Journal of Chromatography A, 658 (1994) 83–90
Elsevier Science B.V., Amsterdam

CHROMSYMP. 2876

Abscisic acid content of salt-stressed *Phaseolus vulgaris* L.

Comparison of high-performance liquid chromatography, gas chromatography with electron-capture detection, enzyme-linked immunosorbent assay and radioimmunoassay

E. Montero, J. Sibole and C. Cabot*

Departament de Biologia Ambiental, Universitat de les Illes Balears, Car. Valldemossa km 7.5, 07071 Palma de Mallorca (Spain)

Ch. Poschenrieder and J. Barceló

Departament de Fisiologia Vegetal, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona (Spain)

ABSTRACT

Sensitivity, reproducibility and cost effectiveness are important parameters to consider in analyses for abscisic acid (ABA). HPLC, GC with electron-capture detection (ECD), enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) were compared to determine the most appropriate method for the determination of ABA acid in salt-stressed bush bean plants. Determination of ABA by HPLC is better suited for samples with high ABA concentrations owing to the low selectivity of the UV detector. GC–ECD, ELISA and RIA are well suited for the assay of large numbers of samples and show good sensitivity for ABA. Analysis by RIA was the least costly method and required no sample purification process.

INTRODUCTION

Abscisic acid (ABA) (Fig. 1) is a phytohormone present in low concentrations in all higher plant organs, and evidence for its presence in algae and fungi has also been reported. The pathway for ABA synthesis in plants has yet to be fully elucidated, but there is considerable evidence to suggest the existence of two different paths. One results in the direct formation of ABA from farnesyl pyrophosphate and the other is an indirect route, presumably via the cleavage of a xanthophyll. Both pathways however, stem from mevalonic acid [1].

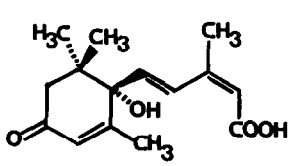

Fig. 1. Structure of (+)-(*S*)-abscisic acid.

ABA has been postulated to be involved in several phases of plant growth and development, although it is difficult to assign a specific role for this hormone owing to the lack of general

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E0727-C

knowledge of the regulation of developmental processes in plants. ABA is considered to be a stress hormone that functions as an endogenous regulator of plant transpiration, ameliorating the effects of water stress [2]. In some species of higher plants there is evidence for an ABA-mediated response to salt stress [3,4].

We are conducting several experiments to determine whether or not ABA is involved in the responses of the glycophyte *Phaseolus vulgaris* L. to conditions of salt stress, with special consideration being given to root–shoot interactions. These studies require extensive analyses of large numbers of samples taken at different time periods, so that we may define more clearly the phytohormone's role in the plant's response and adaptation to saline conditions.

The extraction and determination of ABA are difficult, as the phytohormone is present only in minute concentrations and the extraction and purification procedures are unavoidably extensive and time consuming. In addition, the efficiency of the extraction procedure is very difficult to determine.

ABA can be determined using physico-chemical methods such as HPLC and GC with electron-capture detection (ECD) [5]. Recently, however, the use of enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) methods has gained in popularity for ABA determination, providing a rapid and inexpensive determination of the hormone in plant tissues [6]. These techniques do not require large amounts of plant tissue for the extraction process, and therefore permit the determination of ABA even at the level of an individual plant cell [7]. Nevertheless, these methods share some of the problems that confront the physico-chemical methods: ELISA requires an extensive purification process owing to interferences with other plant compounds, and both ELISA and RIA analyses are occasionally subject to specific interferences that can be difficult to detect. Owing to these problems, ABA determinations via the latter techniques should always be verified by either GC–ECD or GC–MS [8].

The objective of this work was to determine which of the methods tested, HPLC, GC–ECD, ELISA and RIA, was the most suitable for the determination of ABA in tissue samples of bush bean plants.

