Acta Bot. Neerl. 34(1), February 1985, p. 73–82.

ABSCISIC ACID IN THE REPRODUCTIVE ORGANS OF PETUNIA HYBRIDA AND LILIUM LONGIFLORUM

Z. H. YANG[1], G. W. M. BARENDSE[2] and H. F. LINSKENS[2]

[1]Department of Biology, Peking University, China
[2]Department of Botany, University of Nijmegen, The Netherlands

Key words: ABA, Abscisic acid, incompatibility, *Lilium longiflorum*, *Petunia hybrida*

SUMMARY

Styles, ovaries, anthers and pollen of *Petunia hybrida* and pollen of *Lilium longiflorum* were analysed for the presence of abscisic acid (ABA) by means of high performance liquid chromatography (HPLC) combined with UV and fluorometric detection. All these reproductive organs were shown to contain endogenous ABA. However, styles have a particular high content in comparison with that of ovaries, anthers and pollen. In addition the ABA content of styles increased rapidly during flower development prior to anthesis, while in ovaries and anthers after an initial decrease the ABA content remained constant at a comparatively low level. After compatible cross-pollination the ABA content of styles in particular, but also of ovaries, was significantly reduced compared with incompatible self-pollination.
Lilium longiflorum pollen also contained ABA. Upon germination *in vitro* the ABA content of *Lilium* pollen decreased rapidly, while at the same time a large amount ABA was released into the germination medium, possibly due to enzymic hydrolysis of ABA conjugates present in pollen.

1. INTRODUCTION

Abscisic acid (ABA) was first isolated as an accelerator of abscission from cotton fruits (LUI & CARNS 1961). MAYAK et al. (1972) and EEUWENS & SCHWABE (1975) have identified ABA in mature flowers of cotton, rose and pea. The presence of ABA in pine pollen was shown by SHIBUYA et al. (1978). ABA was also shown in ovules and seeds of pears (MARTIN et al. 1977) and in styles of citrus during flower development (GOLDSCHMIDT 1980). These studies indicate that ABA may occur generally in reproductive organs of higher plants. However, it is not known whether ABA plays any role in the fertilization processes.

BARENDSE et al. (1970) have studied the occurrence and possible role of auxins and gibberellins in the reproductive organs of *Petunia hybrida* and of *Lilium* species. This study deals with the presence of ABA in styles and ovaries of *Petunia hybrida* during flower development, and after incompatible self- and compatible cross-pollination, in anthers during flower development, in pollen of several *Petunia hybrida* clones as well as in pollen of *Lilium longiflorum* before and after germination.

2. MATERIAL AND METHODS

2.1. Plant material

Petunia and *Lilium* plants were grown in a computer controlled greenhouse maintained at 20 °C ± 1, however, occasionally higher when the outside temperature exceeded 21 °C. The natural daylight was when necessary supplemented with artificial light at about 120 μmol $m^{-2}s^{-1}$, supplied with 400 W Philips HPLRG lamps, for a 16 h photoperiod. Styles, ovaries and anthers of *Petunia hybrida* Hort., clone W166H with incompatibility alleles S_2S_3, were collected from flowers at 3, 2 and 1 day prior to and at anthesis.

Pollinations were carried out on emasculated flowers of clone W166H one day before anthesis, i.e. incompatible self-pollination ($S_2S_3 \times S_2S_3$) and compatible cross-pollination with pollen of clone W43, incompatibility alleles S_1S_1 ($S_2S_3 \times S_1S_1$). In addition also a cross-pollination was carried out with "dead" pollen, obtained by X-ray irradiation of clone W43 pollen with an ENRAT-Röntgen tube (type OEG-60) at a dose rate of 100 KR/min for 10 min (GILISSEN & LINSKENS 1976).

Styles and ovaries were collected at respectively 4, 8, 16 and 24 hours after pollination.

In addition pollen was collected from the *Petunia hybrida* clones W166H, T2U, and W78B4, and from *Lilium longiflorum* cv. White Europe. In all cases the collected material was weighed, divided into 1 g portions and immediately frozen at –20 °C for future analysis of the ABA content.

