereas blue light regulates the number of axillary buds via cryptochrome, and concluded that light quality influences bud differentiation and interacts with apical dominance.

Although begonias are readily multiplied by vegetative cuttings, they are often propagated using cell culture techniques because they are susceptible to pathogenic bacteria, fungi and nematodes (Takayama 1990). Begonias can be propagated via organogenesis, using leaf or petiole sections, in liquid or on solid medium (Peck and Cumming 1984, Simmonds and Werry 1987) and have the potential to be micropropagated via somatic embryogenesis (Sehgal 1975, Hvoslef-Eide and Sæbø 1991, Castillo and Smith 1997).

In addition to the composition of the culture medium, temperature and photoperiod (Heide 1965, Welander 1977), light quality can also influence the regenerative response of begonias in vitro. Hvoslef-Eide and Sæbø (1991) found that callus induction in Begonia × cheimantha occurred best under red light, whereas culture under white light or preculture in the dark, promoted organogenesis. Castillo and Smith (1997) found that direct somatic embryogenesis from leaf or petiole explants of Begonia gracilis was best under red light and that rhizogenesis occurred when explants were cultured under cool-white fluorescent light at the same intensity.

Although light quality is clearly important for begonia morphogenesis in vitro, little is known of the mechanisms through which light exerts its effects. In a study of organogenesis from Begonia × erythrophylla petiole explants, Burritt and Leung (1996) showed that shoot formation occurs directly from cells of epidermal origin, when petiole explants are cultured on a shoot-inducing medium (SIM) under cool-white fluorescent lamps. This system is especially useful for studying the influence of light quality on organogenesis, because the cells responding to the organogenic stimulus are directly exposed to light and are not buried deep within the explant or callus.

In this study we investigate whether light quality can influence organogenesis from begonia petiole sections and whether phytochrome is involved in controlling regenerative ability. Our findings demonstrate that cultured petiole sections of B. × erythrophylla respond to both red and blue light, and show temporal sensitivity to light quality.

Materials and methods

Plant material

Begonia × erythrophylla J. Neuman, also known as Begonia × Feistii Hort. ex L.H. Bailey, is a hybrid begonia (Begonia manicata × Begonia hydrocorylfolia), probably of garden origin. As the same trends were observed with different clones, all experiments presented in this study were carried out using explants taken from B. erythrophylla plants cloned from a single donor and maintained in vitro as described by Burritt and Leung (1996). Stock plants were grown on half strength Murashige and Skoog’s (MS) salts (Murashige and Skoog 1962), and maintained at 24°C, under continuous light (50 µmol m⁻² s⁻¹), provided by cool-white fluorescent lamps.

Culture procedures and conditions for organogenesis

Explants, 20 per 9 cm plastic tissue culture dish, were cultured on a SIM consisting of MS salts plus myo-inositol 0.55 mM, nicotinic acid 40.6 µM, pyridoxine HCl 2.43 µM, thiamine HCl 1.48 µM, glycine 26.7 µM, folic acid 1.13 µM, biotin 0.2 µM, 0.54 µM naphthaleneacetic acid (NAA), 4.44 µM benzyladenine, 3% sucrose and 0.7% agar (Plant cell culture tested; Sigma, St. Louis, MO, USA) (Burritt and Leung 1996). The medium was adjusted to pH 5.8 prior to autoclaving for 15 min at 121°C. Counting from the shoot apex, petioles from the fourth, fifth and sixth expanded leaves were excised, cut into 5-mm sections and cultured vertically, in 9-cm plastic culture dishes, with the basipetal surface in contact with the medium. The dishes were randomized in the culture chambers every 24-48 h.

