Gartenbauwissenschaft, **65** (6). S. 241–244, 2000, ISSN 0016–478X. © Verlag Eugen Ulmer GmbH & Co., Stuttgart

Determination of Benzyladenine content of *in vitro* propagated dwarf J-TE-F apple rootstock (*Malus* sp.) by High Performance Liquid Chromatography

Die Bestimmung des Benzyladenin-Gehaltes von in vitro vermehrten J-TE-F Apfelunterlagen mittels HPLC

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Summary

Shoots of J-TE-F dwarf apple rootstock were cultured under *in vitro* conditions on MURASHIGE-SKOOG (1962) basal medium, containing 0.25, 0.50 and 1.0 ppm BA. The 6-benzyladenine (BA) concentration of plantlets was determined by HPLC on the 14th and 30th day of subculture. The plant hormone was extracted by ethanol, further cleaned on SEP-PAK cartridge. The effluent was examined by reversed phase High Performance Liquid Chromatography with diode array detection at 270 nm. Recovery and reproducibility assessment indicates good accuracy and acceptable relative standard deviation (RSD) 5 %). Linear responses ($r^2$0.998) for calibration curve was obtained with BA standard in a range of 0.2-30 g·ml^{-1} with a limit of quantification of 0.15 g·ml^{-1}.

The highest BA concentration (5.49 ppm) was measured in the plants, which were cultured on 1.0 ppm BA containing multiplication medium on the 14th day. On the 30th day the BA concentration decreased to ~1 ppm/dry weight.

Zusammenfassung

In unserem Versuch wurde der Gehalt des Benzyladenin (BA) der *in vitro* vermehrten J-TE-F Apfelunterlagen untersucht. Die Unterlagenpflanzen wurden im MURASHIGE-SKOOG (1962) Grundmedium vermehrt, das 0.25, 0.50 und 1.0 ppm BA enthielt. Der BA-Gehalt der Pflänzchen wurde mittels HPLC am 14-ten und am 30-ten Tage der Subkultur bestimmt. Die Pflanzenhormone wurden mit Ethanol extrahiert und weiterhin mittels SEP-PAK-Kartusche gereinigt. Das Eluat wurde mit Hilfe von HPLC bei 270 nm geprüft. Die Experimente zeigten gute Genauigkeit und akzeptierbare relative Standardabweichung RSD 5 %. Die Kalibrationskurve war linear im Bereich von 0.2–30 g·ml^{-1} des Standardes und die Nachweisgrenze war 0.15 g·ml^{-1}.

Die höchste BA Konzentration (5.49 ppm) wurde am 14-ten Tage in den Pflanzen festgestellt, die im Multiplikationssubstrat mit 1 ppm BA Gehalt kultiviert wurden. Nach 30 Tagen fiel die BA Konzentration auf ca. 1 ppm/g in der Trockensubstanz zurück.

Introduction

The plant bioregulators have significant influence on plant vigour, growth and development of new organs. Under natural conditions the content of plant growth regulators have an effect on the plant. The interaction of all bioactive materials determine the kind of plant development.

This bioregulators can be arranged in 4 groups, such as auxin, cytokinin, gibberellin and retardants. The horticultural praxis use all of the above materials during growing.

Recently, our interest has turned to cytokinin, because these plant hormones are able to break the dormant state of summer buds. As a result, on one hand new shoots can develop during the summer, and on the other hand it is very important to use them during the propagation phase of *in vitro* cultured plants.

BA is one of the synthetic products used as bioregulator in a branching agent (BA as an exogenous plant growth regulator has great cytokinin-like effects) in nurseries and in young orchards CODY et al. (1985), BASAK and SOCZEK (1986), WERTHEIM and ESTABROOKS (1994) and HROTKÓ et al. (1996 a, b, 1997).

In the *in vitro* propagation, using plant hormones in the medium is of fundamental importance, because of the control of implants developing. As cytokinin, BA is commonly used, because of its high efficiency in promoting lateral shoots, and developing of adventitious buds. Applied in callus culture SKOOG and MILLER (1957) detected, that using cytokinin and auxin in appropriate concentration, induces the developing of meristems. Having used these plant growth regulators, the regulation of tissue- and organ cultures was also successful (MARÓTI 1979).