EXPERIMENTAL

Plant materials

Eight-day-old plants of *Phaseolus vulgaris* L. var. Contender were germinated in vermiculite and transferred to a 50% modified Hoagland nutrient solution (pH 5.5) [9]. To each solution container either 1 or 25 m*M* NaCl was added. The experiments were conducted in a growth chamber (Conviron 15) under conditions that resembled a typical mediterranean spring day.

Twelve hours after salt addition, samples of roots, leaves and xylem sap were taken. Plant tissue was immediately frozen in liquid nitrogen, freeze-dried and stored at −80°C until analysis.

ABA purification for HPLC and GC–ECD

All procedures for ABA extraction and purification were performed under conditions of low light intensity and temperature to minimize the photodegradation and oxidation of the phytohormone. All solvents used were of chromatographic grade.

ABA extraction and purification were carried out by a modification of a procedure for HPLC reported earlier [10]. Freeze-dried plant tissues were homogenized in liquid nitrogen and extracted overnight at 4°C in 80% aqueous methanol, adjusted to pH 8 with $NaHCO_3$. Butylated hydroxytoluene were added as an antioxidant at a concentration of 20 mg l^{-1}.

The same protocol was followed for GC–ECD with a slight modification as the greater sensitivity for ABA provided by this method permitted the use of smaller amounts of sample and lower solvent volumes. For HPLC, 1 g of leaves of stems or 2 g of root tissue were required. For GC-ECD, only 0.25 g of each type of tissue was needed to perform the above extraction procedure.

Approximately 300 Bq of [^{3}H]ABA (2.55 TBq $mmol^{-1}$) (Amersham International, Amersham, UK) were added to determine purification losses. Extraction of ABA from the plant tissue using water [11] was also performed but proved unfeasable for beans, as it was impossible to

separate the organic and aqueous phases in the partitioning step.

The homogenate was centrifuged at 1545 *g* for 20 min. The supernatant was taken to the aqueous phase *in vacuo* at 35°C. The pH of the aqueous phase was then adjusted to 8 with 6 *M* NaOH and partitioned into ethyl acetate three times. The aqueous fraction was then adjusted to pH 2.5 with 6 *M* HCl and again extracted three times with ethyl acetate. The ethyl acetate fraction was dried over Na_2SO_4 (anhydrous) and evaporated to dryness at 35°C. Methylene chloride (2 ml) was added to the dry extract and the solution was loaded on to a Sep-Pak silica cartridge (Waters), prewashed with 5 ml of methylene chloride. The cartridge was washed with aliquots of organic solvents with increasing polarity: (1) 2 ml of 5% acetone in methylene chloride, (2) 5 ml of 4% methanol in methylene chloride and (3) 3 ml 10% methanol in methylene chloride. The ABA was eluted from the Sep-Pak cartridge by solvents with polarities greater than 4% methanol in methylene chloride. Fractions 2 and 3 were bulked and evaporated to dryness *in vacuo* at 35°C.

Quantification by HPLC

The resulting dry residue from the extraction procedure was dissolved in 0.5 ml of methanol and filtered through a 0.45-μm pore size filter.

ABA was determined with the use of a Beckman System Gold HPLC, equipped with a solvent programmable module (Beckman Model 126) and a variable-wavelength detector (Beckman Model 166), set at 254 nm. The column used was a Beckman Ultrasphere (25 cm × 4.6 mm I.D.) of 5.0-μm particle size. ABA was determined using a 0–100% linear gradient from water to methanol for 15 min at a flow-rate of 1 ml min^{-1}. The column was then flushed with methanol for 15 min to remove any remaining compounds. The retention time of ABA was *ca.* 12 min.

To confirm the authenticity of the ABA peak, authentic *cis,trans*-ABA (Sigma) was co-chromatographed with each sample The ABA content was calculated from a calibration graph obtained with known amounts of ABA (r = 0.99).

