Plant Growth Regulation (2005) 45: 53–61
DOI: 10.1007/s10725-004-5971-z

Light quality influences the polyamine content of lettuce (*Lactuca sativa* L.) cotyledon explants during shoot production *in vitro*

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Received 10 September 2004; accepted in revised form 9 November 2004

Key words: Lettuce, *Lactuca sativa*, Light quality, Polyamines, Organogenesis

Abstract

Light quality has previously been shown to influence morphogenesis in lettuce cotyledon explants, with white or red light promoting adventitious shoot production, and blue light inhibiting it. Endogenous polyamine (PA) concentrations were compared between explants cultured under different light qualities. Explants cultured under white or red light accumulated PAs during shoot primordia production, with a 5.6-fold increase compared to initial concentrations under white light, and 6.7-fold increase under red light. These results suggest polyamines are involved in the formation of shoot primordia. After 18 days in culture PA concentrations decreased under white light, and to a lesser extent under red light, signaling a shift in polyamine metabolism that correlates with shoot expansion, which occurs more readily under white light. Explants cultured under blue light accumulated polyamines for the first 7 days, to a level 1.3 times greater than initial values, followed by a gradual decline during the remainder of the culture period. Explants cultured under blue light also contained a greater proportion of PCA-insoluble conjugated PAs, compared to explants under white or red light, which contained greater proportions of free or PCA-soluble conjugated polyamines. The ratio of putrescine to spermidine was also different with a lower Put:Spd ratio being associated with shoot production under white or red light, and higher Put:Spd ratio being associated with culture under blue light.

Introduction

Polyamines (PAs) are small aliphatic amines, which are regarded as essential cellular components because they are found in all living organisms (Martin-Tanguy 2001), and mutant plants with a reduced ability to synthesize PAs do not develop normally (Galston and Kaur-Sawhney 1995). The three polyamines commonly found in higher plants are putrescine (Put), spermidine (Spd), and spermine (Spm), all of which can exist in free, conjugated (PCA-soluble conjugated) or bound (PCA-insoluble conjugated) forms (Li and Burritt 2003). PAs can either be conjugated to small molecules, such as hydroxycinnamic acids, or bound to larger molecules such as nucleic acids, and it is thought that interconversion between free, and PCA-soluble conjugated or PCA-insoluble conjugated forms, regulates PA concentrations during developmental processes (Li and Burritt 2003).

The biosynthesis of polyamines has long been understood (see Tiburcio et al. 1997), but the direct influence of PAs on plant growth and differentia-

tion has not yet been elucidated. Development of metabolic inhibitors to PA biosynthesis, and molecular techniques facilitating the regulation of PA biosynthetic enzymes have demonstrated that plants require PAs for normal growth and development (Kumar et al. 1997), and indicate that PAs are involved in numerous physiological events. These include cell division (Mad Arif et al. 1994), embryogenesis, morphogenesis (Rafart Pedros et al. 1999), flower development (Song et al. 2001), seed and fruit development (Thu-Hang et al. 2002), and senescence (Marco and Carrasco 2002). In addition, concentrations of endogenous PAs can be altered through the manipulation of environmental factors (Zapata et al. 2003), or the application of plant growth regulators (Cho, 1983), and it has also been suggested that PAs confer stress tolerance (Bouchereau et al. 1999).

Differentiation of plant tissue, in culture, has previously been associated with elevated concentrations of PAs. For example, addition of Spd to the culture medium induced a greater endogenous concentration of Spd in *Panax ginseng* callus tissue, which correlated also with a greater production of somatic embryos (Monteiro et al. 2002). Melon cotyledon sections cultured under shoot inducing conditions accumulated Put during organogenesis, but under root inducing conditions an accumulation of Spd occurred (Lesham et al. 1991). Such results indicate that the absolute concentration of endogenous PAs might influence the degree to which differentiation and morphogenesis occurs *in vitro*, but also suggest that concentrations of individual PAs or the ratio between individual PAs influences the outcome of the differentiation process.

