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Endogenous cytokinin profiles of tissue-cultured and acclimatized 'Williams' bananas subjected to different aromatic cytokinin treatments

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ABSTRACT

Endogenous cytokinin (CK) levels of in vitro-cultured and greenhouse-acclimatized 'Williams' bananas treated with six aromatic CKs were quantified using UPLC–MS/MS. The underground parts had higher endogenous CK levels than the aerial parts. Control plantlets had more isoprenoid CKs while the aromatic-type CKs were predominant in all other regenerants. Following acclimatization of the control and 10 μM CK regenerants, there was a rapid decline in both isoprenoid and aromatic CK in the greenhouse-grown plants. Apart from the control and 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (Me*m*TTHP) treatment with higher level of isoprenoid CK, aromatic CK remain the predominant CK-type across all CK treatments. The most abundant CK forms were *meta*-topolin (*m*T) and benzyladenine (BA) in the micropropagated and acclimatized plants, respectively. Micropropagated plantlets had *cis*-Zeatin (*c*Z) as the major isoprenoid CK-type which was in turn replaced by isopentenyladenine (iP) upon acclimatization. On a structural and functional basis, 9-glucoside, a deactivation/detoxicification product was the most abundant and mainly located in the underground parts (micropropagation and acclimatization). The results establish the wide variation in metabolic products of the tested aromatic CKs during micropropagation and acclimatization. The findings are discussed with the possible physiological roles of the various CK constituents on the growth and development of banana plants.

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Abbreviations: BA, N^6-Benzyladenine; BA9G, N^6-Benzyladenine-9-glucoside; BAR, N^6- Benzyladenine riboside; BAR5′MP, N^6-Benzyladenosine-5′-monophosphate; CDK, Cyclin-dependent kinase; CK, Cytokinin; *c*Z, *cis*-Zeatin; *c*Z9G, *cis*-Zeatin-9-glucoside; *c*Z*O*G, *cis*-Zeatin-*O*-glucoside; *c*ZR, *cis*-Zeatin riboside; *c*ZR5′MP, *cis*-Zeatin riboside-5′-monophosphate; *c*ZR*O*G, *cis*-Zeatin-*O*-glucoside riboside; DHZ, Dihydrozeatin; DHZ9G, Dihydrozeatin-9-glucoside; DHZ*O*G, Dihydrozeatin-*O*-glucoside; DHZR, Dihydrozeatin riboside; DHZR5′MP, Dihydrozeatin riboside-5′-monophosphate; DHZR*O*G, Dihydrozeatin-*O*-glucoside riboside; IAC, Immunoaffinity chromatography; iP, N^6-Isopentenyladenine; iP9G, N^6-Isopentenyladenine-9-glucoside; iPR, N^6-Isopentenyladenosine; iPR5′MP, N^6-Isopentenyladenosine-5′-monophosphate; IPT, Isopentenyltransferase; KIN, Kinetin; KIN9G, Kinetin-9-glucoside; KINR, Kinetin riboside; KINR5′MP, Kinetin riboside-5′-monophosphate; Me*m*T, *meta*-Methoxy topolin; Me*m*TR, *meta*-Methoxy topolin riboside; Me*m*TTHP, 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine; MRM, Multiple reaction monitoring; MS, Murashige and Skoog medium; *m*T, *meta*-Topolin; *m*T9G, *meta*-Topolin-9-glucoside; *m*T*O*G, *meta*-Topolin-*O*-glucoside; *m*TR, *meta*-Topolin riboside; *m*TR5′MP, *meta*-Topolin-5′-monophosphate; *m*TR*O*G, *meta*-Topolin-*O*-glucoside riboside; *o*T, *ortho*-Topolin; *o*T9G, *ortho*-Topolin-9-glucoside; *o*T*O*G, *ortho*-Topolin-*O*-glucoside; *o*TR, *ortho*-Topolin riboside; *o*TR5′MP, *ortho*-Topolin-5′-monophosphate; *o*TR*O*G, *ortho*-Topolin-*O*-glucoside riboside; PGR, Plant growth regulator; PPF, Photosynthetic photon flux density; *p*T, *para*-Topolin; PTC, Plant tissue culture; *p*T*O*G, *para*-Topolin-*O*-glucoside; *p*TR, *para*-Topolin riboside; *p*TR5′MP, *para*-Topolin-5′-monophosphate; *p*TR*O*G, *para*-Topolin-*O*-glucoside riboside; *tZ*, *trans*-Zeatin; *tZ*9G, *trans*-Zeatin-9-glucoside; *tZO*G, *trans*-Zeatin-*O*-glucoside; *tZR*, *trans*-Zeatin riboside; *tZR*5′MP, *trans*-Zeatin riboside-5′-monophosphate; *tZRO*G, *trans*-Zeatin-*O*-glucoside riboside; UPLC, Ultra performance liquid chromatography.

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1. Introduction

Cytokinins (CKs) are naturally occurring N^6-substituted adenine compounds which are vital for the regulation of several developmental and physiological processes in plants [1]. These processes include shoot apical dominance, branching, chlorophyll production and root growth as well as contributing to nutritional signalling and formation of embryo vasculature [2,3]. Based on the side chain configuration, CKs are classified as of the isoprenoid [N^6-2-isopentenyl adenine (iP), *trans*-Zeatin (*tZ*), *cis*-Zeatin (*cZ*) and dihydrozeatin (DHZ)] or aromatic [N^6-benzyladenine (BA), kinetin (KIN) and topolin] types. Together with auxins, CKs affect the basic mechanisms of cell proliferation and differentiation in plants [4].