The efficiency of the extraction process could not be determined as it was not possible to quantify exactly how much of the original *in vivo* hormone pool had been recovered. The efficiency of the purification procedure was determined by scintillation counting of [^{3}H]ABA, removing aliquots of known amount in each step of the purification procedure. Tritium activity was determined with a Beckman LS 1800 liquid scintillation spectrometer. The efficiency for ^{3}H was 81.1 ± 5.56%.

Quantification by GC–ECD

The dry residue obtained at the end of the purification process was methylated with diazomethane (50 μl of methanol + 500 μl of etheral diazomethane). After 15 min the samples were evaporated to dryness in a stream of nitrogen, reconstituted with 0.5 ml of hexane and stored at −30°C for GC–ECD analysis.

The ABA content was determined on a Hewlett-Packard (St. Louis, MO, USA) Model 5790 gas chromatograph equipped with a ^{63}Ni electron-capture detector and a Hewlett-Packard Model 4500 integrator. Splitless injections of 1 μl were made on to a DB-5 capillary column (30 m × 250 mm I.D.) manufactured by J&W Scientific (Folsom, CA, USA). The GC operating conditions were an injector temperature of 250°C with helium as the carrier gas at a flow-rate of 2 ml ml^{-1} and nitrogen as the make-up gas at a flow-rate of 20 ml min^{-1}. The starting oven temperature programme was 100°C and was raised at a rate of 25°C min^{-1} to 240°C, where it was held for 5 min. The retention time of ABA was *ca.* 7 min. The ABA peaks were qualitatively confirmed as *cis,trans*-ABA after chromatography with authentic *cis,trans*-ABA. Peak areas were plotted against known amounts of ABA injected, with a linear working range of 0.5–500 ng for the calibration graph. Based on this calibration curve (r = 0.99), ABA concentrations were calculated, taking into account the specific extraction yield of each particular sample (dpm [^{3}H]ABA recovered).

ABA purification for ELISA

The purification procedure for ELISA was based on methods reported previously [3,4].

Briefly, plant tissue was homogenized in liquid nitrogen for 5 min and extracted overnight at 4°C in 80% aqueous methanol containing 1 mg l^{-1} of butylated hydroxytoluene to avoid oxidation. A trace amount of [^{3}H]ABA was added to each sample to monitor the ABA recovery.

To remove any impurities, the methanolic extract was passed through a Sep-Pak C_{18} cartridge that had been prewashed with 1 ml of 80% methanol. The methanolic phase was removed with a vacuum centrifuge (Savant Speed Vac Plus, Model SC 110A) at 40°C. The resulting aqueous portion was partitioned three times against ethyl acetate, which had previously been adjusted to pH 3.0 with 1 *M* HCl. The ethyl acetate fractions were combined and evaporated to dryness under low pressure. The residue was dissolved in saline Tris buffer (TBS) of pH 7.8 and sonicated for 2–3 min for assay. The efficiency of ABA purification, based on recoveries of added [^{3}H] ABA, was 92 ± 1%.

ABA quantification by ELISA

ABA was determined by ELISA using commercially available ABA assay kits from Idetek (San Bruno, CA, USA), which uses the competitive binding protein method to measure ABA concentrations. In this assay, the sample and the enzyme-coupled antigen must compete for antibody-binding sites.

The ELISA protocol consists of incubation of samples or ABA standards at 4°C for 1 h in a microwell plate precoated with antibodies. A 100-μl volume of tracer (ABA–alkaline phosphatase conjugate) was added to each cell and incubated at 4°C for 3 h. After discarding the solutions, the cells were washed with distilled water and blotted dry with filter-paper. A 200-μl volume of substrate (*p*-nitrophenyl phosphate) was added to the dried cell and incubated at 37°C for 1 h. Absorbances were then read at 405 nm. A series of ABA standards containing from 0.01 to 0.5 ng of ABA ware assayed for each microtitre plate. ABA concentrations were calculated by referring to the calibration graph after linearization using a log–logit function [12]. No evidence of any non-specific interference with the ELISA assay was observed for sample dilutions over a 30-fold range or with the addition of synthetic ABA. Validation of this assay was done by comparing the results with those obtained with physico-chemical methods.

