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Metabolism of Fluorinated Topolin Cytokinins in Micropropagated *Phalaenopsis amabilis*

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Abstract: Fluorinated cytokinins have emerged as promising alternatives to traditional cytokinins in Phalaenopsis plant tissue culture, offering enhanced stability and bioactivity. However, their metabolic fate and impact on endogenous cytokinin profiles remain largely unexplored. This study builds upon previous research to investigate the comparative metabolism of the traditional cytokinin 6-Benzylaminopurine (BA) with the successful alternatives 6-(3-fluorobenzylamino)purine (FmT) and 6-(3-fluorobenzylamino)purine 9-riboside (FmTR). Additionally, this study examines the impact of another crucial factor, the use of ventilated versus closed containers, on metabolic processes. The results revealed the distinct metabolic profiles associated with each treatment, highlighting the complex interplay between exogenous and endogenous cytokinin levels. This study is the first to investigate the effects of these stable, synthetic, and exogenous cytokinins on the naturally occurring cytokinin levels and their metabolites in micropropagated Phalaenopsis. Additionally, we proposed an alternative inactivation pathway involving the conversion of FmTR and BA to pT and pTR. These findings provide valuable insights into the intricate relationship between cytokinin metabolism and plant growth under in vitro conditions.

Keywords: *Phalaenopsis*; orchid; cytokinin metabolism; in vitro tissue culture; micropropagation

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1. Introduction

Cytokinins (CKs), a class of essential plant hormones, play a pivotal role during plant growth in vitro. These phytohormones also regulate numerous developmental processes, including meristem maintenance, cell division, shoot formation, and leaf senescence [1]. During in vitro micropropagation, the exogenous application of CKs is crucial for achieving optimal plant multiplication and quality [2,3]. Plants have evolved complex mechanisms to maintain homeostasis between the exogenous supply and endogenous levels of CKs through intricate metabolic pathways, essential for overall plant growth and development [4]. Despite their widespread use in micropropagation, the comprehensive metabolic profiles of CKs have only been elucidated for a limited number of plant species. Moreover, the relative abundance of various cytokinin metabolites is influenced by the specific type of applied CKs and exhibits interspecies variability [5,6].

Orchid micropropagation through plant tissue culture techniques has been extensively studied and applied over the years [7]. Phalaenopsis micropropagation is crucial for commercial production since traditional propagation methods, such as cutting off-shoots, are characterized by a low multiplication rate [8]. However, the in vitro clonal propagation of *Phalaenopsis* still faces challenges for large-scale production. The choice of CKs significantly influences shoot proliferation, in addition to the culture system and media components. Common CKs used in orchid tissue culture include BA (N6-benzylaminopurine), kinetin,

2iP (2-isopentyladenine), and TDZ (thidiazuron), often combined with auxins like 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (α-naphthaleneacetic acid), and IAA [7,9,10]. In our study, we showed that supplementing in vitro media with fluorinated CKs improved the micropropagation rate compared to traditionally applied BA [11]. Our recent research has demonstrated that fluorinated CKs significantly enhance shoot proliferation in orchids grown in vitro, particularly in hermetically sealed containers [11]. However, the mechanisms behind this phenomenon remain elusive. To explore this subject, we performed a study focusing on the metabolites of the traditionally used 6-benzylaminopurine (BA)-type cytokinin and "second"-generation topolins 6-(3-fluorobenzylamino)purine (FmT) and 6-(3-fluorobenzylamino)purine 9-riboside (FmTR) with supposed improved stability and bioactivity [12]. Understanding the metabolic response of orchids to fluorinated CKs is crucial for developing optimized micropropagation protocols, not only for orchids but potentially for other recalcitrant plant species as well.

Upon absorption from the culture medium, CKs are subjected to extensive metabolic transformations within plant tissues, resulting in a dynamic array of metabolites with varying biological activities [13]. They encompass active forms, predominantly free bases like BA, meta-topolin (mT), 2iP, and zeatin (Z), which directly influence plant growth and development. These active forms are often converted into less active transport forms, including ribosides and ribotides, facilitating their movement within the plant [14]. Additionally, CKs can be reversibly stored as O-glucosides, which are inactive conjugates that can be readily hydrolyzed back to their active free base forms when required. Conversely, N-glucosides (such as N7 glucosides and N9-glucosides) are generally considered to be irreversible and completely inactive storage forms [14,15]. This intricate metabolic network allows plants to tightly regulate cytokinin levels in response to developmental and environmental cues, a critical factor in maintaining plant homeostasis. In the context of in vitro culture, where exogenous cytokinin application and the absence of natural environmental cues can disrupt this delicate balance, understanding and manipulating cytokinin metabolism is important for achieving the optimal conditions for de novo organogenesis and successful micropropagation [15–18].