Lilium pollen was germinated at 25 °C for 2 hours in a medium composed of 0.3 M polyethylene glycol 400 (ZHANG & CROES 1982) and boric acid at 100 μg/ml. The density of pollen in the medium was 5 mg fresh weight per ml. After germination pollen and medium were separated by filtering through three layers of nylon gauze and rinsed with cold medium. Both the germinated pollen and the medium were extracted for ABA determination as describe below.

2.2. Extraction

One gram portions of material were homogenized in precooled, i.e. 4 °C, methanol/water (80:20, v/v) with a Potter-Elvehjem homogenizer. A known aliquot of DL-cis, trans [G-^{3}H[- Abscisic acid (39 Ci/mmol, Amersham) was added as an internal standard for quantification of ABA.

The homogenate was stirred continuously and allowed to extract for 48 hours at 4 °C, then centrifuged for 10 min at 12000 × g. The residue was resuspended in methanol/water, stirred, and after one hour again centrifuged. The combined supernatants were evaporated at 35 °C under vacuum with a Büchi-rotavapor untill the aqueous phase remained, which was adjusted to pH 8.5 with 1 N NaOH in order to minimize isomeridation of ABA to trans-ABA (SAUNDERS 1978), followed by centrifugation at 0 °C for 15 min at 1400 × g. The supernatant was carefully removed, adjusted to pH 2.5 with 1 N HCl and partitioned three times with equal volumes of diethylether. The combined diethylether fraction was evaporated to dryness with nitrogen.

2.3. PVP column chromatography

The residue of the ether fraction was dissolved in 0.5 ml methanol/diethylether (1:1, v/v) and treated dropwise with concentrated ammonium hydroxyde solution under shaking, until the ammonia was in excess (4–5 drops). The solution was then evaporated to dryness.

The polyvinylpyrrolidone (PVP) column, 20 × 0.9 cm, was prepared according to SAUNDERS (1978). The above prepared ammonia salts of the samples were dissolved in 0.5 ml 50 mM KH_2PO_4, containing 200 mg/l Sodium diethyldithiocarbamate as antioxidant. The sample was then brought on the PVP column and eluted with 50 mM KH_2PO_4, pH 8.0. The eluent from 5.5 to 12.5 ml, which contained the ABA fraction, was collected and extracted by partitioning with diethylether. The combined diethylether fraction was evaporated to dryness and kept at –20 °C till further analysis with high performance liquid chromatography (HPLC).