Lettuce is an important crop plant worldwide, being grown on most continents (Michelmore and Eash 1985). In New Zealand, the trade of lettuce annually contributes $40.1 million to the economy, as well as providing a valuable source of potassium, vitamin A, and vitamin C (Kerr et al. 2003). However, lettuce is susceptible to many diseases, pathogens, pests, and post harvest disorders (Pink and Keane 1993), and the effects of these might be reduced using novel genetic techniques. Such improvements would require the reliable regeneration of large numbers of plants utilizing tissue culture methods. A culture medium from which maximal adventitious shoot numbers were obtained from cotyledon explants, taken from a range of lettuce genotypes, has previously been described (Hunter and Burritt 2002). Further to this, it has been demonstrated that the response of lettuce cotyledon explants can be manipulated by the application of different light qualities throughout the culture period (Hunter and Burritt 2004).

Light quality has previously been shown to influence the activity of polyamine biosynthetic enzymes, with red light promoting the activity of arginine decarboxylase (ADC) in pea buds (Dai and Galston 1981), and *S*-adenosylmethionine decarboxylase (SAMDC) in *Pharbitis nil* seedlings (Hirasawa and Shimada 1994). In both instances, the increase in enzyme activity was reversed with the application of far-red light, implicating phytochrome in the regulation of PA biosynthetic enzymes (Dai and Galston 1981; Yoshida et al. 2002). Additionally, blue light also promoted SAMDC activity in *Pharbitis nil*, although this was not reversible with the application of far-red light, indicating the involvement of a separate blue-light receptor (Yoshida et al. 2002).

The purpose of this study was to examine the influence light quality has on the endogenous PA concentration of lettuce cotyledon explants, and to determine whether altered concentrations of PAs might explain differences in the number of adventitious shoots that form under various light qualities.

Materials and methods

Plant material and culture conditions

Seed from the lettuce genotype Greenway was purchased from commercial sources. Seeds were surface sterilized by dipping in 90% ethanol for 30 s, followed by soaking in a 1% sodium hypochlorite solution containing 2 drops of Tween 20 per litre, for 15 min, and then rinsing three times in sterile distilled water.

Seeds were germinated on half-strength MS salts (Murashige and Skoog 1962) solidified with 8 g l^{-1} agar, and cultured at 24 °C under continuous white light (Thorn 2636w840), at approximately 35 μmol m^{-2} s^{-1} PAR. Cotyledons were excised from seedlings 3 days after sterilization, cut in half latitudinally and placed on shoot inducing medium containing full strength MS salts and vitamins (Murashige and Skoog 1962), supplemented with

30 g l^{-1} sucrose, 8 g l^{-1} agar, 0.44 μM benzylaminopurine, and 0.54 μM naphthalene acetic acid (Hunter and Burritt 2002). The pH of the medium was adjusted to 5.8 prior to autoclaving. Ten explants were placed on each culture dish, and cultured at 24 °C under continuous white, red (Phillips TLD 36W/15) or blue (TLD 36W/18) light for 0, 4, 7, 11, 14, or 18 days. Explants were then gently blotted with tissue to remove residual medium, and frozen in liquid nitrogen, followed by storage at −80 °C. For a full description of lighting conditions, and response of explants to light quality, see Hunter and Burritt (2004).

Polyamine extraction

Polyamines were extracted using the method of Smith (1991) with minor modifications (Li and Burritt 2003). Pooled explant tissue (0.1 g) was frozen in liquid nitrogen and ground to a fine powder using a chilled mortar and pestle. The frozen powder was homogenized in cold 5% perchloric acid (PCA) (0.1 g tissue/ml PCA), and the homogenates maintained at 4 °C for 60 min, then centrifuged for 20 min at 25,000 RCF. The supernatant was removed and used for the determination of free polyamines and PCA-soluble conjugated polyamines. The pellets were washed three times with PCA (1 ml) and resuspended in 0.3 ml of PCA by vortexing, providing a suspension for the determination of PCA-insoluble conjugated polyamines. The pellet suspensions and 0.2 ml aliquots of the original supernatants were hydrolyzed overnight with 0.3 ml of 12 M hydrochloric acid, at 100 °C. The hydrolyzates were centrifuged for 20 min at 25,000 RCF and 0.1 ml aliquots of the supernatants were dried *in vacuo*, then dissolved in 0.1 ml of PCA. The above extraction procedure was repeated three times with each extraction considered a replicate.