ABA quantification by RIA

Freeze-dried bean tissue was ground to a fine powder and ABA was extracted overnight with chilled distilled water (0°C).

The RIA analysis was carried out as described [13] using the monoclonal antibody AFRC MAC 62 (supplied by Dr. S. Quarrie of the Institute of Plant Research, Cambridge, UK), which is specific for (+)-ABA. Samples were incubated with [^{3}H]ABA and the monoclonal antibody for 45 min at 5°C. A saturated solution of ammonium sulphate was added, incubated for 4 min and then centrifuged for 5 min at 8800 *g* (Eppendorf centrifuge) to separate the free and bound antigen. The pellet was washed by resuspension in 50% ammonium sulphate solution and then centrifuged for 5 min at 8800 *g*. Radioactivity in the resultant solution was determined with a liquid scintillation counter (Beckman LS 1800). Concentrations of ABA in the samples were calculated from a calibration curve constructed from known concentrations of standard *cis,trans*-(+)-ABA after linearization using a logit transformation [13]. ABA concentrations ranged from 0.125 to 2.0 ng per vial that were present in each standard bath. The lack of non-specific interferences in RIA assays for leaves, roots and xylem sap in crude-tissue extracts of beans has been reported recently [14]. Validation of the RIA assay was performed by comparing the results with those obtained by GC–ECD.

RESULTS AND DISCUSSION

Extraction

An extended extraction and purification process was necessary for all of the methods tested, except for the xylem sap samples in ELISA and all plant tissues analysed by RIA, where no sample purification was required.

The experiments involved the determination of ABA in young plants where, as the total ABA content is very low, a large portion of the plant must be sampled to obtain a detectable level of ABA. Consequently, the extraction of the

phytohormone proved to be a considerable problem, as the 1–2 g dry mass of sample needed for ABA determination required the harvesting of at least four plants. This constraint seriously limited the number of replicates in the different treatments, as the possible number of plants to be cultured was ultimately controlled by the capacity of the growth chamber. In more complex experiments with a greater number of treatments, successive plant cultures would be necessary in order to achieve a significant number of replicates for the hormone analysis.

Although the GC–ECD and HPLC methods for ABA determination used the same purification process, the need for larger amounts of tissue for HPLC necessitated the use of larger solvent volumes, with a resulting increase in cost per sample extraction. It should also be noted that the added difficulty of handling larger amounts of tissue required a greater time expenditure, and as a result it was possible to process only 4–6 samples per day with this method. Moreover, the higher sensitivity of GC–ECD for ABA permitted its determination in tissues of an individual plant, which was impossible with HPLC. Consequently, the tissue savings provided more replications for the same number of plants and thus offered the possibility of a much better statistical significance. Although the determination of ABA by GC–ECD required an extra step in which the hormone had to be methylated with diazomethane, this added manipulation posed no real extra time expenditure or cost increase when account is taken of the general advantages of GC–ECD over HPLC.

With regard to the purification process used for ABA determination by HPLC and GC–ECD, the recovery of [^{3}H]ABA was 81.1 ± 5.56%. Losses of ABA were highest during the partitioning against ethyl acetate, because despite the solvent's high partition coefficient for ABA at both pH 2.5 and 8 [15], ABA losses still amounted to *ca.* 10% (data not shown).

The sample purification process for ELISA had the advantage that only very small amounts of sample were required, which allowed an increase in the number of replicates per treatment and a high [^{3}H]ABA recovery (92 ± 1%).

ABA quantification

The ABA contents in different bush bean plant tissues treated with 1 or 25 m*M* NaCl, determined by the different methods tested, are given in Table I. Extracts from the 25 m*M* NaCl stressed plants contained up to twice the ABA concentration present in the non-stressed 1.0 m*M* NaCl controls. Other workers have also reported increases in the ABA content of salt-stressed plants [3,4].