 Cultures were maintained at 24°C, in the dark (D) or under continuous white (W) (Philips TLD 36 W/33 Philips Lighting Co., Eindhoven, the Netherlands), red (R) (Philips TLD 36 W/15) or blue (B) light (TLD 36 W/18 in combination with a 118 Light Blue Lee filter), at approximately 35 µmol m⁻² s⁻¹ PAR unless otherwise stated. Far-red light (FR) (5 µmol m⁻² s⁻¹) was obtained from Philips 100 W incandescent lamps filtered through KODAK WRATTEN gelatin filters no. 89B (Eastman Kodak Company, Rochester, NY, USA). For the R plus B treatment, cultures were maintained under a 50:50 mixture of R and B giving a total PAR of approximately 35 µmol m⁻² s⁻¹. Spectral outputs from the different light sources were measured with an Optronics OL754 spectroradiometer (Optronics Laboratories Inc., Orlando, FL, USA) fitted with an OL IS-670 6’ integrating sphere and OS-754-0-PMT photomultiplier tube (200-800) and calibrated using an OL 752-10E calibration standard, and are shown in Fig.1. For dose-response experiments, and to regulate the Pfr/Ptot ratios, light intensities were controlled by varying the number of lamps, the distance of the cultures from the lamps and/or by using neutral density filters. The estimated phytochrome photoequilibria (Pfr/Ptot, the amount of the active form of phytochrome over the total amount) under each light source, were calculated from the photoconversion cross-sections as given by Mancinelli (1988), and are shown in Table1.

Pre-treatment experiments

For the light pretreatment experiments, plants were grown in vitro under W, R, B, FR light or in the dark for 14 days, on half-strength MS salts or half strength MS salts plus 3% sucrose, prior to explant excision. Explants were cultured on SIM and were maintained at 24°C under continuous white light.
For the R/FR transfer, explants were cultured on SIM, at 24°C, under continuous R or FR light for 0, 1, 3, 5, 7, 10, 14 or 21 days and then transferred to FR or R light, respectively. The explants were cultured for a total of 35 days to allow the development of all shoot primordia. The same regime was used for the R/B transfer experiments except that FR was replaced with B light.

Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a Pulse Amplitude Modulation (PAM-2000) portable chlorophyll fluorometer (Walz, Effeltrich, Germany). The plants were dark-adapted, at ambient temperature for 30 min. To quantify the efficiency of photon capture by open photosystem II (PSII) reaction centres, the leaf surfaces were exposed to a saturating pulse of light for determination of the ratio of variable ($F_v$) to maximal fluorescence ($F_m$).

Histological procedures

The explants were fixed, dehydrated and infiltrated with LR White acrylic resin (London Resin Co., Berkshire, UK) as detailed in Burritt and Leung (1996). Briefly, explants were cut into 1–2 mm sections and fixed at room temperature for 3–4 h under vacuum in 2.5% glutaraldehyde, in a 0.075 M sodium phosphate buffer (pH 7.2). Tissues were dehydrated for an ethanol series (10–100%) and then infiltrated with increasing concentrations of LR White acrylic resin. Polymerization of the resin was carried out at 4°C using the LR White cold-curing procedure. Transverse sections (2–4 µm) were cut and stained with Methylene blue-azure A (Warmke and Lee 1976).

Determination of shoot formation and statistical methods

The mean number of shoots and the percentage of explants forming shoots were determined for each plate of explants with the aid of a stereomicroscope. Each plate was considered an experimental unit for statistical analysis. All statistical tests were performed using SPSS 10 for the Macintosh. A one-way analysis of variance was performed and Tukey's test used to determine significant differences between the means.

Table 1. Photoequilibria ($P_{fr}/P_{tot}$) for the different light treatments.

<table>
<thead>
<tr>
<th>Light treatments</th>
<th>$P_{fr}/P_{tot}$</th>
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<tbody>
<tr>
<td>Red</td>
<td>0.86</td>
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<tr>
<td>Red + blue</td>
<td>0.82</td>
</tr>
<tr>
<td>White</td>
<td>0.76</td>
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<tr>
<td>Far-red + red</td>
<td>0.43</td>
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<tr>
<td>Blue</td>
<td>0.42</td>
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<tr>
<td>Far-red</td>
<td>0.05</td>
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<tr>
<td>Dark</td>
<td>0.00</td>
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Results and discussion

