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Dissecting the role of two cytokinin analogues (INCYDE and PI-55) on *in vitro* organogenesis, phytohormone accumulation, phytochemical content and antioxidant activity

Adeyemi O. Aremu[a], Wendy A. Stirk[a], Nqobile A. Masondo[a], Lenka Plačková[b], Ondřej Novák[b], Aleš Pěnčík[b], Marek Zatloukal[b], Jaroslav Nisler[b], Lukáš Spíchal[b], Karel Doležal[b], Jeffrey F. Finnie[a], Johannes Van Staden[a,*]

[a] *Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg Campus, P/Bag X01, Scottsville 3209, South Africa*

[b] *Laboratory of Growth Regulators & Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University & Institute of Experimental Botany ASCR, Šlechtitelů 27, CZ-783 71 Olomouc, Czech Republic*

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ABSTRACT

There is a continuous search for new chemical entities to expand the collection of suitable compounds to increase the efficiency of micropropagation protocols. Two cytokinin (CK) analogues, 2-chloro-6-(3-methoxyphenyl)aminopurine (INCYDE) and CK antagonist 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55) were used as a tool to elucidate the auxin-CK crosstalk under *in vitro* conditions in the medicinally important plant, *Eucomis autumnalis* subspecies *autumnalis*. These compounds were tested at 0.01, 0.1 and 10 μM alone as well as in combination with benzyladenine (BA) and naphthaleneacetic acid (NAA). The organogenesis, phytohormone content, phytochemical and antioxidant response in 10 week-old-*in vitro* regenerated *E. autumnalis* subspecies *autumnalis* was evaluated. INCYDE generally favoured shoot regeneration while the effect of PI-55 was more evident in root proliferation. Overall, INCYDE promoted the accumulation of higher concentrations and varieties of endogenous CK relative to the PI-55 treatments. In contrast, higher concentration of indole-3-acetic acid and 2-oxindole-3-acetic acid were generally observed in PI-55-supplemented cultures when compared to plantlets derived from INCYDE. Both CK analogues (individually and in-conjunction with exogenously applied PGRs) significantly influenced the phytochemicals and consequently the antioxidant potential of the *in vitro* regenerants. These results provided insight on how to alleviate root inhibition, a problem which causes considerable loss of several elite species during micropropagation.

Abbreviations: BA, N^6-benzyladenine; BAR, N^6-benzyladenine-9-riboside; BA9G, N^6-benzyladenine-9-glucoside; BAR5′MP, N^6-benzyladenine-9-riboside-5′-monophosphate; CCE, cyanidin chloride equivalents; CKX, cytokinin oxidase/dehydrogenase; CE, catechin equivalents; CK, cytokinin; *c*Z, *cis*-Zeatin; *c*Z9G, *cis*-Zeatin-9-glucoside; *c*ZOG, *cis*-Zeatin-*O*-glucoside; *c*ZR, *cis*-Zeatin-9-riboside; *c*ZR5′MP, *cis*-Zeatin-9-riboside-5′-monophosphate; *c*ZROG, *cis*-Zeatin-*O*-glucoside riboside; DHZ, dihydrozeatin; DHZ9G, dihydrozeatin-9-glucoside; DHZOG, dihydrozeatin-*O*-glucoside; DHZR, dihydrozeatin-9-riboside; DHZR5′MP, dihydrozeatin-9-riboside-5′-monophosphate; DHZROG, dihydrozeatin-*O*-glucoside riboside; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; DW, dry weight; GAE, gallic acid equivalents; HE, harpagoside equivalents; IAA, indole-3-acetic acid; IAAsp, indole-3-acetyl-L-aspartic acid; IAC, immunoaffinity chromatography; IAGlu, indole-3-acetyl-L-glutamic acid; INCYDE, 2-chloro-6-(3-methoxyphenyl)aminopurine (inhibitor of cytokinin oxidase/dehydrogenase); iP, N^6-isopentenyladenine; iP9G, N^6-isopentenyladenine-9-glucoside; iPR, N^6-isopentenyladenine-9-riboside; iPR5′MP, N^6-isopentenyladenine-9-riboside-5′-monophosphate; IPT, isopentenyltransferase; KIN, kinetin; KIN9G, kinetin-9-glucoside; KINR, kinetin riboside; KINR5′MP, kinetin riboside-5′-monophosphate; MRM, multiple reaction monitoring; MS, Murashige and Skoog medium; *m*T, *meta*-Topolin; *m*T9G, *meta*-Topolin-9-glucoside; *m*TOG, *meta*-Topolin-*O*-glucoside; *m*TR, *meta*-Topolin-9-riboside; *m*TR5′MP, *meta*-Topolin-5′-monophosphate; *m*TROG, *meta*-Topolin-*O*-glucoside riboside; NAA, α-naphthaleneacetic acid; *o*T, *ortho*-Topolin; *o*T9G, *ortho*-Topolin-9-glucoside; *o*TOG, *ortho*-Topolin-*O*-glucoside; *o*TR, *ortho*-Topolin-9-riboside; *o*TR5′MP, *ortho*-Topolin-9-riboside-5′-monophosphate; *o*TROG, *ortho*-Topolin *O*-glucoside riboside; OxIAA, 2-oxindole-3-acetic acid; PGR, plant growth regulator; PI-55, 6-(2-hydroxy-3-methylbenzylamino)purine; PPFD, photosynthetic photon flux density; *p*T, *para*-Topolin; PTC, plant tissue culture; *p*TOG, *para*-Topolin-*O*-glucoside; *p*TR, *para*-Topolin riboside; *p*TR5′MP, *para*-Topolin-5′-monophosphate; *p*TROG, *para*-Topolin-*O*-glucoside riboside; *t*Z, *trans*-Zeatin; *t*Z9G, *trans*-Zeatin-9-glucoside; *t*ZOG, *trans*-Zeatin-*O*-glucoside; *t*ZR, *trans*-Zeatin riboside; *t*ZR5′MP, *trans*-Zeatin riboside-5′-monophosphate; *t*ZROG, *trans*-Zeatin-*O*-glucoside riboside; UHPLC, ultra-high performance liquid chromatography.

