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High-performance thin-layer chromatographic and high-performance liquid chromatographic determination of abscisic acid produced by cyanobacteria

HELENA ZAHRADNÍČKOVÁ*
Laboratory of Analytical Chemistry, Czechoslovak Academy of Sciences, Branišovská 31, 370 05 České Budějovice (Czechoslovakia)
BLAHOSLAV MARŠÁLEK
Institute of Soil Biology, Czechoslovak Academy of Sciences, Branišovská 31, 370 05 České Budějovice (Czechoslovakia)
and
MARIE POLIŠENSKÁ
Institute of Plant Molecular Biology, Czechoslovak Academy of Sciences, Branišovská 31, 370 05 České Budějovice (Czechoslovakia)
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ABSTRACT

Methods for the determination of abscisic acid released by cyanobacteria into culture media based on high-performance thin-layer chromatographic densitometry and high-performance liquid chromatography have been developed. Abscisic acid exogenously released from cyanobacterial culture was separated and purified in only one step by using Sep-Pak C_{18} cartridges. In the case of high-performance thin-layer chromatography, a mobile phase of toluene–ethyl acetate–acetic acid (25:15:2, v/v/v) and silica gel plates with fluorescent label were used with absorbance measurements; for high-performance liquid chromatography, a mobile phase methanol–1% acetic acid (1:1, v/v) and a reversed-phase column were used. Comparison of the methods is included. Both methods were applied to the determination of abscisic acid in stressed and non-stressed cultures of the cyanobacteria *Trichormus variabilis* (Kom. Anag.) syn. *Anabaena variabilis* strain A 215. The salt stress (0.05 *M*) significantly increased the release of abscisic acid into the culture medium after only 2 under h the influence of stress.

INTRODUCTION

Abscisic acid (ABA) is an important plant growth hormone involved in various processes during the life cycle of higher plants. However, the occurrence and role of ABA in lower plants remains obscure. It is not at all clear whether ABA synthesized by algae, fungi, bryophyta, etc., is merely a product of secondary metabolism or if it is involved in the regulation of growth and development of lower plants. So far, the majority of ABA determinations in higher plants has been based on gas chromatography (GC) [1,2], GC–mass spectrometry (MS) both with and without single ion monitoring (SIM) [3,4], high-performance liquid chromatography (HPLC) [5–8], radio-

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immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [9–10]. In our previous work we developed a high-performance thin-layer chromatographic (HPTLC) method for the quantification of ABA produced by stressed green algae [11].

Most of the above-mentioned procedures employ an initial extraction of the plant material with an aqueous organic solvent. The non-selective nature of these solvent systems yields a highly complex sample containing many polar and non-polar compounds, including many plant pigments and polyphenolic components [1,12]. The high concentrations of impurities relative to ABA content means that extensive purification is required before most analytical techniques can be performed. In the course of purification of ABA many workers used solvent partitioning and TLC with or without different liquid chromatography systems (open column chromatography and HPLC). Time-consuming purification steps have been greatly simplified by using chromatographic cartridges that reduce the time necessary for sample preparation [1,8]. Purification steps can also be decreased by using water as extraction solvent [11,13].

The aim of this study was to devise a simplified sample preparation method (especially with a shortened purification step) and to develop and compare HPTLC and HPLC in the determination of ABA produced by cyanobacteria and, after comparison of the studied methods, to find the most convenient one.

EXPERIMENTAL

Plant material

Trichormus variabilis (Kom. Anag.) syn. *Anabaena variabilis* strain A 215, obtained from the collection of autotrophic organisms in the Institute of Botany, Czechoslovak Academy of Sciences, Třeboň, was used as the experimental organism. The cyanobacterium was cultivated in Bold's basal medium [14] in suspended culture with a 12-h photoperiod at 21°C for 13 days. After 13 days of cultivation 0.05 *M* sodium chloride was applied to one part of cultures. The stressing factor was applied to the cultures for 2 h, 48 h and 6 days. The fresh weight of cyanobacterium was established by centrifugation of the suspension; the volume of the suspension was measured before the centrifugation.

