HPLC Determination of Benzyladenine Residues in Micropropagated Apple Explants

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A specific reversed-phase high-performance liquid chromatographic (HPLC) method has been developed to determine the concentration of benzyladenine (BA) [*N*-(phenylmethyl)-1*H*-purin-6-amine; CAS 1214-39-7] in micropropagated apple explants. BA is extracted into 95% ethanol, the ethanol evaporated, and the resulting extract residue redissolved in ammonium formate buffer (pH 3.7), which is then washed with petroleum ether. Further cleanup is performed with exchange chromatography (DEAE-cellulose) before the application of the solid-phase extraction procedure. Methanolic eluates of BA are submitted to HPLC determination using a C_8 column and an isocratic system of 30:70 (v/v) water–methanol containing 0.05% acetic acid. Recovery and reproducibility assessments indicate good accuracy (overall mean relative recovery of 87%) and precision (coefficient of variation from 0.4 to 7.7) over the BA concentration range 0.5–300 ppm, with a limit of quantitation of 0.5 ppm.

INTRODUCTION

Benzyladenine (BA) [*N*-(phenylmethyl)-1*H*-purin-6-amine] is an exogenous plant growth regulator having remarkable cytokinin-like effects. Therefore, it is commonly used to promote seed germination and nursery tree production, to control the growth of terminal buds, and to influence various aspects of fruit quality (Tanne and Cantliffe, 1989; Popenoe and Barritt, 1988; Greene et al., 1990; Owen and Aung, 1990). Among cytokinin-like growth regulators, BA is the most commonly used compound in in vitro micropropagation, because of its high efficiency in promoting lateral branching of shoots. Some biochemical responses have been observed following BA treatments, such as enzyme induction, isoenzyme modification, and chlorophyll, DNA, and ATP accumulation (Wang et al., 1987; Gil'manov and Sultanbaev, 1989; Yuri and Feucht, 1990; Soeda et al., 1990; Chernyad'ev and Kozlovskikh, 1990). It is therefore important to determine the amount and persistence of the parent molecule to correlate the physiological and biochemical responses to its presence in plant tissues and to optimize BA treatments in crops.

Despite the numerous reports regarding the effect of BA applications in industrial crop production, no procedures for the quantification of the BA–parent molecule in the crop tissues are reported in the literature. Therefore, the aim of this study was to develop a sensitive and specific procedure for the determination of BA residues. Micropropagated apple explants were used as the plant material matrix because BA is readily taken up in this tissue (Nordström and Eliasson, 1986) and because of the importance of BA utilization in this type of production.

EXPERIMENTAL PROCEDURES

Chemicals. Analytical grade BA and the other components of the nutrient solution reported in Table 1 were purchased from Sigma Chemical Co. (St. Louis, MO). Water, methanol, and acetic acid (HPLC grade) were purchased from BDH (Poole, England). All other reagents were of ACS grade. DEAE-cellulose and Prep-Sep C_{18} (30 mg) cartridges were obtained from Merck (Darmstadt, Germany) and Fisher Scientific (Fair Lawn, NJ), respectively.

Plant Material. Shoots of the apple cv. Starkspur Red (*Malus domestica* Borkh) were obtained from meristems with two or three attached leaf primordia, aseptically dissected, of dormant buds. The explants were propagated in vitro using a usual proliferating medium (Table 1) (Jacoboni and Standard, 1982). As this regeneration step does not produce any roots, all of the BA taken up is present in the shoots. The proliferating procedure (Zimmerman, 1984) was repeated many times to obtain plant material sufficient to begin a culture in a medium with or without BA. Some of the shoots were allowed to grow in 30 jars (15/jar) on medium without BA and the rest in 30 jars on a medium containing 4.44 μM BA.

The cultures were incubated in a growth chamber at 22 ± 0.5 °C with a 16-h photoperiod provided by warm white fluorescent lights at a photon flux density of 50–60 μmol s^{-1} min^{-2}.

Fifteen days from the beginning of the culture the 30 jars of BA-treated explants were divided into three groups of 10 jars each, and the shoots from each group of 10 jars were combined to make a homogeneous subsample. The same procedure was followed for the explants grown without BA. The six subsamples were lyophilized, stored in a freezer at −25 °C, and analyzed.