Dansylation of polyamines and TLC analysis

The extracts were dansylated as described by Smith (1991) with minor modifications. A 0.1 ml aliquot of extract was added to 0.1 ml of saturated sodium carbonate and 0.2 ml dansyl chloride dissolved in acetone (7.5 mg/ml). The mixture was incubated at 60 °C for 30 min in the dark. Excess dansyl chloride was eliminated with the addition of 0.1 ml of proline (0.1 g/ml), and incubation at room temperature for 15 min in the dark. Dansylated polyamines were extracted with 0.25 ml of benzene by vortexing for 1 min and centrifuging for 1 min at 25,000 RCF. The benzene phase was removed and the polyamines analyzed by thin layer chromatography using the method of Torrigiani et al. (1987). Pre-coated plates of Silica gel 60 (Merck) were used and run with ethylacetate:cyclohexane (2:3 v/v) as the eluent. Spots were visualized under UV radiation and those corresponding to Put, Spd, and Spm identified by comparison with dansylated standards. The spots were scraped from the TLC plate, eluted with ethylacetate, and their relative fluorescence measured using a spectroflurometer (Jasco FP 750, Tokyo, Japan). The concentration of PCA-soluble conjugated polyamines was estimated as the concentration of the polyamines in the hydrolyzate of the original supernatant, less that of the free polyamines. Corrections were made for losses during the extraction, dansylation and TLC analysis. Losses were determined by comparison with extracts to which known concentrations of polyamines had been added prior to processing. Recovery of individual polyamines was 90% or greater.

Statistical analysis

Statistical analysis was performed using SigmaStat 2.0. Differences between the means were detected using a one-way analysis of variance, in conjunction with the Tukey test.

Results

Influence of light quality on shoot production

Explants excised 3 days after sterilization and cultured under continuous white or red light for 28 days produced means of 26 and 31 shoots, respectively. There is no significant difference between the numbers of shoots produced under white or red light ($p < 0.01$). When cultured under blue light, explants produced significantly fewer shoots ($P < 0.01$) than explants cultured under white or red light, with a mean of 14 shoots being produced per explant.

Influence of light quality on total polyamine concentrations

Explants cultured under blue light accumulated much lower concentrations of PAs, relative to explants cultured under white or red light, and by day 18 explants cultured under blue light contained lower total PA concentrations than explants immediately following excision (Table 1). A large increase in total PAs was observed in explants cultured under white or red light, with concentrations approximately doubling within the first four days of culture, followed by a steady accumulation until day 14, after which concentrations declined. At all time points, explants cultured under red light contained significantly greater total PA concentrations than explants cultured under white light.

Influence of light quality on the concentrations of individual polyamines

As reflected in the values of total PAs, explants cultured under white or red light contained significantly more free, PCA-soluble conjugated and total Put, Spd and Spm than explants cultured under blue light; with the exception of PCA-soluble conjugated Spm (Figures 1, 2, and 3). The concentrations of PCA-insoluble conjugated Put, Spd, and Spm remained low in explants cultured under all light qualities. There was no significant difference in the concentration of PCA-insoluble conjugated Put when explants cultured under white, red or blue light for 4 or 7 days when compared (Figure 1). Following day 11, explants cultured under red light contained significantly more PCA-insoluble conjugated Put than explants cultured under white or blue light. At day 4, significantly greater concentrations of PCA-insoluble conjugated Spd and Spm were detected in explants under blue light, compared to red light. However, over the remaining time course explants cultured under white, or red light accumulated significantly greater concentrations of PCA-insoluble conjugated Spd and Spm, compared to explants cultured under blue light (Figures 2 and 3). A significant difference in the ratio between Put and Spd was detected, with a lower Put:Spd ratio occurring in explants cultured under red and white light, compared to explants under blue light ($p < 0.01$).