A common characteristic observed for the ABA content with all the methods used was the high error values. This may be due at least in

TABLE I

ABA CONCENTRATIONS IN DIFFERENT TISSUES OF BUSH BEAN PLANTS GROWN WITH 1 OR 25 m*M* NaCl AND DETERMINED BY HPLC, GC–ECD, ELISA AND RIA

Values are means ($n = 4$). No significant differences were found among ABA values determined by the different methods tested (analysis of variance).

Method	ABA concentration					
	Leaves (ng g^{-1} dry mass)		Xylem (μmol m^{-3})		Roots (ng g^{-1} dry mass)	
	1 m*M* NaCl	25 m*M* NaCl	1 m*M* NaCl	25 m*M* NaCl	1 m*M* NaCl	25 m*M* NaCl
HPLC	410 ± 90	770 ± 98	–	–	250 ± 80	310 ± 50
GC–ECD	450 ± 40	830 ± 55	37 ± 3	78 ± 10	320 ± 50	370 ± 30
ELISA	500 ± 10	910 ± 15	42 ± 2	72 ± 7	290 ± 20	330 ± 15
RIA	490 ± 15	850 ± 55	35 ± 5	65 ± 8	278 ± 45	320 ± 20

part to the high phenotypic variability of bean plants. In this respect, analysis by ELISA or RIA would be advantageous, as the analysis of large numbers of samples would be needed to provide a greater statistical significance.

There were no significant differences among ABA values determined by the different methods (analysis of variance).

Fig. 2 shows the typical HPLC (A) ABA standard and bush bean (B) leaf and (C) root samples. ABA determination was achieved with difficulty owing to the large amounts of different products present in the sample, despite the extended purification procedure applied. Root analysis (Fig. 2B) was especially difficult owing to the low ABA content in this tissue and the increase in the amounts of interfering products, as it was necessary to use 2 g dry mass of sample for the extraction. We consider that for ABA determination by HPLC, a more extensively purified sample should be used to increase the ABA resolution, which can be achieved by using a preparative HPLC step prior to quantification [16,17].

Following the same procedure for other tissue samples, the HPLC selectivity was too low to detect the ABA content in the collected samples of xylem sap (Table I), for which purification was necessary. Larger amounts of sap would have be necessary so that interferences from other compounds could be offset by the larger ABA peak areas.

With ABA determination by HPLC, special care should be taken to avoid the accumulation of impurities in the column and solvents, which can seriously affect the ABA resolution. Perhaps owing to a related phenomenon, although proper maintenance and operational procedures were followed, the HPLC columns had a much shorter life span than their GC counterparts.

GC–ECD tracers for ABA are presented in Fig. 3 for (A) standards and (B and C) tissue samples. A superior resolving power was achieved with the capillary GC column compared with HPLC. The electron-capture detector with high sensitivity and selectivity for molecules with high electron affinity [18] proved to be very

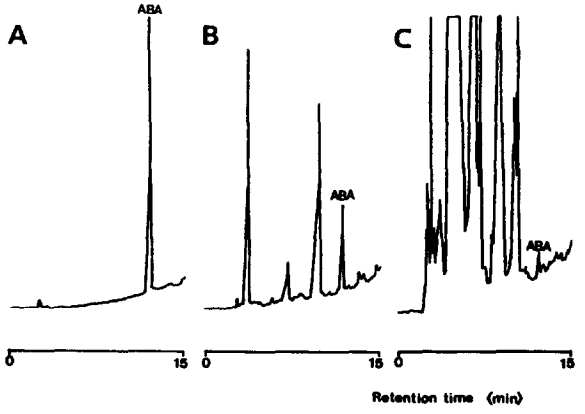

Fig. 2. HPLC of (A) ABA standard (500 ng) and bush bean (B) leaf and (C) root samples.