2.4. HPLC

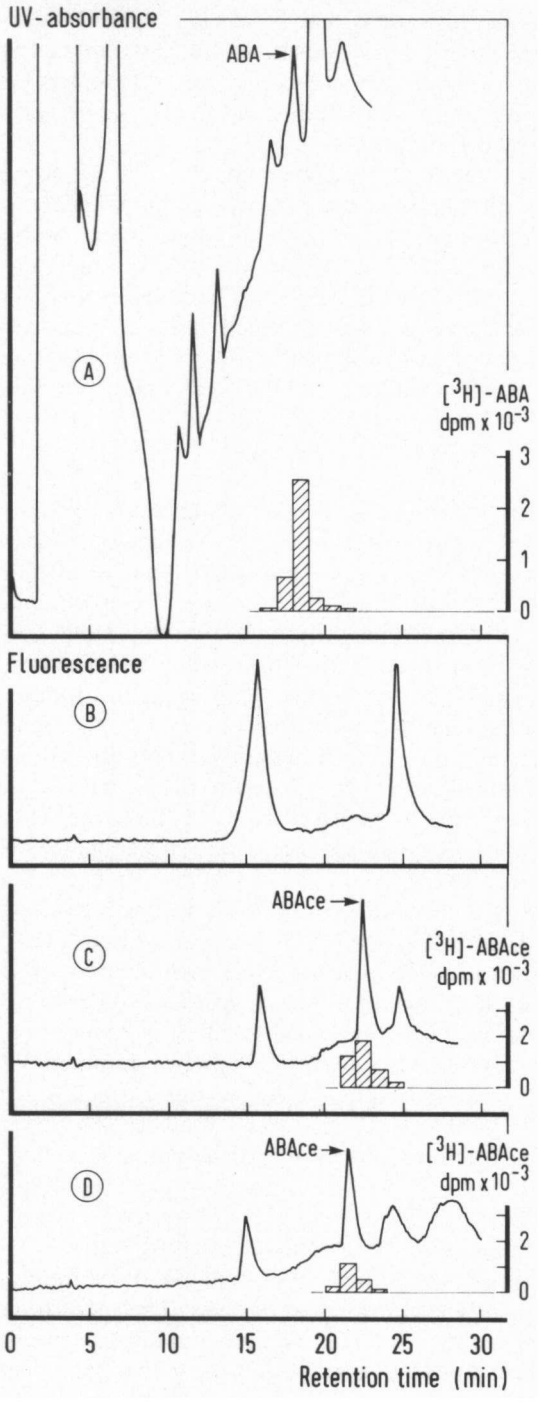
The further purification and separation of the ABA containing samples were carried out on a HPLC system from Waters Associates consisting of two pumps (model 600A), solvent programmer (model 660), universal injector (UK6), variable wavelength detector (model 450), radial compression module (RCM-100) with a Radial-PAK A cartridge containing 10 μ Bondapak C_{18}-porasil B, guard column, Omniscribe recorder (Houston instruments), fraction collector (Pharmacia Frac 3000), and Chromatopac C-R2A integrator (Shimadzu).

The sample to be analysed was dissolved in 200 μl methanol and 10–20 μl were used per injection. For elution a gradient was employed, flow rate 1 ml/min, with methanol/water (15:85, v/v), adjusted to pH 2,8 with acetic acid, as starting solvent, which was linearly displaced by 100% methanol for 15 min. By then the eluens contained 32% methanol and was subsequently kept stationary till the end of the run. ABA was detected by UV absorbance at 254 nm and AUFS 0.04 on the ng level. The identity of the ABA peak was checked by (a) the retention of the added radioactive ABA as internal standard, (b) comparison with retention of authentic ABA, and (c) further identification of the collected ABA fraction by means of spectrofluorometry as described below.

The ABA content of samples was quantified by using the integrator, which compared the integrated ABA peak with a preprogrammed ABA standard concentration curve based on authentic ABA, plus correction for recovery based on the internal [^{3}H]-ABA standard, which varied between 45–55%. The radioactivity of collected fractions was measured by liquid scintillation counting in a Philips PW 4540 LSA.

2.5. Spectrofluorometric identification of ABA

The ABA fraction collected after HPLC and UV detection was adjusted to pH 7.0, and subsequently evaporated to dryness. The methoxycoumaryl ester of ABA (ABAce) was prepared by crown ether catalysis according to the procedures described by CROZIER et al. (1982) for gibberellins. The sample residue


UV-absorbance
ABA
A
[3H]-ABA
dpm x 10-3
3
2
1
0
Fluorescence
B
ABAce
C
[3H]-ABAce
dpm x 10-3
2
0
ABAce
D
[3H]-ABAce
dpm x10-3
2
0
0
5
10
15
20
25
30
Retention time (min)