Chemicals

All chemicals and solvents were of analytical grade. Organic solvents and water were freshly distilled prior to use. Ethyl acetate, methanol, toluene and acetic acid were from Lachema (Brno, Czechoslovakia). (±)-2-*cis*-4-*trans*-Abscisic acid was obtained from Aldrich (Steinheim, Germany) and Sep-Pak C_{18} cartridges from Waters Associates (Milford, MA, USA).

HPTLC system

Apparatus: Linomat IV, CAMAG (Muttenz, Switzerland); TLC Scanner II, CAMAG; integrator SP 4270, Spectra Physics (San Jose, CA, USA); twintrough developing chamber, CAMAG. Chromatographic plates: Precoated silica gel 60 F254 HPTLC plates (10 × 10 cm, Merck, Darmstadt, Germany). Plates were cleaned before use by developing in methanol. The developing system was toluene–ethyl acetate–acetic acid (25:15:2, v/v/v).

HPLC system

A Varian 5500 liquid chromatographic system equipped with a Rheodyne 7126 injection valve with 10-μl loop, DS 604 data station and UV - 200 detector was used. The column used was an analytical Separon TM SGX RPS column (250 mm × 4 mm I.D.), 5 μm (Tessek, Prague, Czechoslovakia).

Purification procedure

The supernatant from centrifuged (2260 *g*, 20 min) cyanobacterial culture (usually 100 ml) was frozen, slowly thawed and centrifuged again (2260 *g*, 10 min). To one half of the supernatant a known amount of ABA standard was added to determine the ABA recovery, and both halves were processed in the same way. The supernatant after adjusting its pH to 2.5 with acetic acid was passed through a Sep-Pak C_{18} cartridge consecutively preconditioned with 10 ml of water 10 ml of methanol and 10 ml of 0.2 *M* acetic acid (rate 1 ml/min, the peristaltic pump was used). The cartridge was washed with 10 ml of 0.2 *M* acetic acid and 10 ml of 10% methanol; ABA was eluted with 5 ml of methanol–0.2 *M* acetic acid (3:2, v/v). The eluate was evaporated at 35°C to dryness with a stream of nitrogen; the residue was dissolved in 500 μl of methanol and used for HPTLC and HPLC ABA determination after centrifugation.

HPTLC method

Samples of 10 μl (triplicate) were applied in 3-mm stripes at 2-mm intervals in a nitrogen stream, at a rate of 5 s per 1 μl, alternating with 15, 10, 5 and 2 μl of standard ABA solution (1 μg/ml methanol, duplicate). Both standards and samples were applied to both halves of the plates, 8 mm from the lower edge of the plate. One-dimensional developments were performed vertically at ambient temperature (approximately 25°C) in saturated twin-trough chambers; the developing path was 5 cm. The developed plates were completely dried in a stream of air and immediately scanned under these conditions: λ = 270 nm (chosen after recording the absorbance spectrum of ABA on HPTLC plates – the optimum wavelength found concurs with that in ref. 15); absorption mode; deuterium lamp; monochromator bandwidth, 10 mm; slit width, 0.4 mm; slit length, 2 mm; scanning speed 1 cm/min. Calibration was based on peak heights obtained from the application of ABA standards to each plate.

HPLC method

Isocratic elution with a solvent mixture of methanol–1% acetic acid (1:1, v/v) was caried out for 11 min, followed by a linear gradient to 100% methanol over 3 min. At 6 min the column was washed isocratically with 100% methanol [16]. The solvents were carefully degassed prior to use. A constant flow of 0.8 ml/min and temperature of 40°C were maintained during the analysis. The injection volume was 10 μl, the detection wavelength 268 nm. Calibration was based on peak heights of standard solutions of ABA (1–15 ng of ABA injected).