Based on many experiments among cytokinin-like growth regulators the BA is used commonly in the micropropagation (JONES et al. 1977, MACHNIK and ORLIKOWSKA 1981, MARN 1988 and YUI et al. 1993). In case of apple rootstocks, BA concentration between 0.5-2 mg·l^{-1} is suggested (YUI et al. 1993). The endogenous hormone content of *in vitro* plantlets is not well-known (JÁMBOR-BENCZÚR 1993).

YOUNG (1977) describes a highly selective method for the detection and identification of cytokinin in plant extract by gas-liquid chromatography. The plants used were *Actinidia arguta* and *Cerasus avium*.

BLAKESLEY et al. (1991) showed that throughout the culture period BA was only present in trace amount within the shoot of *Gerbera* sp. BLAKESLEY and CONSTANTINE (1992) measured the BA-concentration of *in vitro* propagated ornamental plants. They used different types of ^{14}C labelled BA for studying its uptake and metabolism. After HPLC separation liquid scintillation counter was applied for determination of the active component. In that experiment the medium contained 2.22 µM BA, and 0.04–2.46 nmol/fresh g quantities of ^{14}C labelled BA was determined from plant.

Other plant hormons, such as auxin (WODZICKI et al. 1987, CROZIER et al. 1980, RODRIGUEZ and TAMES 1985, NONHEBEL et al. 1983) was measured by gas-liquid chromatography. Gas chromatography was used in the experiments of PENGELLY et al. (1981). Abscic acid, and indol-acetic-acid (IAA) was measured by GUINN et al. (1986) and gibberellin by ODEN et al. (1987) with gas chromatography–mass spectrometry.

The review of literature shows, that cytokinin concentration, such as the usually used BA of *in vivo*- and *in vitro* propagated apple plants, until now has not been fully cleared yet.

In our experiments, connecting to previous application forms of BA, *in vitro* conditions were applied.

Our aim was to clarify what of BA is taken up by micropropagated apple plants from the medium which contains known amount of added BA. This is a simple and informative way to study the bioavailability of benzyladenine. In our experiment – based on our previous experience – a sensitive and reliable isocratic HPLC (DAD) method was applied.

Materials and methods

1. Growing conditions:

In vitro propagated J-TE-F apple (*Malus sp.*) dwarf rootstock plantlets (ANONYM 1987) were used, for plant material cultured on MURASHIGE and SKOOG (1962) basal medium solified with 8 g/l Difco-Bacto agar, the quantity of sucrose was 20 g/l furthermore it contains MURASHIGE and SKOOG (1962) vitamin-stock. The medium contained –depending on examination– 0.25, 0.5 and 1.0 ppm BA (6-benzyladenine) [1214-39-7], and 0.1 ppm IBA [133-32-4] (indole-3-butyric acid) also. The pH was adjusted to 5.6 with 0.1N KOH.

The plantlets were cultured in 400 ml glass-jars, on 80 ml medium, in a growth-room at 22 ± 2 °C with 16/8 photoperiod. The density of photon-flux was 55–70 µmol·s^{-1}·m^{-2}.

The plant material was removed from jars for analysis on the 14th and the 30th day of subculture.

2. Analytical conditions:

Chemicals:

Analytical grade BA and other components of the medium were purchased from Sigma Aldrich Chemical Co. Water, methanol and acetic acid (HPLC grade) were purchased from CARLO ERBA CO./Italy. All other chemicals were of analytical grade. BA was used in a methanolic stock solution (0.01g/50 ml) and a 50x dilution of it was used as working standard in HPLC.

Sample preparation:

The plantlets were dried at 60 °C until constant weight (the dry material content of them was about 15 %), and without storing about 1g of them were extracted with a Soxhlet-apparatus using 250 ml 95 % ethanol for 4 hours.

The extract was evaporated in vacuo at 45–50 °C to dryness, and the residue was dissolved in 8 ml ammonium-formate (0.01M, pH 3.7). Chlorophyll-content was removed by extracting 3 times with petroleum ether (bp. 40 – 70 °C). The extract was further cleaned on a SEP-PAK C18 cartridge, (WATERS) which was activated by 10 ml methanol, BA was eluted by 1 ml methanol, and the eluate, was filtered using a MILLEX-HV 0.45 µm filter unit. The filtered solution was directly injected onto the HPLC.