Discussion

Polyamines have previously been shown to be involved in morphogenic processes (Rafart Pedros et al. 1999), and changes in endogenous PAs have been associated with both somatic embryogenesis (Monteiro et al. 2002) and organogenesis (Lesham et al. 1991). For example, *Euphorbia esula* explants readily produce roots and shoots in culture, but when treated with canaline and canavanine, inhibitors of PA biosynthesis, a decrease in organogenesis corresponded with a marked decrease in Put and Spd concentrations (Davis 1997). In lettuce, light quality influences the degree to which cotyledon explants undergo differentiation and morphogenesis (Hunter and Burritt 2004), and this is the first study to investigate changes in the concentrations of endogenous PAs during culture under light qualities that promote or inhibit shoot production.

Adventitious shoots have previously been shown to appear at the surface of lettuce cotyledons approximately 7 days after excision from the seedling (Hunter and Burritt 2004), when explants are cultured under white or red light. After

Table 1. Changes in the total polyamine content (Put + Spd + Spm; nmol g^{-1} FW) of lettuce cotyledon explants on shoot inducing medium, cultured under white, red or blue light.

	Light Quality		
	White	Red	Blue
Day 0	615 ± 16	615 ± 16	615 ± 16
Day 4	1141 ± 41	1324 ± 17	799 ± 2
Day 7	2353 ± 59	2788 ± 24	820 ± 17
Day 11	3423 ± 82	4119 ± 19	809 ± 12
Day 14	3470 ± 56	4128 ± 70	585 ± 19
Day 18	2183 ± 72	3624 ± 24	384 ± 3

From day 4 onwards, differences between all light qualities were significant ($p < 0.01$), $n = 3$, mean ± SE.

