Plant Cell, Tissue and Organ Culture **32**: 219–225, 1993.

Promotion of petunia (*Petunia hybrida* L.) regeneration *in vitro* by ethylene

Kortessa Dimasi-Theriou, Athanasios S. Economou & Evangelos M. Sfakiotakis
Department of Horticulture, Aristotle University, 54006 Thessaloniki, Greece

Received 14 October 1991; accepted in revised form 13 July 1992

Key words: ethylene, ethylene inhibitors, *in vitro* culture, *Petunia hybrida*, root formation, shoot formation

Abstract

The influence of ethylene on shoot and root formation from petunia leaf explants was studied in cultures in test tubes placed in 5 l glass jars. Reduction of the endogenously produced ethylene by inclusion of ethysorb ($KMnO_4$), an ethylene absorbent, caused a decrease of the number of shoots. On the other hand, supplementing the cultures with ethylene (0.01–10 ppm) caused a marked increase of the number of shoots without, however, any effect on the length and fresh weight. Ethylene treatments (1 ppm) were found to be most effective when they were applied in the second week of culturing of petunia explants. Addition of Co^{++} to the medium resulted in a reduction of the endogenously produced ethylene and concomitantly reduced shoot formation. Similarly, inclusion of Ag^{+}, an inhibitor of ethylene action, resulted in poor shoot formation. Ethylene also appeared to play a role on rooting of petunia microshoots *in vitro* in an auxin-free medium. Ethylene at a concentration of 10 ppm induced adventitious root formation considerably, whereas at low levels (0.01–1 ppm) it had no influence on rooting.

Introduction

It has been reported that cells, tissues and organs of various plant species produce ethylene when cultured *in vitro* (La Rue & Gamborg 1971; Horner et al. 1977; Thomas & Murashige 1979; Gavinlertvatana et al. 1982; De Proft et al. 1985; Koves & Szabo 1987; Sauerbrey et al. 1988). The endogenously produced ethylene which accumulates in the vessel atmosphere or even exogenously supplied ethylene can exert an influence on explant growth and morphogenesis. Ethylene may promote shoot formation in some plant species while in others it may have an inhibitory effect. Also, ethylene may promote or inhibit root initiation and root growth in explants cultured *in vitro*.

In tobacco callus, ethylene accumulation after the fifth day of culture enhanced shoot formation (Huxter et al. 1979). Panizza et al. (1988) observed that the shoot forming ability of stem node and flower spike explants of lavandin (*Lavandula officinalis* × *L. latifolia*) was linked to the degree of ethylene evolution from the cultures. Exogenous application of ethylene early in the culture period of *Lilium speciosum* increased the number of shoots which were formed on bulb-scale explants (Van Aartrijk et al. 1985). Similarly, Kumar et al. (1987) found that ethylene and carbon dioxide which built up during the first 10 to 15 days of culture promoted shoot bud formation in cotyledonary explants of *Pinus radiata*. Ethylene concentrations within limits enhanced shoot formation in tomato cotyledonary explants, while excessive accumulation reduced shoot growth (Mensuali-Sodi et al.

1990). On the other hand, the inhibitory effect of ethylene on shoot formation has been altered by inhibitors of either ethylene action or ethylene production. Thus, incorporation of $AgNO_3$ in the culture medium promoted shoot regeneration from callus of *Brassica oleracea* (Williams et al. 1990). Also, in some other members of the *Cruciferae* family addition of the ethylene inhibitors $AgNO_3$ or amino-ethyoxyvinyl-glycine (AVG) in the culture medium enhanced shoot regeneration from seedling explants (Pua et al. 1990).

Ethylene and ethylene releasing compounds were found to have a promoting effect on rooting of mung bean cuttings (Krishnamoorthy 1970, 1972; Roy et al. 1972; Robbins et al. 1983). However, in cultures of several plant species *in vitro* ethylene did not favour rooting. Thus, exogenous ethylene did not induce adventitious root initiation in juvenile and mature petiole explants of *Hedera helix* (Geneve et al. 1990). Furthermore, the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC) caused a marked reduction in rooting of cherry shoot cultures (Biondi et al. 1990). Absorption of ethylene by mercuric perchlorate in the vessel or inhibition of ethylene action by $AgNO_3$ in the medium, enhanced rooting from tomato leaf explants (Coleman et al. 1980). On the contrary, absorption of ethylene by mercuric perchlorate eliminated rooting of auxin-treated *Phaseolus vulgaris* petiole explants (Linkins et al. 1973).