Pre-treatment of donor plants with different light qualities

In numerous studies, the condition of the donor plant has been shown to be critical for a maximal regenerative response (Santos and Torne 1986, Hess and Carman 1998, Padua et al. 1998, Dahleen 1999, Guo et al. 1999). Pre-treatment by culture in the dark resulted in rapid etiolation and a significant reduction in photosynthetic efficiency of $B. erythrophylla$ donor plants, as measured by chlorophyll fluorescence (Fig. 2). A similar reduction in photosynthetic efficiency was observed for donor plants cultured under FR light (Fig. 2). Pre-treatment under W, B or R light resulted in no significant decline in photosynthetic efficiency (Fig. 2). When the influence of pretreatment on competence for organogenesis was determined, culture in the dark or under FR light greatly reduced the number of shoots produced per explant and significantly reduced the percentage of explants producing shoots. Many explants taken from donor plants grown in the dark or under FR light rapidly produced polyphenols and died during the early stages of culture, resulting in fewer petioles being available to respond to the SIM. Pre-treatment under R light resulted in no significant difference in the number of shoots, or the percentage of explants producing shoots in comparison with W light (Fig. 3A and B). Pre-treatment with B light resulted in a significant decline in the number of shoots produced per explant, but only a small reduction in the percentage of explants producing shoots, with most explants remaining viable. Therefore, the B light pretreatment did not appear to influence explant survival or the photosynthetic efficiency of the donor plants. As the fluences of the W, R and B light pretreatments were all similar, it is unlikely that fluence rate had any effect on the number of shoots produced per explant. Bertram and Lercari (2000) found that exposure of wild-type tomato seedlings to R, FR or W light, prior to culture, produced hypocotyls competent for shoot regeneration, whereas exposure to B light reduced their
competence. With the exception of the FR pretreatment, our results with B. erythrophylla show a similar trend. R or W light promoted competence and B light, although not completely eliminating competence, reduced the ability of explants to produce shoots. Bertram and Lercari (2000) used 8-day-old tomato seedlings in their study, whereas in this study mature B. erythrophylla plants were used. Although the period of preculture under FR light was short (14 days), it was sufficient to reduce the photosynthetic capability of these plants and reduce the survival of explants in culture. This most likely caused the poor shoot production observed. In addition, begonia are considered to be shade-plants and are physiologically adapted for growth in an environment with a low R:FR ratio. In contrast, tomato plants are sun-plants whose seedlings respond to a low R:FR ratio by increasing their rate of elongation, therefore species-related differences could also account for the different results obtained.

No significant differences in photosynthetic efficiency or competence for organogenesis were observed between plants grown on half strength MS salts or half strength MS salts plus 3% sucrose, following pretreatment with W, R or B light (data not shown). Plants cultured on half strength MS salts plus 3% sucrose and pretreated with FR light or grown in the dark had slightly higher Fv/Fm values, 0.61 and 0.55, respectively, than those grown in the absence of sucrose. However, no significant difference in competence for organogenesis was observed (data not shown). These results indicate that the addition of sucrose to the culture medium cannot replace the requirement for R or W light for maximal explant competence.

The influence of light quality during culture
Although R or W light was required for the production of highly competent explants, light was not an absolute requirement for the formation of shoot primordia when explants were cultured on SIM. The explants formed shoots in the absence of light, but fewer shoots were produced in comparison with explants cultured under continuous W or R light (Fig. 4A).

Culture under W or R light resulted in no significant difference in the number of shoots or the percentage of explants producing shoots, with a mean of more than 25 shoots produced per explant for both treatments, respectively (Fig. 4A and B). The FR and B treatments resulted in the lowest numbers of shoots (Fig. 4A), with explants producing small amounts of callus from their basal cut surfaces.

Similar results can be found in the literature, with R light being required for the formation of shoot primordia (Kadkade and Seibert 1977) and B light inhibiting shoot formation (Bertram and Lercari 2000). In addition, B light has been shown to inhibit somatic embryogenesis in quince (D’Onofrio et al. 1998) and the proliferation of embryogenic tissue of Norway spruce (Latkowska et al. 2000).