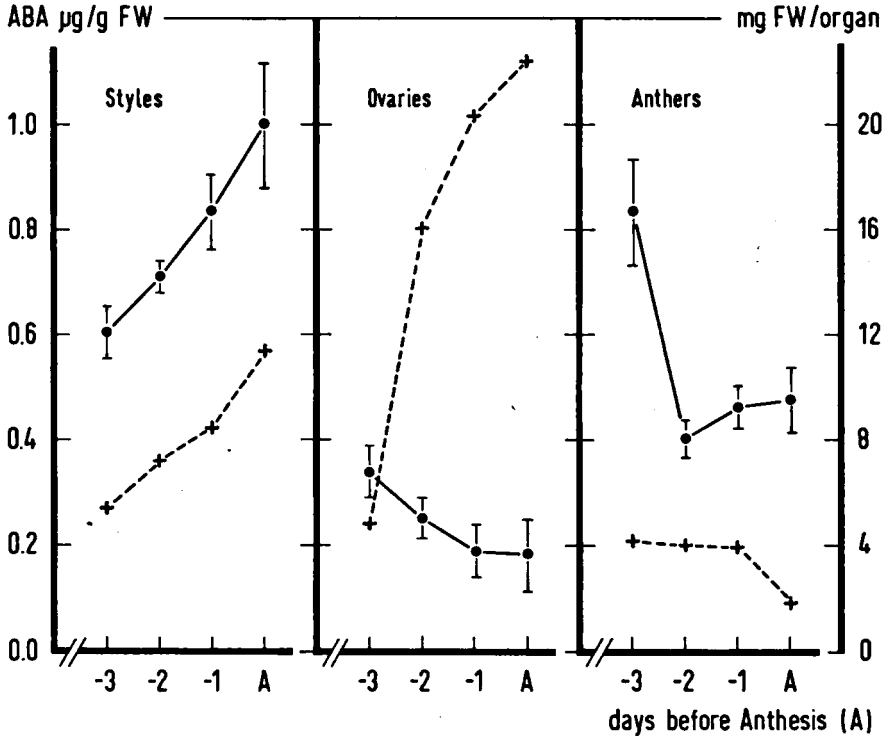


Fig. 2. ABA content and fresh weight (FW) of styles, ovaries and anthers of *Petunia hybrida* during flower development prior to anthesis (A).

was dissolved in 100 µl of dry acetonitrile to which an approximately equimolar amount of 4-bromomethyl-7-methoxycoumarin (BMMC), a one-tenth molar equivalent of 18-crown-6 and a crystal of K_2CO_3 were added.

After incubation at 60 °C for 2 h this yields a highly fluorescent ABAce (λ^{ex}_{max} 320 nm, λ^{ex}_{max} 400) which could be detected on the picogram level after HPLC with a fluorescence spectrometer (Perkin-Elmer LS-3).

Fig. 1. HPLC analysis of ABA in a style extract of *Petunia hybrida*. Column, Radial-PAK A (10 µ Bondapak C_{18}); Mobile phase, 15 min linear gradient, 15–32% methanol in water adjusted with acetic acid to pH 2.8; flow rate, 1 ml min^{-1}. Detectors, UV at 254 nm and 0.04 AUFS (A) and spectrofluorimeter, λ^{ex}_{max} 320, λ^{ex}_{mac} 400 (B, C & D). Radioactivity was determined by fraction collection and liquid scintillation counting.

A: Sample of prepurified style extract plus [^{3}H]-ABA as internal standard.

B: Reaction mixture of BMMC and 18-crown-6.

C: Sample of authentic ABA and [^{3}H]-ABA with BMMC and 18-crown-6 reaction mixture after 2 h at 60 °C.

D: ABA fraction collected from A and reacted with reaction mixture (B) for 2 h at 60 °C.

3. RESULTS

2.1. Determination of ABA in extracts

Preliminary experiments had shown that styles were a particularly rich source of an ABA-like substance. Therefore, style extracts have been purified in different ways, e.g. thin-layer chromatography, PVP column chromatography and Sep-pak C_{18} purification, prior to HPLC, to establish a reliable and convenient assay for the estimation of endogenous ABA. This led to the procedures described in "Material and Methods" which yielded reproducible results with good recovery for detection. An example of a style extract is presented in *fig. 1.*