In this work the effect of the endogenously produced ethylene, as well as the exogenously supplied ethylene, on shoot formation from petunia leaf explants was studied. To achieve these objectives, the ethylene absorbent ethysorb and the ethylene inhibitors Ag^+ ($AgNO_3$) and Co^{++} ($CoCl_2$) were used, while the exogenous application of ethylene was tested for different time periods of explants culture. In addition, the effect of exogenous ethylene on microshoot rooting *in vitro* was investigated.

Materials and methods

New and fully expanded leaves from the top of the shoot of petunia plants (*Petunia hybrida* L. cv. Alderman) were used as explant source for the experiments. Leaf sterilization was that described elsewhere (Economou & Read 1982). Explants, 1×1 cm in cross sections of the midrib of the leaf lamina, were transferred after sterilization onto medium in glass test tubes. Each test tube, of 10×3 cm dimensions, contained 10 ml of medium and was sealed by cotton-wool bungs to permit gas exchange. One leaf explant was incubated per test tube.

The medium for shoot formation was the MS (Murashige & Skoog 1962) supplemented with 1 μM benzyladenine (BA), 20 g l^{-1} sucrose and 6.5 g l^{-1} Difco bacto-agar. The pH of the medium was adjusted to 5.8 before agar addition.

The medium for root formation was the same MS as above described except for the BA which was omitted. The explants were well developed shoots, 1.5–2.0 cm long, from *in vitro* cultures of petunia leaf explants on MS medium with 1 μM of BA.

Ten test tubes with explants, sealed by cotton-wool bungs, were placed in individual and sterilized glass jars of 5 l volume which then were closed hermetically. On the glass jar lids small holes of 0.5 cm in diameter were opened and plugged with rubber closures for air sampling or ethylene injection.

All cultures were maintained under 16 h of cool-white fluorescent light of 45 μmol m^{-2} s^{-1} (400–700 nm) at a temperature of 23 ± 2°C. Shoot formation experiments were terminated after four weeks of culture and root formation after two weeks.

Ethylene production by the leaf explants and its effect on shoot formation were evaluated by using the absorbent ethysorb (potassium permanganate, $KMnO_4$). A small glass vial containing 20 g of ethysorb was inserted inside the glass jars from the start. Air samples of 1 ml were withdrawn from the glass jars every week for analysis of ethylene content.

Exogenous application of ethylene to the cultures were made by injecting different amounts of pure gas, giving final concentrations of 0.01, 0.1, 1, or 10 ppm in the glass jars atmosphere. This was tested for its effect on shoot formation. Besides these treatments, a treatment with ethysorb in the glass jar was also included as a control. Air samples from the glass jars atmos-

phere were taken for the measurement of the ethylene content at the end of the first and second week of culture.

The effect of the time of ethylene application to the cultures on shoot formation was studied by adding 1 ppm of ethylene in the glass jars at the beginning of the first, second or third week of culture. The ethylene treatment lasted one week and before or after this one-week period 20 g of ethysorb was inserted into the glass jars.

The influence of the inhibitors Co^{++} and Ag^{+} on shoot formation was evaluated by adding concentrations of 1, 10, or 100 mg l^{-1} of $CoCl_2$ or $AgNO_3$ to the medium before autoclaving. Air samples from the cultures were withdrawn at the end of the second and third week of culture for measurement of the ethylene content.

The ethylene effect on root formation was tested under concentrations of 0.01, 0.1, 1, or 10 ppm in the glass jars atmosphere. The environmental conditions were the same as mentioned above in cultures for shoot formation.

Ethylene levels in the glass jars atmosphere were determined using a Varian model 2740 gas chromatograph equipped with a flame ionization detector and a 0.32×120 cm column packed with activated alumina. One ml gas-tight hypodermic syringes were used to take samples.

