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Changes in endogenous cytokinins during germination and seedling establishment of *Tagetes minuta* L.

Wendy A. Stirk[1,*], John D. Gold[1], Ondřei Novák[2], Miroslav Strnad[2] and Johannes van Staden[1]
[1]*Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa;* [2]*Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany ASCR, Šlechtitelů 11, 783 71 Olomouc, Czech Republic; *Author for correspondence (e-mail: stirk@ukzn.ac.za; phone: +27-33-260-5135; fax: +27-33-260-5897)*

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Abstract

Endogenous cytokinins were quantified and identified in germinating achenes and developing seedlings of *Tagetes minuta* L. incubated at 25 °C over a 144 h period. The process of germination (radicle emergence) was completed 38 h after commencement of imbibition. Subsequent growth was considered to cover seedling establishment. Eighteen isoprenoid cytokinins, belonging to the zeatin (9), dihyrozeatin (5) and isopentenyladenine (4) groups and one aromatic cytokinin, benzyladenine, were identified. The total isoprenoid cytokinin concentration increased upon imbibition, reached a peak by 48 h and subsequently decreased with seedling development. The individual cytokinin groups and the respective derivatives within each group did, however, not follow such a consistent trend. During the course of the experiment, the ribotides and ribosides were present in the highest concentrations, reaching a peak at 48 h and decreasing thereafter. The free bases and O-glucoside remained at low levels throughout the experiment. Isopentenyladenine-9-glucoside increased dramatically in the developing seedlings and after 144 h was the predominate cytokinin. Benzyladenine was the only aromatic cytokinin detected throughout the experiment. It was present in high concentrations in the dry achenes and declined rapidly upon imbibition.

Abbreviations: ABA – abscisic acid; BA – benzyladenine; *c – cis*; CBP – cytokinin binding protein; DHZ – dihyrozeatin; DHZOG – dihydrozeatin-O-glucoside; DHZ9G – dihydrozeatin-9-glucoside; DHZR – dihydrozeatin riboside; DHZR5MP – dihydrozeatin riboside-5′-monophosphate; DHZROG – dihydrozeatin riboside-O-glucoside; HPLC–MS – high performance liquid chromatography–mass spectrometry; iP – isopentenyladenine; iP9G – isopentenyladenine-9-glucoside; iPR – isopentenyladenosine; iPR5MP – isopentenyladenosine-5′-monophosphate; *t – trans*; Z – zeatin; ZOG – zeatin-O-glucoside; Z9G – zeatin-9-glucoside; ZR – zeatin riboside; ZRED – zeatin reductase; ZR5MP – zeatin riboside-5′-monophosphate; ZROG – zeatin riboside-O-glucoside

Introduction

Germination is regarded as 'events which commence with the uptake of water by a quiescent dry seed and terminates with the elongation of the embryonic axis' (Bewley 1997). The visual cue for the completion of germination and the beginning of seedling establishment is the appearance of the radicle. Germination is considered as a triphasic event comprising an initial period of water uptake, followed by a lag phase during which all the metabolic events leading up to radicle emergence occur and ending with the elongation of the embryonic axis and radicle emergence (Bewley and Black 1982). Imbibition is associated with the activation of enzymes present in the dry seed, the resumption of metabolic activity such as respiration, nutrient mobilization and *de novo* synthesis of newly required enzymes (Bewley 1997).

Plant hormones are essential in all physiological and developmental processes occurring during plant growth. Cytokinins have been isolated from seeds of a number of species (Thomas 1980) and the embryonic axis has been suggested as the major site of cytokinin synthesis in germinating seeds (Villalobos and Martin 1992). Removal of the embryonic axis can be countered by exogenous cytokinin substitution (Letham 1978). There are many reports of cytokinins playing a role in nutrient mobilization during germination in dicotyledonous plants. In chickpea, cytokinins were first detected in the embryonic axis and then moved into the cotyledons where they promoted mobilization of storage reserves. Different cytokinins were able to exert a specific action on reserve metabolism. ZR was most effective in the mobilization of carbohydrates, Z regulated protein and to a lesser extent, carbohydrates while iP was active in lipid mobilization. The O-glucosides were considered storage forms (Villalobos and Martin 1992).

Although cytokinins are generally considered to be involved in nutrient mobilization, it has yet to be established if they have more specific functions during the germination process which also embodies considerable nutrient mobilization. They are generally considered to have a permissive role in germination as applied cytokinins can alleviate the effect of germination inhibitors such as ABA and methyl jasmonate (Singh and Sawhney 1992; Bialecka and Kepczński 2003). There is no conclusive evidence or indication that cytokinins are directly involved in the germination process although cytokinins do regulate cell division (see review by del Pozo et al. 2005) and enlargement. There are many reports of cytokinins being directly involved in post-germination events such as root and hypocotyl growth and chlorophyll synthesis (Singh and Sawhney 1992). It is essential to have a detailed and accurate assessment of the concentration of the various endogenous cytokinins and their fluctuations during germination before a more precise role for them, if any, can be established using a molecular approach. The various cytokinins are known to display different biological activities, conversion rates, solubilities and transport across membranes (Kamínek 1992). Generally, isoprenoid cytokinins are thought to have a greater influence on cell cycling with their primary role being to promote mitotic events during cell division (Zhang et al. 1996). Cytokinins are involved in the regulation of the DNA synthesis (S-phase) and mitosis (M-phase) in the cell cycle (del Pozo et al. 2005). Aromatic cytokinins display higher activity with respect to growth and developmental processes (Holub et al. 1998).