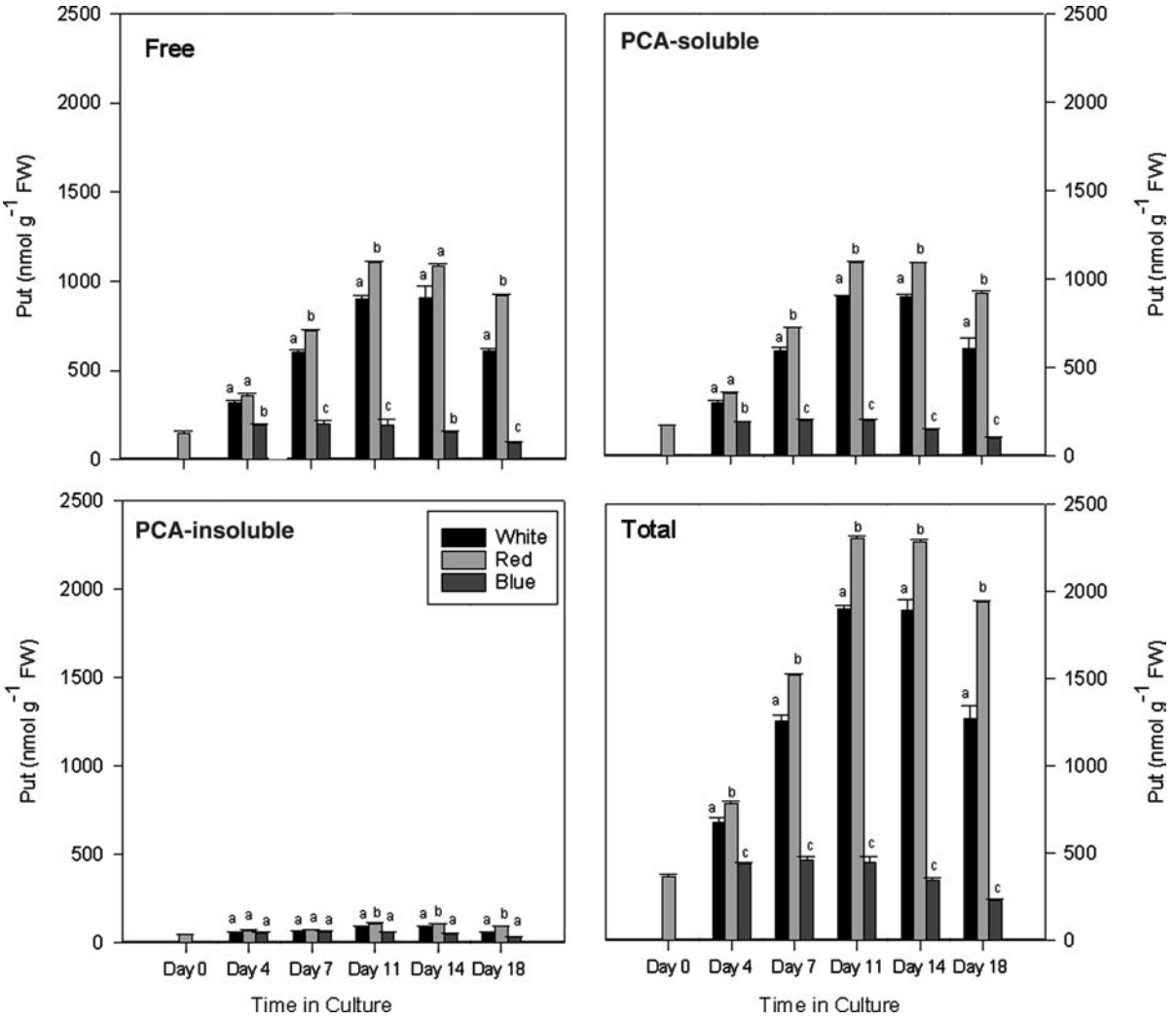

Figure 1. Changes in the endogenous concentration of Putrescine in lettuce cotyledon explants during 18 days of culture under white, red, or blue light. Different letters at each time point indicate significance ($p < 0.01$), $n = 3$, vertical bars are standard error of the mean.

11 days, greater numbers of shoots are observed on explants cultured under white or red light, and shoot expansion begins in explants cultured under white light. These morphogenic processes correspond with the maximal values observed for Put, Spd, and Spm, and for total PAs found in cotyledon explants. Explants cultured under blue light produce few shoots (Hunter and Burritt 2004), and contain relatively low and constant concentrations of Put, Spd, Spm, and total PAs. These results suggest that PA accumulation is induced during the early stages of culture and is required for successful organogenesis, and/or that the process of organogenesis promotes PA accumulation as a result of the rapid increase in cell division and cellular activity.

An accumulation of Put, Spd, and Spm following exposure to red light has previously been reported in pea seedlings (Goren et al. 1982) and *Pharbitis nil* (Yoshida and Hirasawa 1998). In lettuce cotyledon explants, exposure to red light was also associated with a significant increase in total PA concentrations after only 4 days in culture, when compared to white light. However, the concentration of total PAs in explants cultured under white light also increased during the culture