The shoot or root formation parameters measured were the number, length and fresh weight of shoots or roots. Four jars of ten test tubes each were allocated per treatment. All experiments were conducted twice. Statistical analysis of the data was based on the analysis of variance. Comparisons among the treatments were made by the HSD test at the 5% level.

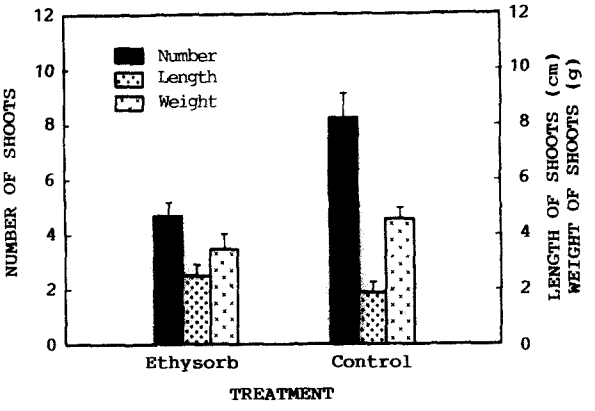

Fig. 1. Shoot formation in petunia leaf explants with or without ethysorb.

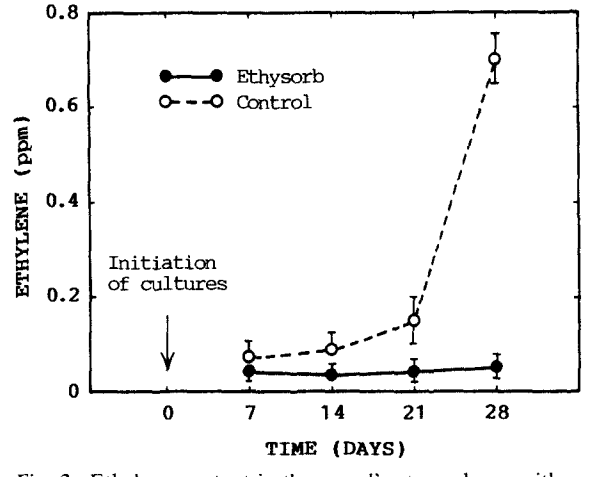

Fig. 2. Ethylene content in the vessel's atmosphere, with or without ethysorb, in cultures of petunia leaf explants.

Results

Cultures of petunia leaf explants in glass jars with the addition of ethysorb produced less shoots than cultures in glass jars without ethysorb (Fig. 1). However, the length and fresh weight of the shoots were not affected by the presence or absence of ethysorb in the glass jar. Ethysorb absorbed up to 90% of the total ethylene and kept the ethylene level steady at about 0.04 ppm (Fig. 2).

By increasing the concentrations of ethylene in the glass jar atmosphere from 0.01 ppm to 10 ppm the number of shoots increased linearly (Fig. 3). However, the shoots which produced at 10 ppm ethylene were of poor quality and had yellow not sturdy leaves. There was no significant difference between the number of shoots at the concentration of 0.01 ppm ethylene and the control treatment where ethysorb was added to the glass jar. On the other hand, the length and the fresh weight of the shoots produced were not significantly different for the various ethylene concentrations tested. The ethylene content of the glass jar atmosphere changed slightly in the first week of culture, whereas during the second week the levels of ethylene increased significantly, with the exception of the 10 ppm ethylene

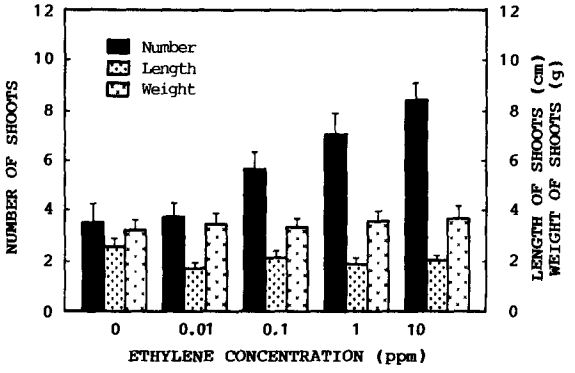

Fig. 3. Shoot formation in petunia leaf explants in response to exogenous application of ethylene in the vessel's atmosphere.

treatment where ethylene content was reduced below the initially applied concentration (Fig. 4).