As fluorinated CKs have shown enhanced shoot proliferation in phalaenopsis, in this study, we aim to understand the response of in vitro plants to these fluorinated CKs. We therefore investigated the metabolic profile of applied CKs. These insights offer a deeper understanding of the intricate relationship between cytokinin metabolism and plant growth in a controlled environment, which could lead to optimized micropropagation protocols (not only for this valuable ornamental plant but also for other challenging crops). We demonstrate the balance between endogenous and exogenous CKs and interaction with the culture conditions.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

An unnamed cultivar of *Phalaenopsis amabilis* served as the model plant for this study. The initial plant material was propagated on a 2.14 g/L MS (Murashige and Skoog, 1962) basal medium supplemented with 10 µM FmTR. Following a two-month growth period, these shoots were transferred to the fresh medium of the same composition, but supplemented with one of three different CKs: FmT, FmTR (Olchemim, Olomouc, Czech Republic), or BA, each at a fixed concentration of 10 µM. Axillary shoots (1 cm) with two young leaves were separated from in vitro grown plants. Five explants were transferred to each 565 mL polypropylene container containing 100 mL of medium. The lids of these recipients were tightly closed or contained a 'Large' filter ('L' = #10 filter) (Microbox™, Eco2box, Geraardsbergen, Belgium). The cultures were maintained for 10 weeks at 22 ± 2 °C in a 16/8 light/dark photoperiod (40 µmol m^{-2} S^{-1} PAR) provided by white fluorescence tubes.

2.2. Cytokinin Analyses

Five plants were randomly selected from each treatment group and weighed before lyophilization. Additionally, leaves from a potted Phalaenopsis plant were collected to serve as a control/reference sample for comparison. All samples were stored at −80 °C

before CK analysis. Cytokinin metabolites were quantified using a modified protocol by Novák et al. [19]. In brief, lyophilized samples were finely ground in liquid nitrogen and 5 mg of dry weight was aliquoted for analysis. Subsequently, 1 µL of cold extraction solution (Modified Bieleski buffer MeOH/H_2O/HCOOH (15/4/1), Bieleski, 1964) and 23 deuterium-labelled cytokinin (CK) internal standards (Olchemim, Olomouc, Czech Republic) were added to each sample. The internal standards, applied at concentrations of 1 pmol (free bases) and 2 pmol (O-glucoside and nucleotides) per sample, served to assess the recovery of target CKs during purification and validate their quantification. Following homogenization in a vibration mill (27 Hz, 3 min), sonication (4 °C, 5 min), and agitation (4 °C, 30 min), samples were centrifuged (20,000 rpm, 10 min, 4 °C) and the resulting pellets were re-extracted using the same procedure. The collected supernatants were then subjected to a multi-step purification process involving octadecyl C18 (100 mg/1 mL) speed SPE cartridges, Oasis MCX (30 mg/1 mL) columns, and immunoaffinity chromatography (IAC). Blank samples were prepared in parallel using an identical procedure.

Detailed purification and quantification procedures of CKs and its metabolites were previously described by Plačková [20]. Briefly, samples were analyzed using an Acquity UPLC™ system (Waters, Milford, CT, USA) coupled with a Xevo™ TQ MS™ (Waters, Milford, CT, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. CK quantification was achieved via the multiple reaction monitoring (MRM) of [M + H]+ and the appropriate product ions. The optimal MRM conditions (dwell time, cone voltage, and collision energy) were determined for each CK. The ratio of CKs to their corresponding labelled standards was used to quantify CK levels based on the known internal standard concentrations [16]. All CK quantifications were performed in triplicate.

2.3. Data Analysis

The experiment followed a completely randomized design with three repetitions for each sample to assess CKs. Each treatment included three replicates, and each replicate consisted of 10 explants per culture vessel. CKs were measured in the leaves of plants grown in vitro for 10 weeks. An independent two-sample *t*-test was conducted across all means using Python 3.10 to compare the means of different sample groups. Significant difference was set at $p < 0.05$. This statistical approach was chosen to determine if there were significant differences between the independent sample groups under various conditions. All tests were performed assuming unequal variances.