The aim of this study was to investigate the changes in the cytokinin profile during germination and early seedling development of *Tagetes minuta* achenes using a method which allowed for detailed identification and quantification of all known endogenous cytokinin forms. The hope was that the results should provide some insight into the possible role of the various cytokinins during germination and seedling establishment. At present we have no conclusive proof for the direct involvement of endogenous cytokinins in the germination process.

Materials and methods

Tagetes minuta L. achenes were collected in June 1998 from a single population in Pietermaritzburg and air dried for 6 weeks. Four samples (2 g achenes) were placed in Petri dishes (55 mm) lined with two pieces of Whatman No. 1 filter paper and imbibed with 5 ml distilled water. The samples were incubated for 24, 48, 96 and 144 h, respectively at 25 °C in continuous low light (15 μmol m^{-2} s^{-1}). Dry achenes (2 g) were used as a control. After treatment, the samples were

immediately frozen in liquid nitrogen and ground to fine powders. Samples were extracted in 70% ice-cold ethanol (Novák et al. 2003). A cocktail of deuterium labeled cytokinin standards were added and the extracts purified by combined DEAE-Sephadex and octadecylsilica column and immunoaffinity chromatography. Cytokinins were identified and quantified by HPLC–MS (Novák et al. 2003).

Results

A germination curve where radicle emergence was recorded every 2 h over a 48 h period was generated using *T. minuta* achenes (five replicates of 25 achenes each) incubated at 25 °C (Figure 1f). Imbibition was rapid with a 50% increase in mass being obtained after 6 h. First radicle emergence was noted at 14 h although the majority of the achenes germinated between 20–30 h (75% germination) with 96% overall germination attained by 38 h (Figure 1f). Earlier reports indicated that metabolic processes in *T. minuta* achenes were initiated rapidly upon imbibition eg. change in the polypeptide profiles was observed after 12 h imbibition (Hills et al. 2001). High concentrations of ABA in dry achenes decreased sharply upon imbibition with the onset of germination (Taylor et al. 2005). Thus, in the present study, the sample collected after 24 h imbibition was considered to be in the process of germination while the samples collected at 48 h and after, represented early stages of seedling development.

Eighteen isoprenoid cytokinin derivatives (9 Z, 5 DHZ and 4 iP) were detected in *T. minuta* achenes during the course of the experiment. The only aromatic cytokinin detected was BA which occurred in high concentrations in the dry achenes, contributing 77% to the total cytokinin concentration (Figure 1e). The BA concentration rapidly decreased upon imbibition and very low concentrations were detected for the remainder of the experiment (Figure 1e). No other BA derivatives and no topolins were detected throughout the experiment.

The total isoprenoid cytokinin concentration was low in the dry controls, contributing only 23% to the total cytokinin pool of which 12% comprised of DHZ-type cytokinins (Figure 1c). The isoprenoid cytokinin content increased upon imbibition with the highest concentration being detected at 48 h during early seedling establishment, contributing 98% to the total cytokinin pool. Although the isoprenoid cytokinins subsequently remained the predomiant cytokinins present, contributing over 98% to the total cytokinin pool, there was a gradual decrease in the total cytokinin concentration after 48 h (Figure 1a–d). The main cytokinin groups detected after imbibition were *c*Z-types which made up 80% of the total cytokinin pool after 48 h imbibition and iP-types which made up 80% of the total cytokinin pool after 144 h imbibiton (Figure 1b and d). DHZ-types were detected throughout the experiment but they never contributed more than 23% to the total cytokinin pool while *t*Z-types never contributed more than 2% to the total cytokinin pool (Figure 1a and c).

Upon imbibition and hence with the commencement of nutrient mobilization, the ribotides rapidly increased in concentration and after 24 h of imbibition, contributed 51% to the total cytokinin pool. The ribotide levels continued to increase, reaching a peak after 48 h imbibition (74% of the total cytokinin pool). This was mainly due to an increase in *c*ZR5MP (Figure 1b), DHZR5MP (Figure 1c) and iPR5MP (Figure 1d). After 48 h, the ribotide concentrations slowly decreased until at 144 h they only made up 6% of the total cytokinin pool (Table 1). Riboside concentrations increased rapidly after radicle emergence, contributing 15% to the total cytokinin pool after 48 h imbibition whereafter concentrations decreased (Table 1). This was due to an increase in *c*ZR (Figure 1b) and iPR (Figure 1d) concentrations. The 9-glucosides increased steadily upon incubation and after 144 h, they made up 88% of the total cytokinin pool (Table 1). This was due to a large increase in iP9G (Figure 1d) and a slight increase in DHZ9G (Figure 1c). O-glucosides and riboside-O-glucosides remained low throughout the experiment, only being detected in the dry achenes and after 24 h imbibition. These concentrations were mainly due to the presence of DHZROG (Table 1, Figure 1c). Concentrations of free bases remained low throughout the entire experiment although there was a slight increase after 48 h imbibition (10% of the total cytokinin pool) due to a slight increase in cZ (Figure 1b).