RESULTS

HPTLC method

The reproducibility of the sample application was checked. Four series of standards, 2, 5, 10 and 15 ng of ABA, two series on one half of the plate, two on the other,

TABLE I

RELATIVE STANDARD DEVIATIONS OF DENSITOMETRIC RESPONSES TO ABA ON HPTLC PLATES

ABA (ng)	Relative standard deviation (%)
2	5.33
5	4.61
10	5.27
15	5.12

were applied to three HPTLC plates. The average relative standard deviations calculated from densitometric responses are listed in Table I. R_F of ABA was 0.43, the detection limit being 2 ng of ABA on the HPTLC plate. In a similar way we measured the reproducibility of the sample application of series of standards of cinnamic acid, which has been used by some authors [5] as an internal standard. Because the results showed great differences and were not easily reproducible, cinnamic acid could not be used to determine the recovery of ABA (in HPTLC or in HPLC).The linear regression equation calculated from densitometric responses on three plates with four series of ABA contents in the range 2–15 ng was as follows: $y = 5.075\,x + 1.681$ ($r^2 = 0.9836$). The data obtained confirmed the linearity of the calibration curves at the concentrations of ABA used.

The repeatability of absorbance measurements in reflectance mode was also evaluated by repeated scanning of strips of ABA standards. From each of three HPTLC plates two strips of different ABA content from different halves of the plate were scanned ten times. Relative standard deviations of the densitometer responses were in the range 0.58–1.69%.

The recovery of ABA by HPTLC was 95 ± 3% (determined from a known added amount of ABA in one half of each sample).

HPLC method

The reproducibility of the peak heights was studied. Four series of standards (1, 5, 10 and 15 ng of ABA in injection) were injected three times in a day and again on

TABLE II

RELATIVE STANDARD DEVIATIONS OF PEAK HEIGHTS OF ABA STANDARDS MEASURED BY HPLC

ABA (ng)	Relative standard deviation (%)			
	Day 1	Day 2	Day 3	Within 3 days[a]
1	0	6.15	3.54	12.59
5	4.11	1.64	3.29	4.20
10	3.82	4.45	3.93	6.83
15	2.09	3.37	0	4.23

[a] Relative standard deviation calculated from all results obtained by measuring ABA standards responses on three successive days.

TABLE III

DETERMINATION OF ABA IN SUPERNATANT FROM SUSPENSION OF *TRICHORMUS VARIABILIS* CULTIVATED UNDER SALT STRESS

Time of stress	Kind of stress	Fresh weight (g)	ABA (ng) per ml susp.		ABA (ng) per g fresh weight		HPLC as a percentage of HPTLC
			HPTLC	HPLC	HPTLC	HPLC	
2 h	None[a]	1.94	1.1	0.85	45.4	35.1	77.3
	0.05 *M* sodium chloride	1.93	1.9	1.55	78.8	64.2	81.6
48 h	None[a]	1.86	0.64	0.48	34.4	25.8	75
	0.05 *M* sodium chloride	1.93	2.8	2.5	145	129	89
6 d	None[a]	1.88	0.6	0.47	31.9	25	78.4
	0.05 *M* sodium chloride	1.91	4.6	3.4	240.8	178	73.9

[a] Control.

two successive days. The relative standard deviations calculated from peak heights are listed in Table II. The retention times of ABA ranged between 7.3 and 7.7 min and the detection limit was 1 ng of ABA injected.

The linearity of the calibration curves was tested using four solutions of ABA of concentrations 0.0001, 0.0005, 0.001 and 0.0015 mg per millilitre of methanol (corresponding to 1, 5, 10 and 15 ng of injected ABA). The results were evaluated by linear regression analysis. The linear regression equation calculated from peak heights measured three times or three successive days was as follows: $y = 5.714\,x + 1.119$ ($r^2 = 0.99497$). The data obtained confirm the linearity of the calibration curves at the concentrations of ABA used.

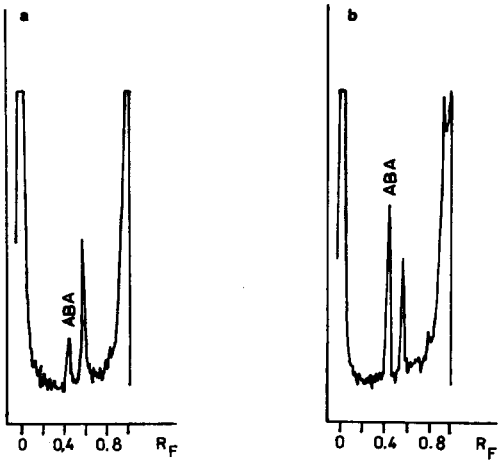

Fig. 1. Densitograms of stressed cyanobacterial samples (48 h, 0.05 *M* sodium chloride). Conditions: detection wavelength, 270 nm; mobile phase, toluene–ethyl acetate–acetic acid (25:15:2, v/v/v). (a) Original sample (2 ng of ABA); (b) sample spiked with 10 ng of ABA.