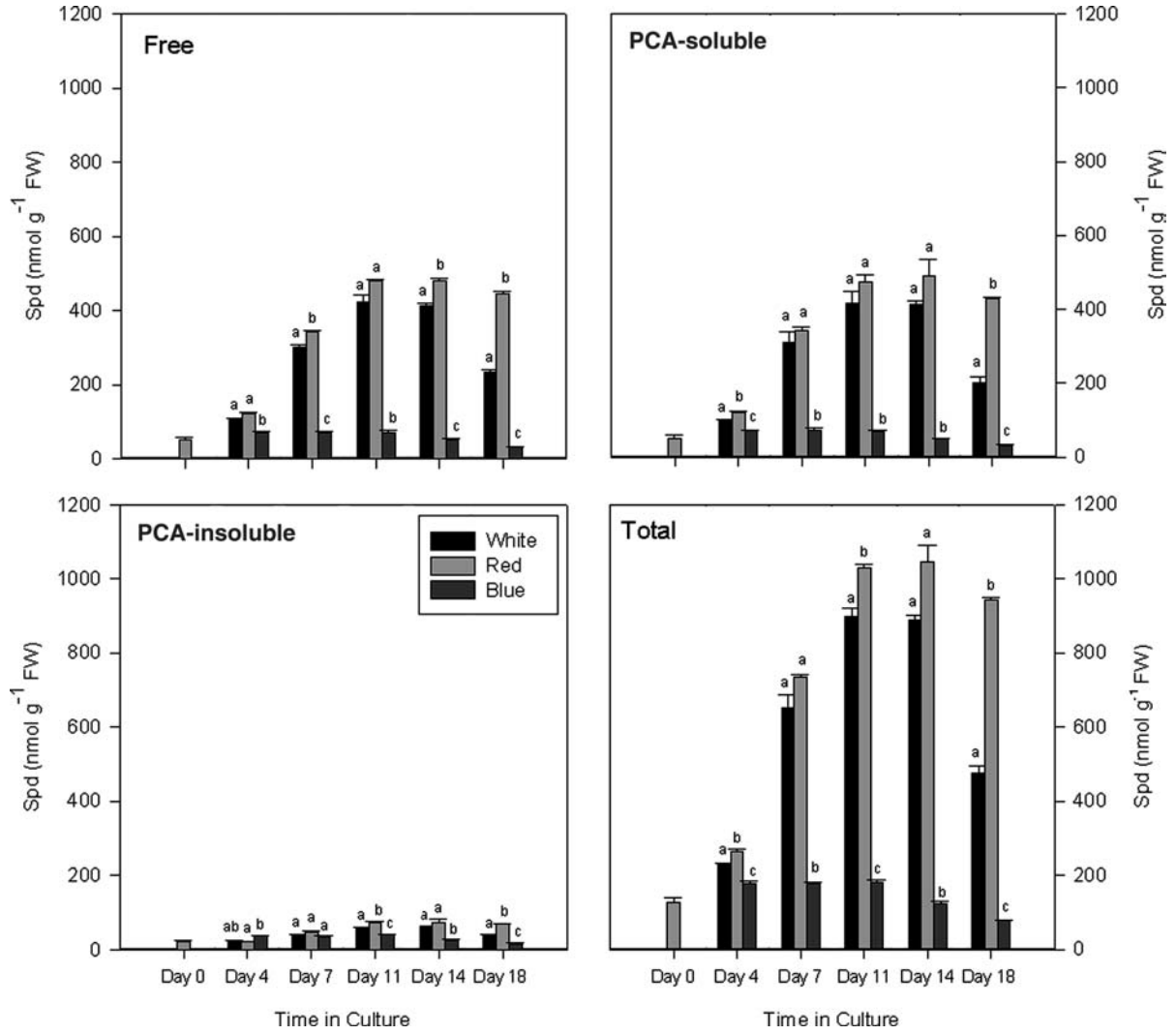

Figure 2. Changes in the endogenous concentration of Spermidine in lettuce cotyledon explants during 18 days of culture under white, red, or blue light. Different letters at each time point indicate significance ($p < 0.01$), $n = 3$, vertical bars are standard error of the mean.

period, although to a lesser extent than under red light. These results suggest that PA accumulation is promoted by phytochrome, and that the degree of PA accumulation might be regulated by different phytochrome photoequilibria. Under white light the ratio between active phytochrome (P_{fr}) and total phytochrome was 0.75, and 0.86 under red light (Hunter and Burritt 2004), demonstrating that the greater phytochrome photoequilibrium achieved under red light, corresponded with a greater accumulation of total PAs.

