ADVENTITIOUS BUD FORMATION FROM BULB-SCALE EXPLANTS OF *LILIUM SPECIOSUM* THUNB. *IN VITRO*

J. van Aartrijk

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Promotor: dr. J. Bruinsma, hoogleraar in de fysiologie der planten

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Voorwoord

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Inhoud

Algemene inleiding

De vermeerdering van vrijwel alle geteelde soorten en rassen van het genus *Lilium* geschiedt vegetatief. Van oudsher is daarbij gebruik gemaakt van de karakteristieke eigenschap van lelieplanten bolletjes te vormen in de oksels van bladeren en van ondergrondse bladachtige plantedelen. Een snellere methode van vermeerdering, het zgn. schubben, is gebaseerd op de adventieve vorming van bolletjes aan de basis van afgebroken bolschubben (Boontjes et al. 1981). Deze methode kan resulteren in een vermeerderingsfactor van 50-100 per periode van drie jaren.

Nog snellere vermeerderingsmethoden zijn gewenst wanneer omvangrijke partijen moeten worden opgebouwd vanuit enkele waardevolle individuen, bijvoorbeeld virusvrij gemaakte planten (Asjes et al. 1974) of nieuwe genotypen. *In vitro* vermeerderingsmethoden zijn bij uitstek geschikt om aan deze wens tegemoet te komen.

Diverse organen van lelieplanten zijn in staat tot adventieve plantvorming *in vitro*. Voorbeelden hiervan zijn de stengel (Bigot 1974), de bladeren (Niimi en Onozawa 1979), de cotylen (Zimmerman en Ascher 1982), diverse bloem-delen (Coquen en Astié 1977, Takayama en Misawa 1979) en bolrokken (Stimart en Ascher 1978). Om een aantal praktische redenen werden explantaten van bolrokken gebruikt voor de in dit proefschrift te beschrijven experimenten.

Het proces van adventieve knopvorming vanuit planteweefsels *in vitro* wordt beïnvloed door tal van weefsel-, voedingsbodem- en omgevingsfactoren, die hun invloed veelal volgens een optimum-verloop uitoefenen (Murashige 1974, Thorpe 1980). De regeneratie vanuit bolweefsel van lelie vormt hierop geen uitzonde-ring. Dit proces wordt o.a. beïnvloed door de plaats van herkomst van het explantaat in de bol (Robb 1957), de oriëntatie van het explantaat op de voe-dingsbodem (Leshem et al. 1982), de voorafgaande bewaaromstandigheden van de bol (Stimart en Ascher 1981), het onderzochte ras (Van Aartrijk en Blom-Barn-hoorn 1981), de hoeveelheden sucrose en zouten in en de pH van de voedingsbodem (Takayama en Misawa 1979), de aanwezigheid, aard en hoeveelheid van groeiregula-toren (Stimart en Ascher 1978) en vitaminen (Van Aartrijk en Blom-Barnhoorn 1980), de kweektemperatuur (Stimart en Ascher 1981) en de lichtomstandigheden (Leshem et al. 1982).

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De effecten van de meeste factoren zijn onderzocht zonder daarbij de mogelijke samenhang ervan met andere in ogenschouw te nemen. Wanneer er interacties bestaan tussen de effecten van diverse factoren, zal de reproduceerbaarheid van de waargenomen effecten afhankelijk kunnen zijn van de, al of niet toevallig gekozen, overige proefomstandigheden. Inzicht in de samenhang van factoren kan derhalve zeer belangrijk zijn, enerzijds om de interpretatie van op het eerste oog tegenstrijdige literatuurgegevens te vergemakkelijken, anderzijds om het empirisch karakter van het *in vitro*-vermeerderingsonderzoek een meer theoretische basis te geven.

In dit proefschrift worden experimenten beschreven die tot doel hadden na te gaan hoe de effecten van een aantal factoren afhankelijk zijn van andere. Bovendien wordt een poging gedaan om de effecten van en de interacties tussen de onderzochte factoren te verklaren vanuit hun invloed op één proces. De vijf hoofdfactoren, die in het onderzoek werden betrokken, zijn:

1) de verwonding van het bolschubweefsel,
2) de auxine-concentratie in het voedingsmedium,
3) de concentratie van de auxine-transport-remmer 2,3,5-triiodobenzoëzuur (TIBA) in het voedingsmedium,
4) de kweektemperatuur en
5) de ethyleenvorming van het bolschubweefsel.

ad 1): Algemeen wordt aangenomen dat het proces van adventieve spruitvorming samenhangt met de reactie van planteweefsel op daarin aangebrachte verwondingen (Aitchison et al. 1977). Bewijzen hiervoor in systemen *in vitro* zijn echter schaars en blijven vnl. beperkt tot een beschrijving van de effecten van variaties in explantaatgrootte (Yeoman et al. 1968, Pierik en Post 1975).

ad 2): De groep van groeiregulatoren met auxine-activiteit speelt in systemen *in vitro* een belangrijke rol. In het algemeen geldt dat auxinen de wortelinductie stimuleren en de spruitvorming remmen. Een andere groep van groeiregulatoren, de cytokininen, bevordert daarentegen veelal de spruitinductie en remmen de wortelvorming (Skoog en Miller 1957, Murashige 1974, Thorpe 1980). Er zijn echter enkele uitzonderingen op deze regel. Deze zijn o.a. beschreven voor enkele bolgewassen uit de familie der Liliaceae (Pierik en Steegmans 1975, Hussey 1976). Toevoeging van auxine aan het voedingsmedium leidde bij deze gewassen tot stimulering van de spruitvorming.

Na complexvorming met specifieke receptoreiwitten kunnen auxinen de RNA-synthese stimuleren (Van der Linde et al. 1984) en celdelingen bevorderen

(Zeroni en Hall 1980).

ad 3): Het proces van adventieve spruitvorming vanuit diverse weefsels van lelie en andere Liliaceae vertoont een basipetale polariteit (Pierik en Ruibing 1973, Niimi en Onozawa 1979). Een dergelijke voorkeur van celdelingsloci voor het basale uiteinde van een explantaat is ook beschreven in andere systemen *in vitro* (Chlyah et al. 1975) en wordt in relatie gebracht met de basipetale transportrichting van auxinen in parenchymatische weefsels (Goldsmith 1977). Verantwoordelijk voor dit polaire transport zijn specifieke carrier-eiwitten, die vnl. gelocaliseerd zijn in de basale celmembraan en die het vermogen hebben auxine-anionen uit de cel te 'pompen' (Jacobs en Gilbert 1983). TIBA is in staat de werking van dit membraansysteem en dus van het basipetale auxine-transport te remmen (Goldsmith 1977). Aangezien de opname van auxine in plantecellen tenminste gedeeltelijk berust op een passief proces, zal remming van het basipetale transport door TIBA kunnen leiden tot intracellulaire accumulatie van auxine (Goldsmith 1982). Literatuurgegevens over de werking van TIBA in vermeerderingssystemen *in vitro* zijn schaars. Bij toepassing leidde het o.a. tot een onderdrukking van de basipetale polariteit van het regeneratie-proces (Pilet 1967).

ad 4): De factor temperatuur speelt een belangrijke rol in alle teeltstadia van het gewas lelie. Het proces van adventieve spruitvorming vormt hierop geen uitzondering (Boontjes et al. 1981). Studies over de effecten van temperatuur op regeneratieprocessen *in vitro* zijn relatief schaars. In het algemeen worden onderzoekingen uitgevoerd in kweekruimten waar een constante temperatuur van $25^{\circ}C$ heerst (Thorpe 1980), hoewel Skoog (1944) reeds aantoonde dat een temperatuur van $18^{\circ}C$ optimaal was voor adventieve spruitvorming bij tabaks-callus.

Binnen het onderzochte temperatuurtraject van $15^{\circ}-27^{\circ}C$ stimuleerde een lagere temperatuur het aantal adventieve spruiten dat *in vivo* gevormd werd op bladstekken van *Begonia cheimantha*, vermoedelijk als gevolg van een beïnvloeding van de endogene concentraties van groeiregulatoren door de temperatuur (Heide 1965). Yeoman et al. (1968) onderzochten het effect van de temperatuur op de celdelingsactiviteit in explantaten van opslagweefsel van *Helianthus tuberosus*. De toename in het aantal cellen gedurende de eerste dagen van de kweekperiode verliep exponentieel voor alle onderzochte temperaturen. Binnen het traject $10^{\circ}-35^{\circ}C$ leidde $30^{\circ}C$ tot de grootste toename in het aantal cellen per tijds-eenheid.

ad 5): In de loop van het in dit proefschrift te beschrijven onderzoek werd

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duidelijk dat de factoren verwonding, auxine, TIBA en temperatuur een zeer overeenkomstige invloed uitoefenen op het regeneratie-proces. Bovendien werden interacties aangetoond tussen de effecten van deze factoren. Deze gegevens leiden tot het vermoeden dat deze factoren inwerken op één centraal fysiologisch proces. Het proces van de ethyleensynthese voldeed in een aantal opzichten aan de eisen die aan zo'n centraal proces gesteld moeten worden.

In de afgelopen jaren is veel bekend geworden over de wijze waarop de ethyleensynthese in planteweefsels verloopt. Algemeen wordt nu aangenomen dat de syntheseroute verloopt vanuit methionine (Yang 1974) via S-adenosylmethionine (SAM; Adams en Yang 1977) en 1-aminocyclopropaan-1-carbonzuur (ACC; Adams en Yang 1979, Lürssen et al. 1979) naar ethyleen. Schematisch:

$$\text{methionine} \longrightarrow \text{SAM} \longrightarrow \text{ACC} \longrightarrow \text{ethyleen.}$$

Van de bestudeerde factoren verwonding en auxine is bekend dat zij de omzettingscapaciteit van SAM naar ACC beïnvloeden via *de novo* synthese van het betrokken enzym, het ACC-synthase (Yu en Yang 1979, Konze en Kwiatkowski 1981). Aminoethoxyvinyl-glycine (AVG) remt de werking van dit enzym (Yu en Yang 1979) en derhalve de ethyleensynthese.

Het mechanisme van de laatste, vrijwel zeker membraangebonden stap van de ethyleensynthese, de omzetting van ACC in ethyleen, is nog niet geheel opgehelderd (Bousquet en Thimann 1984). Wel is o.a. bekend dat deze stap temperatuurgevoelig is en geremd wordt boven circa $30^{\circ}C$ (Yu et al. 1980).

Ethyleen kan, evenals TIBA, het basipetale transport van auxine remmen (Burg en Burg 1967, Osborne en Mullins 1969, Goldsmith 1977).

Literatuur

AARTRIJK, J. VAN, G.J. BLOM-BARNHOORN: Effects of sucrose, mineral salts, and some organic substances on the adventitious regeneration *in vitro* of plantlets from bulb-scale tissue of *Lilium speciosum* Rubrum. Acta Hortic. 109, 297-302 (1980).

AARTRIJK, J. VAN, G.J. BLOM-BARNHOORN: Growth regulator requirements for adventitious regeneration from *Lilium* bulb-scale tissue *in vitro*, in relation to duration of bulb storage and cultivar. Scientia Hortic. 14,261-268 (1981).

ADAMS, D.O., S.F. YANG: Methionine metabolism in apple tissue:implication of S-adenosylmethionine as an intermediate in the conversion of methionine to ethylene. Plant Physiol. 60,892-896 (1977).

ADAMS, D.O., S.F. YANG: Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Natl. Acad. Sci. USA 76,170-174 (1979).

AITCHISON, P.A., A.J. MCLEOD, M.M. YEOMAN: Growth patterns in tissue (callus) cultures. In: STREET, H.E. (ed.): Plant tissue and cell culture, pp. 267-306, Blackwell Scientific Publications, Oxford (1977).

ASJES, C.J., M.H. BUNT, D.H.M. VAN SLOGTEREN: Production of hyacinth mosaic virusfree hyacinths and lily symptomless virusfree lilies by meristem tip culture. Acta Hortic. 36, 223-228 (1974).

BIGOT, C.: Comparaison des aptitudes pour le bourgeonnement de tissus superficiels et de tissus profonds cultivés *in vitro*. Cas de la tige d'un lis hybride, cv. 'Enchantment'. C.R. Acad. Sci. (série D) 278,1027-1030 (1974).

BOONTJES, J., C. KRUYER, P. MANTEL: Wijziging van de temperatuurbehandeling van lelieschubben. Rapport nr. 47, L.B.O., Lisse (1981).

BOUSQUET, J-F., K.V. THIMANN: Lipid peroxidation forms ethylene from 1-aminocyclopropane-1-carboxylic acid and may operate in leaf senescence. Proc. Natl. Acad. Sci. USA 81,1724-1727 (1984).

BURG, S.P, E.A. BURG: Inhibition of polar auxin transport by ethylene. Plant Physiol. 42,1224-1228 (1967).

CHLYAH, H., M. TRAN THANH VAN, Y. DEMARLY: Distribution pattern of cell division centers on the epidermis of stem segments of *Torenia fournieri* during *de novo* bud formation. Plant Physiol. 56,28-33 (1975).

COQUEN, C., M. ASTIÉ: Ontogenèse de bulbilles végétatives obtenues par culture de pièces florales isolées chez le *Lilium candidum* L. Bull. Soc. bot. Fr. 124,51-59 (1977).

GOLDSMITH, M.H.M.: The polar transport of auxin. Ann. Rev. Plant Physiol. 28, 439-478 (1977).

GOLDSMITH, M.H.M.: A saturable site responsible for polar transport of indole--3-acetic acid in sections of maize coleoptiles. Planta 155,68-75 (1982).

HEIDE, O.M.: Interaction of temperature, auxins, and kinins in the regeneration ability of *Begonia* leaf cuttings. Physiol. Plant. 18,891-920 (1965).

HUSSEY, G.: Plantlet regeneration from callus and parent tissue in *Ornithogalum thyrsoides*. J. Exp. Bot. 27,375-382 (1976).

JACOBS, M., S.F. GILBERT: Basal localization of the presumptive auxin transport carrier in pea stem cells. Science 220,1297-1300 (1983).

KONZE, J.R., G.M.K. KWIATKOWSKI: Rapidly induced ethylene formation after wounding is controlled by the regulation of 1-aminocyclopropane-1-carboxylic acid synthesis. Planta 151,327-330 (1981).

LESHEM, B., H. LILIEN-KIPNIS, E. STEINITZ: The effect of light and of explant orientation on the regeneration and subsequent growth of bulblets on *Lilium longiflorum* Thunb. bulb-scale sections cultured *in vitro*. Scientia Hortic. 17,129-136 (1982).

LINDE, P.C.G. VAN DER, H. BOUMAN, A.M. MENNES, K.R. LIBBENGA: A soluble auxin-binding protein from cultured tobacco tissues stimulates RNA synthesis *in vitro*. Planta 160,102-108 (1984).

LÜRSSEN, K., K. NAUMANN, R. SCHRÖDER: 1-aminocyclopropane-1-carboxylic acid-- an intermediate of ethylene biosynthesis in higher plants. Z. Pflanzenphysiol. 92,285-294 (1979).

MURASHIGE, T.: Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25,135-166 (1974).

NIIMI, Y., T. ONOZAWA: *In vitro* bulblet formation from leaf segments of lilies, especially *Lilium rubellum* Baker. Scientia Hortic. 11,379-389 (1979).

OSBORNE, D.J., M.G. MULLINS: Auxin, ethylene, and kinetin in a carrier-protein model system for the polar transport of auxins in petiole segments of *Phaseolus vulgaris*. New Phytol. 68,977-991 (1969).

PIERIK, R.L.M., A.J.M. POST: Rapid vegetative propagation of *Hyacinthus orientalis* L. *in vitro*. Scientia Hortic. 3, 293-298 (1975).

PIERIK, R.L.M., M.A. RUIBING: Regeneration of bulblets on bulb-scale segments of hyacinth *in vitro*. Neth. J. agric. Sci. 21,129-138 (1973).

PIERIK, R.L.M., H.H.M. STEEGMANS:Effect of auxins, cytokinins, gibberellins, ABA, and ethephon on regeneration of bulblets on excised bulb-scale segments of hyacinth. Physiol. Plant. 34,14-17 (1975).

PILET, P.E.: Auxin content and auxin catabolism in relation to the growth polarity. Physiol. Plant. 20,285-290 (1967).

ROBB, S.M.: The culture of excised tissue from bulb scales of *Lilium speciosum* Thunb. J. Exp. Bot. 8,348-352 (1957).

SKOOG, F.: Growth and organ formation in tobacco tissue cultures. Amer. J. Bot. 31,19-24 (1944).

SKOOG, F., C.O. MILLER: Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11,118-130 (1957).

STIMART, D.P., P.D. ASCHER: Tissue culture of bulb-scale sections for asexual propagation of *Lilium longiflorum* Thunb. J. Amer. Soc. Hortic. Sci. 103, 182-184 (1978).

STIMART, D.P., P.D. ASCHER: Developmental responses of *Lilium longiflorum* bulblets to constant or alternating temperatures *in vitro*. J. Amer. Soc. Hortic. Sci. 106,450-454 (1981).

TAKAYAMA, S., M. MISAWA: Differentiation in *Lilium* bulbscales grown *in vitro*. Effect of various cultural conditions. Physiol. Plant. 46,184-190 (1979).

THORPE, T.A.: Organogenesis *in vitro*: structural, physiological, and biochemical aspects. Int. Rev. Cytology, suppl. 11A,71-111 (1980).

YANG, S.F.: The biochemistry of ethylene biogenesis and metabolism. In: RUNECKLES, V.C. et al. (eds.): The chemistry and biochemistry of plant hormones. Recent advances in phytochemistry, vol. 7, pp. 131-164, Academic Press, New York (1974).

YEOMAN, M.M., G.G. NAIK, A.I. ROBERTSON: Growth and differentiation of plant tissue cultures. III. The initiation and pattern of cell division in developing callus cultures. Ann. Bot. 32,301-313 (1968).

YU, Y-B., S.F. YANG: Auxin-induced ethylene production and its inhibition by aminoethoxyvinyl glycine and cobalt ion. Plant Physiol. 64,1074-1077 (1979).

YU, Y-B., D.O. ADAMS, S.F. YANG: Inhibition of ethylene production by 2,4--Dinitrophenol and high temperature. Plant Physiol. 66,286-290 (1980).

ZERONI, M., M.A. HALL: Molecular effects of hormone treatment on tissue. In: MACMILLAN, J. (ed.): Hormonal regulation of development I. Springer Verlag, Berlin (1980).

ZIMMERMAN, T.W., P.D. ASCHER: Tissue culture of the lily embryo cotyledon. HortScience 17,104 (1982).

HOOFDSTUK I

Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*.
Effects of NAA and cytokinins[★]

J. VAN AARTRIJK and G.J. BLOM-BARNHOORN

Bulb Research Centre, 2160 AB   Lisse, The Netherlands

Summary

Effects were studied of the auxin 1-naphthylacetic acid (NAA), the cytokinins N^6-benzyladenine (BA) and N^6-[$\triangle 2$-isopentenyl]-adenine (2iP), and of prior bulb storage at $0^\circ C$, on the process of adventitious bud formation from bulb-scale tissue of *Lilium speciosum* Thunb., cv. Rubrum nr. 10.

Addition of NAA to the nutrient medium influenced the average number of plantlets per explant following an optimum curve. The auxin suppressed the normally basipetal polarity of the regeneration process. The optimal NAA concentration depended on the duration of cold storage of the mother bulbs and on the site of regeneration on the explants, cold-stored tissue and 'basal' sites requiring less auxin than uncooled tissue and 'non-basal' sites.

The cytokinins BA and 2iP did not promote adventitious bud formation.

Introduction

During the last decade, *Lilium* plants free of lily symptomless virus (LSV) were produced in The Netherlands with the technique of meristem culture (Asjes et al. 1974, Van Aartrijk and Blom-Barnhoorn 1979). For a rapid and effective introduction of these LSV-free lilies and of newly bred varieties into commercial culture, a method for rapid vegetative propagation *in vitro* became highly desirable. Several organs of *Lilium* plants have been shown to possess regenerative capability *in vitro*, e.g., bulb scales (Robb 1957, Stimart and Ascher 1978), leaves (Niimi and Onozawa 1979), cotyledons (Zimmerman and Ascher 1982), stem segments (Bigot 1974), and flower parts (Coquen and Astié 1977). For our experiments on rapid vegetative propagation of *Lilium speciosum* Thunb., we chose bulb scales as the explant source.