When 1 ppm of ethylene was applied to the glass jars in the second week of culture the number of shoots which was produced was significantly higher than when ethylene was applied in the first or third week of culture (Fig. 5). Again, the length and the fresh weight of shoots was not affected by the ethylene application.

Addition of $CoCl_2$ and $AgNO_3$ to the medium arrested shoot production from petunia leaf explants. The number of shoots was reduced linearly from the lower to the higher concentrations tested (Fig. 6). Cultures without $CoCl_2$ or $AgNO_3$ in the medium produced the highest number of shoots. There were no differences in shoot length and fresh weight among the various

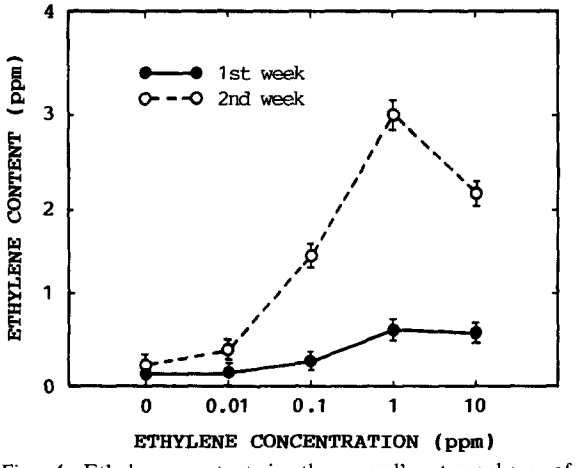

Fig. 4. Ethylene content in the vessel's atmosphere of petunia leaf explant cultures after one and two weeks of ethylene application of various concentrations.

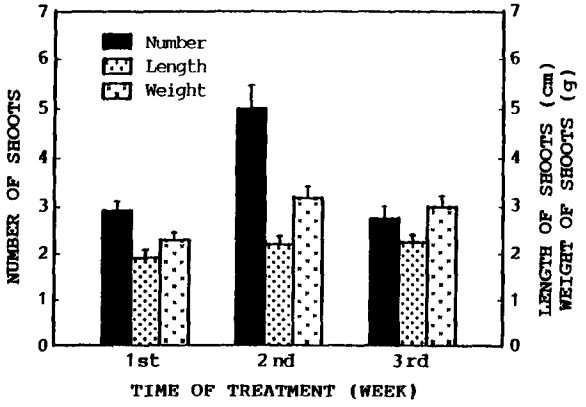

Fig. 5. Effect of time of ethylene application in the culture vessels (1-week duration) on shoot formation in petunia leaf explants.

concentrations of $CoCl_2$, $AgNO_3$ and control treatments. Ethylene levels in the second week remained the same in cultures of the various concentrations of $CoCl_2$ and also remained the

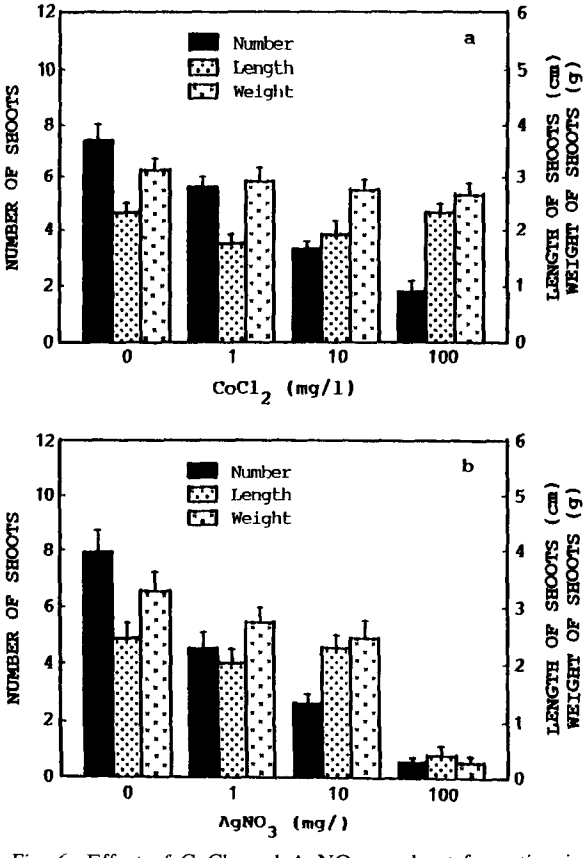

Fig. 6. Effect of $CoCl_2$ and $AgNO_3$ on shoot formation in petunia leaf explants.

