BIOLOGIA PLANTARUM 36 (4): 553-563, 1994

Axillary bud proliferation and ethylene production as controlled by radiation of different spectral composition and exogenous phytohormones

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Abstract

The effect of radiation of different spectral composition on axillary proliferation of lavandin (*Lavandula officinalis* Chaix × *Lavandula latifolia* Villars cv. Grosso) was studied in combination with application of exogenous benzyladenine (BA) and putrescine (Put) and endogenous ethylene production. The effect of BA was predominant over the radiation. Continuous far-red showed a fluence rate-dependent promotion of shoot proliferation in the presence of BA. On BA-free medium, shoot number was enhanced under blue, white, and red radiation, at low photon fluence rates. BA, however, could reduce the inhibiting effect of blue and ultraviolet radiation, at high photon fluence rates. Exogenous Put stimulated axillary bud proliferation under some radiation treatments in the presence of BA. Moreover, Put, analogously to BA, could overcome the detrimental effect of ultraviolet radiation. A positive correlation between biotic ethylene production and shoot formation was evidenced under far-red at high photon fluence rate in the presence of BA, and under white, red and blue radiation at low photon fluence rate in the BA-free medium. However, when abiotic ethylene (released from the agarized medium) was stimulated by UV, no improvement of shoot formation was observed.

Introduction

The importance of duration, photon fluence rate and spectral composition of radiation in the control of the development and differentiation of *in vitro* cultured tissues is well recognized (Economou and Read 1987). However, most of the data deal with adventitious regeneration from both differentiated tissues (Kadkade and Seibert 1977, Economou and Read 1986, Lercari *et al.* 1986) and callus (Weis and Jaffe 1969, Seibert *et al.* 1975, Kadkade and Jopson 1978). More recent, but

Received 23 June 1993, *accepted* 30 September 1993.

Abbreviations: B - blue radiation; D - dark; Fr - far-red radiation; P_{fr} - far red absorbing form of phytochrome; P_{tot} - total phytochrome; Put - putrescine; R - red radiation; UV - ultraviolet radiation; W - white radiation. Suffixes: h - high photon fluence rate; l - low photon fluence rate.

undoubtedly scarcer, are papers about the influence of spectral composition of radiation on axillary bud proliferation (Baraldi *et al.* 1988, Chée and Pool 1989), which is a widespread technique used for commercial micropropagation. Spectral composition of radiation strongly interacts with both endogenous (Butenko *et al.* 1990) and exogenous cytokinins (Baraldi *et al.* 1988), which are deeply involved in breaking apical dominance.

Moreover, polyamines have recently been shown to be involved in the initiation of organized development *in vitro*, also interacting with cytokinins (Kumar and Thorpe 1989, Sanchez-Gras *et al.* 1990). In addition, a link between phytochrome-controlled growth *in vivo* and polyamine titre was found in etiolated pea seedlings following red radiation treatment (Goren *et al.* 1982).

Ethylene has been proved to play an essential role in modulating axillary shoot formation from lavandin explants (Panizza *et al.* 1993). Several studies have shown interactions between spectral composition of radiation and endogenous ethylene production in various phenomena *in vivo*, such as seed germination (Salveit and Pharr 1980), seedling growth (Goeschl *et al.* 1967) and root growth (Eliasson and Bollmark 1988).

To our knowledge, no report is so far available in the literature on: 1) the interaction between radiation quality and exogenous polyamines *in vitro*; 2) radiation-induced changes in ethylene release during shoot formation *in vitro*. The aim of the present work was to investigate the effect of radiation spectral composition on axillary proliferation of lavandin. Particular attention was devoted to the interaction between the different radiation treatments and exogenous growth substances, a cytokinin, benzyladenine (BA), and a polyamine, putrescine (Put), which had previously proved to be effective in regulating this process (Panizza and Tognoni 1988, Panizza *et al.* 1993). The possible influence of radiation on endogenous ethylene production was also investigated, both in the presence and in the absence of the cytokinin.

Materials and methods

Axillary proliferation of lavandin: The micropropagation of lavandin (*Lavandula officinalis* Chaix × *Lavandula latifolia* Villars cv. Grosso) was performed using node explants according to Panizza and Tognoni (1988). Basal medium consisted of Linsmaier and Skoog (1965) medium with 30 g dm^{-3} saccharose and 7.5 g dm^{-3} Difco Bacto agar. BA (1μM) was added to the basal medium before autoclaving, putrescine (Put, 1 and 10 μM) in sterile distilled water to the autoclaved medium while it was still warm.