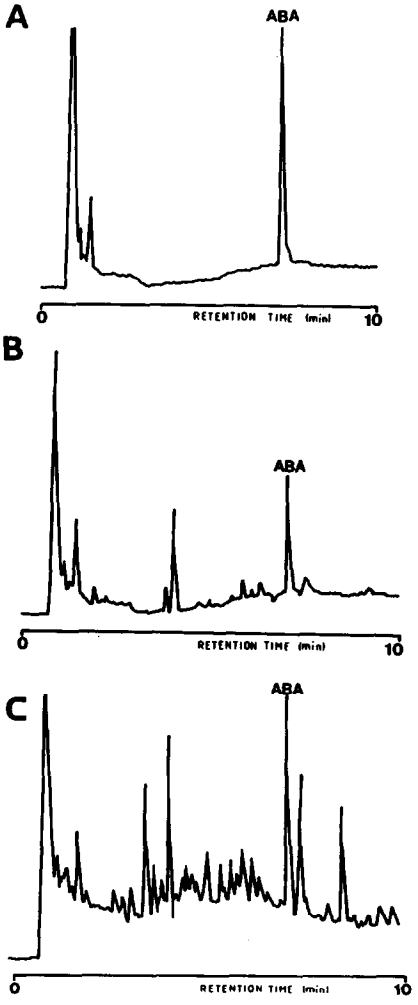

Fig. 3. GC–ECD of (A) ABA standard (500 ng) and bush bean (B) leaf and (C) root samples.

specific for ABA, and hence very few compounds other than the phytohormone were observed.

The standard deviation for ABA determination was not larger in the GC–ECD than the HPLC method. Although higher standard deviations for GC have been reported, these are due to other factors, such as difficulty in achieving reproducible sample injections and the minute injection volume with respect to the sample volume [15]. Concerning sample volume, HPLC had the advantage of using large injection volumes, which made possible the analysis of a greater portion of the total sample for injection. Nevertheless, the standard deviation in this experiment for the HPLC method was larger than that for the GC–ECD method, but this could be due to the heterogeneity of the samples and the smaller number of replicates.

Although the analysis times were similar for the HPLC and the GC–ECD methods, the contamination problem of the HPLC column resulted in an overall greater time expenditure for the HPLC method. Extra flushing and cleaning times were necessary for HPLC, which led to a serious addition of time for the analysis.

Even though the monoclonal antibodies used in the immunoassays are highly specific to the antigen, it is well known that other materials in plant extracts can competitively and non-competitively inhibit the antigen–antibody interaction, given erroneous readings [8,19]. Therefore, there is a need to validate the assay for each type of extract examined. Validation of the ELISA for ABA determination in different tissues of bean plants by comparing the results with those obtained by GC–ECD is shown in Fig. 4A. The good agreement between the ABA values obtained by the ELISA and GC–ECD methods ($r = 0.99$) validated the use ELISA for the determination of ABA in different bush bean plant tissues. The only problem with the extended use of the Idetek kits for this purpose is their high cost.

The RIA assay for ABA was done using a (+)-ABA specific monoclonal that does not bind (−)-ABA or most ABA metabolites and derivatives [13]. For this reason, no purification of the extracts was necessary. The good correlation