same in the third week but were reduced as compared to those of the second week. In both groups of measurements the levels were considerably lower than in the control cultures (Fig. 7a). Similarly, in cultures with $AgNO_3$, in the medium, the ethylene contents were more or less the same for all $AgNO_3$ concentrations for each weekly measurement, with increased levels in the third week measurements (Fig. 7b).

Concentrations of 0.01, 0.1 and 1 ppm ethylene did not influence rooting of petunia microshoots. However, the concentration of 10 ppm of ethylene increased significantly the number of roots as compared to the control and the other ethylene treatments applied (Fig. 8). Also, the fresh weight of the roots was affected significantly at the 10 ppm of ethylene but not the length of the roots which was the same as those of the other ethylene treatments.

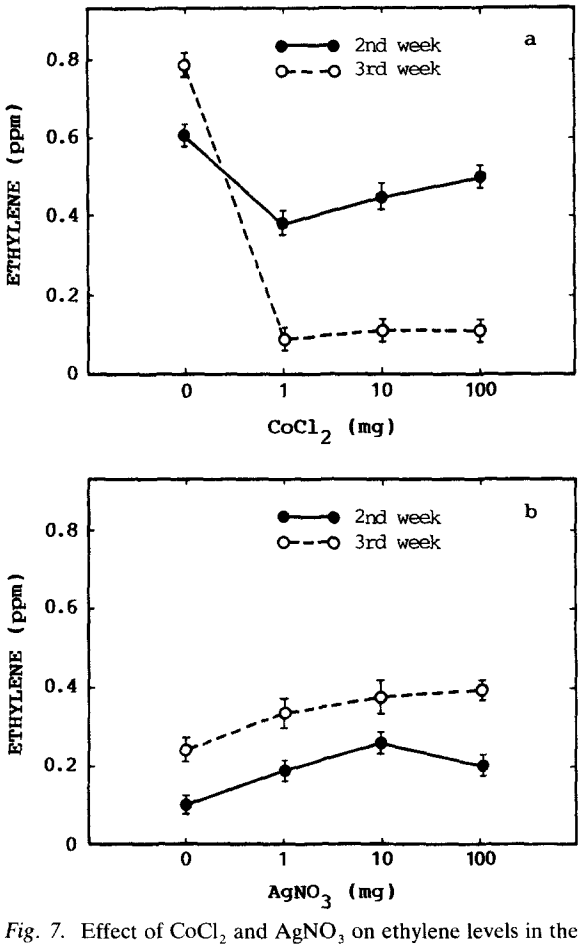

Fig. 7. Effect of $CoCl_2$ and $AgNO_3$ on ethylene levels in the vessel's atmosphere of petunia leaf explant cultures.

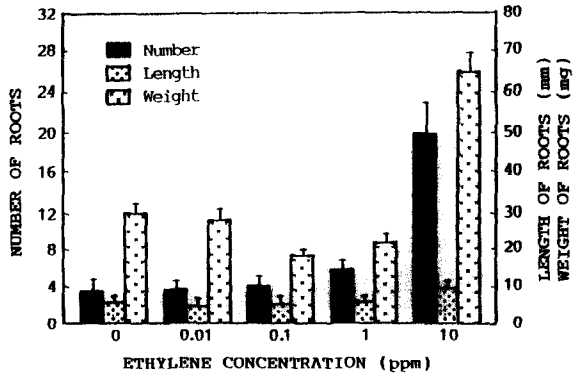

Fig. 8. Effect of ethylene application on root formation *in vitro* from petunia microshoots.

Discussion

From these results it is evident that ethylene influenced the number of shoots produced from leaf explants of petunia without any effect on their length and fresh weight. Ethysorb was efficient in absorbing the ethylene produced by the explant tissue during the culture period. Similarly, absorption of ethylene by mercuric perchlorate in cultures of excised cotyledons of *Pinus radiata* was found to inhibit shoot bud differentiation (Kumar et al. 1987).