HPLC conditions:

A Waters-High Performance Liquid Chromatograph equipped by a 990 Diode array detector (DAD), and a 600 controller were applied. A HICHROM SPHERISORB S5 C8 25 cm x 4.6 mm column was installed. Mobile phase water-methanol 30:70 v/v % containing 0.05 % acetic acid, flow-rate 1cm^3·min^{-1}, the pressure

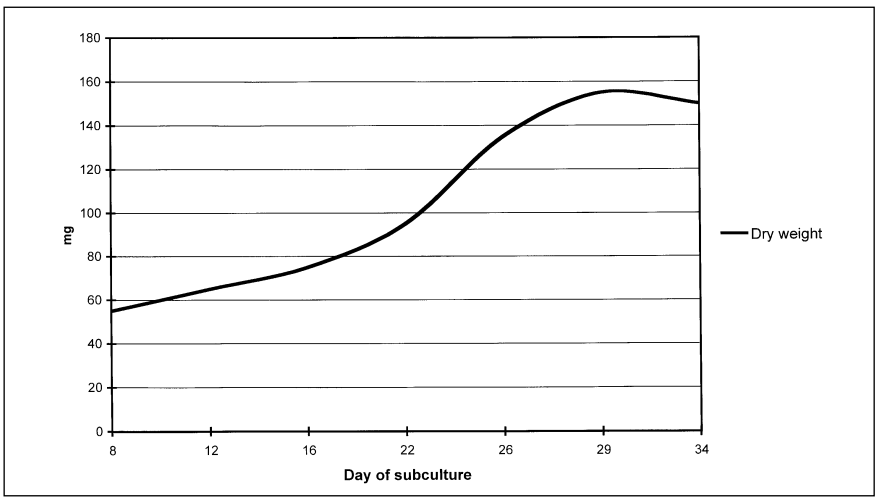


Fig. 1. Development of dry weight during subculture of apple plantlets
Entwicklung des Trockengewichtes während der Subkultur von Apfelsprossen

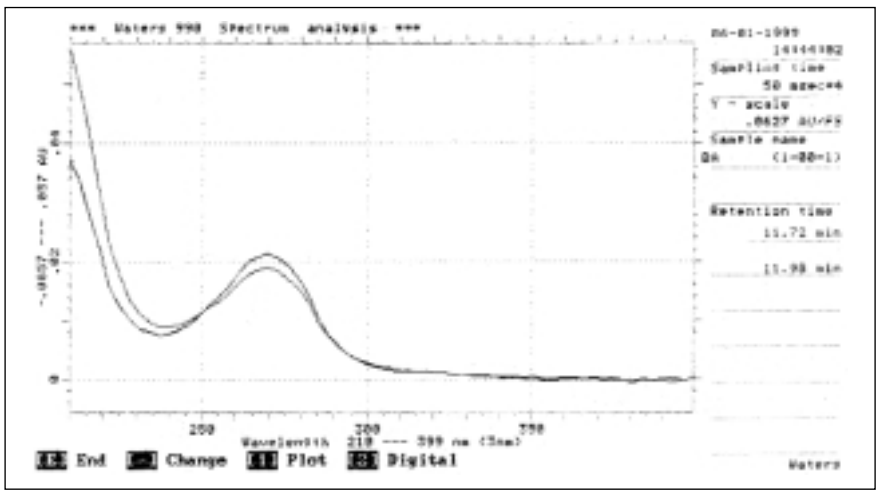


Fig. 2. Characteristical HPLC chromatogram of in vitro propagated apple samples (20 µl).
Ein typisches HPLC-Chromatogramm von Extrakten der in vitro vermehrten Apfelsprosse (20 µl).