It is well known that the process of adventitious bud formation can be controlled, at least partially, by an exogenous supply of plant growth regulators, especially auxins and cytokinins (Skoog and Miller 1957, Murashige 1974). In this paper we report on the effects of exogenous auxin and cytoki-

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Abbreviations: NAA: 1-naphthylacetic acid, BA: N^6-benzyladenine, 2iP: N^6-[$\triangle 2$-isopentenyl]-adenine.

★ Some of the data mentioned in this chapter have been reported previously (Van Aartrijk and Blom-Barnhoorn 1981).

nins on the process of adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, *in vitro*.

In view of the basipetal polarity of the process of regeneration from lily scales *in vivo* (Hartmann and Kester 1959, Rees 1972), from lily leaves *in vitro* (Niimi and Onozawa 1979), and from bulb-scale tissue of other members of the Liliaceae *in vitro* (e.g., Pierik and Ruibing 1973), we investigated the polarity of the regeneration sites on the explants, too.

Lily bulbs can be stored at 0° for at least a year. Because it has been suggested that cold treatment induces changes in the endogenous plant growth regulator activity of bulbs (Tsukamoto 1971, 1974, Rudnicki and Nowak 1976), we also investigated the influence of the duration of cold storage (at 0^oC) of the bulbs on the requirement for exogenous NAA.

Materials and Methods

Plant material: Bulbs of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, circumference 18-20 cm, were harvested in the autumn of 1976, 1977, 1979, and 1981, treated with 0.2% Benlate (active ingredient 50% benomyl) and 1.5% Difolatan (a.i. 48% captafol) for 30 min, and stored at 0^oC until use. Unless stated otherwise, the experiments were performed in the period from April to September with bulbs harvested in the autumn of the year before.

Explants: The outermost scales of the bulbs and the innermost small and leafy scales were discarded. For the experiments, 10-12 scales were taken from each bulb, carefully distributed over the various treatments, disinfected in 1% NaOCl for 30 min, and rinsed twice with sterile water. Two to four explants measuring 7 x 7 mm^2 were cut aseptically from the basal part of each scale, and each explant was placed alone, with the abaxial side down, on 15 ml nutrient medium in a glass tube (150 x 25 mm) provided with a transparent plastic cap (Bellco, Vineland, U.S.A.).

Nutrient media: The media consisted of MS macro- and micro-elements (Murashige and Skoog 1962) enriched with 0.4 mgl^{-1} thiamin (BDH Chem. Ltd.), 100 mgl^{-1} meso-inositol (BDH Chem. Ltd.), 30 gl^{-1} sucrose (J.T. Baker Chem.), 6 gl^{-1} agar (Difco-Noble), and various concentrations of the auxin NAA (BDH, 0.25-25.0 µM), and/or the cytokinins BA (BDH, 0.044-0.44 µM) or 2iP (Sigma Chem. Co., 1.5-15.0 µM). The pH of the medium was 6.0 before 20 min autoclaving at 120^oC.

The number of cultures per treatment was 46. They were incubated in a controlled-temperature room at 20^oC, with a light intensity of 27.9 µEm^{-2}. sec^{-1} (Philips 25W/TL33; 16h photoperiod). After 10 weeks of culture, the polarity and/or the number of plantlets per explant were determined. Plantlets developing at the basal (proximal) end of the explants were designated basal plantlets; all others were termed non-basal. The findings were confirmed by the results of at least one similar experiment. For each result the standard deviation of the mean is reported. Differences were tested for significance (p = 0.05).

Results

The effect of various concentrations of NAA and BA on the average number of

adventitiously formed plantlets per explant is shown in Table 1. Bulbs used in this experiment were stored at $0^{\circ}C$ for c. 10 months. Within the range of concentrations used, BA did not influence this number. A higher concentration of the cytokinin (4.4 µM) did not affect plantlet number either, but plantlet growth was severely reduced (data not shown). Substitution of BA by 2iP did not stimulate plantlet regeneration. A high concentration of this natural cytokinin (15 µM) even inhibited the process, in the presence of 0.5 µM NAA (Table 2).

Table 1: The effect of various concentrations of NAA and BA on the average number of plantlets formed per incubated explant. Temperature was $20^{\circ}C$.

| NAA (µM) | BA (µM) | | | | |
	0.00	0.044	0.22	0.44	Average
0.0	2.7 ± 0.2^{x}	2.1 ± 0.2	2.6 ± 0.3	3.1 ± 0.6	2.6 ± 0.2
0.5	4.9 ± 0.3	4.1 ± 0.4	5.0 ± 0.3	4.2 ± 0.2	4.6 ± 0.1
2.5	3.9 ± 0.4	4.0 ± 0.3	3.0 ± 0.3	3.0 ± 0.3	3.5 ± 0.2
5.0	3.4 ± 0.4	2.9 ± 0.3	3.2 ± 0.5	2.5 ± 0.4	3.0 ± 0.2
Average	3.7 ± 0.2	3.3 ± 0.2	3.5 ± 0.2	3.4 ± 0.2	3.5 ± 0.2

^{x} Standard error of the mean

Table 2: The effect of various concentrations of NAA and 2iP on the average number of plantlets formed per incubated explant. Temperature was $20^{\circ}C$.

| NAA (µM) | 2iP (µM) | | | |
	0.00	1.50	15.00	Average
0.0	2.2 ± 0.2^{x}	3.1 ± 0.3	2.7 ± 0.2	2.6 ± 0.1
0.5	4.7 ± 0.3	4.3 ± 0.2	3.5 ± 0.3	4.2 ± 0.2
Average	3.5 ± 0.2	3.8 ± 0.2	3.1 ± 0.2	3.5 ± 0.2

^{x} Standard error of the mean

On media without NAA, plantlets regenerated predominantly at basal sites (Fig. 1). Basal and non-basal sites responded differently to NAA, the former having a lower optimal NAA concentration than the latter (Fig. 1). At higher NAA concentrations, the basipetal polarity of the regeneration process was suppressed (Fig. 1). Bulbs used in this experiment had been stored at $0^{\circ}C$

for c. 6 months.

Prolonged cold storage of the bulbs (at $0°C$) increased the sensitivity of the scale explants to NAA (Fig. 2). The amount of NAA required to obtain optimal regeneration tended to decrease from 2.5 μM for uncooled bulbs to 0.25 μM for bulbs stored for 12 months.
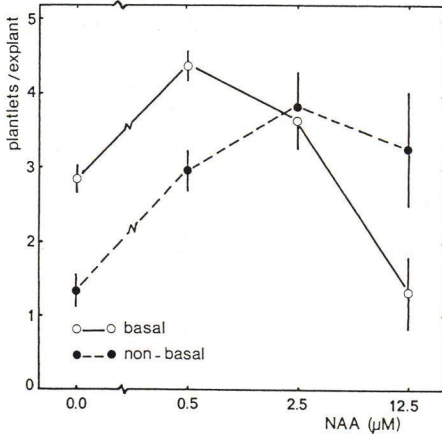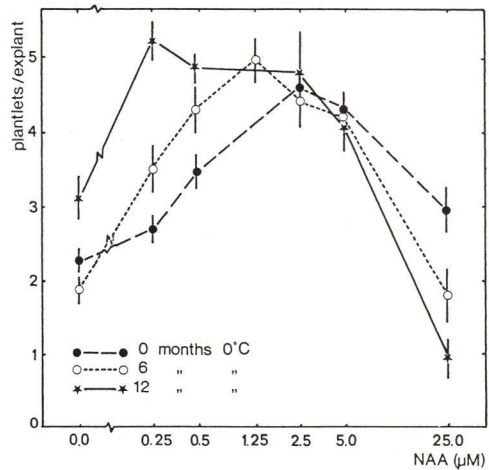

Fig. 1: The average number of basal and non-basal plantlets per explant as a function of the NAA concentration in the nutrient medium. Temperature was $20°C$.

Fig. 2: Effect of the duration of cold storage (at $0°C$) of bulbs on the NAA demand for adventitious bud formation from bulb-scale explants *in vitro*.

Discussion

The auxin NAA had distinct effects on the average number of plantlets per explant. This agrees well with findings of Stimart and Ascher (1978) and of Leshem et al. (1982), both working with *L. longiflorum*.

Under the conditions used, an exogenous supply of the cytokinins BA or

2iP did not stimulate adventitious bud formation from bulb-scale tissue of *L. speciosum* Thunb. A high concentration of 2iP inhibited bud regeneration in the presence of NAA (Table 2). Both cytokinins induced aberrant growth of the regenerated plantlets, even at the lowest concentrations used (Van Aartrijk and Blom-Barnhoorn 1981), which shows that these growth regulators were biologically active. Stimart and Ascher (1978) described a similar inability of the cytokinins 2iP and kinetin to stimulate plantlet regeneration from bulb-scale tissue. The relative insensitivity of this process to cytokinins and the stimulatory effect of low NAA concentrations do not fit well into the classic concept of organ formation as originally stated by Skoog and Miller (1957). This deviating behaviour was also found in studies *in vitro* on storage tissues of other species (Pierik and Steegmans 1975, Hussey 1976, 1982, Yeoman and McLeod 1976). To explain the results they obtained with hyacinth tissue, Pierik and Steegmans (1975) assumed a high endogenous cytokinin level in their explants. Evidence against a similar assumption for our lily system may be provided by the formation of adventitious roots in the absence of NAA and the inability of the cytokinins to stimulate the regeneration process at supra-optimal NAA concentrations.

The auxin NAA acted according to an optimum curve, low concentrations having a stimulatory and high concentrations (>0.5 µM) an inhibitory effect. Inhibitory effects of high auxin concentrations have generally been related to auxin-induced ethylene synthesis (Lieberman 1979). Whether ethylene is involved in this regeneration process is dealt with in chapters IV and V of this thesis.

This process of adventitious bud formation from *Lilium* bulb-scale tissue *in vitro* shows an essentially basipetal polarity (Van Aartrijk and Blom-Barnhoorn 1983). Addition of the auxin NAA to the nutrient medium suppressed this polarity (Fig. 1), the effect depending on the concentration. It cannot be excluded that auxin-induced ethylene production was responsible for this effect, since this gas is known to inhibit basipetal auxin transport (Osborne and Mullins 1969, Goldsmith 1977). The essentially basipetal polarity of the regeneration process and the effects of exogenous auxin suggest that an important role is played by auxin and its distribution within the tissue, as already postulated by Pilet (1977), and Cassells et al. (1982).

Prolonged duration of cold storage of the lily bulbs led to a lower auxin requirement for optimal adventitious bud formation (Fig. 2). Similarly, the NAA optima differed for basal and non-basal regeneration on the bulb-scale explants (Fig. 1). These facts strongly suggest that auxin interacts with

some other endogenous factor(s) to influence this regeneration process. The shift in NAA optimum,induced by cold storage of the bulbs, might suggest a modulation of auxin action by the low-temperature treatment, either by an increase of the endogenous auxin level, or by a decrease of the level of auxin antagonist(s), or by changes in the affinity of the tissue for auxin(s) or auxin antagonist(s).

It is not known whether there is a relationship between the remarkably low endogenous IAA level of our lily tissue, (see Chapter III of this thesis), the inability of cytokinins to stimulate bud regeneration from this tissue, and the stimulatory action of NAA on this process.

Acknowledgements

References

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Some influences of α-naphthylacetic acid on the differentiation of meristems of *Lilium speciosum* 'Rubrum' nr. 10 *in vitro*. Acta Hortic. 91, 269-279 (1979).

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Growth regulator requirements for adventitious regeneration from *Lilium* bulb-scale tissue *in vitro*, in relation to duration of bulb storage and cultivar. Scientia Hortic. 14, 261-268 (1981).

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*. Effects of wounding, TIBA, and temperature. Z. Pflanzenphysiol. 110, 355-363 (1983).

ASJES, C.J., M.H. BUNT and D.H.M. VAN SLOGTEREN: Production of hyacinth mosaic virus-free hyacinths and lily symptomless virus-free lilies by meristem tip culture. Acta Hortic. 36, 223-228 (1974).

BIGOT, C.: Comparaison des aptitudes pour le bourgeonnement de tissus superficiels et de tissus profonds cultivés *in vitro*. Cas de la tige d'un lis hybride, cv. 'Enchantment'. C.R. Acad. Sci. 278, 1027-1030 (1974).

CASSELS, A.C., R.D. LONG and D.M.A. MOUSDALE: Endogenous IAA and morphogenesis in tobacco petiole cultures. Physiol. Plant. 56, 507-512 (1982).

COQUEN, C. and M. ASTIÉ: Ontogenèse de bulbilles végétatives obtenus par culture de pièces florales isolées chez le *Lilium candidum*. L. Bull. Soc. bot. Fr. 124, 51-59 (1977).

GOLDSMITH, M.H.M.: The polar transport of auxin. Ann. Rev. Plant Physiol. 28, 439-478 (1977).

HARTMANN, H.T. and D.E. KESTER: Plant propagation, principles and practices. Prentice-Hall Inc., Englewood Cliffs, New Jersey (1959).

HUSSEY, G.: Plantlet regeneration from callus and parent tissue in *Ornithogalum thyrsoïdes*. J. Exp. Bot. 27, 375-382 (1976).

HUSSEY, G.: *In vitro* propagation of *Narcissus*. Ann. Bot. 49, 707-719 (1982).

LESHEM, B., H. LILIEN-KIPNIS and B. STEINITZ: The effect of light and of
     explant orientation on the regeneration and subsequent growth of bulblets
     on *Lilium longiflorum* Thunb. bulb-scale sections cultured *in vitro*. Scientia
     Hortic. 17, 129-136 (1982).
LIEBERMAN, M.: Biosynthesis and action of ethylene. Ann. Rev. Plant Physiol.
     30, 533-591 (1979).
MURASHIGE, T. and F. SKOOG: A revised medium for rapid growth and bioassays
     with tobacco tissue cultures. Physiol. Plant. 15, 473-497 (1962).
MURASHIGE, T.: Plant propagation through tissue cultures. Ann. Rev. Plant
     Physiol. 25, 135-166 (1974).
NIIMI, Y. and T. ONOZAWA: *In vitro* bulblet formation from leaf segments of
     lilies, especially *Lilium rubellum* Baker. Scientia Hortic. 11, 379-389 (1979).
OSBORNE, D.J. and M.G. MULLINS: Auxin, ethylene and kinetin in a carrier-protein
     model system for the polar transport of auxins in petiole segments of
     Phaseolus vulgaris. New Phytol. 68, 977-991 (1969).
PIERIK, R.L.M. and M.A. RUIBING: Regeneration of bulblets on bulb-scale segments
     of hyacinth *in vitro*. Neth. J. agric. Sci. 21, 129-138 (1973).
PIERIK, R.L.M. and H.H.M. STEEGMANS: Effect of auxins, cytokinins, gibberellins,
     abscisic acid and ethephon on excised bulb-scale segments of hyacinth.
     Physiol. Plant. 34, 14-17 (1975).
PILET, P.E.: Auxin content and auxin catabolism in relation to the growth
     polarity. Physiol. Plant. 20, 285-290 (1967).
REES, A.R.: The growth of bulbs. Acad. Press., London (1972).
ROBB, S.M.: The culture of excised tissue from bulb scales of *Lilium speciosum*
     Thunb. J. Exp. Bot. 8, 348-352 (1957).
RUDNICKI, R.M. and J. NOWAK: Studies on the physiology of hyacinth bulbs. J. Exp.
     Bot. 27, 303-313 (1976).
SKOOG, F. and C.O. MILLER: Chemical regulation of growth and organ formation in
     plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11, 118-130 (1957).
STIMART, D.P. and P.D. ASCHER: Tissue culture of bulb-scale sections for asexual
     propagation of *Lilium longiflorum* Thunb. J. Amer. Soc. Hortic. Sci. 103,
     182-184 (1978).
TSUKAMOTO, Y.: Changes of endogenous growth substances in Easter lily as
     affected by cooling. Acta Hortic. 23, 75-81 (1971)
TSUKAMOTO, Y.: Changes in endogenous regulators and dormancy in plants.
     Proc. XIX Int. Hortic. Congr., Warszawa, vol. 4, p. 293-296 (1974).
YEOMAN, M.M. and A.J. MACLEOD: Tissue (callus) cultures - techniques. In: STREET,
     H.E. (ed.): Plant Tissue and Cell Culture, p. 31-59, Blackwell Scientific
     Publications, London (1977).
ZIMMERMAN, T.W. and P.D. ASCHER: Tissue culture of the lily embryo cotyledon.
     HortScience 17, 104 (abstract) (1982).

HOOFDSTUK II

Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*.
Effects of wounding, TIBA, and temperature

J. VAN AARTRIJK and G. J. BLOM-BARNHOORN

Bulb Research Centre, NL-2160 AB Lisse, The Netherlands

Summary

Effects of tissue wounding, TIBA, and temperature on the process of adventitious bud formation *in vitro* from bulb-scale explants of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, were studied.

Removal of the abaxial epidermis from the explants led to an increase in the number of plantlets formed at the adaxial surface and to suppression of the normally basipetal polarity of the regeneration process. It is concluded that the actual wounding itself was responsible for these effects and that wounding must have given rise to a transmittable stimulus.

When incorporated into the nutrient medium, TIBA had an effect similar to that of wounding: the number of plantlets per explant increased and the polarity was suppressed. However, at concentrations higher than 2.10^{-6} M, TIBA inhibited the regeneration process.

Within the range of 15°–25 °C, a rise of the temperature led to more plantlets per explant and to (partial) suppression of basipetal polarity. The results are discussed with special attention to the similarity in the action of these three factors.

Key words: Lilium speciosum, adventitious bud formation, bulbscale, wounding, TIBA, temperature, auxin transport.

Introduction

Methods for rapid vegetative propagation can be valuable tools in horticulture. For lilies such a method, based on repeated adventitious bud formation from bulb-scale tissue, has been developed (Van Aartrijk and Blom-Barnhoorn 1981).

Many factors are known to influence this process, including tissue factors and chemical and physical growing conditions (Robb 1957, Stimart and Ascher 1978, Takayama and Misawa 1979, Van Aartrijk and Blom-Barnhoorn 1980). We recently reported on the effects of plant growth regulators on this process (Van Aartrijk and Blom-Barnhoorn 1981).

This paper reports a study on the effects of tissue wounding, TIBA, and temperature on the number of regenerating plantlets per explant of *Lilium speciosum* Thunb., cv. Rubrum nr. 10. In view of the effect of the auxin, NAA, on this process (Van Aartrijk and Blom-Barnhoorn 1981) and the basipetal character of auxin transport in

Abbreviations: NAA: 1-Naphthylacetic acid, TIBA: 2,3,5-Triiodobenzoic acid.

parenchymous tissues (Goldsmith 1977), the polarity of the regeneration sites on the explants was also studied.

Materials and Methods

Plant material: Bulbs of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, (circumference 18–20 cm) were harvested in the autumns of 1978, 1979, and 1980, treated with 0.2 % Benlate (active ingredient 50 % benomyl) and 1.5 % Difolatan (a.i. 48 % captafol) for 30 min, and stored at 0 °C until use. Unless stated otherwise, the experiments were performed in the period April to August of a year using bulbs harvested in the autumn of the year before.

Explants: The outermost scales of the bulbs were discarded. For the experiments 10–12 scales were taken from each bulb. The innermost small and leafy scales were not used. The scales were carefully distributed over the various treatments (in order to avoid that differences between individual bulbs and between scales within one bulb influenced the experiments), disinfected in 1 % NaOCl for 30 min, and rinsed twice in sterile water. Unless stated otherwise, two to four explants measuring 7×7 mm were cut aseptically from the basal part of each scale and each explant was placed with the abaxial side down on 15 ml nutrient medium in a glass tube (150×25 mm), provided with a transparent plastic cap (Bellco, U.S.A.). One explant was used per tube.

Nutrient media: The basal nutrient medium consisted of MS macro- and micro-elements (Murashige and Skoog 1962) enriched with 0.4 mg l^{-1} thiamin (BDH Chem. Ltd.), 100 mg l^{-1} meso-inositol (BDH Chem. Ltd.), 30 g l^{-1} sucrose (J. T. Baker Chem.), 6 g l^{-1} agar (Difco-Noble) and 0.5 μM NAA (BDH Chem. Ltd.). The pH of the medium was 6.0 before autoclaving (20 min at 120 °C).