Exogenous application of ethylene to the culture environment increased the number of shoots linearly from the lowest to the highest concentration tested. In tomato cotyledonary explants, shoot formation was also enhanced by ethylene concentrations, within limits, while excessive ethylene application reduced shoot differentiation (Mensuali-Sodi et al. 1990).

Application of 1 ppm ethylene was more effective in bringing about shoot formation when it was applied during the second week of culture than during the first or third week. Van Aartijk et al. (1985) also reported an increase of the number of differentiated buds per cultured explant of *Lilium speciosum* bulb-scales by ethylene provided during the first 3 to 7 days of the culture period. Similarly, in *Pinus radiata* cotyledonary explants, the highest number of shoot buds were obtained when ethylene accumulation reached an appropriate level in the first 10 to 15 days of culture (Kumar et al. 1987). Furthermore, ethylene accumulation enhanced shoot formation from callus of *Nicotiana*

tabacum (Huxter et al. 1979). It seems that petunia tissue is susceptible to ethylene injection during the second week after transfer which was parallel with the possible shoot bud initiation (Rao et al. 1973) while the first visible shoot buds appeared during the third week of culture.

The inclusion of $AgNO_3$ or $CoCl_2$ markedly reduced shoot formation from petunia leaf explants. Both cobalt and silver ion are known to inhibit ethylene synthesis (Co^{++}) or prevent ethylene action (Ag^{+}) (Beyer 1976; Lau & Yang 1976). The ethylene levels in the culture container on medium with $CoCl_2$ were lower than in the control cultures, whereas in the presence of $AgNO_3$ the ethylene levels were similar to the control cultures. Thus, either reduction of the ethylene content in the culture atmosphere by $CoCl_2$ or inhibition of the ethylene action by $AgNO_3$ resulted in poor shoot formation from leaf explants of petunia.

Ethylene also appeared to play a role on root formation *in vitro* of petunia microshoots. Although ethylene at low levels of exogenous applications (0.01–1 ppm) did not influence rooting of petunia microshoots in an auxin-free medium, it induced adventitious root formation considerably at a concentration of 10 ppm without any effect on the root length. The contradictory experimental results in the literature (Krishnamoorthy 1970, 1972; Roy et al. 1972; Coleman et al. 1980; Robbins et al. 1983; Geneve et al. 1990) support the hypothesis that the influence of exogenous ethylene on adventitious root formation varies with the plant species and the physiological and environmental conditions (Moncousin et al. 1989; Mudge 1989).

Acknowledgements

This work was supported in part by a grant from the General Secretariat of Research and Technology of Greece.

References

Beyer EM, Jr (1976) A potent inhibitor of ethylene action in plants. Plant Physiol. 58: 268–271

Biondi S, Inglesias I, Diaz T & Bagni N (1990) Polyamines and ethylene during rooting of wild cherry shoot cultures. In: Abstracts VIIth Intl. Congress on Plant Tissue and Cell Culture, Amsterdam, June 24–29, 1990: 277

Coleman WK, Huxter TJ, Reid DM & Thorpe TA (1980) Ethylene as endogenous inhibitor of root regeneration in tomato leaf discs cultured *in vitro*. Physiol. Plant. 48: 519–525

De Proft MP, Maene LJ & Debergh PC (1985) Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured *in vitro*. Physiol. Plant. 65: 375–379

Economou AS & Read PE (1982) Effect of NAA on shoot production *in vitro* from BA-treated petunia leaf explants. J. Amer. Soc. Hort. Sci. 107: 504–506

Gavinlertvatana P, Read PE, Wilkins HF & Heins R (1982) Ethylene levels in flask atmospheres of *Dahlia pinata* Cav. leaf segments and callus cultured *in vitro*. J. Amer. Soc. Hort. Sci. 107: 3–6

Geneve RL, Hackett WP & Swanson BT (1990) Ethylene production in deblated petioles from the juvenile and mature phases of English ivy in relation to adventitious root initiation. J. Amer. Soc. Hort. Sci. 115: 123–127