In the current study, light quality also influenced the form in which the PAs were present. Lettuce cotyledon explants cultured under blue light contained a greater proportion of PCA-insoluble conjugated Put, Spd, and Spm, compared to explants cultured under white or red light, which contained greater proportions of free and PCA-soluble conjugated PAs. Moysset et al. (2002) demonstrated that pulses of FR light, which causes a decrease in the phytochrome photoequilibrium, results in an increase in PCA-insoluble conjugated PAs in *Araujia sericifera* explants, and FR light pulses have also been shown to cause a decrease in somatic embryogenesis (Torné et al. 2001). Therefore, it is likely that the greater proportion of

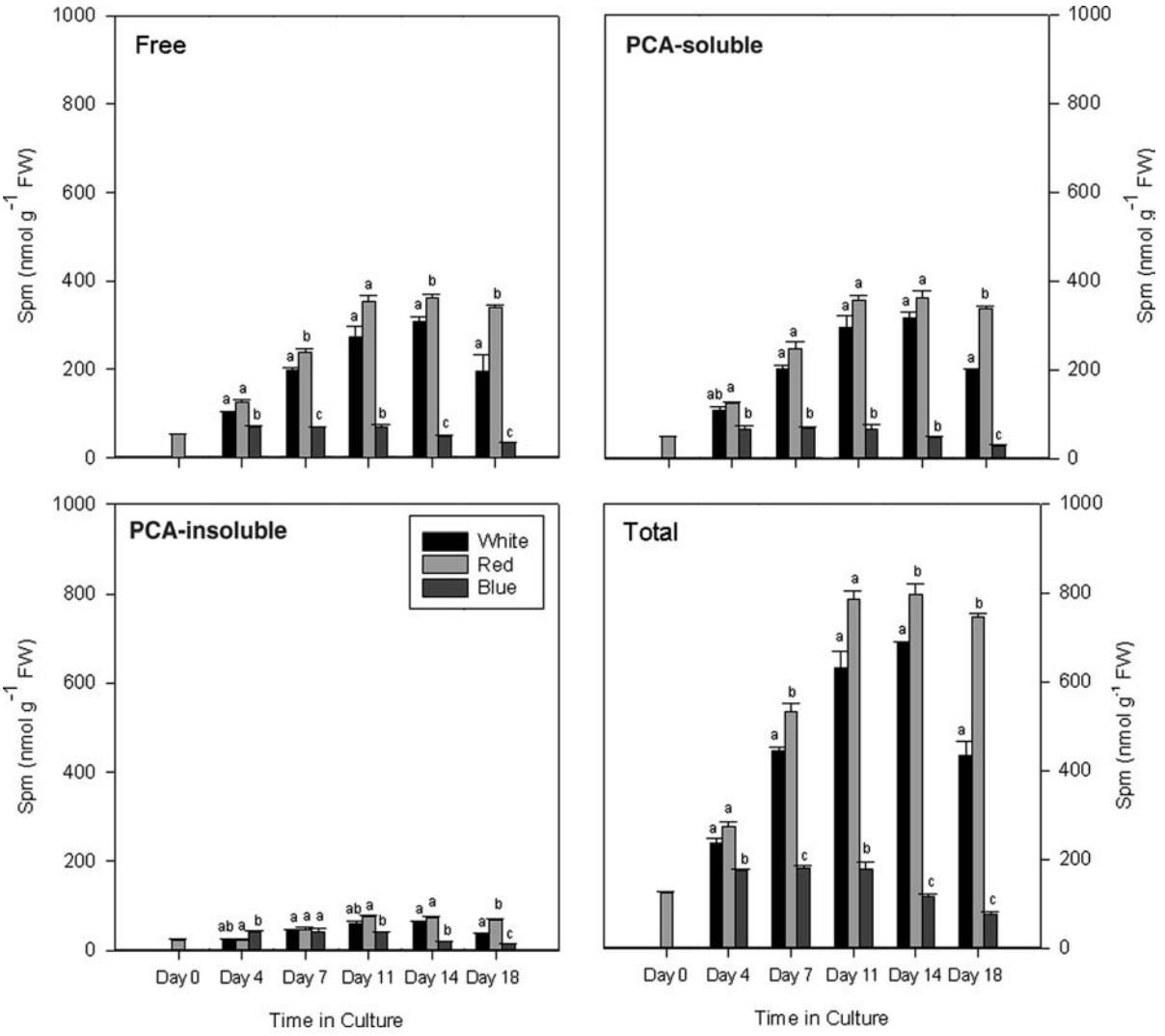

Figure 3. Changes in the endogenous concentration of Spermine in lettuce cotyledon explants during 18 days of culture under white, red, or blue light. Different letters at each time point indicate significance ($p < 0.01$), $n = 3$, vertical bars are standard error of the mean.