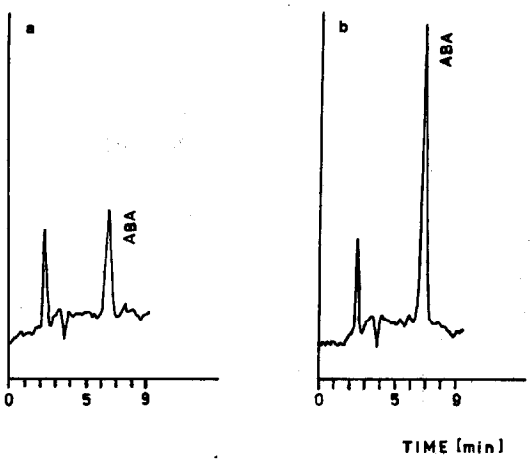

Fig. 2. HPLC chromatograms of stressed cyanobacterial samples (48 h, 0.05 *M* sodium chloride). Conditions: detection wavelength, 268 nm; mobile phase, methanol–1% acetic acid (1:1, v/v). (a) Original sample (5 ng); (b) sample spiked with 10 ng of ABA.

Both HPTLC and HPLC were used for ABA determination in cyanobacterium suspension cultures placed under stress by the addition of sodium chloride solution. The results (the averages of three analyses of three parallel determinations, *i.e.* of nine values) are listed in Table III. Examples of the densitograms and HPLC chromatograms of sample from cyanobacterial suspension after sodium chloride stress are given in Fig. 1a and b (densitograms) and Fig. 2a and b (HPLC chromatograms) both without (Figs. 1a, 2a) and with (Figs. 1b, 2b) spiked ABA.

DISCUSSION

The detection limit was nearly the same in HPTLC (2 ng on HPTLC plates) and HPLC (1 ng injected). The relative standard deviations of chromatographic responses ranged in HPTLC from 4.61% to 5.33% (Table I) and in HPLC from 0 to 6.12% in one day and from 4.20% to 12.59% (Table II) after several days. It is evident from Table II that measurements of the lowest standard concentration (1 ng) are subject to the greatest errors. As concerns the speed of analyses, HPTLC has a significant advantage over HPLC. The application of nine samples and two series of standards on both sides of an HPTLC plate, development and densitometric scanning takes at most 3 hours, while in HPLC one analysis lasts half an hour, and so to measure the same number of samples takes the whole day. In HPTLC we could apply more than 10 μl of sample (25 μl was possible without any deterioration of ABA differentiation) if the peak height was too small. The HPLC column was overloaded by impurities when more than 10 μl was injected. In HPTLC the impurities mostly remain at the start of chromatogram and do not interfere with peak of ABA; in HPLC columns the impurities must be washed away after each analysis. The results obtained by HPLC were lower than those obtained with HPTLC, - HPLC results being 82 ± 7% of HPTLC ones. A decrease in ABA as determined by HPLC has also been reported by other investigators [17]. In biological materials the comparison of results obtained by

different methods is very difficult due to different errors in the measurements. From to our results we consider HPTLC to be better than HPLC for the determination of ABA produced by cyanobacteria.

The results in Table III indicate that the cyanobacterium *Trichormus variabilis* is able to produce ABA. Cyanobacteria subjected to sodium chloride stress for six days increased their production of ABA six times; after two days of stress the production of ABA was increased 3–4 times. These methods are able to determine ABA production after only 2 hours (usually an 80% increasing in ABA production). This phenomenon —the occurence of ABA in lower plants– is under investigation and results will be published in the near future. Our finding, that cyanobacteria are able to produce ABA, is very interesting, because it is known that bacteria do not contain ABA [18]. Moreover, in another cyanobacterium, *Nostoc muscorum*, exogenous application of 10 mg/l ABA stimulated a response (exogenous ABA increased the utilization of molecular nitrogen from air six times) [19]. These facts indicate that ABA in cyanobacteria may also play some role in regulatory processes.

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