* Corresponding author. Tel.: +27 33 260 5130.
E-mail address: rcpgd@ukzn.ac.za (J. Van Staden).

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1. Introduction

Globally, productivity enhancement remains a research priority for meeting the surging demands for plants and their associated products by an increasing population. To attain this goal, plant growth regulators (PGRs) are vital tools commonly used in various agriculture processes [1–3]. Generally, PGRs including naturally-occurring phytohormones, synthetic compounds, analogues and related compounds modify plant growth and developmental patterns as well as exerting a profound influence on many physiological processes [4–7]. Auxins and cytokinins (CK) are regarded as the most important PGRs with a strong capacity to affect critical aspects of plant growth. Based on pioneering work by Skoog and Miller [8], a high auxin/CK ratio induces root regeneration, whereas a low ratio favours shoot induction. A considerable body of knowledge exists for both auxins and CKs including the molecular basis for their influence on developmental processes [9–12], although the mechanisms involved in auxin-CK crosstalk are not completely elucidated [13–15].

In vitro organogenesis and general plant regeneration are crucial processes in plant biotechnology [16,17]. Although auxins and CKs influence this process, the underlying molecular mechanisms regulating their functions are not fully understood [9]. The majority of the current knowledge is related to *Arabidopsis* and is largely based on responses in *Arabidopsis* mutants. However, the application of such findings on other plant species with more complex genomes and physiology may produce diverse results [18]. Thus, experimentation on plants with considerable economic value remains essential.

An approach to improve plant growth and development is via the regulation of metabolic pathways of PGRs. CK homeostasis and signalling components have emerged as engineering targets for manipulating plant growth and development [1,12,19,20]. For instance, modulating the CK status with inhibitors of CK perception and/or degradation may influence the general physiology of the plant. Based on this concept, Spíchal, Werner, Popa, Riefler, Schmülling and Strnad [21] identified the first known molecule that antagonizes the activity of CKs at the receptor level, thus reducing the physiological responses of CKs. The compound, 6-(2-hydroxy-3-methylbenzylamino)purine was designated PI-55. Structurally, PI-55 is closely related to benzyladenine (BA), with additions at *meta* (CH_3) and *ortho* (OH) positions of the aromatic side chain which strongly diminished its CK activity and accounts for the antagonistic property [21]. The compound accelerated early seedling development after the germination of *Arabidopsis* seeds and stimulated root growth. PI-55 also helped improve rooting of two medicinal plant subjected to heavy metal stress [22].