PCA-insoluble conjugated PAs, detected in lettuce cotyledon explants cultured under blue light, has an adverse influence on adventitious shoot formation.

It has been suggested that the ratio between individual PAs has a greater influence on regeneration *in vitro*, than the absolute concentrations of individual PAs. For example, varieties of indica rice that were successfully regenerated via somatic embryogenesis contained a Put:Spd ratio of 2.3, but those varieties recalcitrant in culture had a high Put:Spd ratio of 10 (Shoeb et al. 2001). In lettuce, explants cultured under red and white light had lower Put:Spd ratios during shoot formation (days 7–14) than explants cultured under blue light, which were inhibited from shoot formation, suggesting that a low Put:Spd ratio is a marker of shoot formation. By day 18, explants cultured under white light had a relatively high Put:Spd ratio, compared to explants cultured under red light.

Yoshida et al. (1998) determined that *Pharbitis nil* leaves contain at least two genes that regulate the activity of SAMDC, which is involved in the conversion of Put to Spd. Further to this, it was discovered that red and blue light promote

SAMDC activity, and thus influence PA levels, via different signal transduction pathways (Yoshida et al. 1999). This is apparently not the case in lettuce cotyledon cultures. Shoots produced under red light remain very small and unexpanded compared to the broad and elongated shoots that develop under white light (Hunter and Burritt 2004). An increasing Put:Spd ratio later in culture might facilitate or signal primordia growth and expansion, as was observed in explants cultured under white light. Davidonis (1995) suggests that cell proliferation is associated with high concentrations of free PAs, and Cvikrová et al. (1999) states that plant growth and development involves coordination of cell division and cell expansion, processes with inverse requirements for free PAs. The shift observed in the Put:Spd ratio in lettuce cotyledon explants cultured under white light might reflect the switch from differentiation to shoot expansion.

The mechanism by which light can influence PA concentrations has only recently been investigated. In *Arabidopsis thaliana*, light induces gene expression of both the *ADC1* and *ADC2* genes (Hummel et al. 2004). However, in mutant plants that over-expressed ethylene, light induced expression of both *ADC* genes was inhibited. The production of both PAs and ethylene is dependant on the availability of *S*-adenosyl methionine, and Hummel et al. (2004) clearly demonstrate that PA metabolism is influenced by ethylene production. Further to this, Pierik et al. (2004) demonstrate that low fluence blue light induces an increase in ethylene production in tobacco plants. Therefore, the culture of lettuce cotyledon explants under low fluence blue light might also promote ethylene production, at the expense of PA metabolism and shoot primordia development. Alternatively, Pierik et al. (2004) demonstrate that a high R:FR ratio promotes normal vegetative growth, whereas blue light induces shoot elongation as a means of shade avoidance. Under red light, lettuce explants might be induced to proliferate shoots, but the production of ethylene promoted by low fluence blue light could cause the production of significantly fewer shoots, with an elongated morphology, as has previously been observed (Hunter and Burritt 2004), as a response to perceived crowding.

The present study has demonstrated that the production of adventitious shoot primordia on lettuce cotyledon explants is associated with increasing concentrations of PAs, and that concentrations decline as shoot primordia develop. It is not clear whether PAs act as regulatory factors in the formation and development of shoot primordia, or whether changes in PA metabolism occur as a consequence of these processes. Further work monitoring the activities of enzymes involved in PA metabolism, combined with inhibitors of PA biosynthesis, and exogenous application of PAs might help answer this question.

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