3. Results

Effect of Exogenously Administered CKs on Total Quantified Cytokinin Levels and Their Metabolic Profile

The total content of CKs (expressed as pmol/1 g FW) was determined in eight-week-old in vitro shoots of *Phalaenopsis* grown in the presence of 10 µM BA, 10 µM FmT, and 10 µM FmTR, and was compared between plants grown in ventilated or hermetically sealed containers. A total of 48 different CKs and their metabolites were detected in varying concentrations and are listed in Table 1. The concentrations of different cytokinin types vary depending on the treatment and container type; both aromatic CKs (such as BA, ortho-topolin (oT), meta-topolin (mT), para-topolin (pT), and their derivatives) and isoprenoid CKs (such as trans-zeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DHZ), isopentenyladenine (iP), and its derivatives) were detected, with diverse abundance across treatments and container types.

The presence and abundance of specific cytokinin metabolites varied significantly under different treatment and culture conditions. For instance, tZ7G, present in trace amounts under BA treatment, was completely absent in plants grown in closed containers and supplemented with FmT or FmTR. Additionally, the abundance of cZ and its riboside form, cZR, exhibited distinct patterns depending on the treatment and container type. While cZ remained detectable at minimal concentrations in BA-treated plants, it fell below the limit of detection (LOD) in FmT- or FmTR-treated plants. Intriguingly, cZR was consistently abundant across all treated plants.

Table 1. Concentrations of CKs (pmol/g FW) and its metabolites in *Phalaenopsis* shoots grown in ventilated and closed containers in vitro.

	Cytokinin Treatment					
	BA		FmT		FmTR	
Cytokinin	Container Type					
Metabolites	Ventilated	Closed	Ventilated	Closed	Ventilated	Closed
tZ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
tZOG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
tZR	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
tZROG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
tZ7G	20.2 ± 2.07 a	4.9 ± 0.2 b	1.6 ± 0.05 c	<LOD	<LOD	<LOD
tZ9G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
tZR5′MP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
cZ	20.8 ± 2.5 a	11.09 ± 1.4 b	<LOD	<LOD	<LOD	<LOD
cZOG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
cZR	2059.5 ± 96.3 a	1296.1 ± 9.8 b	743.5 ± 56.5 c	893.7 ± 77.0 d	1362.8 ±129.3 b	270.5 ± 21.8 e
cZROG	26.1 ± 1.7 a	11.3 ± 0.5 b	<LOD	3.85 ± 0.2 d	<LOD	4.1 ± 0.1 c
cZ7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
cZ9G	<LOD	<LOD	<LOD	8.6 ± 0.2 a	<LOD	6.1 ± 0.4 b
cZR5′MP	<LOD	74.6 ± 5.3 a	<LOD	40.7 ± 3.6 c	<LOD	66.1 ± 3.9 b
DHZ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
DHZR	5.7 ± 0.8 a	3.8 ± 0.3 b	3.4 ± 0.06 b	1.5 ± 0.2 c	<LOD	<LOD
DHZROG	164.1 ± 4.8 b	<LOD	296.6 ± 20.1 a	<LOD	34.9 ± 1.1 c	<LOD
DHZ7G	<LOD	3.3 ± 0.07 b	<LOD	2.4 ± 0.2 c	<LOD	3.8 ± 0.1 a
DHZ9G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
DHZR5′MP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
iP	8.6 ± 0.1 a	5.2 ± 0.1 b	1.8 ± 0.1 d	3.1 ± 0.2 c	3.1 ± 0.3 c	2.5 ± 0.2 c
iPR	272.9 ± 4.7 a	125.8 ± 5.3 b	53.2 ± 1.1 d	22.1 ± 0.4 f	87.07 ± 3.8 c	23.8 ±1.2 e
iP7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
iP9G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
iPR5′MP	<LOD	21.5 ± 2.03 a	<LOD	14.4 ± 1.3 b	<LOD	20.01 ± 0.8 a
BA	1398.3 ± 151.1 b	4159.68 ± 497.2 a	9.03 ± 0.6 d	15.4 ± 3.1 c	<LOD	<LOD
BAR	84.04 ± 2.9 a	61.4 ± 1.3 b	0.5 ± 0.09 e	<LOD	1.1 ± 0.08 d	3.1 ± 0.3 c
BA7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
BA9G	106.5 ± 3.4 a	37.3 ± 1.3 b	<LOD	<LOD	<LOD	<LOD
BAR5′MP	<LOD	19.1 ± 0.4	<LOD	<LOD	<LOD	<LOD
mT	30.8 ± 1.9 a	16.1 ± 0.3 b	<LOD	9.1 ± 1.0 d	<LOD	19.4 ± 1.3 c
mTR	67.2 ± 6.3 a	17.5 ± 0.9 e	27.1 ± 1.3 c	7.8 ± 0. 2 f	32.2 ± 2.0 b	26.6 ± 1.8 d
mT7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
mT9G	<LOD	<LOD	<LOD	16.8 ± 0,1 a	<LOD	15.1 ± 1.3 b
oT	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
oTR	17.1 ± 1.5 a	4.7 ± 0.4 b	<LOD	<LOD	<LOD	<LOD
oT7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
oT9G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
pT	147.6 ± 9.4 b	27.8 ± 2.3 c	140.7 ± 10.6 b	<LOD	196.5 ± 6.1 a	<LOD
pTR	1242.2 ± 27.8 b	251.9 ± 17.8 d	1142.1 ± 24.9 c	21.6 ± 0.8 f	1346.1 ± 74.4 a	25.8 ±1.3 e
pT7G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
pT9G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
K	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KR	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
K9G	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
FmT	99.2 ± 11.2 f	140.8 ± 11.4 e	1952.3 ± 220.9 c	7836.4 ± 713.3 a	326.1 ± 25.4 d	2350.7 ± 360.5 b
FmTR	844.4 ± 107.2 c	804.2 ± 88.6 d	627.4 ± 44.5 e	490.7 ± 41.03 f	4478.6 ± 150.7 b	12,156.5 ± 1712.8 a