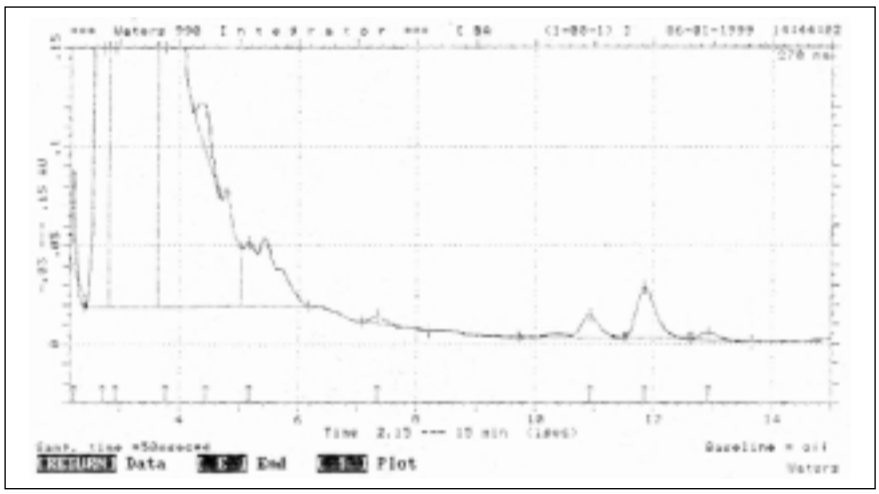


Fig. 3. DAD-UV spectra of the peak at 11.84 min.
DAD-UV Spektrum des Peaks bei 11.84 min.

on the column was about 1200 psi. The injected volume was 20 µl.

The BA was monitored at a wavelength of 270 nm. The retention time of BA in standard solution was 11.87 min.

Each sample was analysed in a triplicate.

Results and discussion

The aim of this work was to consider to which extent micropropagated apple is able to take up BA from the medium. BA content of medium are well known as they are made with analytical accuracy.

There were only few hints in the relevant literature pertaining the BA content of micropropagated plants.

At first we measured the quantity of dry weight of developed biomass during the subculture (Figure 1). The starting biomass was 50 mg, and finally on the 30th day it was 155 mg.

In vitro cultured mother plants, which were used further as subject in the experiments at all three treatments, were grown for 30 days on MS-medium which contained 0.25 ppm BA. These plants were analysed by HPLC, and the BA content of them was measured at 0.81 ± 0.02 ppm. Afterwards these plants were planted into the experimental media. Analyzing the plantlets by HPLC the following results can be observed (Figure 2, Table 1).

Figure 2 shows the chromatogram of 20 µl plant extract derived from 0.25 ppm BA contained medium. Peak at 11.84 min represents a BA concentration of 2.26 ppm. With the help of diode array detection it can be stated, that the peak is pure, there is no interfering materials (Figure 3).

Table 1 summarises the BA content measured at the 14th and 30th day of growth on media containing different amounts of BA and the propagation rate at the 28th day. The RSD value of the determination was about 5 %. (n = 5).

Table 1. The BA concentration of dried plants at 14th and 30th day, in relation to the BA content in the medium
BA Gehalt getrockneter Pflanzen nach 14 oder 30 Tagen, in Abhängigkeit von der BA-Konzentration des Mediums

BA concentration (ppm) (in the medium)	Day of subculture		Propagations rate on the 28th day
	14th day	30th day	
	Measured BA (in the plants, ppm)		
0.25	2.26	0.84	3
0.50	3.21	1.24	5
1.0	5.49	1.08	9

It can be stated, that the BA content of plants depends on BA concentrations in the medium. Until the 14th day, BA level increases, following this on the 30th day significant decrease can be observed.

According to our results, plants are able to take up all of the available BA from the medium, and they accumulate it. To clarify this problem, it might also be necessary to analyse the BA residue in the medium during subculture.

Possible cause of the drop is that after two weeks the plantlet is unable to take up more BA, or the increase of plant weight might cause a decrease in relative BA content. The latter is based on the fact, the starting dry weight of the plantlet is gaining from 50 mg to 150 mg (4 week). Our data complement to the literature values as BLAKESLEY *et al.* (1991) showed also the decline of BA level in *Gerbera sp.* shoots by the 24th day.

The above figures confirm that the applied procedure is sensitive enough to evaluate the BA content of micropropagated apple tissues.

Our experiment will be extended in two ways, on the one hand more frequent (every 3 days) sampling is necessary, on the other hand it would be interesting to study the BA circulation and distribution in case of *in vivo* propagated horticultural plants.

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Eingegangen: 21. 6. 1999 / 17. 11. 1999

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