Light quality not only influences the number of begonia shoot primordia produced, but also influences primordia expansion. Explants cultured under continuous R light produced numerous shoot primordia that developed and expanded slowly, whereas those cultured under W light developed and expanded more rapidly. Histological examination of 18-day-old explants confirmed this, with primordia formed under W light expanding more
rapidly than those under R light (Fig. 5A and B). Examination of 18-day-old explants cultured under B light revealed mostly random cell divisions (Fig. 5C) with few primordia. Interestingly, the primordia produced under continuous B light expanded rapidly, suggesting that the small amount of B light in the W light treatment (approximately 3 μmol m⁻² s⁻¹ between 425 and 475 nm) although not sufficient to inhibit primordia formation, is important for primordia expansion. Light is an important regulator of leaf expansion, but the influence of light quality on expansion has been shown to be species-dependent. In chrysanthemum and *Azorina vidali* (Mortensen and Strømme 1987, Moreira da Silva and Debergh 1997) B light reduces leaf expansion and hence leaf area, whereas in *Betula pendula* B light enhances it (Søbø et al. 1995).

The influence of the phytochrome equilibrium state on shoot regeneration

The results discussed so far demonstrate that organogenesis from begonia petiole explants is sensitive to both R and B light, suggesting the involvement of a phytochrome and possibly a B-light-absorbing cryptochrome.
To determine whether the phytochrome photoequilibrium state is important for shoot initiation, explants were cultured under different combinations of lighting, providing different photoequilibrium states (Table 1). The highest level of organogenesis was observed in the R and W light treatments (Fig. 4A and B), both of which had $P_{fr}/P_{tot}$ greater than 0.75, with the number of shoots per explant falling as $P_{fr}/P_{tot}$ was reduced by the addition of FR light (Fig. 4A and B). This suggests that the presence of activated phytochrome is important for the initiation of shoot meristems and that the more active phytochrome present, the better the response. When explants were exposed to a mixture of R and B light, despite the photoequilibrium being almost the same as under R light alone ($P_{fr}/P_{tot}$: 0.82), the number of shoots produced was significantly less than that of explants cultured under R or W light. However, the percentage of explants producing shoots remained similar to that of explants cultured under R or W light. This suggests that B light is exerting a negative effect on meristem initiation and/or development and that this is independent of the $P_{fr}/P_{tot}$.

As mentioned, B light has a negative effect on somatic embryogenesis in quince (D’Onofrio et al. 1998). When quince explants were cultured under R plus B light, resulting in a high $P_{fr}/P_{tot}$ (0.84), the inhibition caused by B light was attenuated although not completely overcome (Bellocci et al. 2001). In contrast, although the percentage of begonia petiole explants producing shoots under R plus B light increased in comparison with the B light treatment, the mean number of shoots produced per explant was similar to that of explants cultured under continuous B light, in which the percentage of explants producing shoots was significantly reduced. These results suggest that in begonia a high $P_{fr}/P_{tot}$ results in limited attenuation of B light inhibition. Histological examination of explants cultured under R plus B light for 18 days showed many small meristems (Fig. 5D), but as very few shoots were observed these meristems clearly failed to develop. As few meristems were observed under B light alone, the presence of meristems under R plus B light suggests that R light and a high $P_{fr}/P_{tot}$ are required for meristem initiation. An interaction between the positive influence of R light and the negative influence of B light could regulate subsequent meristem and shoot primordia development. Muleo et al. (2001) investigated shoot proliferation in plum and demonstrated that light quality was an important regulator of bud differentiation and interacted with apical dominance.

Dose dependence of promotion and inhibition

Organogenesis showed no dose dependence at R light levels above 5 $\mu$mol m$^{-2}$ s$^{-1}$, with doses of 9–35 $\mu$mol m$^{-2}$ s$^{-1}$ being equally effective in promoting organogenesis (Fig. 6A). In contrast, the inhibitory effect of B light was dose dependent. As the levels of B light were increased, the number of shoots produced per explant decreased significantly, demonstrating a dose-dependent inhibition (Fig. 6B). No dose-dependent influence of W light was observed at fluences between 5 and 35 $\mu$mol m$^{-2}$ s$^{-1}$ (data not shown). These results and those presented above suggest that although both R and B light interact to regulate shoot formation, B light inhibition might involve a developmentally sensitive mechanism independent of $P_{fr}/P_{tot}$.