The cultures were grown at 25 °C. The explants (4 nodes per bottle) were cultured in 30 cm^3 glass bottles (Pyrex, France) closed by plastic screw caps with holes supplied with caoutchouc rubber septa. Each bottle contained 5 cm^3 agarized medium. The containers were cultivated on devices constructed to prevent uneven illumination of the cultures due to radiation adsorption by the black plastic cap.

The effects of the different treatments, on both qualitative (percentage of nodes with shoots) and quantitative (mean shoot number per explant and mean shoot length) parameters related to axillary budding, were estimated at the end of 30 d in culture to allow sufficient growth of the proliferated shoots.

Radiation treatments: Each experiment was carried out either under continuous radiation or in darkness (D). Cool white fluorescent radiation (W) was provided by *Philips* (*Eindhoven*, The Netherlands) *TL 40 W/33 RS* fluorescent tubes. Blue radiation (B) was produced by *Philips TL 40 W/18* fluorescent tubes filtered through 3-mm *PG 627 Plexiglass* (*IMPLA* S.p.A., Napoli, Italy). Red radiation (R) was obtained by filtering the radiation from *Philips TL 40 W/15* fluorescent tubes through a 3-mm *PG 501* filter (*IMPLA* S.p.A.). Far-red radiation (Fr) was obtained from *Phillinea* (*Philips*) *120 W/230-240* incandescent tubes filtered through a *PG 501/3-mm*, a 3-mm *PG 627* (*IMPLA* S.p.A.) and a *KG* 3/2-mm filter (*Schott and Gen.*, Mainz, Germany). A combination of ultraviolet A and B radiation (UV) was obtained from black-radiation fluorescent tubes (*Sylvania GTEF20 T12 BLB20W*, Milan, Italy).

End-of-day treatments with Fr (FrD) or Fr plus R (FrRD) followed by continuous D were also accomplished as described in Table 1.

Table 1. Values for the fluence rates and phytochrome photoequilibria established by the different radiation treatments used for axillary budding of lavandin.

Radiation treatment	Photon fluence rate* [μmol m^{-2} s^{-1}]		P_{fr}/P_{tot}** [%]
	high (h)	low (l)	
W (white)	66	7	70
R (red)	7	1	86
Fr (far-red)	8	2	3
FrD (25 min Frh + 30 d dark, D)	-	-	3
FrRD (25 min Frh + 10 min Rh + 30 d D)	-	-	86
B (blue)	13	1.5	45
UV (a mixture of UV A and B)	62	5	55

* Measured with a *LICOR 1800* spectroradiometer.
** Calculated according to Lercari and Deitzer (1987).

Ethylene determination: For ethylene determination, the containers were sealed from the beginning of the experiments and gas samples were analyzed at intervals over 14 d, a time sufficient for the commitment of lavandin explants to produce lateral shoots. Ethylene was determined by gas chromatography as previously described (Mensuali-Sodi *et al.* 1992).

The term "total ethylene" refers to the total ethylene content [μl l^{-1}] measured in the vessel atmosphere, *i.e.* the sum of abiotic and biotic ethylene minus ethylene losses. Biotic ethylene production was estimated according to Mensuali-Sodi *et al.*

(1992), quantifying ethylene losses and abiotic contributions to ethylene accumulation in the culture system.

Statistical analysis: Each treatment was replicated 5 times and data are means of duplicate experiments. Analysis of variance and least significant difference at probability level of 0.05 were used.

Results

Effect of radiation spectral composition and photon fluence rate on axillary proliferation in relation with exogenous BA: The effect of BA on the proliferation percentage was in general predominant over the influence of radiation. No difference could be found between D and W either on BA-supplemented or basal medium. In the presence of the cytokinin, Frh significantly promoted shoot proliferation, while Frl inhibited it. However, on the basal medium, Fr at both photon fluence rates impaired shoot formation. Bh and UVh strongly inhibited axillary proliferation, being somewhat counteracted by BA; in fact, in the presence of the cytokinin, shoot formation still took place, albeit at a lesser extent (Fig.1).