While isopentenyltransferase (IPT) is an important enzyme implicated in CK biosynthesis, CK oxidase/dehydrogenase (CKX) accounts for most of the CK catabolism and inactivation (mainly isoprenoid type) in a single enzymatic step [20,23]. Thus, the inhibition of CKX offers a target to modulate CK levels in plants, resulting in higher endogenous CK levels. Among the compounds which have been identified as potent inhibitors of CKX, is 2-chloro-6-(3-methoxyphenyl)aminopurine, designated INCYDE and it is highly effective in the inhibition of *Arabidopsis* CKX [24]. Recently, evidence on the potential of INCYDE on different aspects of plant growth and development were reported [22,25–27].

Even though the application of both PI-55 and INCYDE is steadily gaining interest, their use in micropropagation protocols especially for valuable and highly utilized plant species are not fully explored [26]. The benefits associated with *in vitro* propagation cannot be overemphasized and the appropriate choice (type and concentration) of PGRs determine the success of such endeavours [5,17]. Application of exogenous PGRs especially auxins and CKs, are commonly used to enhance shoot and root proliferation during micropropagation [5,9]. These exogenously applied PGRs often modify the metabolic pathways and the sensitivity of endogenous PGRs which results in highly variable and sometimes unpredictable outcomes [5,28–30]. At any particular developmental stage, the quantity and quality of endogenous PGRs is critical and determines the resultant regeneration of the initiated explants. As exemplified in several *in vitro* propagation protocols, the potential crosstalk between auxin and CK determine the organogenetic responses. From a physiological perspective, *in vitro* organogenesis provides a powerful system to study the mechanisms of hormonal crosstalk during plant organogenesis [14]. Thus, exogenous application of CK analogues that directly affect endogenous CK levels and through crosstalk, may affect auxin levels can potentially have an indirect effect on the phytochemical composition of the plant. This is particularly important in *in vitro* grown medicinal plants because their acceptability is often a function of the quantity and quality of accumulated phytochemicals which inevitably determines the bioactivities [31].

In the current study, *Eucomis autumnalis* subspecies *autumnalis* (Asparagaceae), a bulbous species that is widely used in traditional medicine and possesses ornamental value was used as a model plant. The effect of two CK analogues (INCYDE and PI-55) applied alone or in combination with the commonly-used PGRs for organogenesis of the model species during *in vitro* propagation was evaluated. As crosstalk between auxins and CKs during developmental processes of plants is well-described, the endogenous CK and auxin content was quantified in order to provide vital clues on the resultant organogenesis of the *in vitro* regenerants. Due to the medicinal value of *E. autumnalis* subspecies *autumnalis*, the phytochemical content and antioxidant activity in the *in vitro* regenerants were also quantified.

2. Materials and methods

2.1. Source of chemicals

Both PI-55 and INCYDE were prepared as described by Spíchal, Werner, Popa, Riefler, Schmülling and Strnad [21] and Zatloukal, Gemrotová, Dolezal, Havlícek, Spíchal and Strnad [24], respectively. BA and α-naphthaleneacetic acid (NAA) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Explant source and in vitro organogenesis experimental design

Aseptically-obtained leaves of *E. autumnalis* subspecies *autumnalis* derived from primary bulb regenerants sub-cultured on PGR-free media were used for all experiments [32]. Three leaf explants, each measuring approximately 1 cm × 1 cm were inoculated in screw-cap jars (110 × 60 mm, 300 ml volume) containing 30 ml Murashige and Skoog (MS) medium [33]. For the different treatments, the media were supplemented with varying concentrations (0, 0.01, 0.1 or 10 μM) of either the INCYDE or PI-55, combined with BA (5 μM), NAA (4 μM) or combination of BA and NAA. Controls consisted of MS only (PGR-free), BA, NAA and BA + NAA. The choice of the BA and NAA concentration was based on a previous study [34]. The MS media were supplemented with PI-55, INCYDE and PGRs, adjusted to pH of 5.8 and solidified with gellan gum (3 g/l) prior to autoclaving at 121 °C for 20 min. Each treatment had 15 explants and the experiments were conducted twice. The cultures were incubated in 16/8 h light/dark conditions with a photosynthetic photon flux density (PPFD) of 45 μmol m^{-2} s^{-1} at 25 ± 2 °C for 10 weeks. Thereafter, parameters including shoot (number and length) and root (number and length) growth were recorded.