Fig. 1A shows a HPLC elution profile with UV detection after injection of a sample prepurified by PVP column chromatography and containing [^{3}H]-ABA as internal standard. The gradient, which more than doubles the methanol concentration within 15 min, causes a considerable baseline shift, however, in this way a good separation of ABA from other polar compounds in the sample is obtained reproducibly and within a reasonable time. At least 5 such injections were made per extract and a minimum of 3 extracts were made per treatment. The ABA peak was collected at least three times for liquid scintillation counting to determine recovery and once or twice to prepare the ABAce for further identification by fluorometry. In *fig. 1B* the fluorescence elution profile after HPLC of the reaction mixture, containing BMMC and 18-crown-6, for preparing the ABAce is given. *Fig. 1C* shows the fluorescence profile of authentic ABAce and [^{3}H]-ABAce. In *fig. 1D* the profile is given of the ABA peak, collected from the style extract of *fig. 1A*, after esterification.

Fig. 1D demonstrates that the ABA peak eluted after UV detection (*fig. 1A*) is homogenous and apparently identic to authentic ABA.

The ABA peaks collected after HPLC with UV detection were used for quantification of ABA, since in that case better reproducibility was obtained than with ABA determination after esterification. It was shown with authentic ABA that esterification with BMMC is not always complete and, therefore, this method, although much more sensitive, was only employed for qualitative identification of ABA fractions collected after UV detection of the different extracts.

3.2. ABA in styles, ovaries and anthers of Petunia during flower development

The ABA content of styles, ovaries and anthers of the self-incompatible clone W166H and analysed during flower development prior to anthesis, following the above described procedures. Styles have a relative high ABA content (*fig. 2*) which increases during development concurrent with the increase in fresh weight. The ABA content of ovaries is relatively low compared to styles and even decreases somewhat during the rapid increase in fresh weight of ovaries. In anthers the relative high content in ABA at 3 days before anthesis decreases rapidly to a low, more or less constant level till anthesis, while the fresh weight remains constant except for the drop at anthesis due to desiccation prior to release of pollen.

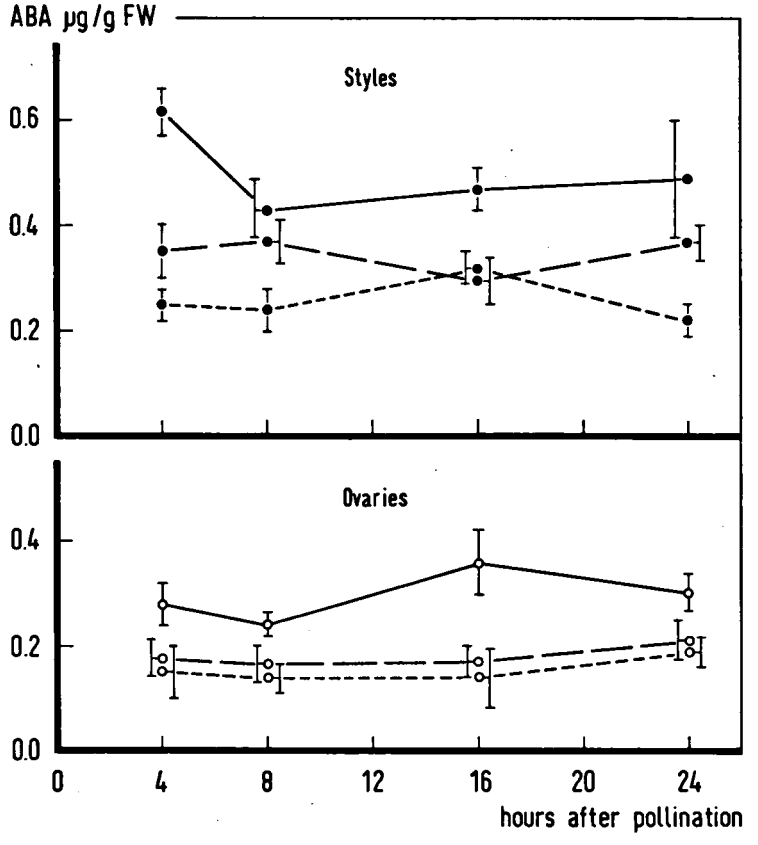


Fig. 3. ABA content of styles and ovaries of *Petunia hybrida* after incompatible self- and compatible cross-pollination. —: self-pollination; – – –: cross-pollination; ---: cross-pollination with irradiated pollen.