Horner M, McComb JA, McComb AJ & Street HE (1977) Ethylene production and plantlet formation by *Nicotiana* anthers cultured in the presence and absence of charcoal. J. Exp. Bot. 28: 1365–1372

Huxter TJ, Reid DM & Thorpe TA (1979) Ethylene production by tobacco (*Nicotiana tabacum*) callus. Physiol. Plant. 46: 374–380

Koves E & Szabo M (1987) Ethylene production in habituated and auxin-requiring tobacco callus cultures. Does ethylene play a role in the habituation? Physiol. Plant. 69: 351–355

Krishnamoorthy HN (1970) Promotion of rooting in mung bean hypocotyl cuttings with ethrel an ethylene releasing compound. Plant Cell Physiol. 11: 979–982

Krishnamoorthy HN (1972) The effects of ethrel, auxin and maleic hydrazide on the rooting of mung bean hypocotyl cuttings. Z. Pflanzenphysiol. 66: 273–274

Kumar PP, Reid DM & Thorpe TA (1987) The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata in vitro*. Physiol. Plant. 69: 244–252

La Rue TAG & Gamborg OL (1971) Ethylene production by plant cell cultures: Variations in production growing cycle and in different plant species. Plant Physiol. 48: 394–398

Lau O & Yang SF (1976) Inhibition of ethylene production by cobaltous ion. Plant Physiol. 58: 114–117

Linkins AE, Lewis LN & Palmer RL (1973) Hormonally induced changes in stem and petiole anatomy and cellulase enzyme patterns in *Phaseolus vulgaris*. Plant Physiol. 52: 554–560

Mensuali-Sodi A, Tognoni F & Serra G (1990) Effects of ethylene on *in vitro* culture of tomato cotyledonary explants. In: Abstracts VIIth Intl. Congress on Plant Tissue and Cell Culture, Amsterdam, June 24–29, 1990: 282

Moncousin C, Favre J-M & Gaspar T (1989) Early changes in auxin and ethylene production in vine cuttings before adventitious rooting. Plant Cell Tiss. Org. Cult. 19: 243–256

Mudge K (1989) Effect of ethylene on rooting. In: Davis TD,

Haissig BE & Sankhla N (Eds) Adventitious Root Formation in Cuttings (pp 150–161). Dioscorides Press, Portland

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Panizza M, Mensuali-Sodi A & Tognoni F (1988) *In vitro* propagation of lavandin: Ethylene production during plant development. Acta Hortic. 227: 334–339

Pua EC, Chi GL & Barfield DG (1990) Effect of ethylene inhibitors on plant regeneration from seedling explants and somatic embryos of recalcitrant genotypes in *Cruciferae*. In: Abstracts VIIth Intl. Congress on Plant Tissue and Cell Culture, Amsterdam, June 24–29, 1990: 284

Rao PS, Handro W & Harada H (1973) Hormonal control of differentiation of shoots, roots and embryos in leaf and stem cultures of *Petunia inflata* and *Petunia hybrida* Physiol. Plant. 28: 458–463

Robbins JA, Kay SJ & Dirr MA (1983) Enhanced rooting of wounded mung bean cuttings by wounding and ethephon. J. Amer. Soc. Hort. Sci. 108: 325–329

Roy BN, Basu R & Bose TK (1972) Interaction of auxins with growth-retarding, -inhibiting, and ethylene producing chemicals in rooting of cuttings. Plant Cell Physiol. 12: 1123–1127

Sauerbrey E, Grossmann K & Jung J (1988) Ethylene production by sunflower cell suspensions. Plant Physiol. 87: 510–513

Thomas DS & Murashige T (1979) Volatile emissions of plant tissue cultures. *In Vitro* 15: 654–658

Van Aartrijk J, Blom-Barnhoorn GJ & Bruinsma J (1985) Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb *in vitro*: Effects of aminoethoxyvinyl-glycine, 1-aminocyclopropane-1-carboxylic acid, and ethylene. J. Plant Physiol. 117: 401–410

Williams J, Pink DAC & Biddington NL (1990) Effect of silver nitrate on long-term culture and regeneration of callus from *Brassica oleracea* var. 'gemmifera'. Plant Cell Tiss. Org. Cult. 21: 61–66