Wounding experiments: Additional wounding of explants was performed by aseptically cutting away the abaxial epidermis and the outermost sub-epidermal layers. Such explants were designated wounded explants. Others are termed normal. In order to analyze the effects of additional wounding experiments were performed:
- with wounded or normal explants placed with their abaxial side on media varying in medium strength and/or NAA-content. All medium constituents except agar and NAA (which was varied separately) were varied. The pH was kept at 6.0.

  This experiment was carried out in the early spring of 1979. Because of the interaction between duration of cold storage of the bulbs and the demand for exogenous NAA (Van Aartrijk and Blom-Barnhoorn 1981), the NAA concentration of the control medium was chosen 1.0 μM NAA.
- with wounded or normal explants placed vertically with their apical end on basal medium, and
- with normal explants of various sizes (15×15 mm, 15×7.5 mm, 7.5×7.5 mm, 5×5 mm) incubated with their abaxial side on basal medium.

TIBA experiments: Normal explants (7×7 mm) were placed with their abaxial side on a basal medium (or on a similar medium without NAA) supplied with TIBA (Serva Chem.) in concentrations varying between 2.10^{-8} M and 2.10^{-5} M. TIBA was co-autoclaved.

The cultures were incubated in controlled temperature rooms. Standard temperature was 20 °C. For the temperature experiments similar rooms held constantly at 15 °C or 25 °C were used. The fluorescent light intensity in all growing rooms amounted to 5.2 W m^{-2} (420–680 nm, Philips 25 W/TL 33; 16 h photoperiod).

The number of cultures per treatment was 46. After 10 weeks of culture, the percentage of regenerating explants and the number and polarity of plantlets per explant were determined. Those plantlets developing at the basal (proximal) end of the explants were designated basal plantlets. All others were termed non-basal. The reported data are confirmed by the results of at least one similar experiment. For each result the standard deviation of the mean is reported. Differences were tested for significance (p = 0.01).

Results

Wounding of tissue

Removal of the outermost abaxial cell layers of the explants led to an increase in the average number of buds per explant (Fig. 1). This effect was also found on media containing more nutrients (Table 1). An interaction between removal of the abaxial cell layers and the concentration of nutrients seems to exist (see treatments 1 and 3 of Table 1). Wounded explants placed vertically with the apical end on the medium also produced more plantlets than similarly incubated, normal explants (Fig. 1). Simply
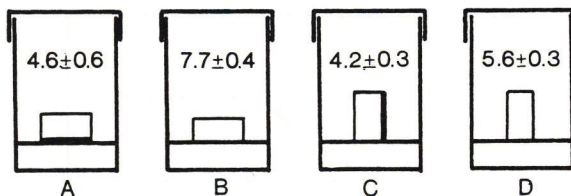

Fig. 1: Average number of plantlets on normal explants (A and C) or wounded (B and D) explants, placed on the medium with the abaxial side (A and B) or the apical end (C and D) down. Temperature was 20 °C. All explants formed plantlets.
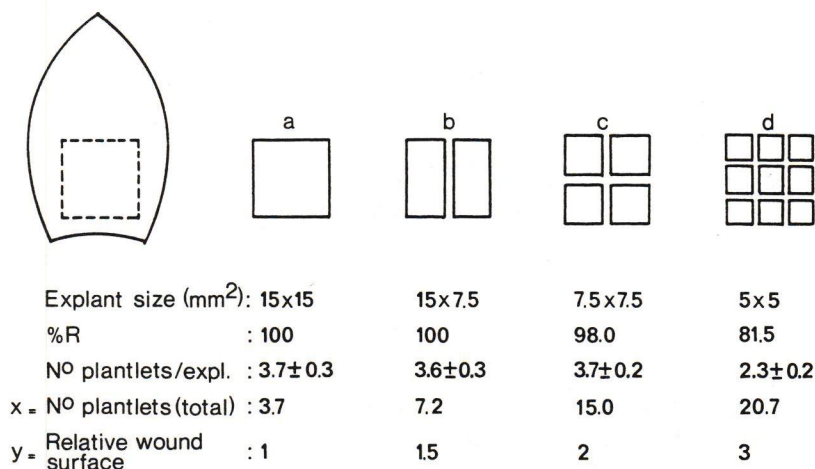

	a	b	c	d
Explant size (mm^2):	15×15	15×7.5	7.5×7.5	5×5
%R :	100	100	98.0	81.5
N^o plantlets/expl. :	3.7±0.3	3.6±0.3	3.7±0.2	2.3±0.2
x = N^o plantlets (total) :	3.7	7.2	15.0	20.7
y = Relative wound surface :	1	1.5	2	3

Fig. 2: Effects of explant size on the percentage of regenerating explants (% R) and the number of plantlets per explant. Temperature was 20 °C. Relative wound surface is based on the wound surface of explant a equals 1.

cutting a certain piece of scale tissue into smaller explants led to an enormous increase in the number of plantlets obtained from that piece of tissue (Fig. 2). When cut smaller than 7×7 mm, the number of explants that gave rise to new plantlets decreased and the originally exponential increase in plantlet number ceased. The plantlet-inducing efficiency per wound was not constant but varied, as shown in Fig. 3. The calculated line intersects the abscissa very close to 0.

Table 1: Effect of additional wounding of explants on the mean number of plantlets per explant as influenced by medium strength and NAA-content. Temperature was 20 °C.

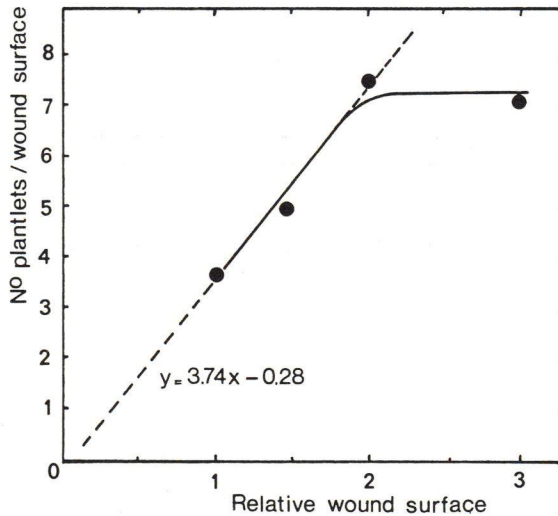| Treatment	NAA (μM)	strength of medium	normal explants	wounded explants	factor
1.	1.0	1.0	5.9±0.3	8.5±0.4	1.46
2.	1.5	1.5	6.5±0.4	10.7±0.6	1.63
3.	1.0	2.0	7.2±0.4	11.4±0.9	1.59
4.	2.0	2.0	7.4±0.4	14.8±0.9	2.00

Fig. 3: Relative plantlet-inducing efficiency per wound, calculated with data from Fig. 2: x/y.

It is noteworthy that the removal of the outermost *abaxial* cell layers led to increased plantlet formation from the *adaxial* surface (Fig. 4).

The process of regeneration from bulb-scale tissue of *Lilium speciosum* is essentially polar in character: plantlets are formed primarily at the basal (proximal) end of the explants (Fig. 5). The additional plantlets induced by removal of the outermost abaxial cell layers arose at non-basal sites (Table 2).

TIBA

Addition of TIBA to the nutrient medium led to an increase in the number of plantlets formed (Fig. 6), the optimal concentration being 2.10^{-6} M. At 2.10^{-5} M, TIBA inhibited the process. Almost all additional plantlets induced by TIBA were situated at non-basal sites. Apparently, TIBA suppressed the basipetal polarity of the system (Table 3). On media without NAA, leading to fewer basal plantlets, this TIBA-induced suppression also occurred, although it was less pronounced (Table 3).
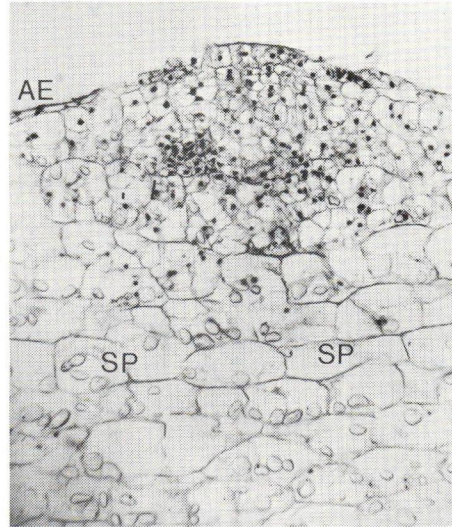
Fig. 4: Section through a bulb-scale explant showing cell divisions at the adaxial epidermal region (after 2 weeks of culture). AE = adaxial epidermis; SP = storage parenchyma.
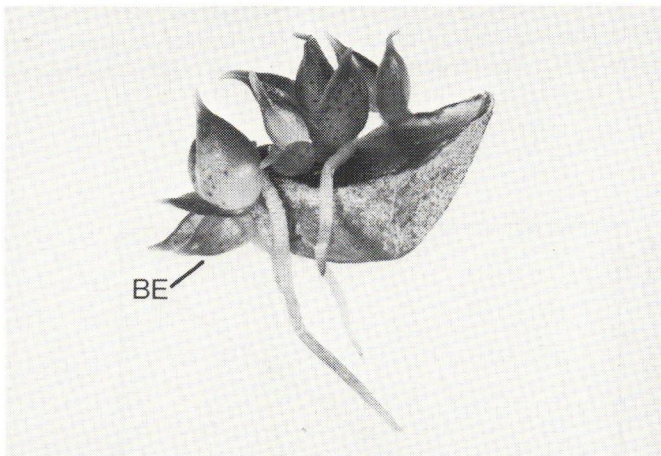

Fig. 5: Adventitious bud formation from *Lilium* bulb-scale tissue, showing basipetal polarity. BE = basal end.

Temperature

Within the range 15–25 °C a higher incubation temperature of the cultures resulted in more plantlets per explant (Fig. 7). Though the values found did not significantly deviate from a straight line, at temperatures above 25 °C this linearity was lost (results not shown). The plantlets induced by the temperature effect arose at basal as well as non-basal sites, the relative increase in non-basal plantlets exceeding that of basal plantlets (Table 2).
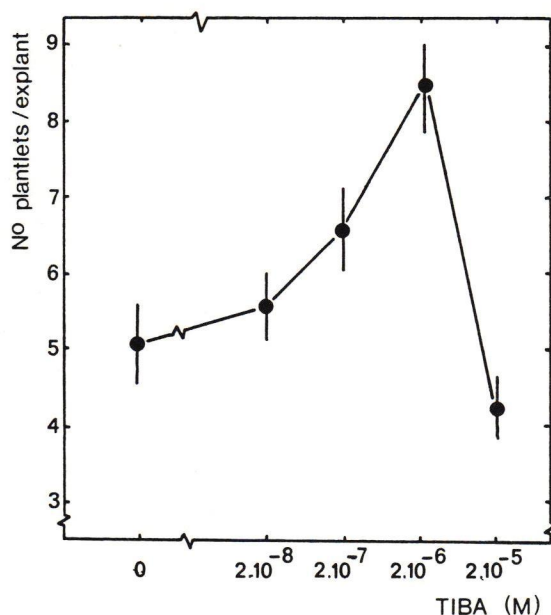
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Fig. 6: Effect of the TIBA concentration in the medium on the mean number of plantlets per explant. Temperature was 20 °C. The medium contained 0.5 μM NAA. Normal explants were used.
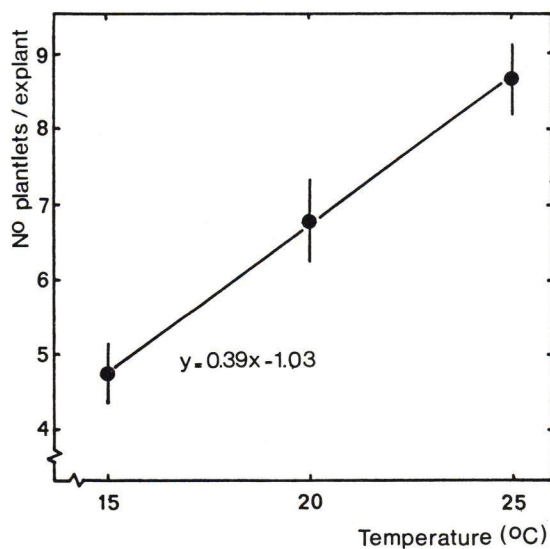

Fig. 7: Effect of the incubation temperature on the number of plantlets per explant. NAA concentration in the medium was 0.5 μM. Normal explants were used. All explants regenerated.

Table 2: Effects of wounding and temperature on the mean number of plantlets per explant and on the site of their development from the explant. NAA concentration was 0.5 μM. All explants regenerated.

Temp. (°C)	Explant	Average numbers of plantlets per explant		
		Total	Basal	Non-basal (% of total)
15	normal	4.7±0.3	3.1±0.2	1.6±0.2 (33.5)
	wounded	6.2±0.4	3.5±0.2	2.7±0.3 (43.8)
20	normal	6.8±0.4	4.5±0.2	2.3±0.3 (33.8)
	wounded	8.4±0.4	4.1±0.3	4.2±0.2 (50.6)
25	normal	8.6±0.4	5.2±0.2	3.4±0.3 (39.3)
	wounded	11.4±0.4	5.5±0.2	5.8±0.4 (51.3)

Table 3: Effects of TIBA on the mean number of plantlets per explant and on the site of their development from the explants. Temperature was 20 °C. All explants regenerated.

Exp.	TIBA (M)	Average numbers of plantlets per explant		
		Total	Basal	Non-basal (% of total)
A	0	7.2±0.3	5.2±0.2	2.0±0.2 (27.5)
	2.10^{-6}	12.7±0.5	5.6±0.2	7.0±0.5 (55.5)
B	0	4.5±0.3	3.2±0.2	1.3±0.2 (29.3)
	2.10^{-7}	5.9±0.3	3.5±0.2	2.4±0.2 (40.3)

Exp. A: NAA-concentration 0.5 μM, normal explants.
Exp. B: No NAA, wounded explants.

Discussion

Removal of the outermost abaxial cell layers of the explants promoted plantlet regeneration considerably. Analogous results were found by Hackett (1969) with *Lilium longiflorum* ‹Croft›. Theoretically, this increase might be explained by:
1) the wound itself,
2) the removal of the abaxial layers if some inhibitory substance were produced by this tissue, and
3) facilitated diffusion of stimulating substances from the medium into the tissue due to the absence of the epidermis and/or adjacent cells as barrier.

If facilitated diffusion were responsible, we would have expected no effect of additional wounding in explants incubated vertically on the nutrient medium (Fig. 1), and a decreasing effect of additional wounding in explants incubated on media containing more nutrients (Table 1). Since the opposite was found, the third possibility can be rejected. The stimulating effect on plantlet production of cutting a given piece of tissue into more and smaller explants cannot be explained by the last two possibilities. Therefore, it can be concluded that wounding of bulb-scale tissue of *Lilium speciosum* Thunb. exerts a stimulatory influence on adventitious plantlet formation by

that tissue. That this wound effect was more pronounced for horizontally than for vertically incubated explants might be explained by the difference in wound area in direct contact with the medium, especially with the auxin present in it, since it is known (Barckhausen 1978, Rosenstock and Kahl 1978) that wound reactions and their morphogenetic responses are stimulated by, especially, auxins.

Removal of the *abaxial* epidermal layers stimulated cell divisions in the tissue below the *adaxial* epidermis. Apparently, a transmittable stimulus must have arisen as a result of wounding. The fact that the plantlet-inducing efficiency per wound was not constant but dependent on the ratio between the wound surface and the tissue volume (Fig. 3), suggests that a certain amount of stimulus has to be produced before optimal regeneration can occur.

The earliest responses to wounding described in the literature include a loss of membrane lipids (Laties 1978), occurring within seconds to minutes after wounding, and ethylene formation (Saltveit and Dilley 1978 a, b, Yang and Pratt 1978, Yu and Yang 1980), occurring within minutes to hours. Whether one or both of these factors represent this stimulus in the lily regeneration system is under present investigation.

Incorporation of TIBA into the nutrient medium in concentrations up to 2.10^{-6} M led to an increase in the number of plantlets. Almost all TIBA-induced extra plantlets were located at non-basal sites. Comparable effects of TIBA in combination with IAA on the distribution of callus on tobacco stem segments were reported by Skoog as early as 1954. The auxin dependency of the process (Van Aartrijk and Blom-Barnhoorn 1981), its predominantly polar character, as well as the polarity-suppressing effect of TIBA, a specific inhibitor of basipetal auxin transport, strongly indicate that auxin and its distribution in the tissue are involved in regeneration from bulb-scale tissue. Similar conclusions were drawn by Pilet (1967) for callus cultured from root fragments of carrot.

The polarity-suppressing effect of TIBA and of wounding were strikingly similar, and one might speculate whether polar auxin transport was inhibited as a result of wounding. To our knowledge, no direct relationships between tissue wounding and polar auxin transport have been described. De la Fuente and Leopold (1966) showed that the polarity of auxin transport was exponentially related to the length of the tissue fragments traversed. Their data, unfortunately, do not permit discrimination between wound effects and other possibilities as explanation.

Within the range 15°–25 °C, higher temperatures influenced both plantlet number and the polarity of the system. Thus, the polarity of the regeneration sites on the explants was suppressed by wounding, TIBA, and temperature. This phenomenon was independent of the number of basal plantlets formed (Tables 2 and 3), which strongly indicates that ‹crowding› (stimulation of non-basal regeneration as a result of saturation of basal sites) did not play a role.

It can be concluded that tissue wounding, TIBA, and temperature influence both the number of regenerated plantlets from bulb-scale explants of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, and the polarity of their sites. The remarkably similar

effect of these three dissimilar factors seems to suggest some kind of interaction. Experiments based on this hypothesis are in progress.

Acknowledgements

References

AARTRIJK, J. VAN and G. J. BLOM-BARNHOORN: Effects of sucrose, mineral salts, and some organic substances on the adventitious regeneration *in vitro* of plantlets from bulb-scale tissue of *Lilium speciosum* Rubrum. Acta Hortic. *109*, 297–302 (1980).

– – Growth regulator requirements for adventitious regeneration from *Lilium* bulb-scale tissue *in vitro*, in relation to duration of bulb storage and cultivar. Scientia Hortic. *14*, 261–268 (1981).

BARCKHAUSEN, R.: Ultrastructural changes in wounded plant storage tissue cells. In: KAHL, G. (ed.): Biochemistry of wounded plant tissues, pp. 1–42. Walter de Gruyter, Berlin, New York, 1978.

DE LA FUENTE, R. K. and A. C. LEOPOLD: Kinetics of polar auxin transport. Plant Physiol. *41*, 1481–1484 (1966).

GOLDSMITH, M. H. M.: The polar transport of auxin. Ann. Rev. Plant Physiol. *28*, 439–478 (1977).

HACKETT, W. P.: Aseptic multiplication of lily bulblets from bulb-scales. Proc. Int. Plant Propag. Soc. Ann. Meet., 105–108 (1969).

LATIES, G. G.: The development and control of respiratory pathways in slices of plant storage organs. In: KAHL, G. (ed.): Biochemistry of wounded plant tissues, pp. 421–466. Walter de Gruyter, Berlin, New York, 1978.

MURASHIGE, T. and F. SKOOG: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. *15*, 473–497 (1962).

PILET, P. E.: Auxin content and auxin catabolism in relation to the growth polarity. Physiol. Plant. *20*, 285–290 (1967).

ROBB, S. M.: The culture of excised tissue from bulb-scales of *Lilium speciosum* Thunb. J. exp. Bot. *8*, 348–352 (1957).

ROSENSTOCK, G. and G. KAHL: Phytohormones and the regulation of cellular processes in aging storage tissues. In: KAHL, G. (ed.): Biochemistry of wounded plant tissues, pp. 623–671. Walter de Gruyter, Berlin, New York, 1978.

SALTVEIT, M. E. and D. R. DILLEY: Rapidly induced wound ethylene from excised segments of etiolated *Pisum sativum* L., cv. Alaska. 1. Characterization of the response. Plant Physiol. *61*, 447–450 (1978 a).