3.3. ABA in styles and ovaries following self- and cross-pollination

The ABA content of styles and ovaries was determined after incompatible self-pollination of clone W166H and compatible cross-pollination of W166H with pollen of clone W43. Clone W166H was also pollinated with irradiated W43 pollen; "dead" pollen.

Fig. 3 shows the ABA determinations at 4, 8, 16 and 24 hours after pollination. In general, there is little change in the ABA content between 4 and 24 hours after pollination. However, the overall ABA content of both styles and ovaries is reduced after incompatible cross-pollination compared with incompatible self-pollination. In both cases, like in developing flowers, the ABA content of styles is higher than of ovaries. Cross-pollination with "dead" pollen also caused a reduced ABA content of styles and ovaries similar to pollination with non-irradiated pollen.

3.4. ABA in pollen from Petunia clones and Lilium

The ABA content of air dry pollen available from three *Petunia* clones was determined. In conjunction with the ABA analysis the viability of the pollen was determined by germination for 2 hours at 25 °C. The results presented in *table 1* show that the ABA content of pollen is comparatively low and varies between clones, which in turn vary in viability of pollen. In *Lilium longiflorum* cv. White Europe the air dry pollen as well as pollen germinated for 2 h at 25 °C, germination percentage 49.0% ± 1.8, were used for ABA determinations. In addition also the medium in which the pollen was germinated was analysed for ABA. Also *Lilium* pollen appeared to have a relatively low ABA content (*table 2*). Upon germination there is a large decrease in ABA, however, a comparatively high amount of ABA is released into the medium by the germinating pollen.

4. DISCUSSION

This study has clearly demonstrated the presence of endogenous ABA in the reproductive organs of *Petunia hybrida* and in pollen of *Lilium longiflorum*. Earlier studies have shown the presence of auxin and gibberellin-like substances in the reproductive organs of *Petunia* and *Lilium* spec. (BARENDSE et al. 1970). There is little doubt that growth regulators are in some way involved in reproductive processes (see GOODWIN 1978), however, their precise role remains to be elucidated. In the case of compatible cross-pollination in *Petunia* the pollen tubes reach the ovary within 24 hours after pollination, while with incompatible self-pollination the pollen tube growth into the style is retarded and stops about half-way the style (LINSKENS 1955).

This study has shown that compatible cross-pollination in *Petunia* resulted in a decrease in ABA content of styles compared with that of styles at anthesis, while the ABA content of ovaries remained constant at the low level reached at anthesis. The same occurs after cross-pollination with "dead" pollen which

Table 1. ABA content of pollen from three *Petunia hybrida* clones.

clone	% germination	ABA: ng/g fresh weight
W166H	26 ± 8	109 ± 10
T2U	12 ± 6	79 ± 6
W78B4	7 ± 6	54 ± 8

Table 2. ABA content of pollen from *Lilium longiflorum* cv. White Europe.

material	ABA: ng/g fresh weight
pollen	181 ± 8
germinated pollen	81 ± 18
germination medium	712 ± 51

indicates that germination of pollen is not required for the cross-reaction with regard to ABA levels. With incompatible self-pollination the ABA content of styles, though showing a little decrease between 4 and 8 hours after pollination, remained at a high level while the ABA content of ovaries is higher than of ovaries after cross-pollination and also higher than the ABA content of ovaries reached at anthesis. These results indicate a possible involvement of ABA in the incompatibility reaction. No such an involvement could be established for auxins or gibberellins (BARENDSE et al. 1970).