In addition to the benefit of mass propagation, genetic improvement and conservation of several plant species, the plant tissue culture (PTC) technique is an avenue for exploring and understanding the often species-specific factors that control plant growth and development. In PTC, the use of exogenous CKs is necessary to stimulate various developmental and physiological processes [5]. Growth and development in vitro are regulated by the interaction and balance between the exogenously supplied and endogenously produced plant growth regulators (PGRs) [6,7]. In plant tissues, these exogenous supplied CKs are often metabolized to different forms such as products of ring substitution (ribosides, ribotides, *N*-glucosides) and side chain substitution (*O*-glucosides) or cleavage (adenine, adenosine, adenosine-5′-monophosphate) [3,8]. These metabolites and conjugates act as storage, transport or biologically inert forms of CKs which are responsible for the physiological and developmental plasticity observed in plants [3,9]. Changes in the levels of endogenous CKs alter the regulation of these physiological processes and influence plant architecture. Apart from stimulating shoot proliferation in vitro, many PTC problems such as shoot-tip necrosis [10], hyperhydricity [11] and rooting inhibition as well as acclimatization failure [12,13] have been partly associated with the level of endogenous CKs in plant tissue at any given time.

In view of the increasing human population and associated problem of food security, the value and economic importance of bananas (*Musa* spp.) as a food and fruit crop cannot be overemphasized. The use of PTC has significantly improved the quality of planting materials and increased the productivity of bananas [14]. Even though considerable success has been achieved, banana remains one of the most highly prioritized research crops. Attempts at optimizing micropropagation protocols has prompted the use of the different CK derivatives, particularly topolins. We have evaluated the role of different aromatic CKs (BA in comparison to topolins) on shoot and root proliferation [15], photosynthetic pigment production [16] as well as on the phytochemical and subsequently ex vitro acclimatization competency of bananas [17]. Despite the recognized value of endogenous hormone levels on these aforementioned processes, limited studies have been reported in the case of bananas [18,19]. In the current study, the effect of six exogenous application of aromatic CKs on the endogenous CK profiles after micropropagation and greenhouse acclimatization of 'Williams' bananas (*Musa* spp. cv. 'AAA') was evaluated. The current study was aimed at understanding the previously reported growth and physiological data in relation to the quantified endogenous CK content. Details of our previous findings have been published [15–17] and the same plant materials were used for the current study.

2. Materials and methods

2.1. Chemicals

The topolins used [*meta*-Topolin (*m*T), *meta*-Topolin riboside (*m*TR), *meta*-Methoxy topolins (Me*m*T), *meta*-Methoxy topolin riboside (Me*m*TR), and 6-(3-Methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (Me*m*TTHP)] were prepared as described previously [20–22]. N^6-benzyladenine was obtained from Sigma-Aldrich (Steinheim, Germany). Gelrite was purchased from Labretoria, Pretoria, South Africa while ^{23}deuterium-labelled CK internal standards were obtained from Olchemim Ltd (Olomouc, Czech Republic). All chemicals used in the study were of analytical grade.

2.2. Micropropagation and greenhouse experiments

The source of initial plant materials, explant initiation, medium composition and growth conditions were as described previously [15]. Aseptically-obtained explants were transferred onto modified Murashige and Skoog [23] medium supplemented with six aromatic CKs at 10, 20 or 30 μM. A control without any PGR was also included in the experiment. Cultures were incubated for 42 days in a growth room under 16 h light/8 h dark conditions and photosynthetic photon flux (PPF) of 45 μmol m^{-2} s^{-1} at 25 ± 2 °C.

Due to ease of rooting, 10 plantlets each from the 10 μM CK treatments and the control were washed and potted in 12.5 cm pots containing sand, soil, vermiculite (1:1:1, v/v/v) treated with 1% Benlate® (Du Pont de Nemours Int., South Africa). Plantlets were maintained in the mist-house with day/night temperatures of 30/12 °C, relative humidity of 80–90% and a 10 s misting at 15 min intervals. After 3 months, the plantlets were transferred to a greenhouse with 30–40% relative humidity, day/night temperatures of 30/15 °C with an average PPF of 450 μmol m^{-2} s^{-1}. Photoperiod during the experiment was that of prevailing natural conditions (summer-12 h). Plants were harvested after two months in the greenhouse for endogenous CK quantification.

2.3. Extraction, purification and quantification of endogenous cytokinins

Harvested-micropropagated and greenhouse-maintained plants (five in each case) were washed and separated into aerial and underground sections. Plant material was immediately frozen in liquid nitrogen, freeze-dried and lyophilized. Prepared samples were extracted and purified using the methods [24,25] outlined for bananas in an earlier study [26].

The samples were analyzed by ultra-performance liquid chromatography (Acquity UPLC™; Waters) coupled to a Xevo™ TQ MS™ ESI (Waters) triple quadrupole mass spectrometer equipped with an electro-spray interface. Further experimental details are as outlined previously [26]. Endogenous CK quantification was achieved by multiple reaction monitoring (MRM) of $[M+H]^+$ and the appropriate product ion. For selective MRM experiments, optimal conditions (dwell time, cone voltage, and collision energy in the collision cell) corresponding to exact diagnostic transition were optimized for each CK [25]. Quantification was performed with Masslynx software using a standard isotope dilution method. The ratio of endogenous CK to appropriate labelled standard was determined and subsequently used to quantify the level of endogenous CKs in the original banana plant extract, based on the known concentration of internal standard added [24].

3. Results

3.1. Endogenous CK content in micropropagated plantlets

The total pool of all CKs quantified ranged from approximately 1.9–810 nmol g^{-1} FW as detected in control and the 10 μM *m*TR treatment, respectively (Table 1). With the exception of the control that had more isoprenoid CKs, aromatic CK were generally higher than the isoprenoid-type in all the treatments. In

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