Table 1. Composition of the Nutrient Solution Used for Apple Explant Culture

component	concn (mg/L)	component	concn (mg/L)
KNO_3	1800	sucrose	30000
$Ca(NO_3)_2 \cdot 4H_2O$	1200	*meso*-inositol	100
NH_4NO_3	400	*p*-aminobenzoic acid	1.0
$MgSO_4 \cdot 7H_2O$	360	benzyladenine	1.0
KH_2PO_4	270	nicotinic acid	1.0
NaEDTA	37.25	thiamin hydrochloride	1.0
$FeSO_4$	27.85	gibberellic acid	0.5
$ZnSO_4 \cdot 7H_2O$	8.6	D-pantothenic acid	0.5
H_3BO_3	6.2	adenine sulfate	0.1
$MnSO_4 \cdot 4H_2O$	1.00	biotin	0.1
$Na_2MoO_4 \cdot 2H_2O$	0.25	indole-3-butyric acid	0.1
KI	0.08	folic acid	0.01
$CoCl_2 \cdot 6H_2O$	0.025		
$CuSO_4 \cdot 5H_2O$	0.025	agar	0.7%

Table 2. Extraction Efficiency of BA from Tissues of Micropropagated Apple Culture with Different Solvent Systems

	solvent (mean ± SD) (n = 6)			
BA concn (ppm)	CH_3OH	C_2H_5OH	CCl_4[a]	CH_3COCH_3[b]
0.50	90 ± 4	93 ± 5	68 ± 7	67 ± 6
25.00	88 ± 3	91 ± 3	72 ± 2	65 ± 4
300.00	88 ± 4	90 ± 2	70 ± 4	53 ± 3

[a] With the addition of 0.8% acetic acid. [b] With the addition of 0.4% acetic acid.

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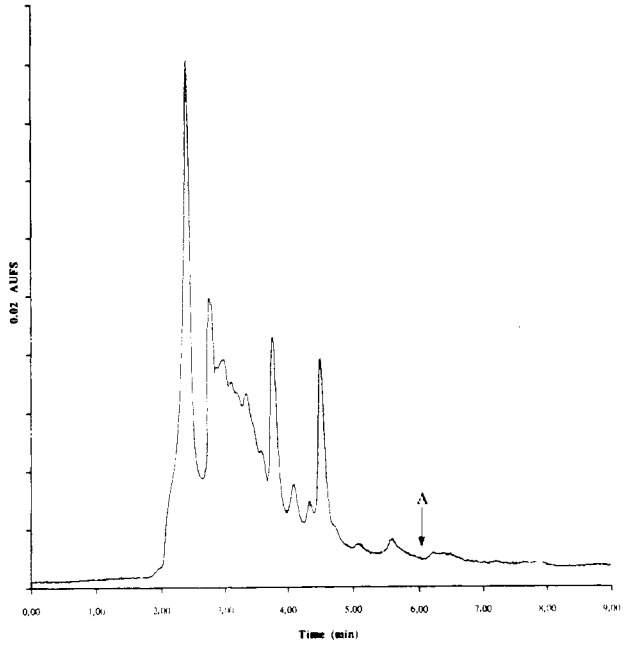

Figure 1. Representative chromatogram of micropropagated apple explants (1.0 g) with no BA. Detection was at 255 nm and 0.02 AUFS. "A" indicates the retention time of BA.

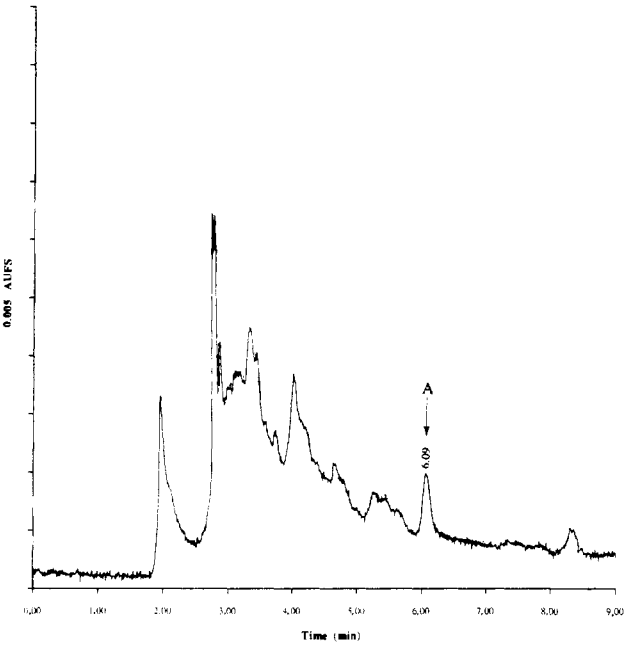

Figure 2. Representative chromatogram of micropropagated apple explants (0.5 g) grown in the presence of BA. The amount detected was 0.5 ppm. Detection was at 255 nm and 0.005 AUFS. "A" indicates the retention time of BA.