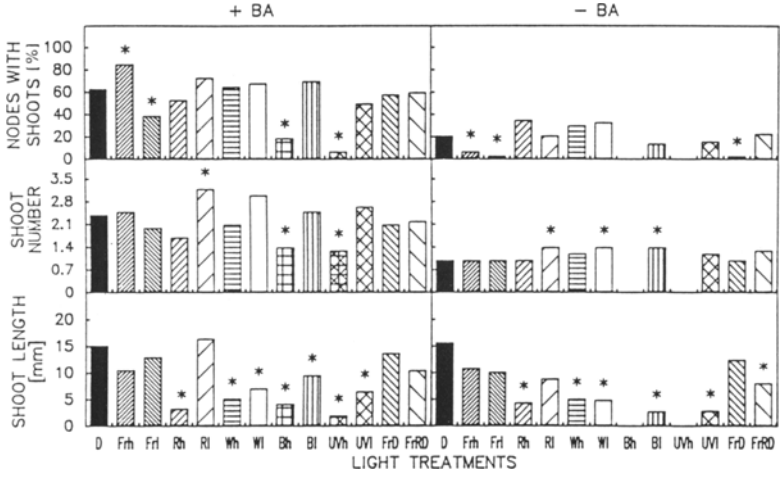

Fig. 1. Effect of continuous radiation treatments (D, dark; Fr, far-red; R, red; W, white; B, blue; UV, ultraviolet; all at both high, h and low, l, photon fluence rates) and end-of-day treatments (FrD, 25 min Fr plus continuous D; FrRD, 25 min Fr plus 10 min R plus continuous D) on axillary bud proliferation of lavandin, both in the presence and absence of 1 μM benzyladenine (BA). Values marked by an asterisk differ significantly from the control (D) at $P = 0.05$.

As regards shoot number, again D and W gave similar responses. A stimulating effect was noticed under Rl on both media, while Wl and Bl promoted shoot number in the absence of BA only. Once more, Bh and UVh negatively affected shoot number.

All radiation treatments, except Fr at both photon fluence rates and Rl impaired shoot elongation, regardless of BA.

As regards the end-of-day treatments (Fig. 1), shoot proliferation was affected on the basal medium only. FrD exerted an inhibitory effect which was completely reversed by an immediately following R treatment (FrRD). This last treatment also negatively affected shoot elongation.

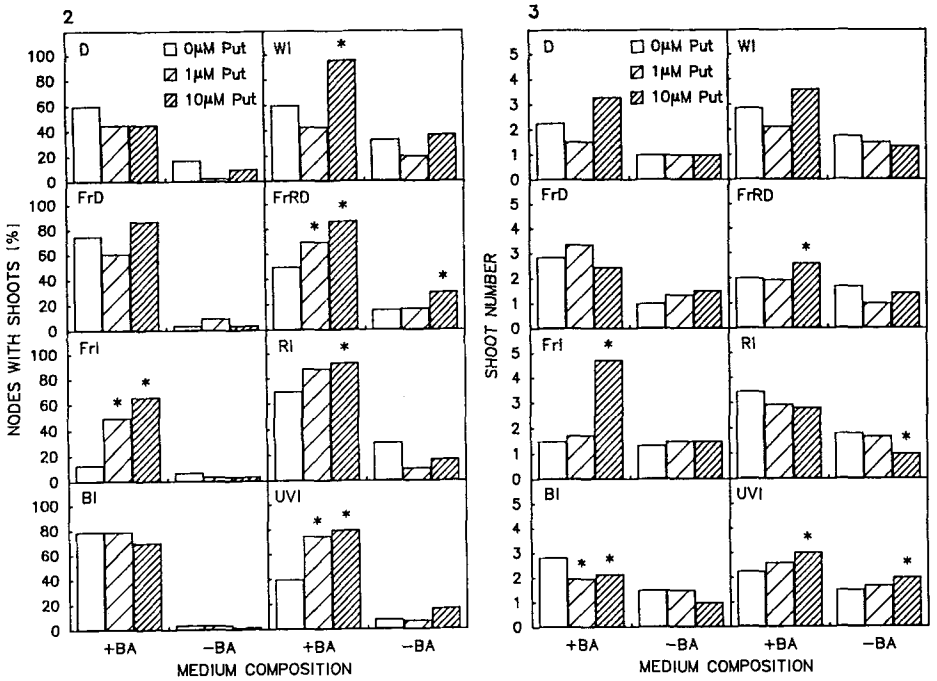

Fig. 2. Effect of putrescine (Put) in combination with various radiation treatments (D, dark; FrD, 25 min far-red plus D; FrRD, 25 min far-red plus 10 min red plus D; Wl, white, Frl, far-red, Rl, red, Bl, blue, UVl, ultraviolet, all at low photon fluence rate) on the percentage of shoot formation from lavandin nodes, both in the presence and absence of 1 μM benzyladenine (BA). Within each culture medium (+ BA or - BA) values marked by an asterisk differ significantly from the control (medium without Put) at $P = 0.05$.