– – Rapidly induced wound ethylene from excised segments of etiolated *Pisum sativum* L., cv. Alaska. 2. Oxygen and temperature dependency. Plant Physiol. *61*, 675–679 (1978 b).

SKOOG, F.: Chemical regulation of growth in plants. In: BOELL, E. J. (ed.): Dynamics of growth processes, pp. 148–183. Princeton University Press (1954).

STIMART, D. P. and P. D. ASCHER: Tissue culture of bulb-scale sections for asexual propagation of *Lilium longiflorum* Thunb. J. Am. Soc. Hortic. Sci. *103*, 182–184 (1978).

TAKAYAMA, S. and M. MISAWA: Differentiation in *Lilium* bulb-scales grown *in vitro*. Effect of various cultural conditions. Physiol. Plant. *46*, 184–190 (1979).

YANG, S. F. and H. K. PRATT: The physiology of wound ethylene. In: KAHL, G. (ed.): Biochemistry of wounded plant tissues, pp. 595–622. Walter de Gruyter, Berlin, New York, 1978.

YU, Y. B. and S. F. YANG: Biosynthesis of wound ethylene. Plant Physiol. *66*, 281–285 (1980).

HOOFDSTUK III

Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*.
Interacting effects of NAA, TIBA, wounding, and temperature

J. VAN AARTRIJK and G.J. BLOM-BARNHOORN

Bulb Research Centre, 2160 AB Lisse, The Netherlands

Summary

The present study was performed to find out whether the effects of the factors NAA, TIBA, tissue-wounding, and temperature interact during the process of adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, *in vitro*. The results refer to the number of buds formed and the polarity of the regeneration sites.

The effects of TIBA and wounding were similar and additive and depended on the presence of NAA in the nutrient medium. The influence of temperature depended on the presence of the auxin and on the degree of wounding of the scale tissue.

The results are discussed with special attention to the low endogenous IAA level of bulb-scale tissue and to a possible role of ethylene in the regeneration process.

Key words: Lilium speciosum, adventitious bud formation, bulb-scale, NAA, temperature, TIBA, wounding.

Introduction

Adventitious bud formation from plant tissues *in vitro* has been reported for a large number of species. Generally, the processes leading to the formation of buds are thought to be under the control of growth substances, a relatively high ratio of cytokinins to auxins being particularly favourable (Skoog and Miller 1957, Murashige 1974).

Exceptions to this general concept have been reported for such bulbous plants as hyacinth (Pierik and Steegmans 1975), *Ornithogalum thyrsoides* (Hussey 1976), and *Lilium speciosum* Thunb. (Van Aartrijk and Blom-Barnhoorn 1981). In these plants adventitious bud formation from storage tissues was stimulated by an exogenous supply of auxin. For *L. speciosum* Thunb. other factors besides auxin, viz. degree of wounding, prevailing temperature, and TIBA, have been shown to act on this regeneration process, too (Van Aartrijk and Blom-Barnhoorn 1983). Both the average number of buds formed per explant and the polarity of the regeneration sites were influenced by these four factors in a strikingly similar way. This remarkable similarity led to the

Abbreviations: NAA: 1-Naphthylacetic acid, TIBA: 2,3,5-Triiodobenzoic acid.

hypothesis that some kind of interaction should exist between the effects of these factors in bud regeneration.

In this paper we report a set of experiments performed to find out whether NAA, TIBA, wounding, and temperature, indeed act in a similar way in the process of adventitious bud formation from bulb-scale tissue of *Lilium speciosum* Thunb. *in vitro*.

Since endogenous auxin levels were reported to be an important factor in the regeneration capacity of tissues (Pilet, 1967, Cassells et al. 1982), we also determined the endogenous IAA level within *Lilium* bulb-scale tissue.

Materials and Methods

Plant material: Bulbs of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, circumference 18-20 cm, harvested in the autumn of 1979, 1980, and 1982, were treated with 0.2% Benlate (active ingredient 50% benomyl) and 1.5% Difolatan (a.i. 48% captafol) for 30 min and stored at $0^{o}C$ until use. The experiments were performed between April and September with bulbs harvested during the preceding autumn.

Explants: 10-12 scales were taken from each bulb. The outermost scales and the innermost small and leafy scales were discarded. To avoid an influence of differences between individual bulbs and of scales within one bulb, the scales were carefully distributed over the various treatments, subsequently disinfected in 1% NaOCl for 30 min, and rinsed twice in sterile water. Two to four explants measuring 7 x 7 mm^2 were then cut aseptically from the basal part of each scale. Such explants are designated 'normal'. 'Wounded' explants were obtained by the removal (by cutting) of the abaxial epidermis with the outermost sub-epidermal layers of the normal explants. Each explant was placed with the abaxial side down on 15 ml nutrient medium in a glass tube (150 x 25 mm) that was closed with a transparent plastic cap (Bellco, Vineland, U.S.A.). One explant was used per tube.

Nutrient media: The nutrient media consisted of MS macro- and micro-elements (Murashige and Skoog 1962) to which were added 0.4 mgl^{-1} thiamin (BDH Chem. Ltd.), 100 mgl^{-1} meso-inositol (BDH Chem. Ltd), 30 gl^{-1} sucrose (J.T. Baker Chem.), 6 gl^{-1} agar (Difco-Noble), and, depending on the experiments, various concentrations of the auxin, NAA (BDH Chem. Ltd.; 0.5-2.0 μM), and/or the inhibitor of polar auxin transport, TIBA (Serva Chem., 2.10^{-8}-2.10^{-5}M). The pH of the media was 6.0 before autoclaving. The media were autoclaved at $120^{o}C$ for 20 minutes.

Culture conditions: The cultures were incubated in controlled-temperature rooms held at 15°, 20°, or $25^{o}C$, the fluorescent-light intensity in all growing rooms being 27.9 μEm^{-2}. sec^{-1} (Philips 25W/TL 33; 16h photoperiod). The number of cultures per treatment was 40-46. After 10 weeks of culture, the number of plantlets per explant was determined. Unless stated otherwise, the polarity of the regeneration sites was also assessed. Plantlets developing at the basal end of the explants were called basal plantlets; all others were termed non--basal. The reported data were confirmed by results of at least one similar experiment. Unless stated otherwise, the standard deviation of the mean is reported. Differences were tested for significance (p = 0.05).

IAA determinations: A number of bulb scales equivalent to 8 g fresh weight was used to determine the content of free IAA according to Knegt and Bruinsma (1973). The content of conjugated IAA was determined after hydrolysis in

1 N KOH overnight at room temperature. Duplicate measurements were made, using bulbs of *L. speciosum* harvested in the autumn of 1979 and stored at $0°C$ for circa 4 months.

Results

Interacting effects wounding-NAA: Normal and wounded explants were cultured on media with various NAA concentrations. Wounded explants showed consistently more plantlets than normal explants did (Fig. 1). Within the investigated range
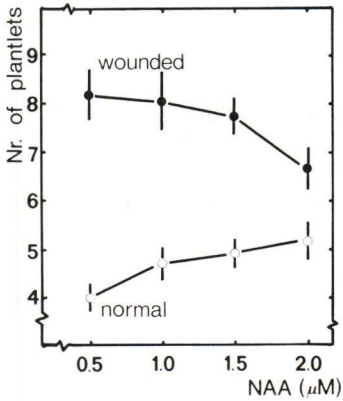

Fig. 1: Effect of NAA on the average number of plantlets per wounded or normal bulb-scale explant. Temperature was $20°C$. No TIBA.
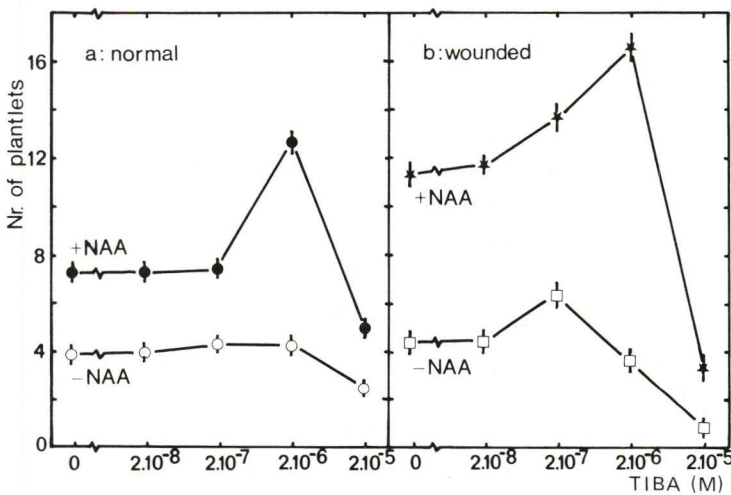

Fig. 2: Effect of TIBA on the average number of plantlets per wounded or normal bulb-scale explant, cultured in the presence of absence of NAA. Temperature was $20°C$.

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of NAA concentrations, the higher amount of auxin stimulated adventitious bud formation on normal explants but inhibited the process on wounded explants (Fig. 1).

Interacting effects NAA-TIBA: The effect of TIBA on the number of plantlets per (wounded) explant was studied in the presence or absence of 0.5 μM NAA (Fig. 2b). In both cases optimum curves were found. Explants cultured on NAA--containing media regenerated more plantlets than those incubated without auxin. TIBA optima were found at 2.10^{-7} M for explants cultured without NAA and at 2.10^{-6} M for those grown in the presence of 0.5 μM NAA. The stimulating effect of TIBA, added in its optimal concentration, was more pronounced when auxin was present in the medium. Experiments with normal explants led to

Table 1: Effect of TIBA on the polarity of regeneration sites on bulb-scale explants of *L. speciosum* Thunb., as influenced by explant type and NAA. Temperature was 20°C.

TIBA (M)	Explant	NAA (μM)	Average numbers of plantlets per explant			
			Total	Basal	Non-basal	(% of total)
0	wounded	0	4.4 ± 0.4	2.8 ± 0.3	1.6 ± 0.3	(36.4)
2.10^{-8}	wounded	0	4.4 ± 0.5	2.7 ± 0.3	1.7 ± 0.3	(38.6)
2.10^{-7}	wounded	0	6.3 ± 0.5	3.5 ± 0.3	2.8 ± 0.3	(44.4)
2.10^{-6}	wounded	0	3.7 ± 0.5	2.2 ± 0.3	1.5 ± 0.3	(40.5)
2.10^{-5}	wounded	0	0.9 ± 0.3	0.6 ± 0.2	0.3 ± 0.6	(33.3)
0	wounded	0.5	11.1 ± 0.5	4.9 ± 0.2	6.3 ± 0.4	(56.8)
2.10^{-8}	wounded	0.5	11.6 ± 0.4	5.0 ± 0.2	6.6 ± 0.4	(56.9)
2.10^{-7}	wounded	0.5	13.6 ± 0.5	5.4 ± 0.3	8.2 ± 0.5	(60.3)
2.10^{-6}	wounded	0.5	16.4 ± 0.5	5.4 ± 0.3	11.0 ± 0.5	(67.1)
2.10^{-5}	wounded	0.5	3.4 ± 0.6	1.9 ± 0.3	1.4 ± 0.3	(41.2)
0	normal	0	3.9 ± 0.2	3.3 ± 0.2	0.6 ± 0.1	(15.4)
2.10^{-8}	normal	0	4.0 ± 0.2	3.2 ± 0.2	0.9 ± 0.1	(22.5)
2.10^{-7}	normal	0	4.3 ± 0.3	3.3 ± 0.2	1.0 ± 0.1	(23.3)
2.10^{-6}	normal	0	4.2 ± 0.3	3.2 ± 0.1	1.1 ± 0.1	(26.2)
2.10^{-5}	normal	0	2.6 ± 0.3	1.7 ± 0.1	0.9 ± 0.1	(34.6)

comparable results (Fig. 2a), although no exact TIBA optimum could be established in the absence of NAA.

Interactions were also found between TIBA and NAA influencing the polarity of the regeneration sites (Table 1). The additional plantlets formed in the presence of TIBA were almost exclusively induced at non-basal sites. The percentage of non-basal plantlets depended on the TIBA concentration, 2.10^{-7} M being optimal in the absence of NAA, and 2.10^{-6} M in the presence of the auxin.

Interacting effects TIBA-wounding: The data in the Figs. 2a, b also show the effects of TIBA on bud number as influenced by the explant type. When cultured without NAA, wounded explants regenerated somewhat more plantlets than normal ones at TIBA concentrations $\leqslant 2.10^{-7}$ M. However, wounded explants were more sensitive toward the inhibitory action of a supra-optimal TIBA concentration $(2.10^{-5}$ M). In the presence of 0.5 μM NAA, comparable interactions between the effects of TIBA and wounding were found.

The polarity of the system was also under the interacting influence of TIBA and wounding. In Table 1 results are listed referring to explants cultured in
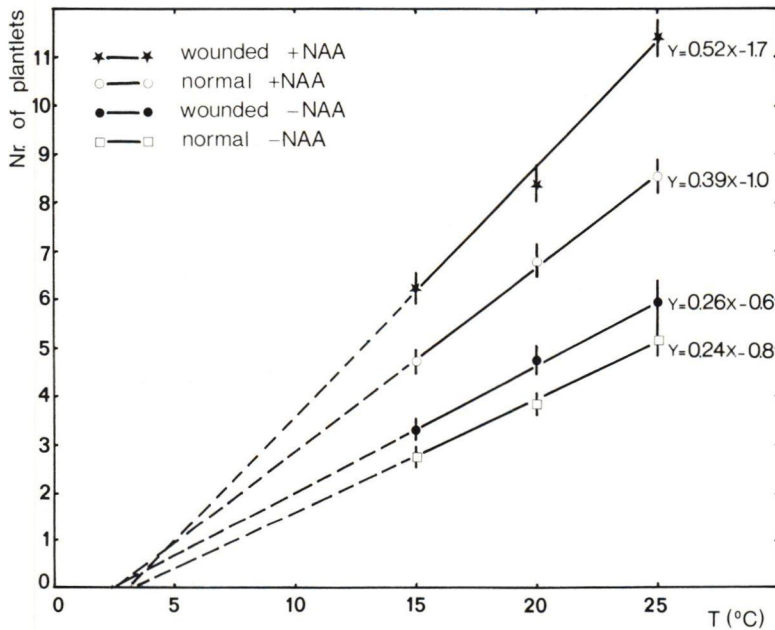

Fig. 3: Effect of the culture temperature on the average number of plantlets per bulb-scale explant, as influenced by NAA and the degree of wounding of the explants.

the absence of NAA. For normal explants, the relative polarity showed no TIBA-optimum as opposed to wounded explants.

Interacting effects temperature-wounding-NAA: In one experimental design normal and wounded explants were cultured without or with 0.5 μM NAA at several temperatures (15°, 20°, 25^OC). The number of plantlets formed per explant under these conditions is shown in Fig. 3. The curves did not significantly deviate from straight lines. The slopes of these lines were found to depend on wounding and NAA. When extrapolated, the lines intersected the abscissa within 2.4^O and 3.6^O. The polarity of the regeneration sites was influenced by all three factors (Fig. 4). Significant interactions occurred between the effects of temperature and auxin and between those of auxin and wounding.
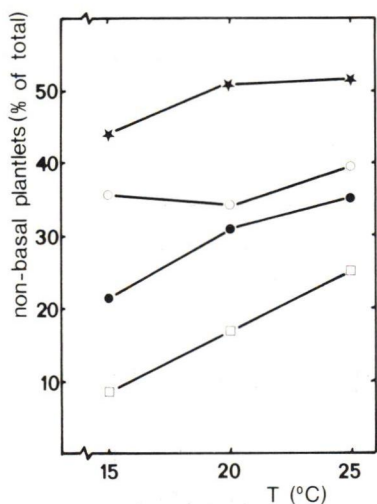

Fig. 4. Effect of the culture temperature on the polarity of the regeneration sites (expressed as the percentage of non-basal plantlets), as influenced by NAA and the degree of wounding of the explants. For explanation of the symbols, see Fig. 3.

IAA determinations: The concentration of free IAA in scales of bulbs stored at 0^OC for circa 4 months was 1.1 ± 0.1 ngg^{-1} fresh weight. The total amount of free and conjugated IAA was 11.9 ± 1.3 ngg^{-1}.

Discussion

The effects of NAA, TIBA, wounding, and temperature on the process of adventitious bud formation from bulb-scale tissue of *Lilium speciosum* Thunb. proved to be closely interrelated: distinct interactions were demonstrated between the effects of these four factors.

The interactions found between TIBA and NAA (Fig. 2a, b, Table 1) are in

agreement with data in the literature on basipetal auxin transport and its inhibition by TIBA. The shift of the TIBA optimum toward a higher concentration in the presence of NAA can be attributed to the competitive character of the auxin-transport inhibition caused by TIBA (Thomson et al. 1973, Goldsmith 1977). An explanation for the rapid decline in bud number at high TIBA concentrations is a matter of speculation. A suggestion might be that intracellular accumulation of auxin, due to the saturation of the proposed auxin-anion carrier by TIBA (Goldsmith, 1982), is responsible for this decline. Inhibition of bud regeneration by high NAA concentrations (> c. 1 μM) has been described for this regeneration system (Van Aartrijk and Blom-Barnhoorn 1981). The increase in bud number due to the action of TIBA at its optimal concentration was more pronounced when NAA was present in the nutrient medium (Fig. 2).

These data indicate that auxin is important for bud regeneration from bulb scales. The relatively small effect of TIBA in media without NAA suggests that bulb-scale tissue of *L. speciosum* Thunb. contains only small amounts of endogenous auxin serving as a 'substrate' for TIBA action. The measured endogenous level of free and conjugated IAA in this tissue were, indeed, found to be extremely low when compared to these levels in other tissues (Bandurski and Schulze 1977, Sweetser and Swartzfager 1978). The presence of auxin-like substances other than IAA cannot be excluded.

Additional wounding of the tissue led to effects on bud number and polarity of regeneration sites closely resembling those described for TIBA (Van Aartrijk and Blom-Barnhoorn 1983). A comparison between wounded and normal explants (Figs. 2a, b, Table 1) show that there are also interactions between the effects of these two factors. The similarity of the action of these factors and the apparent shift of the TIBA optima toward a lower concentration after additional wounding suggest that the two factors act additively. This suggestion is strengthened by the observation that both factors influence the action of auxin in a similar way. The stimulating effects of wounding as well as of TIBA were more pronounced in the presence of 0.5 μM NAA than without the auxin (Figs. 2, 3). Since only two explant types ('normal' and 'wounded') were examined in these experiments, it is not known whether a shift in 'wounding optimum' similar to a shift in TIBA optimum (Fig. 2) was induced by exogenous NAA. Wounding and NAA interacted in influencing the regeneration process (Fig. 1). Since the biological action of auxins as a function of their concentration generally follows an optimum curve, the results suggest that additional wounding caused a shift of the NAA optimum toward a lower concentration.

A triple interaction, i.e., between the effects of temperature, wounding, and NAA, was also established (Figs. 3, 4). The obtained plots were linear, although it is known that at 28°C this linearity is lost (Van Aartrijk and Blom-Barnhoorn, unpublished results) and, for *Lilium longiflorum*, even turns into a sharp decline above 30°C (Stimart and Ascher 1981). Interactions between the effects of IAA and temperature were described by Heide (1965) for bud regeneration from *Begonia* leaf cuttings: this system showed suppression of bud formation with increasing temperature and IAA concentration. Although his results seem to be in conflict with ours, it must be kept in mind that regeneration processes are influenced by temperature and auxin following optimum curves. Whether added auxin or increased temperature will lead to more or less bud formation might therefore depend on the internal status of the tissue, e.g., the endogenous auxin level.

It is intriguing that the four lines (Fig. 3) intersected the abscissa at roughly the same temperature (c. 3°C), which happens to be the temperature most suitable for breaking the dormancy of lily bulbs. The data suggest that wounding, temperature, and NAA act by influencing a single biological process that is inactive below c. 3°C. The only process known to us which is influenced by all these factors is that of ethylene biosynthesis (Lürssen et al. 1979), Adams and Yang 1979, Yu and Yang 1979, Yu et al. 1980, Konze and Kwiatkowski 1981).

Experiments, designed to show whether ethylene plays a role in the process of adventitious bud formation from bulb-scale tissue of *Lilium speciosum* Thunb., will be reported in forthcoming articles.