Apparatus. The HPLC instrument was assembled from the following modular components: two Perkin-Elmer Series 410 LC pumps, a Rheodyne Model 7125-075 injector, a Perkin-Elmer Model LC 235 diode array detector, interfaced with an Omega 2 analytical chromatographic workstation (version 2.50 software), and an Omega 235 software upgrade kit (PE Nelson). The apparatus was equipped with a LC column adsorbosphere C_8 (25 cm × 4.6 mm i.d., 5-μm particle size) and with an adsorbosphere C_8 Direct-Connect cartridge guardcolumn (Alltech Associates).

Extraction and Cleanup Procedure. Each lyophilized subsample of plant material (0.5–1.0 g) was extracted with a Soxhlet apparatus using 200 mL of 95% ethanol for 3 h. The ethanolic extract was evaporated in vacuo at 40 °C to dryness, and the residue was dissolved with 8 mL of ammonium formate buffer (0.01 M, pH 3.7). The solution was extracted three times with 5-mL aliquots of petroleum ether (bp 50–70 °C). The buffer

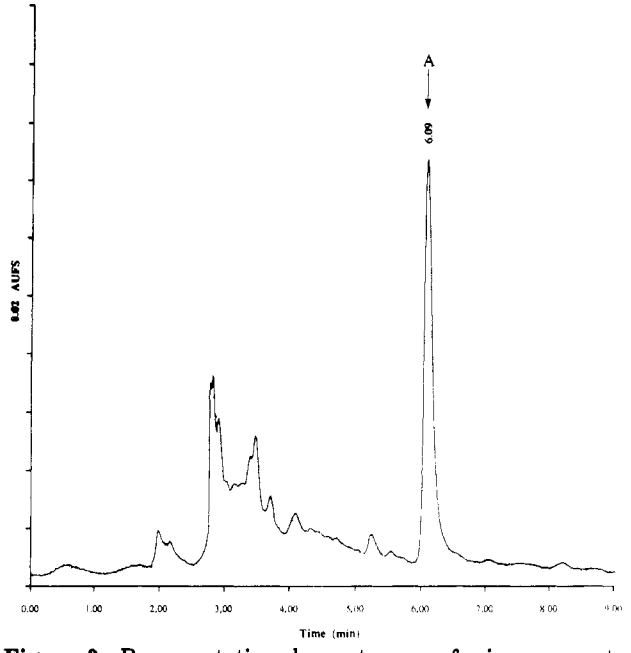

Figure 3. Representative chromatogram of micropropagated apple explants (0.5 g) spiked with 10.00 ppm of BA. Detection was at 255 nm and 0.02 AUFS. "A" indicates the retention time of BA.

Table 3. Statistics and Regression Output for the Standard Curve

r^2	0.999	slope	1.451
y-intercept	0.1	standard error of slope	±0.007
standard error of y-intercept	±0.9	no. of observations	7[a]
		degrees of freedom	5

[a] Represents seven concentrations for each standard curve.

phase was adjusted to pH 5.6 with a 5% NH_4OH solution and quantitatively transferred to a column (10 mm i.d.) containing DEAE-cellulose (15 cm), previously activated by subsequent washings with deionized water, 0.5 N HCl, 0.5 N NaOH, and ammonium formate buffer (0.01 M, pH 5.6; elution buffer).

The column was eluted with 190 mL of the elution buffer; the first 40 mL was discharged, and the subsequent 150 mL was collected and passed through Prep-Sep cartridges prepared as recommended by the manufacturer. Cartridges were washed with 60 mL of elution buffer and eluted with 50 mL of methanol. The methanolic eluates were evaporated to dryness under a stream of dry nitrogen; the residues were redissolved in 1.0 mL of methanol and used for HPLC analysis.

Chromatographic Conditions. The following isocratic system was employed: mobile phase, water–methanol (30:70 v/v), both containing 0.05% acetic acid; flow rate, 1.0 mL/min; injection volume, 6 μL. BA was monitored at a wavelength of 255 nm and 0.2–0.005 adsorbance units full scale (AUFS), showing a retention time of 6.09 min.

Sample Fortification Procedure. From a methanolic solution containing 300.0 μg/mL BA, six subsolutions of the following concentrations were prepared: 150.0, 75.0, 25.0, 10.0, 2.0, and 0.5 μg/mL. One milliliter of each solution was added to a certain amount of lyophilized plant material which was grown in the absence of BA and corresponded to 1 g of fresh weight. The higher fortification level corresponded to the theoretical maximum amount of BA expected on the basis of the volume of nutrient solution utilized, which was approximately 300 mL/g of fresh weight. The suspensions were evaporated to dryness under a gentle stream of dry nitrogen and then submitted to extraction and cleanup procedures. A calibration curve of peak area vs BA concentration was constructed and used for quantification of BA in micropropagated samples.