Fig. 3. Effect of putrescine (Put) in combination with various radiation treatments (D, dark; FrD, 25 min far-red plus D; FrRD, 25 min far-red plus 10 min red plus D; Wl, white; Frl, far-red; Rl, red; Bl, blue; UVl, ultraviolet; all at low photon fluence rate) on mean shoot number per node explant of lavandin, both in the presence and absence of 1 μM benzyladenine (BA). Within each culture medium (+ BA or - BA) values marked by an asterisk differ significantly from the control (medium without Put) at $P = 0.05$.

Interaction between radiation spectral composition and exogenous Put: Put affected shoot formation in a radiation-dependent manner (Fig. 2). No influence was evident under D and FrD, but FrRD enabled Put to enhance shoot proliferation both in the presence and absence of BA, though only at the higher concentration in the basal medium. However, the positive effect of Put on shoot formation under Wl, Rl and

UVl required the presence of BA. Furthermore, Put completely reversed the negative effect of Frl on shoot formation.

Shoot number (Fig. 3) was improved by Put in combination with BA under FrRD, Frl and UVl, but it was inhibited under Bl. On the basal medium under UVl, 10 μM Put resulted in a shoot number at the same level as 1 μM BA. On the contrary, an adverse effect was observed under Rl.

In the presence of BA, Put stimulated shoot elongation under Wl, Frl and Rl, whilst it showed an inhibitory effect in the D and FrD (Fig. 4). In the presence of Put on the basal medium, the shoots appeared shorter under FrD and Rl, but Put was able to improve shoot elongation under Wl, Bl and UVl, though at different concentrations.

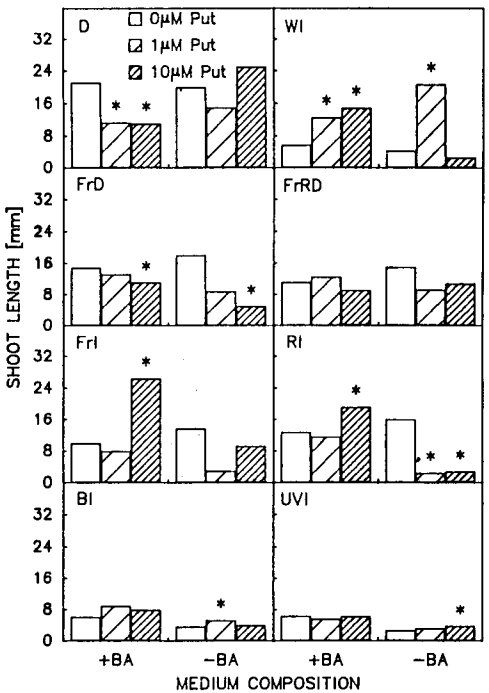

Fig. 4. Effect of putrescine (Put) in combination with various radiation treatments (D, dark; FrD, 25 min far-red plus D; FrRD, 25 min far-red plus 10 min red plus D; Wl, white; Frl, far-red; Rl, red; Bl, blue; UVl, ultraviolet; all at low photon fluence rate) on mean shoot length per node explant of lavandin, both in the presence and absence of 1 μM benzyladenine (BA). Within each culture medium (+ BA or - BA) values marked by an asterisk differ significantly from the control (medium without Put) at $P = 0.05$.

Interaction between radiation spectral composition and ethylene: For all treatments with the exception of UVl, only biotic ethylene production is reported, because it was closely parallel to total ethylene content, the abiotic release being very small under the experimental conditions (data not shown). No difference could be observed between the end-of-day treatments and the D control, either in the presence or absence of BA (Fig. 5).

On BA-provided medium (Fig. 5), Frh strongly raised the biotic emanation, while Rl, Bl and Wl were ineffective. Without BA (Fig. 5), on the contrary, Frh did not yield any effect, but Rl, Wl and Bl stimulated ethylene production.