References

AARTRIJK, J. VAN and G.J. BLOM–BARNHOORN: Growth regulator requirements for adventitious regeneration from *Lilium* bulb-scale tissue *in vitro*, in relation to duration of bulb storage and cultivar. Scientia Hortic. 14, 261-268 (1981).
AARTRIJK, J. VAN and G.J. BLOM–BARNHOORN: Adventitious bud formation from bulb--scale explants of *Lilium speciosum* Thunb. *in vitro*. Effects of wounding, TIBA, and temperature. Z. Pflanzenphysiol. 110, 355-363 (1983).

ADAMS, D.O. and S.F. YANG: Ethylene biosynthesis: Identification of
    1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion
    of methionine to ethylene. Proc. Natl. Acad. Sci. U.S.A. 76, 170-174 (1979).
BANDURSKI, R.S. and A. SCHULZE: Concentration of indole-3-acetic acid and its
    derivatives in plants. Plant Physiol. 60, 211-213 (1977).
CASSELLS,A.C., R.D. LONG and D.M.A. MOUSDALE: Endogenous IAA and morphogenesis
    in tobacco petiole cultures. Physiol. Plant. 56, 507-512 (1982).
GOLDSMITH, M.H.M.: The polar transport of auxin. Ann. Rev. Plant Physiol. 28,
    439-478 (1977).
GOLDSMITH, M.H.M.: A saturable site responsible for polar transport of indole-
    3-acetic acid in sections of maize coleoptiles. Planta 155, 68-75 (1982).
HEIDE, O.M.: Interaction of temperature, auxins, and kinins in the regeneration
    ability of *Begonia* leaf cuttings. Physiol. Plant. 18, 891-920 (1965).
HUSSEY, G.: Plantlet regeneration from callus and parent tissue in *Ornithogalum*
    thyrsoides. J. Exp. Bot. 27, 375-382 (1976).
KNEGT, E. and J. BRUINSMA: A rapid, sensitive, and accurate determination of
    indolyl-3-acetic acid. Phytochem. 12, 753-756 (1973).
KONZE, J.R. and G.M.K. KWIATKOWSKI: Rapidly induced ethylene formation after
    wounding is controlled by the regulation of 1-aminocyclopropane-1-carboxylic
    acid synthesis. Planta 151, 327-330 (1981).
LÜRSSEN, K., K. NAUMANN and R. SCHRÖDER: 1-aminocyclopropane-1-carboxylic acid.
    An intermediate of the ethylene biosynthesis in higher plants. Z. Pflanzen-
    physiol. 92, 285-294 (1979).
MURASHIGE, T. and F. SKOOG: A revised medium for rapid growth and bioassays
    with tobacco tissue cultures. Physiol. Plant. 15, 473-497 (1962).
MURASHIGE, T.: Plant propagation through tissue cultures. Ann. Rev. Plant
    Physiol. 25, 135-166 (1974).
PIERIK, R.L.M. and H.H.M. STEEGMANS: Effect of auxins, cytokinins, gibberellins,
    abscisic acid and ethephon on regeneration and growth of bulblets on excised
    bulb-scale segments of hyacinth. Physiol. Plant.34, 14-17 (1975).
PILET, P.E.: Auxin content and auxin catabolism in relation to the growth
    polarity. Physiol. Plant. 20, 285-290 (1967).
SKOOG, F. and C.O. MILLER: The biological action of growth substances. Symp.
    Soc. Exp. Biol. 11, 118-131 (1957).
STIMART, D.P. and P.D. ASCHER: Developmental responses of *Lilium longiflorum*
    bulblets to constant or alternating temperatures *in vitro*. J. Amer. Soc.
    Hort. Sci. 106, 450-454 (1981).
SWEETSER, P.B. and D.G. SWARTZFAGER: Indole-3-acetic acid levels of plant
    tissue as determined by a new high performance liquid chromatographic
    method. Plant Physiol. 61, 254-258 (1978).
THOMSON, K.S., R. HERTEL and S. MÜLLER: 1-Naphtylphthalamic acid and
    2,3,5-Triiodobenzoic acid; *in vitro* binding to particulate cell fractions
    and action on auxin transport in corn coleoptiles. Planta 109, 337-352 (1973).
YU, Y-B and S.F. YANG: Auxin-induced ethylene production and its inhibition by
    aminoethoxyvinyl glycine and cobalt ion. Plant Physiol. 64, 1074-1077 (1979).
YU, Y-B, D.O. ADAMS and S.F. YANG: Inhibition of ethylene production by
    2,4-dinitrophenol and high temperature. Plant Physiol. 66, 286-290 (1980).

HOOFDSTUK IV

Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro.*
Production of ethane and ethylene

J. VAN AARTRIJK[*], G.J. BLOM-BARNHOORN[*] and J. BRUINSMA[**]

[*] Bulb Research Centre, 2160 AB Lisse, The Netherlands
[**] Agricultural University, 6703 BD Wageningen, The Netherlands

Summary

We describe the influence of NAA, temperature, wounding, and AVG, an inhibitor of ethylene biosynthesis, on the production of ethylene and ethane by bud-regenerating bulb-scale explants of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, *in vitro.*

The production rates of the two hydrocarbon gases depended on culture stage and conditions, and their changes were opposite. An initial ethane production decreased at increasing ethylene evolution. The exponential increase of this evolution during the first 1-2 weeks was followed by a decline during subsequent bud differentiation.

We conclude that the number of regenerated plantlets per explant was closely related to the speed of recovery and the measure of ethylene production during the initial period of membrane repair.

Key words: adventitious bud formation, auxin transport, AVG, bulb scale, differentiation, ethane, ethylene, Lilium speciosum, NAA, temperature, wounding.

Introduction

In previous reports effects were described of NAA, temperature, wounding, and TIBA on the process of adventitious bud formation *in vitro* from bulb-scale explants of *Lilium speciosum* Thunb. (Van Aartrijk and Blom-Barnhoorn 1981, 1983). The number of buds formed per explant and the polarity of the regeneration sites were influenced by these factors in a strikingly similar way. Moreover, the effects of all factors in this regeneration system were shown to interact closely (Van Aartrijk and Blom-Barnhoorn 1984).

These and other data led us to hypothesize that at least part of the action of NAA, temperature, and wounding was exerted via one single biochemical process, viz., that of ethylene biosynthesis (Van Aartrijk et al. 1982, Van Aartrijk and Blom-Barnhoorn 1984. The evaluation of this hypothesis requires information on:

- ethylene production by the scale tissue in relation to bud formation,
- the effects of exogenous ethylene on bud formation, and
- the effects of inhibition of ethylene biosynthesis on bud formation.

Ethylene has been shown to be produced by *in vitro* cultured plant cells (McKenzie and Street 1970, La Rue and Gamborg 1971, Lieberman et al. 1979), anthers (Horner et al. 1977), calluses (Huxter et al. 1979, 1981), and tissue fragments (Bender and Neumann 1978). Correlations were described between ethylene production and growth (La Rue and Gamborg 1971, Bender and Neumann 1978, Gavinlertvatana et al. 1980) and between a reduction in ethylene production and differentiation (Huxter et al. 1979, 1981, Grady and Bassham 1982).

Besides ethylene, plant tissues under stress conditions can also produce ethane. Generally, the evolution of this gas is correlated with the degree of damage to the tissue (Konze and Elstner 1976, Elstner and Konze 1978, Kimmerer and Kozlowski 1982).

In this article we report on the production of ethylene and ethane by regenerating bulb-scale explants of *Lilium speciosum* Thunb., as influenced by NAA, temperature, wounding, and AVG, an inhibitor of ethylene biosynthesis. Effects of exogenous ethylene and AVG on the regeneration process itself will be reported elsewhere.

Materials and Methods

The experiments were carried out in the period June-December of the years 1981 and 1983, using explants of bulbs of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, circumference 18-20 cm, harvested in the autumn of 1980 and 1982, respectively.

Bulb storage conditions, and preparation of culture media and bulb-scale explants were described by Van Aartrijk and Blom-Barnhoorn (1983,1984). Two to four explants (7×7 mm^2) were cut aseptically from the basal part of bulb scales. Such explants are designated normal. Wounded explants were obtained by the removal, by cutting, of the abaxial epidermis with the outermost sub-epidermal layers of the normal explants. Explants were placed with the abaxial side down on a nutrient medium, and grown at $15^{\circ}C$ or $25^{\circ}C$. The quantum flux density in all growing rooms was 27.9 $\mu Em^{-2}sec^{-1}$ (Philips 25 W/TL 33; 16h photoperiod). Nutrient media consisted of MS macro- and micro-elements (Murashige and Skoog 1962) to which were added 0.4 mgl^{-1} thiamin, 100 mgl^{-1} meso-inositol, 30 gl^{-1} sucrose, 6 gl^{-1} agar, and various concentrations of NAA (0.5-10.0 μM). Filter-sterilized AVG was added to autoclaved media to a final concentration of 30 μM.

Ethylene measurements during the culture period: At intervals during the course of the culture period, explants were taken from the culture tubes and transferred aseptically to serum flasks (40 ml) each containing 15 ml of identical medium. Six explants were incubated per serum flask. In the last 3 weeks of the culture period conical flasks (100 ml) with 30 ml medium were

used instead of serum flasks. After flushing with sterile, ethylene-filtered (Purafil, Borg Warner Comp., Washington, Virginia, U.S.A.) air for 2 minutes, the flasks were closed gas-tight and incubated in the environment where the explants had been cultured before the transfer. The fresh weight of the explants was determined by weighing the flasks before and after transfer of the explants. After an incubation period of 24h, 1 ml gas samples were taken from the flasks and injected into a Perkin-Elmer nr. 900 gas chromatograph equipped with a Porapak Q column (column temperature $60^{o}C$) and a flame-ionization detector (temperature $150^{o}C$), connected to a Shimadzu C-R1A Chromatopac Integrator. Similar air-flushed serum flasks or conical flasks with identical medium but without explants served as controls. No corrections were made for ethylene dissolved in the media. The reported data represent the average production of 2-4 flasks each containing 6 explants $\pm$ S.E. of the mean. Authentic C_2H_4 (1 $\pm$ 0.05 ppm) in air (L'Air Liquide, Liège, Belgium) served as a standard.

Concurrently with the ethylene-production experiments, explants were cultured similarly and routinely (n = 46). At the end of the 10-week culture period the number of plantlets, the fresh weight of the bulblets, the number and fresh weight of the leaves, and the fresh weight of the roots were determined.

Ethane and ethylene measurements during days 1-4: Explants were excised and either placed directly in serum flasks (for ethane and ethylene production at day 1), or routinely grown in culture tubes and then transferred, after 1, 2, or 3 days, to similar serum flasks (for gas production at days 2, 3, and 4). Experimental conditions varied as to explant type (normal or wounded), medium composition (with or without NAA or AVG), and temperature (15° or $25^{o}C$). In each serum flask of 106 ml, containing 40 ml medium, 32 explants were incubated. After 2 minutes flushing with sterile, ethylene-filtered air, the flasks were closed gas-tight and incubated for 24h. Then 1 ml gas samples were taken from the flasks and injected into a Perkin-Elmer Sigma 3B gas chromatograph equipped with a Porapak Q column (column temperature 100°) and a flame ionization detector (temperature $150^{o}C$), connected to a Shimadzu C-R1A Chromatopac Integrator. Serum flasks with identical medium but without explants served as controls. No corrections were made for ethane or ethylene dissolved in the media. The reported data represent the average production of 3 flasks $\pm$ S.E. of the mean. The whole experiment was repeated once. Authentic ethylene or ethane in air (L'Air Liquide, Liège, Belgium) served as a standard.

Unless stated otherwise, results were tested for significance at p = 0.05.

Results

Fig. 1 presents results on ethylene production by and number of plantlets formed on bulb-scale explants during the culture period as affected by NAA, wounding, temperature, and AVG. In spite of large differences in the absolute amounts of ethylene produced, similarities in the production patterns were found. The ethylene production was low in the first few days of the culture period, and increased afterwards to reach a peak at day 8 (Fig. 1D), at days 10-12 (Fig. 1A, B, C), or at day 14 (Fig. 1E). In the presence of AVG, no clear ethylene peak could be established (Fig. 1F). In these first few weeks of the culture period, the ethylene production per gram fresh weight closely resembled that measured per 6 explants. Wounded explants cultured at $25^{o}C$ in
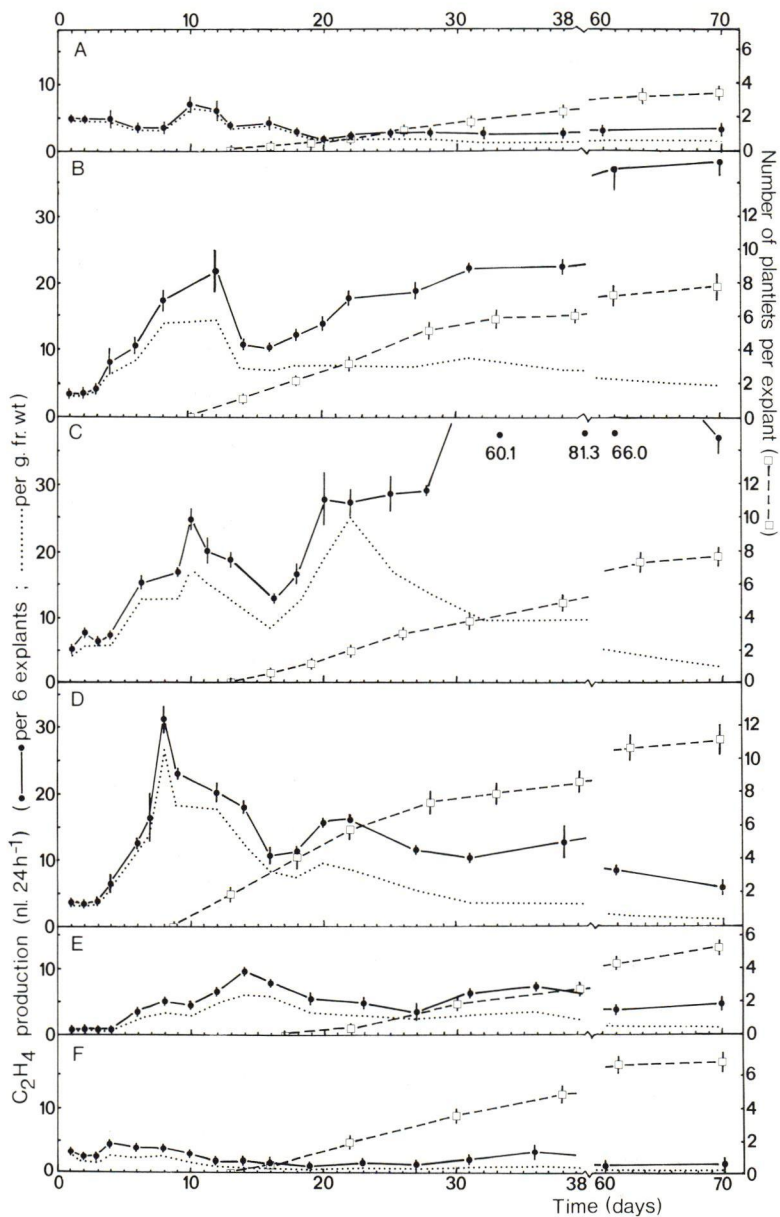
Fig. 1: Course of ethylene production by and plantlet development on bulb-scale explants, as influenced by explant type and culture conditions.

A: normal explants; 0.0 μM NAA; 25°C; no AVG :☆
B: normal explants; 0.5 μM NAA; 25°C; no AVG :★
C: normal explants;10.0 μM NAA; 25°C; no AVG :○
D: wounded explants; 0.5 μM NAA; 25°C; no AVG :●
E: wounded explants; 0.5 μM NAA; 15°C; no AVG :□
F: wounded explants; 0.5 μM NAA; 25°C; 30 μM AVG :✪

the presence of 0.5 μM NAA produced the highest amount of ethylene (Fig. 1D),
whereas in the absence of NAA or in the presence of AVG low production rates
were found (Figures 1A, F). A close linear correlation (c.c. 0.97) existed
between the maximal ethylene production of the explants during the first two
weeks and the ultimate number of plantlets per explant (Fig. 2). The AVG
treatment deviated from this correlation.

A second rise in ethylene production by the tissue was found in all
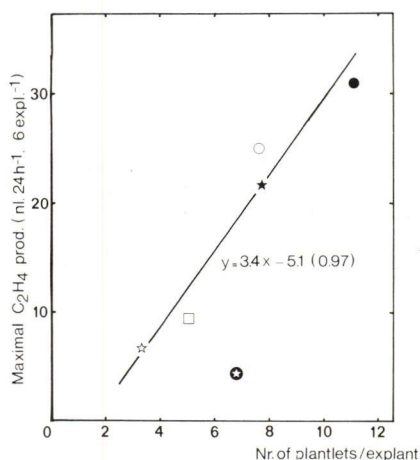treatments containing NAA, except for the AVG treatment. This second rise was

Fig. 2: Relationship between the maximal
ethylene production by explants of
L. speciosum Thunb. during the first two
weeks of culture and the ultimate number
of adventitiously formed plantlets per
explant. The calculated line does not
include the AVG treatment. For explana-
tion of the symbols: see Fig. 1.

found from c. day 18 (Figures 1B, C, D) or c. day 27 (Fig. 1E), and can be
ascribed mainly to increasing fresh weight of the explants, since, with an
exception for the 10 μM NAA treatment, this ethylene production remained
almost constant on a fresh-weight basis.

The first adventitious buds (defined as a meristem with one primordium)
visible by stereomicroscope, were invariably found shortly after the first
ethylene peak (Fig. 1). The second rise in ethylene production correlated
well with the time of root formation, and, its maximal value was related to
the ultimate fresh weight of the roots (c.c. 0.93; data not shown).

The ethylene production by the explants in the first four days of the culture
period as a function of the culture conditions was measured in more detail
(Table 1; Fig. 3). The 10 μM NAA treatment was omitted for practical reasons.
From day 2 onward, the ethylene production by the explants showed an exponential
increase for all treatments (Fig. 3). Both the ethylene-production levels at
day 2 and the slopes of the lines depended on the type of explant and on the

Fig. 3: Ethylene production by explants of *L. speciosum* Thunb. during days 2-4 of the culture period, as influenced by explant type and culture conditions. For explanation of the symbols: see Fig. 1.
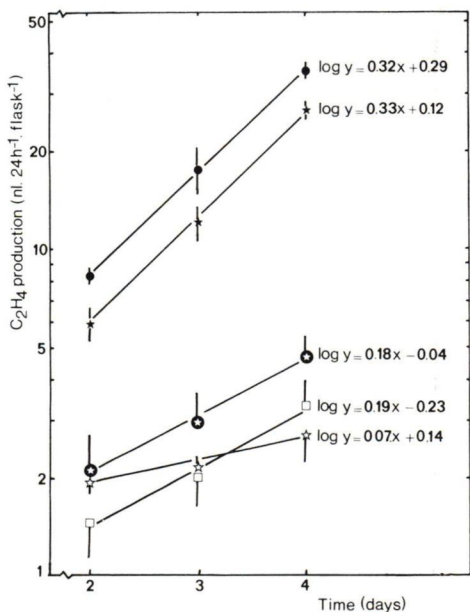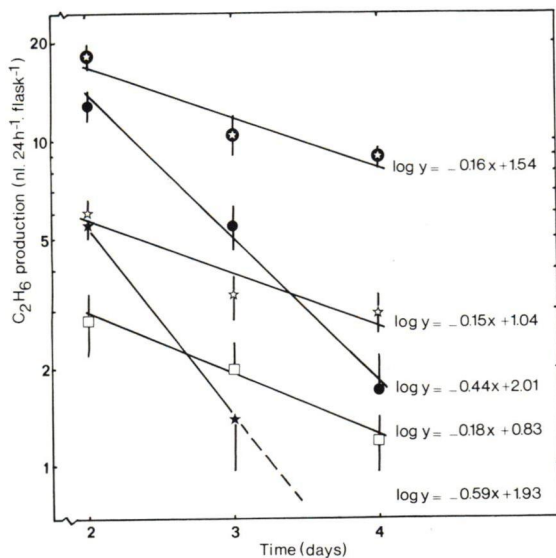

Fig. 4: Ethane production by explants of *L. speciosum* Thunb. during days 2-4 of the culture period, as influenced by explant type and culture conditions. For explanation of the symbols, see Fig. 1. Normal explants cultured at 25°C with 0.5 µM NAA did not produce ethane at day 4.

culture conditions. Additional wounding affected the production level at day 2 but not the slope, whereas temperature, NAA, and AVG influenced both the slopes of the lines and the production levels at day 2. Correlations were found between the ultimate number of plantlets per explant obtained for the various conditions (data derived from Fig. 1) and the slopes of the lines (c.c. 0.87), and between the ultimate number of plantlets and the ethylene production at day 2 (c.c. 0.89).