Values are presented as mean ± SD; (n = 3) LOD = below detection limit. Means in the same row followed by different letters are significantly different ($p \leq 0.05$) according to the t-test.

Endogenous isoprenoid metabolites, specifically the reversible storage form dihydrozeatin-O-glucoside riboside (DHZROG) and the transport form isopentenyladenine riboside (iPR), exhibited higher concentrations in ventilated containers. BA treatment generally led to elevated concentrations of BA, BAR, and their derivatives, while FmT and FmTR treatments led to the accumulation of fluorinated CKs. Notably, less glucosylation of BA occurred in closed containers, with higher levels of free BA observed. Further, small amounts of FmT were transformed into BA in both ventilated and closed containers, unlike FmTR. pT and pTR were observed as metabolites of mF and mFR, implying that mFR might degrade into BA and BAR, and then further convert into pT and pTR.

To better understand how treatment and culture conditions influence Phalaenopsis development in vitro, we analyzed the absolute cytokinin concentrations across different treatments. The highest total cytokinin level was found in FmTR-treated plants grown in closed containers. This suggests that the added FmTR remained stable and constituted the largest portion of the total cytokinin pool across all treatments (Figure 1a). Interestingly, culture conditions not only affected the total cytokinin levels but also the ratio between isoprenoid CKs (iP and zeatins) and aromatic CKs (BA and topolins) [21] (Figure 1b). The concentrations of isoprenoid and aromatic CKs varied depending on the treatment. For example, the highest isoprenoid cytokinin concentration, making up 38.97% of the total cytokinin content, was found in BA-treated shoots grown in ventilated containers, while the lowest concentration, about 2.65% of total CKs, was observed in the FmTR-treated plants grown in closed containers.