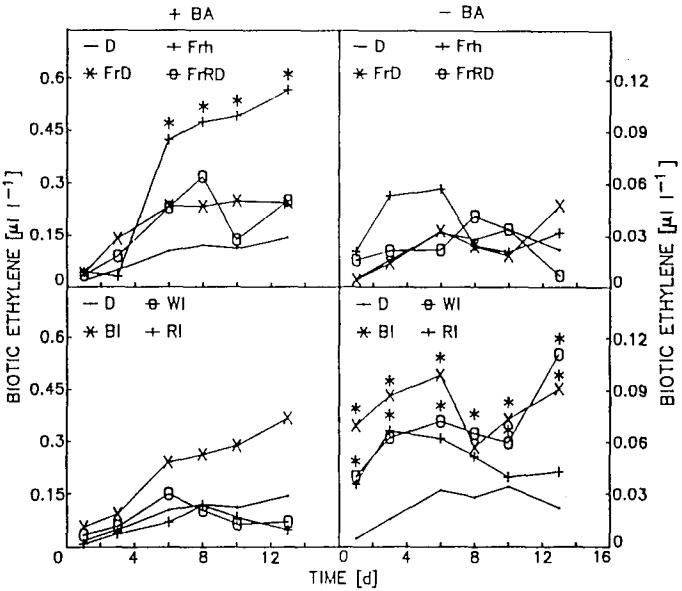

Fig. 5. Effect of various radiation treatments (D, dark; FrD, 25 min far-red plus D; FrRD, 25 min far-red plus 10 min red plus D; Frh, far-red at high photon fluence rate; Wl, white; Rl, red; Bl, blue; all at low photon fluence rate) on biotic ethylene production by lavandin node explants, both in the presence and absence of 1 µM benzyladenine (BA). Values marked by an asterisk differ significantly from the control (D) at $P = 0.05$.

An unexpected phenomenon was observed under UVl (data not shown). Total ethylene content was strongly enhanced both in the presence and absence of BA, but biotic ethylene was unaffected. This discrepancy was caused by the high abiotic ethylene release from the agar-containing medium under UVl.

Discussion

The present results outline the importance of BA in controlling axillary shoot proliferation in lavandin node explants and an interaction between radiation and exogenous growth substances. No radiation treatment could entirely substitute for BA in this process, the effect of the cytokinin being overwhelming. In fact, the action of BA did not require the presence of radiation. On the contrary, both radiation and BA proved to be indispensable factors in the development of *Prunus* microcuttings *in vitro* (Baraldi *et al.* 1988) and in adventitious shoot formation from *Pinus radiata* cotyledons (Villalobos *et al.* 1984).

The promotion of shoot number under Rl and the inhibition of shoot formation under Frl, both in the presence and absence of BA (Fig. 1), suggested phytochrome

involvement through a low energy response. This was confirmed, in the BA-free medium only, by the end-of-day treatments showing a correlation between the extent of shoot proliferation (Fig. 1) and P_{Fr} level (Table 1). Analogously, cytokinin incorporation into the culture medium negated the promoting effect of R in axillary bud proliferation from azalea shoot tips (Economou and Read 1987) and adventitious bud regeneration from petunia leaf segments (Economou and Read 1986). Phytochrome-regulated organogenesis *in vitro* was also reported for lettuce (Kadkade and Seibert 1977).

Phytochrome involvement in lavandin axillary proliferation was also suggested in the absence of BA, by the improvement of shoot number under W1 and B1 (Fig. 1), which established quite high phytochrome photoequilibria (Table 1). A promoting effect of B on organogenesis was also found in tobacco pith cultures (Weis and Jaffe, 1969) and in grapevine shoot cultures (Chée and Pool 1989), in both cases photoinactivation of IAA being postulated.

In the BA-enriched medium, phytochrome also seemed to be involved through a high irradiance response, as indicated by the photon fluence rate dependency of the promotion of shoot proliferation under Fr (Fig. 1). A positive effect of continuous Fr on tomato cotyledonary explants (Lercari *et al.* 1986) was also ascribed to the high irradiance response of the phytochrome system.

For all the radiation treatments excepting Fr, the low photon fluence rate proved to be better for the cultures, especially under B and UV. BA partly counteracted the detrimental effects of Bh and UVh. This agrees with Fridborg and Eriksson (1975) who showed partial reversal by cytokinin of the inhibition of callus morphogenesis induced by near-UV radiation.