Table 1: Ethylene and ethane production by explants of *L. speciosum* Thunb. in the first 24h of the culture period, as influenced by explant type and culture conditions.

Explant	Temperature (^{o}C)	NAA (μM)	AVG (μM)	C_2H_4 (nl. $24h^{-1}$. $flask^{-1}$)	C_2H_6
normal	25	0.0	0	-0.7 ± 0.3 b^x	3.6 ± 0.9 p
normal	25	0.5	0	5.3 ± 1.7 d	8.0 ± 1.1 q
wounded	25	0.5	0	0.6 ± 0.1 c	13.5 ± 1.3 r
wounded	15	0.5	0	-1.8 ± 0.1 a	1.2 ± 0.4 p
wounded	25	0.5	30	-0.4 ± 0.2 b	11.9 ± 1.2 r

^{x} Values scored with the same letter are not significantly different at p = 0.05

Table 1 shows the ethylene production by the explants in the first 24h of the culture period as a function of the culture conditions. None of these data fitted well into the exponential production rates found from day 2 onward. In contrast with the situation at day 2, additionally wounded explants (25°C; 0.5 μM NAA) produced less ethylene than did normal explants. The presence of AVG, the absence of NAA, and lower temperature (15°C) led to ethylene production levels consistently lower than the controls, i.e., negative production figures. During the gas-chromatographic measurement of ethylene we invariably found a second peak in the chromatograms, the retention time of which was identical with that of authentic ethane (Table 1). Additional wounding, the presence of NAA, and higher temperature led to an increase in the ethane production in this early phase of the culture period. The presence of AVG, however, had no effect on ethane production in this period. In contrast with the ethylene production in the first 24h, the ethane production during this part of the culture period was correlated with the number of plantlets (c.c. 0.82).
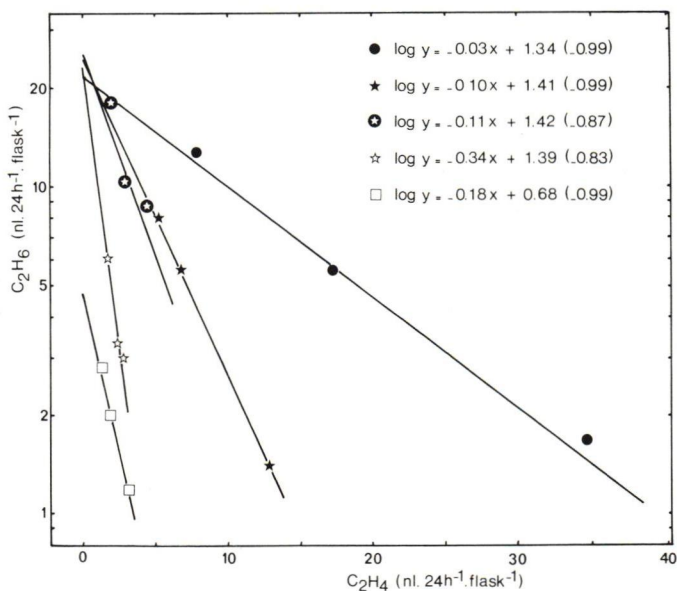
Fig. 5: Correlations between the production of ethane and ethylene by explants of *Lilium speciosum* Thunb. during days 2-4 of the culture period, as influenced by explant type and culture conditions. For explanation of the symbols, see Fig. 1.
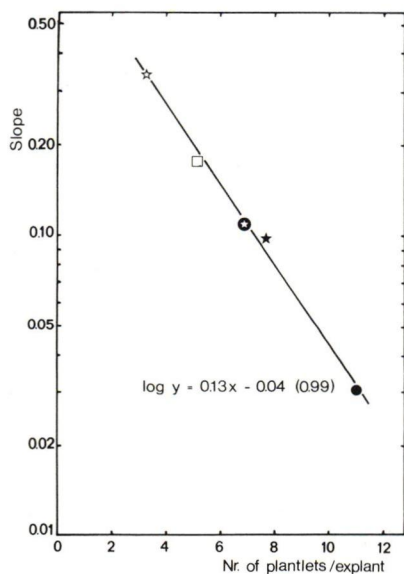
Fig. 6: Correlation between the slopes of the various ethane-ethylene correlation lines (see Fig. 5) and the number of adventitiously formed plantlets per explant (data derived from Fig. 1). For explanation of the symbols, see Fig. 1.

From day 2 onward, the measured ethane levels decreased in all treatments (Fig. 4). This decrease was most prominent for explants cultured at 25°C in the presence of NAA. In the presence of AVG the ethane production at day 2 was higher than at day 1 and the reduction in ethane production went slower.

Negative correlations were found between the logarithms of the ethane production and the ethylene production for all treatments (Fig. 5). When extrapolated, the four lines referring to a culture temperature of 25°C intersected at roughly one ethane concentration (c. 23 nl. $24h^{-1}$. $flask^{-1}$) at a corresponding ethylene production of 0. The line referring to 15°C intersected at ethylene = 0 at a lower ethane concentration (c. 5 nl. $24h^{-1}$. $flask^{-1}$).

A highly significant correlation (P< 0.0005) was found between the slopes of the obtained ethane-ethylene regression lines and the average number of plantlets per explant under the various conditions (Fig. 6).

Discussion

Bulb-scale explants of *L. speciosum* Thunb. produced both ethylene and ethane in an ordered pattern, the amounts depending on culture conditions and culture stage.

In the first 24h of the culture period ethane was detected in all treatments (Table 1). Ethane production by plant tissues has been reported to result from wound-induced free-radical-mediated peroxidation of membrane fatty acids (Elstner and Konze 1976, Konze and Elstner 1976, John and Curtis 1977, Kimmerer and Kozlowski 1982). The increased ethane production brought about by additional wounding of our explants corresponds well with this view. Explanations for the actions of NAA and temperature on ethane production can only be speculative. The temperature effect might be due merely to its effect on reaction kinetics of the ethane-forming system. If ethane production and membrane damage are related, then, indeed, NAA may be regarded as a membrane-damage-stimulating agent, at least in these early hours after wounding of the tissue.

Ethylene production by the explants in the first 24h after excision was generally very low (Table 1). Except for normal explants (25°C; 0.5 μM NAA) the ethylene production levels were close to zero. The negative ethylene production by explants, especially those cultured at 15°C, suggests some kind of absorption by these explants of atmospheric ethylene. Ethylene has been shown to be metabolized by plant tissues (Beyer 1979, Dodds et al. 1983) to simple oxidation products, but there is doubt as to whether this ethylene oxidation is related to ethylene action (Abeles 1984). We do not know whether

such processes occur in our bulb-scale explants.

Interestingly, in these first 24h wounded explants produced less ethylene than normal ones, although wounding is known to stimulate ethylene production by its action on ACC synthesis (Yu and Yang 1980, Konze and Kwiatkowski 1981). However, from day 2 this situation was reversed, wounded explants producing more ethylene than normal ones. An explanation for these data may be that freshly wounded tissues, with their damaged membranes (Galliard 1978), are unable to convert ACC into ethylene, and that more severe wounding leads to more damage and prolonged impairment of the ACC-conversion ability. It is well known that the enzymatic conversion of ACC to ethylene is membrane--associated (Mayak et al. 1981, Apelbaum et al. 1981) and thus likely to be vulnerable to membrane damage. This explanation would imply accumulation of ACC in wounded tissues, followed by a rapid conversion of this precursor into ethylene after restoration of membrane integrity. Recently Evensen (1984) described such a rise in ACC content of freshly excised potato disks prior to a rise in ethylene production.

From day 2 onward, the ethylene production by the explants increased exponentially under all culture conditions (Fig. 3), while at the same time the ethane production levels decreased (Fig. 4). Inverse production rates of the two gases have been described before (Elstner and Konze 1976, Lieberman 1979, Evensen 1984). The negative correlations between ethane and ethylene production (Fig. 5), and the observation that four of the obtained lines intersected at roughly one ethane concentration suggest that the processes leading to ethane and ethylene formation are coupled. Both processes are generally believed to be free-radical-mediated (Kimmerer and Kozlowski 1982, Apelbaum et al. 1981). The ethylene precursor, ACC, might act as a radical--scavenger preventing further radical-mediated peroxidation of membrane fatty--acid and ethane production. The ethane concentration at which the regression lines (Fig. 5) intersected, may represent an irreparable, i.e. lethal amount of membrane damage, this critical damage, which abolishes ethylene evolution, apparently being temperature-dependent.

Except for the AVG treatment, a probably autocatalytic increase in ethylene production of the explants was found from day 2 on until a maximum was reached between days 8 and 14. (Fig. 1). In this period cell divisions occur at the regeneration sites. The correlations between the numbers of plantlets per explant on the one hand, and the ethylene productions on the other hand support the hypothesis (Van Aartrijk and Blom-Barnhoorn 1984) that ethylene is involved

in the processes that ultimately lead to adventitious bud formation from *Lilium* bulb-scale explants.

Especially the highly significant correlation between plantlet number and slope of the ethane/ethylene correlations (Fig. 6) deserves further attention. Our interpretation of these ethane/ethylene ratios is that they represent, as suggested above, the radical-scavenging power of the tissue, possibly the endogenous ACC concentration. Thus, the results suggest that a correlation may exist between the number of ultimately formed plantlets and the logarithm of the endogenous ACC concentration within the tissue in the early culture stages. To verify this hypothesis, determinations of endogenous ACC would be required. Our data do not permit a conclusion as to the causative role of ethylene in the process. More elaborate experiments on the effects of AVG, ACC, and exogenous ethylene are necessary before such a conclusion can be drawn.

The first visible buds consistently appeared shortly after an ethylene peak, but roots appeared after an ethylene minimum. Such an opposite relationship between bud and root formation was also found with respect to the peroxidase activity of regenerating tissues: bud formation was always preceded by an increase in peroxidase activity (Thorpe 1978, Kevers et al. 1981, Gaspar et al. 1982), whereas the appearance of roots was found to be preceded by a decrease in peroxidase activity after an induction period characterized by high activity of the enzymes (Gaspar et al. 1982). The action of these peroxidases was thought to be on auxin catabolism (Gaspar et al. 1982). Moreover, peroxidase activity has also been described to be stimulated by ethylene (Imaseki 1970, Haard and Marshall 1976, Gaspar et al 1982). We do not know whether changes in peroxidase activity occur in our bulb-scale tissue of *Lilium*.

In conclusion, ethylene and ethane were produced by bulb-scale explants in amounts depending on culture conditions and culture stage. The number of regenerated plantlets from bulb-scale tissue of *Lilium speciosum* turned out to be related to the speed of recovery and the measure of ethylene production during repair of membrane damage.

References

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Growth regulator requirements for adventitious regeneration from *Lilium* bulb-scale tissue *in vitro*, in relation to duration of bulb storage and cultivar. Scientia Hortic. 14: 261-268 (1981).

AARTRIJK, J. VAN, G.J. BLOM-BARNHOORN and J. BRUINSMA: Interactions between NAA, wounding, temperature, and TIBA in their effects on adventitious sprout formation on *Lilium* bulb tissue. In: FUJIWARA, A (ed.): Plant Tissue Culture pp. 131-132. Jap. Assoc. Plant Tissue Culture, Tokyo, Japan (1982).

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Adventitious bud formation from bulb--scale explants of *Lilium speciosum* Thunb. *in vitro*. Effects of wounding, TIBA, and temperature. Z. Pflanzenphysiol. 110: 355-363 (1983).

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Adventitious bud formation from bulb--scale explants of *Lilium speciosum* Thunb. *in vitro*. Interacting effects of NAA, TIBA, wounding, and temperature. J. Plant Physiol. (1984 in press).

ABELES, F.B.: Role of ethylene oxidation in the mechanism of ethylene action. In: FUCHS, Y. and E. CHALUTZ (eds.): Ethylene: biochemical, physiological, and applied aspects, Martinus Nijhoff, The Hague, (1984).

APELBAUM, A., A.C. BURGOON, J.D. ANDERSON, T. SOLOMOS, and M. LIEBERMAN: Some characteristics of the system converting 1-aminocyclopropane-1-carboxylic acid into ethylene. Plant Physiol. 67: 80-84 (1981).

BENDER, L. and K.H. NEUMANN: Ethylene production and growth of carrot root tissue cultures (*Daucus carota*) as influenced by β-indole-3-acetic acid and kinetin. Z. Pflanzenphysiol. 88: 227-234 (1978).

BEYER, E.M.: ^{14}C-Ethylene metabolism during leaf abscission in cotton. Plant Physiol. 64: 971-974 (1979).

DODDS, J.H., A.R. SMITH and M.A. HALL: The metabolism of ethylene to ethylene oxide in cultivars of *Vicia faba*: relationship with waterlogging resistance. Plant Growth Regulation 1: 203-207 (1983).

ELSTNER, E.F. and J.R. KONZE: Effect of point freezing on ethylene and ethane production by sugarbeet leaf disks. Nature 263: 351-352 (1976).

EVENSEN, K.B.: Calcium effects on ethylene and ethane production and 1-aminocyclopropane-1-carboxylic acid content in potato disks. Phyisol. Plant. 60: 125-128 (1984).

GALLIARD, T.: Lipolytik and lipoxygenase enzymes in plants and their action in wounded tissues. In: KAHL, G. (ed.): Biochemistry of wounded plant tissues, pp. 155-202, Walter de Gruyter, Berlin, New York, 1978.

GASPAR, Th., Cl. PENEL, T.A. THORPE and H. GREPPIN: Peroxidases 1970-1980, pp. 97-100. Univ. of Genève, Switzerland, 1982.

GAVINLERTVATANA, P., P.E. READ and H.F. WILKINS: Control of ethylene synthesis and action by silver nitrate and rhizobitoxin in *Petunia* leaf sections cultured *in vitro*. J. Amer. Soc. Hortic. Sci. 105: 304-307 (1980).

GRADY, K.L. and J.A. BASSHAM: 1-aminocyclopropane-1-carboxylic acid concentrations in shoot-forming and non-shoot-forming tobacco callus cultures. Plant Physiol. 70: 919-921 (1982).

HAARD, N.F. and M. MARSHALL: Isoperoxidase changes in soluble and particulate fractions of sweet potato root resulting from cut injury, ethylene, and black-rot infection. Physiol. Plant Pathol. 8: 195-205 (1976).

HORNER, M., J.A. McCOMB, A.J. McCOMB and H.E. STREET: Ethylene production and plantlet formation by *Nicotiana* anthers cultured in the presence and absence of charcoal. J. Exp. Bot. 28: 1365-1372 (1977).

HUXTER, T.J., D.M. REID and T.A. THORPE: Ethylene production by tobacco (*Nicotiana tabacum*) callus. Physiol. Plant. 46: 374-380 (1979).

HUXTER, T.J., T.A. THORPE and D.M. REID: Shoot initiation in light- and dark-grown tobacco callus: the role of ethylene. Physiol. Plant. 53: 319-326 (1981).

IMASEKI, H.: Induction of peroxidase activity by ethylene in sweet potato. Plant Physiol. 46: 172-174 (1970).

JOHN, W.W. and R.W. CURTIS: Isolation and identification of the precursor of ethane in *Phaseolus vulgaris* L. Plant Physiol. 59: 521-522 (1977).

KEVERS, Cl., M. COUMANS, W. DE GREEF, M. JACOBS and Th. GASPAR: Organogenesis in habituated sugarbeet callus: auxin content and protectors, peroxidase pattern and inhibitors. Z. Pflanzenphysiol. 101: 79-87 (1981).

KIMMERER, T.W. and T.T. KOZLOWSKI: Ethylene, ethane, acetaldehyde, and ethanol production by plants under stress. Plant Physiol. 69: 840-847 (1982).

KONZE, J.R. and E.F. ELSTNER: Ethylene and ethane formation in leaf disks, plastids and mitochondria. Ber. Deutsche Bot. Ges. 89: 547-553 (1976).

KONZE, J.R. and G.M.K. KWIATKOWSKI: Rapidly induced ethylene formation after wounding is controlled by the regulation of 1-aminocyclopropane-1-carboxylic acid synthesis. Planta 151: 327-330 (1981).

LARUE, T.A.G. and O.L. GAMBORG: Ethylene production by plant cell cultures. Plant Physiol. 48: 394-398 (1981).

LIEBERMAN, M.: Biosynthesis and action of ethylene. Ann. Rev. Plant Physiol. 30: 533-591 (1979).

LIEBERMAN, M., S.Y. WANG and L.D. OWENS: Ethylene production by callus and suspension cells from cortex tissue of postclimacteric apples. Plant Physiol. 63: 811-815 (1979).

MAYAK, S., R.L. LEGGE and J.E. THOMPSON: Ethylene formation from 1-aminocyclopropane-1-carboxylic acid by microsomal membranes from senescing carnation flowers. Planta 153: 49-55 (1981).

McKENZIE, I.A. and H.E. STREET: Studies on growth in culture of plant cell. I. Production of ethylene by suspension cultures of *Acer pseudoplatanus*. J. Exp. Bot. 21: 824-834 (1980).

MURASHIGE, T. and F. SKOOG: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497 (1962).

THORPE, T.A.: Physiological and biochemical aspects or organogenesis *in vitro*. In: THORPE, T.A. (ed.): Frontiers of Plant Tissue Culture, pp. 49-66, Int. Ass. Plant Tissue Culture, Calgary (1978).

YU, Y.B. and S.F. YANG: Biosynthesis of wound ethylene. Plant Physiol. 66: 281-285 (1980).

HOOFDSTUK V

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. VAN AARTRIJK[*], G.J. BLOM-BARNHOORN[*] and J. BRUINSMA[**]

[*]Bulb Research Centre, 2160 AB Lisse, The Netherlands
[**]Agricultural University, 6703 BD Wageningen, The Netherlands

Summary

We studied the effects of AVG, ACC, and ethylene on the process of adventitious bud formation *in vitro* from bulb-scale explants of *Lilium speciosum* Thunb., cv. Rubrum nr. 10.

AVG inhibited plantlet regeneration, especially at non-basal sites. The effects of AVG were counteracted by ACC and TIBA.

Ethylene, applied in the first 3 or 7 days of the culture period in a concentration of 1 or 10 ppm, caused an increase in bud number per explant and suppressed the predominantly basipetal polarity of the regeneration sites. Ethylene increased the sensitivity of the tissue for exogenous auxin.

A model is proposed, showing the influence of ethylene, its biosynthetic pathway, and the other modifying factors on the regulation of plantlet induction in bulb-scale explants by auxin.

Key words: Lilium speciosum, adventitious bud formation, auxin transport, AVG, bulb scale, ethylene, NAA, temperature, TIBA, wounding.

Introduction

Of the factors known to influence the process of adventitious bud formation from bulb-scale tissue of *Lilium speciosum* Thunb. *in vitro*, four have been studied in more detail, viz., NAA, wounding, temperature, and TIBA (Van Aartrijk and Blom-Barnhoorn 1981, 1983, 1984, Van Aartrijk et al. 1984). The strikingly similar and interacting effects of these factors on the number of buds formed per explant and on the polarity of their regeneration sites led to the hypothesis that these factors act, at least partly, through one biochemical process, possibly that of ethylene biosynthesis (Van Aartrijk and Blom-Barnhoorn 1984).