Stage specificity of light-dependent promotion or inhibition

To test the influence of R and B light at different developmental stages, transfer experiments similar to those detailed by Christianson and Warnick (1983, 1988) were carried out, except that instead of transfer from a permissive to a non-permissive medium, explants were transferred from one light treatment to another.
Inhibition of organogenesis by FR light was stage specific. When explants were transferred from R to FR light, inhibition only occurred in explants exposed to R light for less than 10 days. In the reciprocal transfer experiment (FR to R), no reduction in shoot number was observed until explants were exposed to FR light for more than 10 days (Fig. 7A). It is during the first 10–14 days of culture that most meristems are initiated and become determined for organogenesis (Burritt and Leung 1996). These results suggest that active phytochrome plays a role in promoting the initiation of adventitious meristems and that although FR light can inhibit the initiation of meristems, it does not appear to inhibit their development once formed. In addition, FR light does not significantly influence the competence of excised explants for organogenesis until they have been in culture for 10 days or more. Culture on a basal medium without hormones resulted in a similar loss of competence in begonia petiole explants, but after only 3 days in culture (Burritt and Leung 1996).

Explants exposed to R light for 14 days and then transferred to B light produced similar numbers of shoots to those cultured under continuous R light. As most shoot primordia are initiated in the first 14 days of culture (Burritt and Leung 1996), B light does not appear to inhibit primordia development, but rather inhibits some aspect of meristem formation. The opposite trend was observed when explants were cultured under B light prior to transfer to R (Fig. 7B). Even a brief exposure to B light caused a significant decline in the number of shoots produced and the subsequent ability of explants to form shoots. However, unlike the effects of FR light, which could be reversed within the first 14 days of culture, a short exposure to B light reduces the competence of explants for meristem initiation under permissive conditions. This reduction in organogenesis is similar to that observed when donor plants are precultured under blue light before culture of explants under permissive conditions. It would appear that in *B. erythrophylla* the mechanism by which B light inhibits organogenesis is more complex than a simple change in $P_{f1}/P_{tot}$ as a reduction in explant competence is involved. It is possible that in *B. erythrophylla*, R and B light do not directly interact to influence organogenesis, but instead regulate different processes or genes involved in organogenesis, or the same processes or genes via signal-transduction pathways with different components.

In a recent study on *Pharbitis nil*, Yoshida et al. (2002) demonstrated that R and B light both regulate expression of the S-adenosylmethionine decarboxylase gene (*SAMDC*) via calcium and calmodulin. However, despite calcium and calmodulin being involved in both R and B light responses, treatment of seedlings with the protein phosphatase inhibitor okadaic acid only inhibited the R light up-regulation of *SAMDC* expression. These results indicate possible differences in the components of the R and B light signal transduction chains regulating the expression of *SAMDC* (Yoshida et al. 2002).

**Concluding remarks**

To our knowledge, this is the first study to demonstrate a temporal sensitivity to light quality with respects to direct organogenesis in vitro. Although a more detailed study of the roles of phytochrome and cryptochrome in begonia organogenesis is required before further conclusions can be drawn, our results suggest that R light plays a role in meristem initiation and that B light is antagonistic to meristem formation, but is important for primordia development.

Potentially the ability to perturb organogenesis by manipulating light quality could be a useful tool to study the process in vitro. Development could be manipulated without the added complication of changing the composition of the culture medium, simply by moving explants from one light environment to another. In addition, manipulation of the light environment might be a useful tool for the micropropagation of plants using tissue culture technology. For example, meristem
formation could be suppressed to allow the development of existing shoot primordia without the expense of moving explants from one culture medium to another.

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