RESULTS AND DISCUSSION

From preliminary analyses performed on BA-grown

Table 4. Within-Day Reproducibility and Accuracy of BA Determination in Apple Tissue Culture

actual concn (ppm)	day 1 mean ± SD (n = 3)	RSD (%)	assay accuracy (%)	day 2 mean ± SD (n = 3)	RSD (%)	assay accuracy (%)	day 3 mean ± SD (n = 3)	RSD (%)	assay accuracy (%)
0.50	0.52 ± 0.04	7.7	104	0.52 ± 0.02	3.8	104	0.50 ± 0.03	6.0	100
2.00	1.88 ± 0.14	7.4	94	1.92 ± 0.11	5.7	96	1.83 ± 0.14	7.7	92
10.00	8.07 ± 0.07	0.9	81	8.11 ± 0.56	6.9	81	7.99 ± 0.09	1.1	80
25.00	19.8 ± 0.7	3.5	79	20.3 ± 0.6	2.9	81	20.7 ± 0.6	2.9	83
75.00	62.7 ± 0.8	1.3	84	61.7 ± 0.8	1.3	82	61.3 ± 1.3	2.1	82
150.00	123 ± 1	0.8	82	120 ± 2	1.7	80	124 ± 2	1.6	82
300.00	261 ± 1	0.4	87	263 ± 3	1.1	88	265 ± 2	0.7	88

Table 5. Between-Day Assay Reproducibility and Accuracy of BA Determination in Apple Tissue Culture

actual concn (ppm)	concn determinated mean ± SD (n = 9) (ppm)	RSD (%)	assay accuracy (%)
0.50	0.52 ± 0.03	5.9	102
2.00	1.90 ± 0.12	6.3	95
10.00	8.10 ± 0.09	1.1	81
25.00	20.5 ± 0.7	3.4	82
75.00	62.0 ± 1.0	1.6	83
150.00	122 ± 2	1.6	81
300.00	263 ± 2	0.8	88

samples, the extraction with the Soxhlet apparatus for 3 h was the most efficient. Among the solvent systems able to dissolve BA, methanol, ethanol, carbon tetrachloride acidified with 0.8% CH_3COOH, and acetone acidified with 0.4% CH_3COOH were chosen because of their solving capacity and then investigated for optimum selectivity and extraction efficiency. There were no significant differences between ethanol and methanol in their extraction efficiency of BA from apple micropropagated tissue (Table 2). However, the extraction selectivity of methanol was poor because some interference peaks occurred due to the extraction of other vegetable components. Figure 1 reports the chromatogram of an untreated sample showing that the zone where BA is eluted is free of any interference peaks. Therefore, ethanol was the solvent of choice. Figure 2 shows the chromatogram of a sample obtained from a 15-day old apple micropropagated tissue grown in the presence of BA. Peak A was adequately resolved from endogenous peaks of the control. Figure 3 gives further confirmation that peak A, observed in Figure 2, is that of BA; in fact, the retention time and UV absorption spectra taken at the apex of the BA peaks for the fortified and unfortified samples were the same.

Calibration curve data are shown in Table 3. An average correlation coefficient (r^2) of 0.999 indicates very good fit to the least-squared weighted linear regression model. The mean slope data were associated with a coefficient of variation (RSD) of 1.5%, showing good between-day reproducibility.

The reproducibility and accuracy of the method were determined by analyzing the plant material samples spiked with seven different amounts of BA. The data, considered both within-day and between-day, are summarized in Tables 4 and 5, respectively. The mean relative standard deviations (RSD) for both within- and between-day analyses did not exceed 7.7%, while the accuracy of the assay ranged from 79% to 104%. The reproducibility and accuracy of this experiment obtained by the straight calibration curve method were quite reliable. Therefore, an internal standard in the method was not used.

The limits of detection and quantification calculated according to the ACS Committee on Environmental Improvement (1980) were 0.15 and 0.50 ppm, respectively. These values confirm that the described procedure is sensitive enough to evaluate the persistence of BA in micropropagated apple tissues. The content found in tissues of explants micropropagated for 15 days was 0.6 ± 0.04 ppm (n = 3). The good results of selectivity suggest the feasibility of applying this procedure to other cultures micropropagated or grown in a greenhouse; however, the BA elution pattern from the cellulose column would have to be determined for each plant tissue.

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