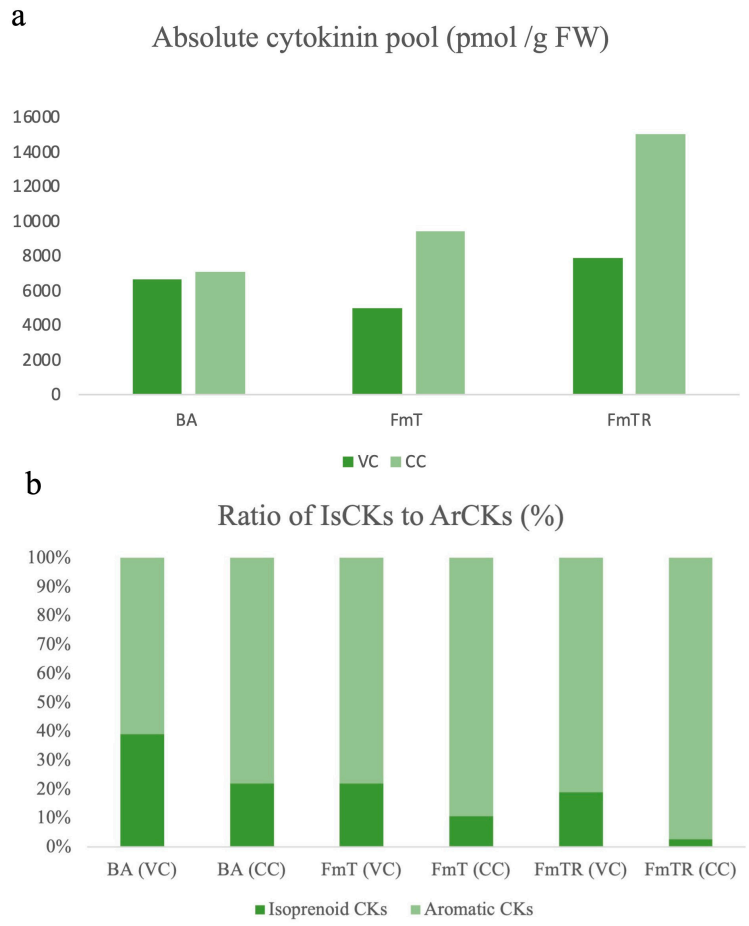

Figure 1. Cytokinin pool in *Phalaenopsis* axillary shoots under different treatments. Shoot grown on media supplemented with BA, FmT, and FmTR, with two types of culture vessels: closed container (CC) and ventilated container (VC). (**a**) Absolute cytokinin pool and (**b**) the ratio (%) of isoprenoid CKs (IsCKs) to aromatic CKs (ArCKs).

Figure 2 illustrates the distribution of CK metabolites in *Phalaenopsis* under different treatments and container types. In filter containers, ribosides constituted the majority of the total CK content across all treatments, consistent with their role as long-distance transport forms capable of interconversion into active forms [1,14]. Free bases represented the second largest proportion, with varying percentages depending on the applied cytokinin (BA: 26%; FmT: 42%; and FmTR: 7%). In closed containers, free bases predominated, except in the case of FmTR treatment.