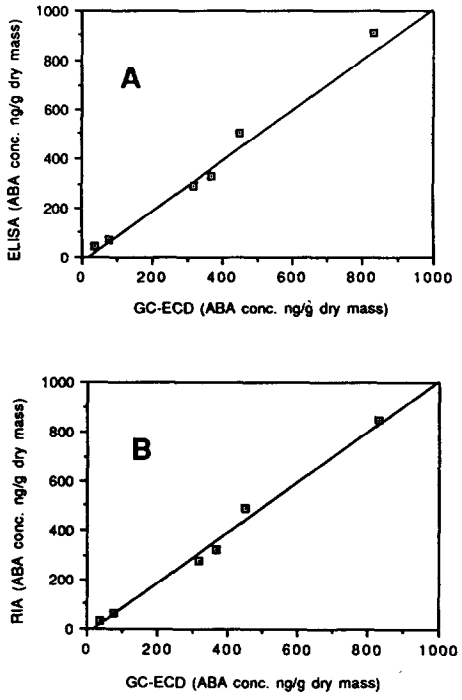

Fig. 4. Comparison of ABA concentrations obtained by (A) RIA and (B) ELISA *versus* GC–ECD. Linear regression estimates are: (A) $y = -26.894 + 1.1057x$; $R^2 = 0.988$; (B) $y = -22.876 + 1.0433x$; $R^2 = 0.988$.

found for ABA contents in the different bean tissues analysed by GC–ECD and RIA validated this method. Because no purification was needed for RIA, this method proved to be the least time consuming, permitting the assay of over 100 samples in duplicate within 1 day.

In conclusion, the determination of ABA in bush bean plants by GC–ECD and RIA was the most suitable with regard to selectivity and cost. Once validated, RIA proved to be the fastest of the four methods. ELISA had the inconvenience of high cost, but was the most sensitive for ABA detection. HPLC was best suited for samples with dry masses greater than 1 g and with high ABA contents, owing to the low selectivity of the UV detector.

ACKNOWLEDGEMENTS

The authors thank the DGCIYT (projects PB88-0234 and PB91-0664) for financial support.

REFERENCES

1 J.A.D. Zeevart and R.A. Creelman, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 39 (1988) 439.
2 R.A. Creelman, *Physiol. Plant.*, 75 (1989) 131.
3 W.O. Wolf, W.D. Jeschke and W. Hartung, *J. Exp. Bot.*, 41 (1990) 593.
4 Z. Kefu, R. Munns and R.W. King, *Aust. J. Plant Physiol.*, 18 (1991) 17.
5 A.D. Parry and R. Horgan, in W.J. Davies and H.G. Jones (Editors), *Abscisic Acid Physiology and Biochemistry*, BioScientific Publishers, Oxford, 1991, pp. 5–22.
6 V.C. Pence and J.L. Caruso, in P.J. Davies (Editor), *Plant Hormones and Their Role in Plant Growth and Development*, Martinus Nijhoff, Dordrecht, 1987, pp. 240–256.
7 E. Brinckmann, W. Hartung and M. Wartinger, *Physiol. Plant.*, 80 (1990) 51.
8 M. Bopp, *Plant Growth Substances*, Springer, Berlin, 1985, pp. 35–43.
9 D.R. Hoagland and D.I. Arnon, *The Water-Culture Method for Growing Plants Without Soil (California Agricultural Experiment Station, Circular 347)*, California Agricutural Experiment Station, Berkeley, CA, 1950.
10 C. Cabot, Ch. Poschenrieder and J. Barceló, *J. Liq. Chromatogr.*, 13 (1986) 2977.
11 F. Addicott, *Abscisic Acid*, Praeger Publishing, New York, 1983, pp. 36–47.
12 *Idetek Phytodetek-ABA, Technical Brochure*, Idetek, San Bruno, CA, 1985.
13 S.A. Quarrie, P.N. Whitford, N.E.J. Appleford, T.L. Wang, S.K. Cook, I.E. Henson and B.R. Loveys, *Planta*, 173 (1988) 330.
14 C.L. Trejo and W.J. Davies, *J. Exp. Bot.*, 42 (1991) 1507.
15 A.J. Ciha, M.L. Brenner and W.A. Brun, *Plant Physiol.*, 59 (1977) 821.
16 R.A. Creelman and J.A.D. Zeevart, *Plant Physiol.*, 77 (1985) 25.
17 J.A.D. Zeevart, T.G. Heath and D.A. Gage, *Plant Physiol.*, 91 (1989) 1594.
18 J.A.D. Zeevart, *Plant Physiol.*, 66 (1980) 672.
19 H. Belefant and F. Fong, *Plant Physiol.*, 91 (1989) 1467.