In contrast with what was reported in *Prunus* (Baraldi *et al.* 1988), the elongation of lavandin shoots was not inhibited by BA, maybe due to the low concentration used in our experiments. All radiation treatments, with the exception of R1, suggested an inverse relationship between shoot length (Fig. 1) and phytochrome photoequilibrium (Table 1), which was also confirmed, in the basal medium only, by the end-of-day treatments (Fig. 1). However, both in the presence and absence of BA, no inhibition was observed under R1 (Fig. 1), though it established a high phytochrome photoequilibrium (Table 1). Similarly, R promoted shoot elongation in *Pelargonium* shoots *in vitro* (Appelgren 1991).

Exogenous Put also showed a strong requirement for the presence of the cytokinin: indeed Put was more effective in the presence of BA. This may be caused by increased uptake of Put induced by BA, as reported by Kumar and Thorpe (1989) in cotyledons of *Pinus radiata*. It is noteworthy that neither BA alone nor Put alone could counterbalance the negative effect of Fr1 on shoot formation, but they were highly effective when used in combination (Fig. 2), significantly improving also shoot number and length (Fig. 3 and 4). An interaction between Put and BA was also evidenced under R1, as the effect of Put on shoot length shifted from stimulatory to inhibitory according to the presence of the cytokinin (Fig. 4).

Moreover, under UV1, Put could substitute for BA in improving shoot number (Fig. 3), suggesting that it exerted a regulatory function similar to the cytokinin. No such effect could be detected under W1. However, previous data (Panizza *et al.*

1993) indicated that Put mimicked the action of BA in lavandin cultures under W at a higher photon fluence rate than in the present work, suggesting a dependence on irradiance. A close relationship between cytokinin and Put was also indicated by the increased content of endogenous Put in lettuce cotyledons after BA treatment (Cho 1983) and tobacco leaf explants (Malfatti *et al.* 1983). Further evidence is given in a recent paper (Feray *et al.* 1992) reporting that Put and BA have a similar regulatory role in long-distance transport.

In general, Put had a beneficial influence on the cultures in the presence of radiation. P_{fr} requirement for Put action was suggested by the end-of day treatments, the short R treatment being critical to allow the positive effect of Put on shoot formation (Fig. 2 and 3) and to nullify its negative effect on shoot elongation (Fig. 4). Nevertheless, no clear relationship could be found between phytochrome photo-equilibrium and the action of Put, which was also promoting (Fig. 2, 3 and 4) at the low value of P_{fr}/P_{tot}.

The effect of the radiation treatments on ethylene production reflected the influence of radiation on the proliferation process. The increased ethylene content measured under Frh in the presence of BA, due to biotic production (Fig. 5), suggested a possible involvement of ethylene in the high irradiance response of the phytochrome, postulated for the improvement of shoot proliferation. Similarly in sorghum (Craker *et al.* 1973), it was suggested that ethylene production under B and Fr was induced through a high irradiance response of the phytochrome.

It is interesting to note that the high ethylene content induced by UV1 (data not shown), similar to the level under Frh (Fig. 5) but due to high abiotic production, was not accompanied by increased proliferation. This observation lends support to the conclusion that the ethylene that is functional in proliferation control is probably that within the cell. Thus the measurement of total ethylene content in the culture atmosphere, often reported in the literature, with no evaluation of the biotic production, may give misleading information on the action of ethylene. This is consistent with the observation that externally applied ethylene had no effect on adventitious root formation in mustard seedlings (Pfaff and Schopfer 1980), while changes in endogenous ethylene altered the susceptibility of this morphogenetic process to phytochrome control.

In the BA-free medium, further evidence of the correlation between biotic ethylene and the proliferation process was given by the observation that the biotic emanation (Fig. 5) increased under those radiation treatments (R1, B1 and W1) which also improved shoot number (Fig. 1). In contrast with the present results, R-promoted (Fr-reversible) inhibition of ethylene production was evidenced in excised rice coleoptiles (Imaseki *et al.* 1971) However, when a number of species were compared (Vangronsveld and Van Poucke 1989), it was found that the inhibiting effect of R on ethylene production was species- and age-dependent. Furthermore, in the present work, no significant change in ethylene levels was induced by the end-of-day treatments (Fig. 5).

In accordance with our results, the stimulation of ethylene production under W was also reported in germinating cucumber seeds (Saltveit and Pharr 1980). In a more recent work on intact green seedlings of several plant species (Weckx and Van

Poucke 1989), it is demonstrated that W acts by promoting the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene, while inhibiting the malonylation.

In conclusion, in the evaluation of the responsiveness of a tissue to radiation *in vitro*, great care should also be devoted to radiation-induced changes in the abiotic environment (*e.g.* ethylene release).

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