Pollen of the different *Petunia hybrida* clones and of *Lilium longiflorum* were also shown to contain endogenous ABA, however, on a fresh weight basis their ABA content is low compared to styles, and thus it seems that the contribution of pollen ABA can be neglected with pollinations. However, when *Lilium* pollen was germinated it was found that the medium contained relatively high amounts of ABA, which when related to the fresh weight of the germinated pollen, are comparable to the ABA content of styles. It is very likely that this release of ABA from germinating pollen into the medium represents ABA derived from enzymatic hydrolysis of ABA conjugates, since pollen release many enzymes, including glucosidases, upon germination (STANLEY & LINSKENS 1974). That pollen may accumulate ABA conjugates can also be inferred from the sharp drop in "free" ABA content of anthers between 3 and 2 days prior to anthesis (*fig. 2*). In addition these results are very similar to those previously reported for gibberellins (BARENDSE et al. 1970). Whether such a release of ABA upon pollen germination plays any role during growth of the pollen tube into the style remains to be elucidated.

REFERENCES

BARENDSE, G. W. M., A. S. RODRIGUES, P. A. BERKERS, F. M. DRIESSEN, A. VAN EYDEN-EMONS & H. F. LINSKENS (1970): Growth hormones in pollen, styles and ovaries of Petunia hybrida and of Lilium species. *Acta Bot. Neerl.* **19**: 175–186.

CROZIER, A., J. B. ZAERR & R. O. MORRIS (1982): Reversed- and normal-phase high performance liquid chromatography of gibberellin methoxycoumaryl esters. *J. Chromatography* **238**: 157–166.

EEUWENS, C. J. & W. W. SCHWABE (1975): Seed and pod wall developments in Pisum sativum L. in relation to extracted and applied hormones. *J. Exp. Bot.* **26**: 1–14.

GILISSEN, L. J. W. & H. F. LINSKENS (1975): Pollen tube growth in styles of self-incompatible Petunia pollinated with irradiated pollen and with foreign pollen mixtures. In: D. L. MULCAHY (ed.): *Gamete competition in plants and animals.* North-Holland Publishing Company, Amsterdam, p. 201–205.

GOLDSCHMIDT, E. D. (1980): Abscisic acid in citrus flower organs as related to floral development and function. *Plant & Cell Physiol.* **21**: 193–195.

GOODWIN, P. B. (1978): Phytohormones and fruit growth. In: D. S. LETHAM, P. B. GOODWIN & T. J. V. HIGGINS (eds.): *Phytohormones and related compounds, a comprehensive treatise,* volume II. Elsevier/North-Holland, Amsterdam. p. 175–214.

LINSKENS, H. F. (1955): Physiologische Untersuchungen der Pollenschlauchkeimung selbststeriler Petunien. *Zeitschr. Bot.* **43**: 1–44.

LUI, W. C. & H. R. CARNS (1961): Isolation of abscisin and abscission accelerating substance. *Science* **134**: 384–385.

MARTIN, G. C., F. G. DENNIS, J. MACMILLAN & P. GASKIN (1977): Hormones in pear seeds: I levels of gibberellins, abscisic acid, phaseic acid, dihydrophaseic acid, and two metabolites of dihydrophaseic acid in immature seeds of Pyrus communis L. *J. Amer. Soc. Hort. Sci.* **102**: 16–19.

MAYAK, S., A. H. HALEVY & M. KATZ (1972): Correlative changes in phytohormones in relation to senescence processes in rose petals. *Physiol. Plant.* **27**: 1–4.

SAUNDERS, P. F. (1978): The identification and quantitative analysis of abscisic acid in plant extracts. In: J. R. HILLMAN (ed.): *Isolation of plant growth substances*. Cambridge University Press, p. 115–134.

SHIBUYA, T., M. FUNAMIZU & Y. KITAHARA (1978): Abscisic acid from Pinus densifora pollen. *Phytochemistry* **17**: 322–232.

STANLEY, R. G. & H. F. LINSKENS (1974): *Pollen; Biology, Biochemistry, Management*. Springer-Verlag (Berlin, Heidelberg, New York), p. 193–222.

ZHANG, H. Q. & A. F. CROES (1982): A new medium for pollen germination in vitro. *Acta Bot. Neerl.* **31**: 113–119.