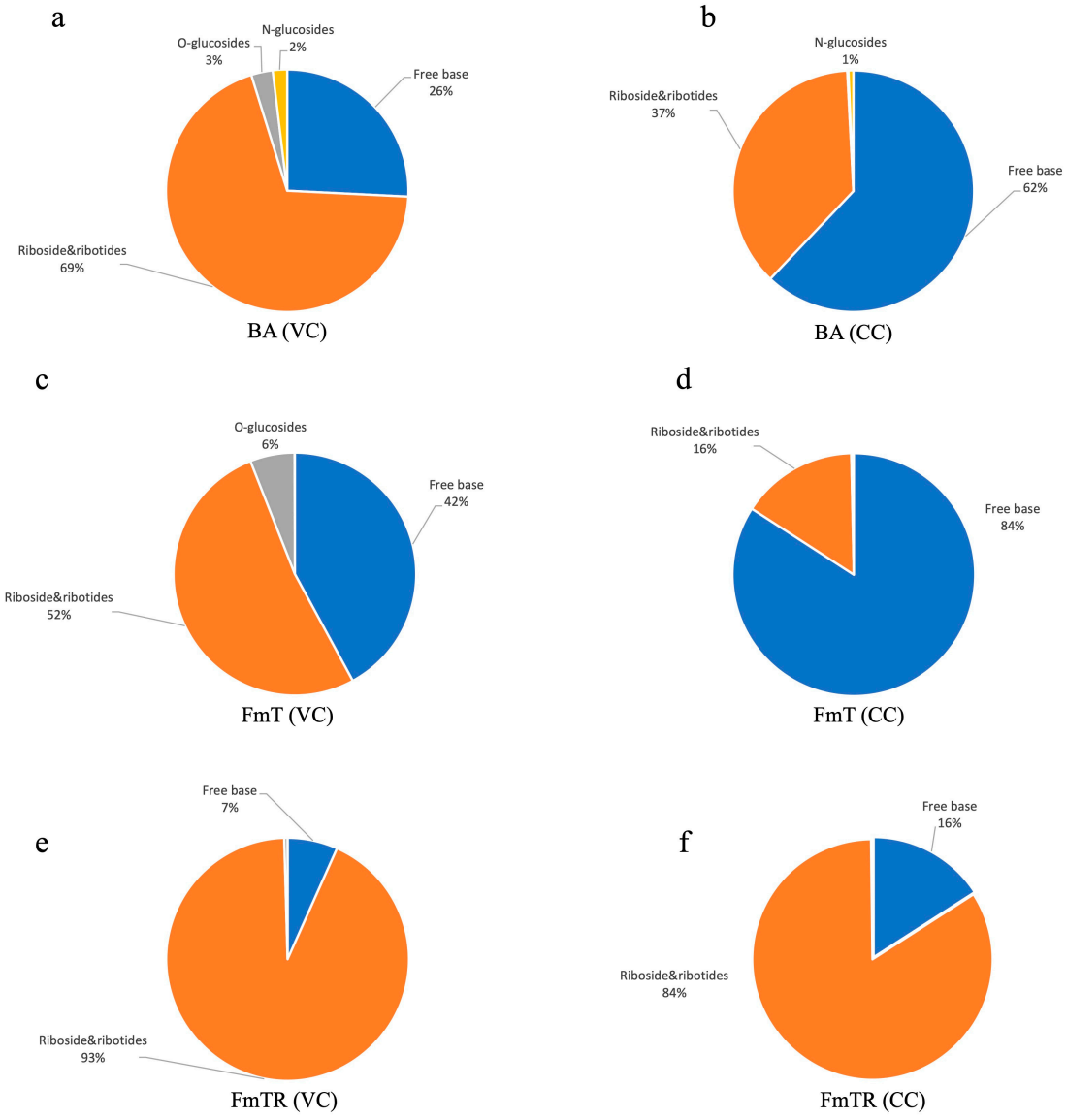

Figure 2. Distribution of cytokinin metabolites in Phalaenopsis shoots treated with BA, FmT, or FmTR and grown in either closed containers (CCs) or ventilated containers (VCs). The metabolites are grouped into free bases (cZ, IP, BAP, mT, oT, pT, and FmT), ribosides (FmTR, pTR, oTR, mTR, BAR, iPR, cZR, and DHZR), and O-glucoside ribosides and N-glucosides (DHZROG, tZ7G, DHZ7G, cZ9G BAP9G, and mT9G). Panels (**a**), (**c**), and (**e**) show the distribution in ventilated containers for BA, FmT, and FmTR treatments, respectively. Panels (**b**), (**d**), and (**f**) illustrate the distribution in closed containers for BA, FmT, and FmTR treatments, respectively.

O-glucosides, detectable only in FmT- and BA-treated plants, accounted for a small proportion of the total CK pool (3% with BA and 6% with FmT). These reversible storage forms can contribute to the active CK pool upon hydrolysis [22–24]. In closed containers, only negligible amounts of cZROG were found. N-glucosides were largely below the

detection limit, except in BA-treated plants where tZ7G and BAP9G were present. The predominance of BAP9G suggests that 9-glycosylation is a primary BA deactivation pathway in *Phalaenopsis*. Notably, FmT-treated plants exhibited extremely low levels of N-glucosides (Table 1).

4. Discussion

Our study reveals a complex interplay between exogenous cytokinin application, headspace aeration, and the resulting endogenous cytokinin profiles in *Phalaenopsis*. The differential accumulation of specific metabolites under varying treatments and container types underscores the dynamic nature of cytokinin metabolism in this orchid species. Notably, the absence of tZ7G in plants treated with FmT or FmTR, particularly in closed containers, suggests the potential inhibitory effect of these fluorinated CKs on the N7-glucosylation pathway. Furthermore, the unexpected abundance of cZR across all treatments, despite the low or undetectable levels of its precursor cZ, raises questions surrounding the biological significance of these compounds in *Phalaenopsis*. cis-Zeatin was previously considered biologically inactive, potentially as a byproduct of tRNA degradation; however, recent studies challenge this notion and suggest its involvement in various stages of plant development [25]. Notably, neither cZ nor its metabolites were detected in control leaves from a potted plant. Li et al. [26] described the dynamics of endogenous CKs in TDZ-treated *Phalaenopsis* plants, demonstrating that TDZ promotes the biosynthesis of CK-ribosides, particularly trans-zeatin riboside (tZR) and isopentenyladenosine riboside (iPR), with a small increase in the level of cis-zeatin riboside (cZR). Contrary to their findings, our study observed an increase in cZR levels under fluorinated cytokinin treatment. These findings indicate that not only can the metabolism of endogenous CKs be strongly influenced by exogenous CKs, but also that endogenous CK biosynthesis can vary between species of the same genus. While in Phalaenopsis aphrodite, TDZ promoted shoot proliferation via CK-ribosides, in *P. amabilis*, we did not observe an increase in tZR or iPR, and cZR was not detected in control *P. amabilis*.