Recently, we reported on ethane and ethylene production by *Lilium* bulb-scale explants, as influenced by NAA, wounding, temperature, and AVG, an inhibitor of ethylene biosynthesis (Van Aartrijk et al. 1984). We found a highly significant

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Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid, AVG: aminoethoxy-vinyl glycine, DNA: deoxyribonucleic acid, NAA: 1-naphthylacetic acid, RNA: ribonucleic acid, SAM: S-adenosylmethionine, TIBA: 2,3,5-triiodobenzoic acid

relationship between the ultimate number of plantlets per explant and the speed of recovery and measure of ethylene production during repair of membrane damage. However, these results did not permit to distinguish whether ethylene synthesis was merely the result of the regeneration process or a causal factor in it. Such a conclusion requires further information on the effects on plantlet induction of modifying ethylene biosynthesis and of exogenous ethylene.

Data on the effects of ethylene in systems *in vitro* are scarce. Bouriquet (1972) and Lefebvre (1972) reported increased bud formation on root fragments of *Cichorium intybus* as a result of the application of 100-200 ppm ethylene early in the culture period. Huxter et al. (1979, 1981), studying callus cultures of *Nicotiana tabacum*, established a correlation between callus growth and ethylene production. Treatments with inhibitors of ethylene synthesis fitted well into this correlation. Moreover, the ethylene or ACC, the immediate precursor in ethylene biosynthesis, stimulated shoot regeneration, if applied within a critical period of the culture. Recently, Everett (1982) described an inhibitory effect of AVG on plantlet regeneration from tobacco cotyledons.

In this paper we describe experiments on the effects of AVG, ACC, and ethylene on the process of adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb.

Materials and Methods

Plant material: Bulbs of *Lilium speciosum* Thunb., cv. Rubrum nr. 10, circumference 18-20 cm, were harvested in the autumn of 1980, 1981 and 1982, treated with 0.2% Benlate (active ingredient 50% benomyl) and 1.5% Difolatan (a.i. 48% captafol) for 30 min, and stored at $0^{o}C$ until use. The experiments were performed in the period April to September with bulbs harvested in the preceding autumn.

Explants: The preparation of the explants (7×7 mm^2) was as described (Van Aartrijk and Blom-Barnhoorn 1983). Additional wounding of explants was performed by aseptically cutting away the abaxial epidermis and the outermost sub-epidermal layers. Such explants were designated wounded explants. Others were termed normal. Each explant was placed with the abaxial side down on 15 ml nutrient medium in a glass tube (150 x 25 mm) that was closed with a transparent plastic cap (Bellco, Vineland, U.S.A.). One explant was used per tube.

Nutrient media: The composition and preparation of the media were as reported by Van Aartrijk and Blom-Barnhoorn (1983). AVG (obtained from Dr. R. Maag AG, Dielsdorf, Switzerland) and ACC (Calbiochem-Behring Corp., La Jolla, U.S.A.) were added to the media after separate filter sterilization. TIBA (Serva Chem., Heidelberg, F.R.G.) was co-autoclaved at $120^{o}C$ for 20 min.

Exposure to ethylene: Cultures were placed in an exsiccator, volume 10 l, and flushed at 10 lh^{-1} with sterile ethylene gas, 1 or 10 ppm in air, during the first 3 or 7 days of the culture period. Cultures, flushed at 10 lh^{-1} with sterile, ethylene-filtered (Purafil, Borg-Warner Comp., Washington, W. Virginia,

U.S.A.) air, served as controls of the ethylene treatments. Explants cultured routinely in a growing room were used to check the effect of flushing the cultures.

All experiments were performed in a controlled-temperature room at 20°C. The quantum flux density was 27.9 μEm^{-2}. sec^{-1} (Philips 25 W/TL 33; 16h photoperiod). The number of cultures per treatment was 46. After 10 weeks of culture, the number and polarity of plantlets per explant were determined, unless stated otherwise. Plantlets developing at the basal (proximal) end of the explants were designated basal plantlets. All others were termed non-basal. The reported data were confirmed by the results of at least one similar experiment. For each result the standard deviation of the mean is reported. Differences were tested for significance at p = 0.05.

Results

Addition of AVG to the nutrient medium resulted in a concentration-dependent decrease of the number of plantlets formed on both normal and wounded explants (Fig. 1). Concentrations $\geqslant$0.1 mM led to further reduction of plantlet regeneration and were toxic to the explants, especially to the additionally wounded ones (data not shown). AVG affected both basal and non-basal plantlet regeneration, the effect on non-basal plantlets consistently being more pronounced (Table 1).
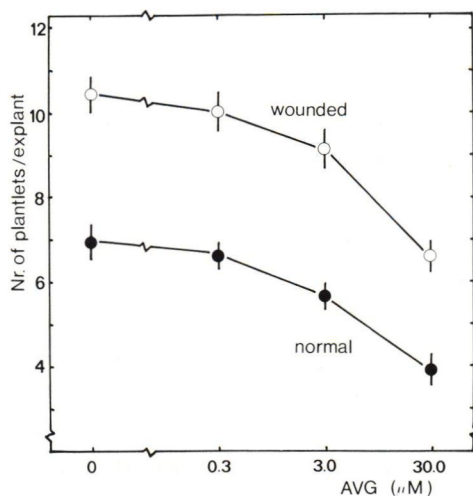

Fig. 1: Effect of AVG on the average number of plantlets per normal or wounded bulb-scale explant of *Lilium speciosum* Thunb. NAA-concentration: 0.5 μM. All explants regenerated.

Table 1: Effects of AVG on the average number of plantlets per normal explant ($\bar{x}$) and on the polarity of the regeneration sites. NAA concentration was 0.5 μM. All explants regenerated.

AVG (μM)	0	0.01	1	10
$\bar{x}$	6.9 + 0.3	6.9 + 0.4	5.7 + 0.4	5.0 + 0.4
% basal	54	55	58	62

Table 2: Effects of TIBA and AVG on the average number of plantlets per normal explant and on the polarity of the regeneration sites. Between brackets: % of the plantlets at basal sites. NAA concentration was 0.5 μM. All explants regenerated.

TIBA (M)	AVG (μM)	
	0	10
0	6.7 + 0.4 (75)	4.2 + 0.5 (86)
2.10^{-7}	8.7 + 0.5 (68)	6.0 + 0.7 (80)
2.10^{-6}	10.3 + 0.5 (59)	6.2 + 0.4 (73)

The presence of TIBA in the nutrient medium caused an increase in the number of plantlets formed both in the absence and in the presence of AVG (Table 2). Most of the TIBA-induced plantlets were situated at non-basal sites.

ACC, in concentrations up to 50 μM, enhanced the number of plantlets per explant (Table 3), and suppressed the polarity of their sites. When applied together, ACC nullified the effect of AVG on plantlet number and counteracted the effect of AVG on the polarity of the regeneration (Table 3).

Ethylene (1-10 ppm in air), flushed through the cultures in the first 3 or 7 days of the culture period, promoted the number of plantlets per explant, particularly at non-basal sites, but to a smaller extent than ACC (Table 4). We did not establish any significant effect of the ethylene concentration or of the duration of the treatment. The increase in plantlet number upon 7 days flushing with air was found to be consistent; since it cannot be ascribed to ethylene, it may depend on removal of a gaseous component as, e.g., CO_2.

Fig. 2 shows the effects of NAA on the number of plantlets per explant, as influenced by a 3-day treatment with 1 ppm ethylene. The effect of ethylene depended on the amount of NAA present in the nutrient medium. In the absence of NAA, ethylene caused a small but consistent increase in the number of plantlets. At 0.5 μM NAA, ethylene clearly promoted plantlet induction, whereas

Table 3: Effects of AVG and ACC on the average number of plantlets per normal explant and on the polarity of the regeneration sites. Between brackets: % of the plantlets at basal sites. NAA concentration was 0.5 μM. All explants regenerated.

ACC (μM)	AVG (μM)	
	0	30
0	5.6 + 0.3 (66)	3.7 + 0.3 (78)
10	8.1 + 0.6 (57)	5.0 + 0.4 (66)
50	8.3 + 0.6 (55)	5.7 + 0.4 (58)

Table 4: Effects of ethylene (1 or 10 ppm), applied in the first 3 or 7 days of the culture period, on the average number of plantlets per normal explant and on the polarity of the regeneration sites. Between brackets: % of the plantlets at basal sites. NAA concentration was 0.5 μM. All explants regenerated.

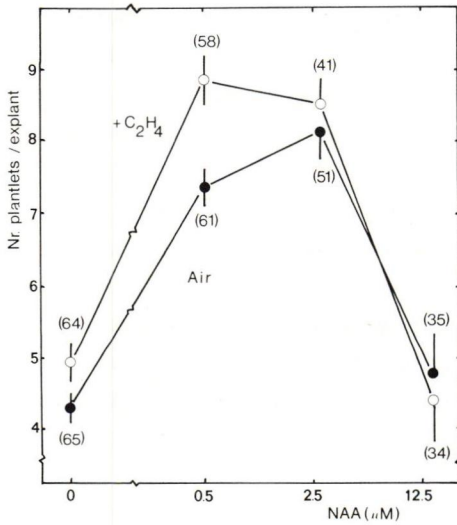| Ethylene (ppm)	Days of flux		
	0	3	7
0	5.0 + 0.3 (62)	4.9 + 0.4 (61)	5.9 + 0.3 (63)
1	–	6.2 + 0.4 (56)	6.9 + 0.4 (58)
10	–	6.9 + 0.3 (54)	7.2 + 0.4 (57)

Fig. 2: Effect of NAA on the average number of plantlets per bulb-scale explant of *Lilium speciosum* Thunb., as influenced by ethylene (1 ppm in air), supplied during the first 3 days of the culture period. Normal explants were used. Between brackets: % of plantlets located at basal sites.

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at higher NAA concentrations this promotive effect was lost. The reduction in the number of plantlets at these supra-optimal concentrations is ascribed to membrane damage, the shift of the curve to lower auxin concentration by ethylene is ascribed to intracellular auxin accumulation (see Discussion).

Discussion

Striking similarities between the effects of NAA, wounding, and temperature on the number of plantlets and the polarity of their sites (Van Aartrijk and Blom-Barnhoorn 1981, 1983), and the interactions between the effects of these factors (Van Aartrijk and Blom-Barnhoorn 1984) led to the hypothesis that they act, at least partly, through one biochemical process, possibly that of ethylene biosynthesis. The significant correlations between the ethylene production by and the plantlet development on bulb-scale explants (Van Aartrijk et al. 1984), and the effects of AVG, ACC, and exogenous ethylene (Fig. 1, Tables 1, 2, 3, 4) allow the conclusion that, indeed, the pathway of ethylene biosynthesis plays a pivotal role in the process of adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. The counteracting effects of ACC and AVG (Table 3) indicate that ethylene biosynthesis in the tissue proceeds through the common pathway: methionine $\longrightarrow$ SAM $\longrightarrow$ ACC $\longrightarrow$ ethylene (Adams and Yang 1979, Lürssen et al. 1979).

TIBA counteracts the effects of AVG (Table 2) and acts similarly to ACC and ethylene (Tables 3, 4). These data, and the fact that both ethylene and TIBA are known to inhibit basipetal auxin transport (Burg and Burg 1967, Osborne and Mullins 1969, Goldsmith 1977), indicate that all these factors affect the process of polar auxin translocation. The effects of TIBA, wounding, and temperature were more pronounced in the presence of 0.5 µM NAA than in the absence of the auxin (Van Aartrijk and Blom-Barnhoorn 1984). The effects of ethylene and NAA also interact (Fig. 2), and indicate an ethylene-induced shift of the NAA optimum toward a lower concentration. It may be noteworthy to relate this shift in auxin optimum with the oppositely directed one found by Mulkey et al. (1982) for the elongation of maize roots after treatment with AVG and cobalt ions. Based on the above-mentioned data we conclude that auxin and auxin transport (as affected by TIBA and/or ethylene) are the key factors regulating the process of adventitious plantlet induction in bulb-scale explants of *Lilium speciosum* Thunb.

In an effort to explain the interacting effects of NAA, wounding, temperature, TIBA, ACC, and AVG, on the number of plantlets and the polarity of their

sites in this regeneration system, we like to propose the following model
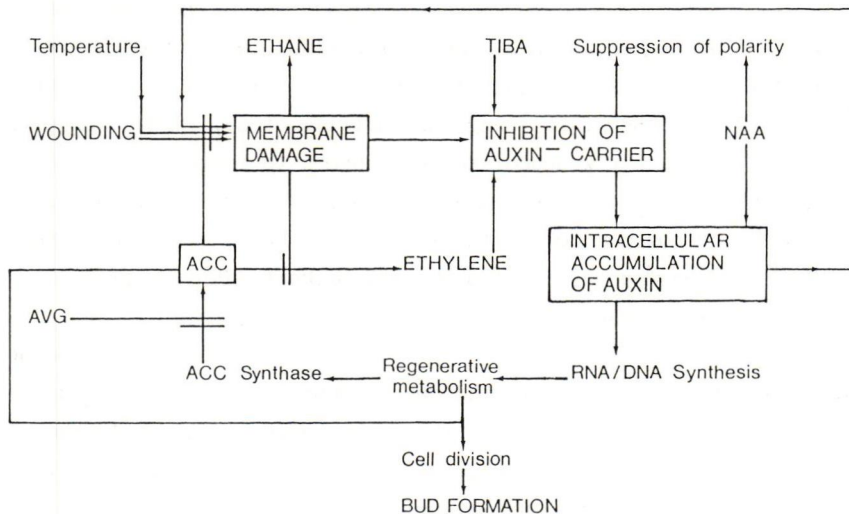(Fig. 3):

Fig. 3: Model of bud induction in bulb-scale explants of *Lilium speciosum*
Thunb., cv. Rubrum nr. 10, *in vitro*.

Wounding is thought to initiate the sequence of events. Wound effects may
be found up to 2-5 mm from the cut surfaces (Laties 1978, Tanaka and Uritani
1979), i.e., in the major part of our up to 5 mm thick explants, especially
the additionally wounded ones. This role for wounding is in agreement with
literature data (Aitchison et al. 1977, Rosenstock and Kahl 1978), and with
the observation that the extrapolated line representing the plantlet-inducing
efficiency per wound roughly goes through zero (Van Aartrijk and Blom-Barnhoorn
1983, Fig.3).

Cutting the tissue inevitably causes *membrane damage* (Galliard 1978, Laties
1978). Free-radical-mediated peroxidation of polyunsaturated fatty acids, re-
leased from the membranes, is set in motion, leading to, a.o., the production
of *ethane* (John and Curtis 1977, Galliard 1978, Kimmerer and Kozlowski 1982).
The amount of ethane produced, considered to represent the amount of membrane
damage, was enhanced by additional wounding, *higher temperature*, and,
remarkably, by *AVG* and *auxin* (Van Aartrijk et al. 1984, Fig.4, Table 1). This,
and the observation by John and Curtis (1977) that young, meristematic tissue
of bean seedlings had the greatest ability to liberate ethane after wounding,

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indicate that membrane damage upon wounding is especially pronounced in auxin-
-rich tissue.

As a consequence of the membrane damage, the membrane potential is changed
(Starrach et al. 1984), and membrane-bound processes may be impaired: Laties
(1982) related the disorganization of the CN-resistant, alternate respiratory
path in fresh slices of storage organs to the loss of mitochondrial membrane
activity. Similarly, Evensen (1984) and Van Aartrijk et al. (1984) suggested
a reduced ability of freshly wounded storage tissues to convert ACC into
ethylene, a process known to be membrane-associated (Mayak et al. 1981).

Critical in the proposed model is the assumption that the action of the
membrane-linked *auxin-anion carrier* (Jacobs and Gilbert 1983) is also
inhibited, which in the presence of auxin may result in *intracellular
accumulation of auxin* (Goldsmith 1982). An increased level of IAA in wounded
root tissue of sweet potato prior to the development of wound-induced enzyme
systems was reported by Uritani (1982). The accumulated auxin may further
enhance membrane damage (Van Aartrijk et al. 1984), but also stimulate the
synthesis of RNA and DNA (Yeoman and Mitchell 1970, Van der Linde 1984),
coding possibly for, e.g., proteins involved in regenerative metabolism
(Laties 1978, Abraham and Reinhold 1980). *ACC synthase* might be formed, too,
since auxin and wounding are known to initiate *de novo* synthesis of ACC synthase
(Yu et al. 1979, Konze and Kwiatkowski 1982).

Several observations suggest that the radical scavenger, ACC, may well be
involved in the process of membrane recovery. First, the productions of
ethylene and ethane were negatively correlated, and the ethane/ethylene
regression lines, referring to a culture temperature of 25° C, intersected
each other at a corresponding ethylene production of 0 (Van Aartrijk et
al. 1984, Fig. 5). Recent data of Bousquet and Thimann (1984) suggest that
the ACC-oxidizing system and the ethane-forming process (John and Curtis 1977,
Kimmerer and Kozlowski 1982) may have steps in common. Second, the inhibition
of ACC synthase by AVG led to increased ethane production (Van Aartrijk et
al. 1984, Fig. 4, 5, 6). Thirdly, ACC is more effective in bud regeneration
than ethylene (Tables 3, 4), indicating a function of ACC other than merely
an intermediate one in ethylene synthesis. The *ethylene* formed may further
inhibit basipetal auxin efflux from the cells (Goldsmith 1977), which results
in the maintenance of a high intracellular auxin concentration, which in turn
will lead to ethylene formation, etc., thus establishing an autocatalytic
process. Ethylene is produced by the bulb-scale explants in an apparently

autocatalytic process, except when AVG was present in the medium (Van Aartrijk et al. 1984, Fig. 1). Ethylene, applied in the first 3 or 7 days of the culture period stimulated bud number and suppressed the polarity, particularly in the presence of 0.5 μM NAA (Fig. 2).

The presence of NAA in the nutrient medium also suppressed the polarity of bud formation, possibly because of the overriding effect of passive, lateral auxin translocation (Fig. 2). The effects caused by *TIBA* can be explained by its inhibitory action on basipetal auxin transport (Jacobs and Gilbert 1983). This also accounts for the interacting and additive effects of TIBA and AVG (Table 2) and of other factors (Van Aartrijk and Blom-Barnhoorn 1984).

The model, the hypothetical aspects of which need further investigation, designates auxin and its translocation as the regulatory factors for bud initiation in bulb-scale explants, when the inevitable membrane damage is alleviated by the auxin-dependent ethylene-synthesizing system.

Acknowledgements

References

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Growth regulator requirements for adventitious regeneration from *Lilium* bulb-scale tissue *in vitro*, in relation to duration of bulb storage and cultivar. Scientia Hortic. 14,261-268 (1981).

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*. Effects of wounding, TIBA, and temperature. Z. Pflanzenphysiol. 110,355-363 (1983).

AARTRIJK, J. VAN and G.J. BLOM-BARNHOORN: Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*. Interacting effects of NAA, TIBA, wounding, and temperature. J. Plant Physiol. (1984; in press).

AARTRIJK, J. VAN, G.J. BLOM-BARNHOORN and J. BRUINSMA: Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*. Production of ethane and ethylene. J. Plant Physiol. (1984; submitted for publication).

ABRAHAM, G. and L. REINHOLD: Mechanism of effect of aging on membrane transport in leaf strips of *Centranthus ruber*: possible ethylene involvement in cutting shock. Planta 150,380-384 (1980).

ADAMS, D.O. and S.F. YANG: Ethylene biosynthesis: identification of 1-amino-cyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Natl. Acad. Sci. USA 76,170-174 (1979).

AITCHISON, P.A., A.J. MACLEOD and M.M. YEOMAN: Growth patterns in tissue (callus) cultures. In: STREET, H.E. (ed.): Plant Tissue and Cell Culture, pp. 267-306, Blackwell Scientific Publications, London (1977).

BOURIQUET, R.: Action de l' éthylène sur le bourgeonnement et la floraison *in vitro* de fragments de racines d'endive. C.R. Acad. Sci. (série D) 275, 33-34 (1972).

BOUSQUET, J-F. and K.V. THIMANN: Lipid peroxidation forms ethylene from 1-amino-cyclopropane-1-carboxylic acid and may operate in leaf senescence. Proc. Natl. Acad. Sci. USA 81,1724-1727 (1984).

BURG, S.P. and E.A. BURG: Inhibition of polar auxin transport by ethylene. Plant Physiol. 42,1224-1228 (1967).

EVENSEN, K.B.: Calcium effects on ethylene and ethane production and 1-amino-cyclopropane-1-carboxylic acid content in potato disks. Physiol. Plant. 60, 125-128 (1984).