Interestingly, isoprenoid metabolites DHZROG and iPR were found at higher concentrations in filter pots compared to closed containers, contrary to the observation that closed containers typically yield more shoots [11]. This finding suggests that the fluorinated CKs FmT and FmTR, as well as headspace aeration, may modulate isoprenoid cytokinin biosynthesis or metabolism. Additionally, the de-fluorination of FmT to BA was proposed, putatively observed in both ventilated and closed containers but not measured with FmTR, highlights the potential for metabolic interconversions between different cytokinin types and underscores the importance of considering the stability and metabolic fate of synthetic CKs in plant tissue culture.

The abundance of total cytokinin content in closed containers suggests that a hermetically sealed environment might prevent the degradation of certain CKs, potentially explaining the earlier reported increased shoot proliferation under closed containers [11]. However, the exact mechanism behind this action remains unclear. Interestingly, we also observed that within the same treatment group, a higher concentration of aromatic CKs was linked to an increased number of shoots, particularly in plants grown in closed containers. This finding suggests that aromatic CKs may have a positive impact on shoot development in *Phalaenopsis*. Our previous work [11] showed that in vitro *Phalaenopsis* treated with FmTR produced more shoots (average shoot per explant = 25.3) compared to FmT (average shoots per explant = 14.6) and BA (average shoots per explant = 7), with smaller leaves when treated with FmTR or FmT, compared to BA treatment. In addition, while BA and FmT treatment did not affect rooting, FmTR completely prevented root formation during micropropagation.

Notably, the hydroxylated metabolites of BA, such as mT, oT, and pT [27–29], were observed in our study, despite not being detected in greenhouse-grown *Phalaenopsis*. Since para-topolin and its derivatives are not very biologically active [29], this pathway could be an alternative inactivation method, as aromatic CKs are known to be less easily broken

down by CKX enzymes [30]. pT and pTR have also been found in *Handroanthus guayacan*, *Tabebuia rosea*, and *Pistacia vera* L. [17,31], though as metabolites of mTR. However, high-resolution mass spectrometry is needed to confirm the identity of these products. The decline of pT and pTR levels in closed containers may be linked to altered metabolic flux under these conditions. The presence of FmT and FmTR in BA-treated plants is not surprising, as their previous subculture involved FmTR, and its stability allows for carryover. These observations underscore the importance of understanding the dynamics of CK metabolism and the effects of exogenous CKs (such as TDZ, BA, FmT, or FmTR), which can help optimize micropropagation protocols. By manipulating CK levels through genetic or chemical means, it is possible to enhance shoot proliferation, thereby improving the efficiency of orchid propagation.

5. Conclusions

In conclusion, our investigation into the CK metabolism of *Phalaenopsis* under varying exogenous CK treatments and culture conditions has revealed a complex and dynamic system. The differential accumulation of specific metabolites highlights the intricate interplay between exogenous and endogenous CK levels, influenced by both the chemical structure of the applied CK and the culture environment.

The observed metabolic alterations, such as the unexpected abundance of cZR and the proposed de-fluorination of FmT, underscore the need for a deeper understanding of cytokinin metabolism in orchids. The identification of a potential novel metabolic pathway involving pT and pTR further emphasizes the complexity of this system.

These findings have significant implications for optimizing *Phalaenopsis* micropropagation protocols. By tailoring cytokinin treatments and culture conditions to specific metabolic profiles, we can potentially enhance shoot proliferation, improve plant quality, and overcome recalcitrance in orchid tissue culture. Moreover, our results contribute to a broader understanding of CK metabolism in plants and its role in regulating growth and development. Further research is warranted to elucidate the precise mechanisms underlying these metabolic processes and to explore their potential applications in plant biotechnology.

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