EVERETT, N.: The determination phase of differentiation. In: FUJIWARA, A. (ed.): Plant tissue culture 1982. Jap. Ass. Plant tissue culture, Tokyo, Japan (1982).

GALLIARD, T.: Lipolytik and lipoxygenase enzymes in plants and their action in wounded tissues. In: KAHL, G. (ed.): Biochemistry of wounded plant tissues, pp. 155-201, Walter de Gruyter, Berlin, New York (1978).

GOLDSMITH, M.H.M.: The polar transport of auxin. Ann. Rev. Plant Physiol. 28, 439-478 (1977).

GOLDSMITH, M.H.M.: A saturable site responsible for polar transport of indole-3--acetic acid in sections of maize coleoptiles. Planta 155,68-75 (1982).

HUXTER, T.J., D.M. REID and T.A. THORPE: Ethylene production by tobacco (*Nicotiana tabacum*) callus. Physiol. Plant. 46,374-380 (1979).

HUXTER, T.J., T.A. THORPE and D.M. REID: Shoot initiation in light- and dark--grown tobacco callus: the role of ethylene. Physiol. Plant. 53, 319-326 (1981).

JACOBS, M. and S.F. GILBERT: Basal localization of the presumptive auxin transport carrier in pea stem cells. Science 220,1297-1300 (1983).

JOHN, W.W. and R.W. CURTIS: Isolation and identification of the precursor of ethane in *Phaseolus vulgaris* L. Plant Physiol. 49,521-522 (1977).

KIMMERER, T.W. and T.T. KOZLOWSKI: Ethylene, ethane, acetaldehyde, and ethanol production by plants under stress. Plant Physiol. 69,840-847 (1982).

KONZE, J.R. and G.M.K. KWIATKOWSKI: Rapidly induced ethylene formation after wounding is controlled by the regulation of 1-aminocyclopropane-1-carboxylic acid synthesis. Planta 151,327-330 (1981).

LATIES, G.G.: The development and control of respiratory pathways in slices of plant storage organs. In: KAHL, G. (ed.): Biochemistry of wounded plant tissues, pp. 421-466, Walter de Gruyter, Berlin, New York (1978).

LATIES, G.G.: The cyanide-resistant, alternative path in higher plant respiration. Ann. Rev. Plant Physiol. 33,519-556 (1982).

LEFEBVRE, R.: Effets comparés d'un traitement anaérobie et de l'éthylène sur le bourgeonnement et la synthèse des pigments foliaires. C.R. Acad. Sci. (série D)275,193-195 (1972).

LINDE, P.C.G. VAN DER, H. BOUMAN, A.M. MENNES and K.R. LIBBENGA: A soluble auxin-binding protein from cultured tobacco tissues stimulates RNA synthesis *in vitro*. Planta 160,102-108 (1984).

LÜRSSEN, K., K. NAUMANN and R. SCHRÖDER: 1-aminocyclopropane-1-carboxylic acid -- an intermediate of ethylene biosynthesis in higher plants. Z. Pflanzenphysiol. 92,285-294 (1979).

MAYAK, S., R.L. LEGGE and J.E. THOMPSON: Ethylene formation from 1-aminocyclopropane-1-carboxylic acid by microsomal membranes from senescing carnation flowers. Planta 153,49-55 (1981).

MULKEY, T.J., K.M. KUZMANOFF and M.L. EVANS:Promotion of growth and shift in the dose/response relationship in maize roots treated with the ethylene biosynthesis inhibitors aminoethoxyvinyl glycine and cobalt. Plant Sci. Lett. 25, 43-48 (1982).

OSBORNE, D.J. and M.G. MULLINS: Auxin, ethylene, and kinetin in a carrier--protein model system for the polar transport of auxins in petiole segments of *Phaseolus vulgaris*. New Phytol. 68,977-991 (1969).

ROSENSTOCK, G. and G. KAHL: Phytohormones and the regulation of cellular pro-
    cesses in aging storage tissues. In: KAHL, G. (ed.): Biochemistry of
    wounded plant tissues, pp. 623-671, Walter de Gruyter, Berlin, New York
    (1978).
STARRACH, N., C. FREUDLING, W.E. MAYER and D. GRADMAN: Aged preparations are
    electrophysiologically more representative for intact tissue than are freshly
    excised preparations. Planta 160,88-90 (1984).
TANAKA, Y. and I. URITANI: Polar transport and content of indole-3-acetic acid
    in wounded sweet potato root tissues. Plant & Cell Physiol. 20,1087-1096
    (1979).
URITANI, I.: Biochemical approaches to general principles in plants underlying
    plant disease phenomena. In: ASADA, Y. et al. (eds.): Plant Infection: The
    physiological and biochemical basis, pp. 345-358, Jap. Sci. Soc. Press,
    Tokyo, Japan (1982).
YEOMAN, M.M. and J.P. MITCHELL: Changes accompanying the addition of 2,4-D
    to excised Jerusalem artichoke tuber tissue. Ann. Bot. 34,799-810 (1970).
YU, Y-B. and S.F. YANG: Auxin-induced ethylene production and its inhibition
    by aminoethoxyvinyl glycine and Cobalt ion. Plant Physiol. 64,1074-1077
    (1979).

Samenvatting

Adventieve plantvorming *in vitro* vanuit bolschubweefsel van *Lilium speciosum* Thunb.

In dit proefschrift worden de interagerende effecten beschreven van een aantal weefsel-, voedingsbodem- en omgevingsfactoren op de adventieve plant- vorming *in vitro* vanuit bolschubweefsel van *Lilium speciosum* Thunb., cv. Rubrum nr. 10. Voorts worden resultaten weergegeven van experimenten ter toet- sing van de hypothese, dat de effecten van en de interacties tussen enkele van de onderzochte factoren te verklaren zijn vanuit hun invloed op een centraal fysiologisch proces. De belangrijkste resultaten van het onderzoek worden hieronder puntsgewijs weergegeven:

In Hoofdstuk I worden de effecten onderzocht van het auxine 1-naphthylazijn- zuur (NAA) en van de cytokininen N^6-benzyladenine (BA) en N^6-[$\triangle$2-isopentenyl]- -adenine (2iP).
   -De beide cytokininen hadden geen invloed op het aantal plantjes dat gevormd werd per explantaat. Wel remden zij de (bol)groei van de geïnduceerde plantjes.
   -Lage concentraties auxine ($\leqslant$0,5 μM NAA) bevorderden het aantal geïnduceerde plantjes, maar hogere concentraties niet.
   -Bewaring bij 0^oC van de uitgangsbollen leidde tot een verschuiving van de optimale NAA concentratie naar een lagere waarde.
   -Vanuit het basale uiteinde van de explantaten vond optimale regeneratie van plantjes bij een lagere NAA concentratie plaats dan vanuit het niet- basale deel van het explantaat.
   -Toediening van NAA aan het voedingsmedium leidde tot een onderdrukking van de basipetale polariteit van het regeneratieproces, d.w.z., het aantal niet- basaal geïnduceerde plantjes nam toe ten opzichte van het totale aantal per explantaat.

Hoofdstuk II beschrijft hoe de factoren verwonding, TIBA en temperatuur het regeneratieproces beïnvloeden.
   -Het verwijderen van de abaxiale epidermis van de explantaten resulteerde, bij aanwezigheid van 0,5 μM NAA in het voedingsmedium, in verhoging van het aantal plantjes per explantaat. Dit effect kon worden toegeschreven aan de verwonding

zelf en niet aan de afwezigheid van die abaxiale epidermis of aan gewijzig-
de diffusie tussen het weefsel en de voedingsbodem.

-De toevoeging van lage concentraties 2,3,5-triiodobenzoëzuur (TIBA) aan het
voedingsmedium ($\leqslant$2 μM), in de aanwezigheid van 0,5 μM NAA, stimuleerde de
adventieve plantvorming. Hogere concentraties remden het proces.

-In het onderzochte traject van 15°-25°C leidde een temperatuurverhoging,
bij aanwezigheid van 0,5 μM NAA, tot een lineaire toename van het aantal
plantjes per explantaat.

-Verwijdering van de abaxiale epidermis, TIBA en temperatuurverhoging
verminderden de basipetale polariteit van het regeneratieproces.

-De overeenkomstige effecten van de factoren NAA, verwonding, TIBA en tempe-
ratuur leidden tot de hypothese dat er interacties bestaan tussen de effecten
van deze factoren.

In Hoofdstuk III worden deze interacties tussen de effecten van de factoren
NAA, verwonding, TIBA en temperatuur nader onderzocht.

-*verwonding en NAA:* het auxine-optimum lag voor extra verwonde explantaten
bij een lagere concentratie NAA dan voor normale explantaten.

-*NAA en TIBA:* het stimulerende effect van TIBA op de plantinductie was veel
sterker bij aanwezigheid van 0,5 μM NAA dan bij afwezigheid van het auxine.

-*TIBA en NAA:* om het optimale effect van TIBA te bewerkstelligen moest bij
aanwezigheid van 0,5 μM NAA meer TIBA worden toegediend dan bij afwezigheid
van NAA.

-*verwonding en TIBA:* extra verwonde explantaten waren gevoeliger voor de
effecten van TIBA dan normale explantaten.

-*temperatuur, verwonding en NAA:* temperatuurverhoging leidde tot een lineaire
toename van het aantal plantjes per explantaat. De richtingscoëfficiënt van
de verkregen lijnen was afhankelijk van de verwondingsgraad van de explan-
taten en van de aanwezigheid van 0,5 μM NAA in het voedingsmedium. Na extra-
polatie sneden deze lijnen elkaar op de X-as (aantal plantjes per explantaat
= 0) bij ca. 3°C.

-Het effect van elk van de factoren NAA, TIBA, verwonding en temperatuur op de
basipetale polariteit van het regeneratieproces bleek afhankelijk te zijn van
de andere.

-De endogene concentratie vrij IAA in bolschubweefsel van *Lilium speciosum*
was zeer laag, nl. 1,1 $\pm$ 0,1 ngg^{-1} vers gewicht.

-De hypothese werd gesteld, dat de werking van de factoren NAA, verwonding

en temperatuur tenminste gedeeltelijk berust op de beïnvloeding van één fysiologisch proces, vermoedelijk dat van de ethyleenbiosynthese. Deze hypothese wordt getoetst in de Hoofdstukken IV en V.

Hoofdstuk IV geeft de effecten weer, die de factoren NAA, verwonding, temperatuur en aminoethoxyvinyl glycine (AVG) hebben op de ethyleen- en ethaanproduktie door bolschubexplantaten gedurende het verloop van het regeneratieproces.

-Ethyleen en ethaan werden geproduceerd door de explantaten. De gemeten hoeveelheden waren afhankelijk van de kweekomstandigheden en van de fase waarin het regeneratieproces zich bevond.

-In de eerste twee weken van de cultuurperiode, d.w.z. gedurende de periode van celdelingen, werd een toename van de ethyleenproduktie gemeten. Deze periode werd gevolgd door een fase, gekenmerkt door een dalende ethyleen-produktie, die samenviel met de differentiatie van groeipunten.

-Vanaf dag 2 tot en met dag 4 van de cultuurperiode werden, voor alle onderzochte behandelingen, een exponentiële toename van de ethyleenproduktie, en een exponentiële afname van de ethaanproduktie gemeten. Voor alle behandelingen werd een negatieve, lineaire correlatie gevonden tussen de logarithme van de ethaanproduktie en de ethyleenproduktie.

-Correlaties werden gevonden tussen het aantal plantjes per explantaat aan het einde van de cultuurperiode en de ethyleen-, resp. de ethaanproduktie door het weefsel. Zeer significant ($p < 0,0005$) was de correlatie tussen het uiteindelijke aantal plantjes per explantaat en de hellingshoeken van de waargenomen ethaan/ethyleen regressielijnen.

Hoofdstuk V beschrijft de effecten van AVG, 1-aminocyclopropaan-1-carbonzuur (ACC), de onmiddellijke bron van ethyleen in de biosyntheseroute, en van toegediend ethyleen op de spruitregeneratie.

-Toediening van 0,3-30 μM AVG aan het voedingsmedium leidde tot een concentratie-afhankelijke remming van de adventieve spruitvorming. De basipetale polariteit van het regeneratieproces werd versterkt.

-Toevoeging van 10-50 μM ACC aan het voedingsmedium stimuleerde daarentegen de adventieve plantvorming en onderdrukte de basipetale polariteit van het proces. 50 μM ACC deed de effecten van 30 μM AVG teniet.

-Ethyleen (1-10 dpm in lucht), toegediend gedurende de eerste 3 of 7 dagen van de cultuurperiode, stimuleerde de adventieve plantvorming, bij aanwezigheid van 0,5 μM NAA, en onderdrukte de basipetale polariteit van het proces.

Aan het slot van Hoofdstuk V wordt een aantal conclusies getrokken:

1)Het proces van ethyleenbiosynthese speelt een belangrijke rol bij de adventieve plantvorming vanuit bolschubweefsel van *Lilium speciosum* Thunb. *in vitro*.

2)De effecten van TIBA, verwonding, temperatuur en ethyleen zijn afhankelijk van de aanwezigheid van auxine.

3)Auxine en het basipetale transport ervan, beïnvloed door TIBA en ethyleen, zijn de belangrijkste factoren die het proces van adventieve plantvorming vanuit het bolschubweefsel reguleren.

Een model wordt voorgesteld ter verklaring van de waargenomen interagerende effecten (zie Hoofdstuk V, Fig. 3). Het model geeft aan dat auxine en auxine-transport de belangrijkste factoren zijn die het regeneratieproces reguleren, mits de bij de explantaatbereiding geïnduceerde membraanschade kan herstellen onder invloed van het proces van ethyleenbiosynthese.

Summary

Adventitious bud formation *in vitro* from bulb-scale explants of *Lilium speciosum* Thunb.

In this thesis the interactive effects are described of tissue, medium, and other environmental factors on the process of adventitious bud formation *in vitro* from bulb-scale explants of *Lilium speciosum* Thunb. Besides, results are presented of experiments designed to test the hypothesis that the effects of some of the factors can be explained by their action on one physiological process. Some important results from the investigations are stated below:

Chapter I presents effects of the auxin, 1-naphthylacetic acid (NAA), and of the cytokinins, N^6-benzyladenine (BA) and N^6-[$\triangle 2$-isopentenyl]-adenine (2iP).
-The two cytokinins did not influence the number of adventitiously formed plantlets per explant, but severely reduced the bulblet growth of these plantlets.
-Low concentrations ($\leqslant 0.5$ μM) of NAA led to an increase in the number of plantlets per explant. Higher concentrations were less effective.
-Explants of cold-stored bulbs (at 0°C) were more sensitive to the action of NAA than those of uncooled bulbs.
-The regeneration process on bulb-scale explants showed basipetal polarity. Addition of NAA to the nutrient medium suppressed this polarity.
-The optimum response to NAA of 'basal' plantlets was found at a lower concentration than that for 'non-basal' plantlets.

Effects of the factors wounding, TIBA, and temperature on plantlet number and polarity are described in Chapter II.
-Additional wounding of the explants by removal of the abaxial epidermis led to an increase in the number of plantlets per explant. This effect, observed when explants were cultured in the presence of 0.5 μM NAA, could be ascribed to the act of wounding itself and not to the absence of the abaxial epidermis or to altered diffusion conditions between the explant and the medium.
-Addition of low concentrations ($\leqslant 2$ μM) of 2,3,5-triiodobenzoic acid (TIBA) to the medium, in the presence of 0.5 μM NAA, resulted in an increase in the number of plantlets per explant. Higher concentrations were inhibitory.

-Within the range of 15°-25°C, a higher temperature during the culture period led to a linear increase in the number of plantlets per explant.

-Additional wounding, TIBA, and higher temperature suppressed the basipetal polarity of the regeneration sites in the explants.

-The similarities in the effects of NAA, wounding, TIBA, and temperature, led to the hypothesis that interactions exist between the effects of these factors.

In Chapter III these interactions between the effects of NAA, wounding, TIBA, and temperature, are analyzed.

-*wounding and NAA:* additionally wounded explants regenerated more plantlets than normal ones, and were more sensitive to the action of NAA.

-*NAA and TIBA:* the stimulatory effect of TIBA on the number of plantlets, observed in the presence of 0.5 µM NAA, was absent or much smaller in the absence of the auxin. In the presence of 0.5 µM NAA, TIBA exerted its optimal effect at a higher concentration than without NAA.

-*wounding and TIBA:* additionally wounded explants were more sensitive to the action of TIBA than normal explants.

-*temperature, wounding, and NAA:* within the range of 15°-25°C, a higher culture temperature caused a linear increase in plantlet number. The slope of the line depended on the degree of wounding of the explants and on the presence of 0.5 µM NAA. When extrapolated, the lines intersected the abscissa ($\sim$ number of plantlets per explant=0) at c. 3°C.

-The effects of each of the factors NAA, TIBA, wounding, and temperature, on the polarity of the regeneration sites in the explants, depended on the other ones.

-The endogenous concentration of free IAA in bulb-scale tissue of *Lilium speciosum* Thunb. was established at 1.1 $\pm$ 0.1 ngg^{-1} fresh weight.

-The hypothesis was made that the factors NAA, wounding, and temperature, acted, at least partly, by affecting one central physiological process, possibly that of ethylene biosynthesis.

In Chapter IV effects of the factors NAA, wounding, temperature, and aminoethoxyvinyl glycine (AVG) are described on the production of ethylene and ethane by bulb-scale explants during the course of the culture period.

-Ethylene and ethane were produced by the explants, the amounts depending on condition and stage of the culture.

-The first 1-2 weeks of the culture period, i.e., the stage of cell divisions, were characterized by an increase in the production of ethylene. The subsequent decrease in ethylene production by the explants corresponded with a phase of bud differentiation.

-During days 2-4 of the culture period, an exponential increase in ethylene production by the explants was established for all culture conditions, concomitantly with an exponential decrease of the ethane production. For all culture conditions, a negative linear relationship was found between the logarithm of the ethane production and the ethylene production.

-Various correlations were established between the ultimate number of plantlets per explant and the ethylene and ethane productions by the explants. A highly significant ($p<0.0005$) relationship was found for all culture conditions between the ultimate number of plantlets per explant and the slope of the ethane/ethylene regression lines.

Chapter V describes effects of AVG, 1-aminocyclopropane-1-carboxylic acid (ACC), and ethylene.

Addition of 0.3-30 μM AVG to the nutrient medium caused a concentration-dependent decrease of the number of plantlets per explant and reinforced the basipetal polarity of the process.

-On the contrary, addition of 10-50 μM ACC to the medium led to an increase in the number of regenerated plantlets and suppressed the basipetal polarity. 50 μM ACC nullified the inhibitory effect of 30 μM AVG.

-In the presence of 0.5 μM NAA, ethylene (1-10 ppm in air), supplied during the first 3 or 7 days of the culture period, caused an increase in the number of plantlets per explant and suppressed the polarity of the process.

At the end of Chapter V, some conclusions are drawn:

1) Ethylene biosynthesis plays a key role in the process of adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb.

2) The effects of TIBA, wounding, temperature, and ethylene depend on the presence of 0.5 μM NAA in the nutrient medium.

3) The process of adventitious bud formation from bulb-scale explants of *Lilium speciosum* is regulated by auxin and its basipetal transport, as influenced by, e.g., TIBA and/or ethylene.

A model sysytem (see Chapter V, Fig. 3) is proposed, which accounts for the observed effects. It designates auxin and its translocation as the regulating

factors for bud initiation in bulb-scale explants, when the inevitable membrane damage is alleviated by the auxin-dependent ethylene-synthesizing system.

Curriculum vitae

Jan van Aartrijk werd op 12 maart 1951 te 's Gravenhage geboren. Hij behaalde in 1969 het einddiploma gymnasium β aan de Christelijke Scholengemeenschap te Amstelveen en begon in hetzelfde jaar met de studie biologie aan de Vrije Universiteit te Amsterdam. In november 1972 werd het kandidaatsexamen afgelegd. Het doctoraalexamen werd afgelegd in maart 1976 met als hoofdvak radiobiofysica en als bijvakken plantenfysiologie en cytodifferentiatie.

Sedert april 1976 is hij als wetenschappelijk ambtenaar werkzaam bij de Stichting Laboratorium voor Bloembollenonderzoek te Lisse.