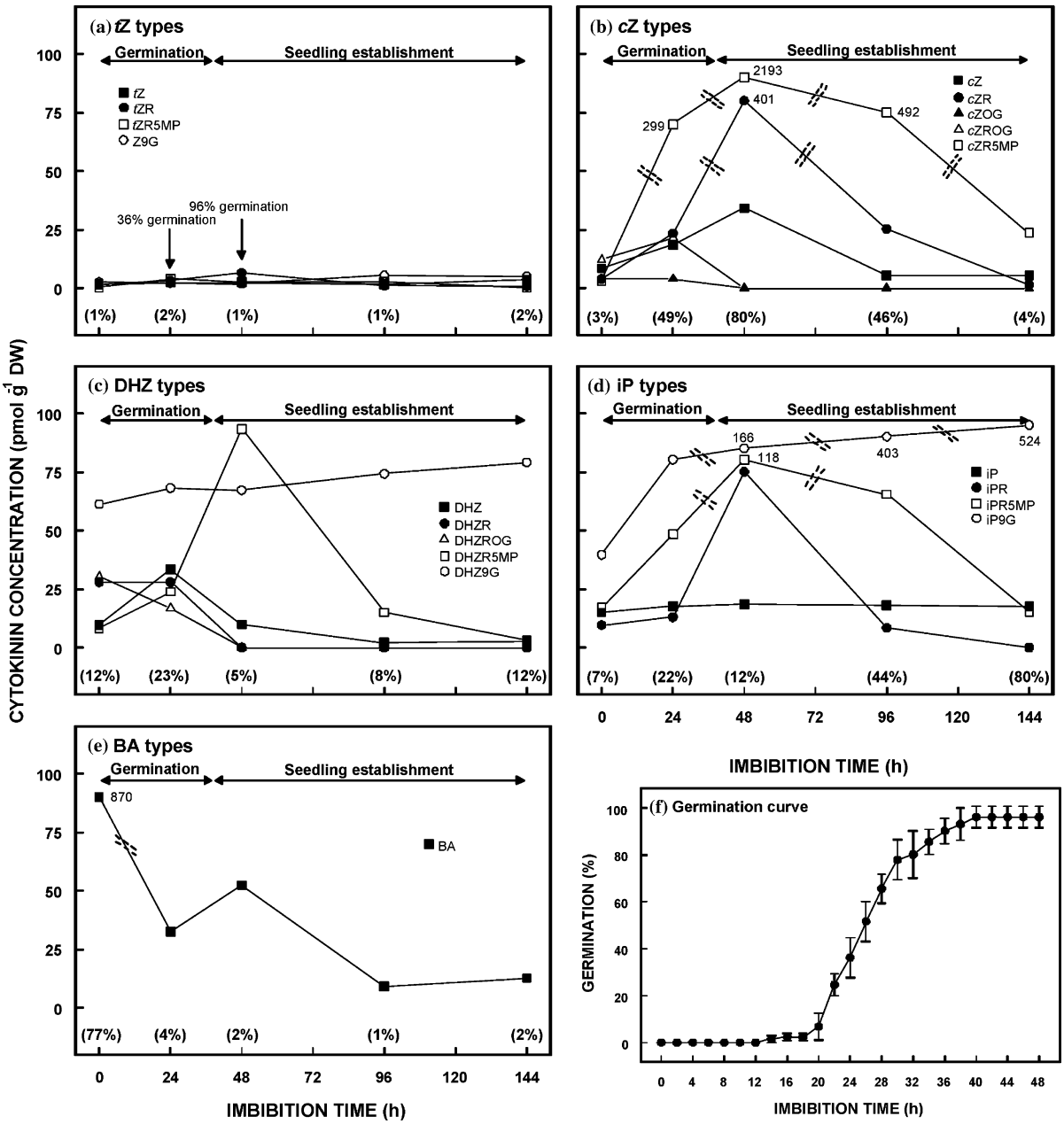

Figure 1. Changes in concentrations of (a) *t*Z, (b) *c*Z, (c) DHZ, (d) iP and (e) BA cytokinin types detected in *T. minuta* achenes germinated at 25 °C. Figures in brackets represent the percentage of the cytokinin group to the overall cytokinin pool; (f) germination recorded as radicle emergence for *T. minuta*.

Discussion

The different trends in cytokinin concentration changes for the various cytokinin groups during germination and seedling development of *T. minuta* supports the view that particular cytokinins have specific roles in the various physiological processes in plants. Cytokinin conjugates are classed as (1) active forms that evoke a growth response, (2) translocation forms, (3) storage forms, and (4) detoxification products that are formed when endogenous cytokinin concentrations

Table 1. Contribution of cytokinin type to the total cytokinin concentration (pmol g^{-1} DW) in *T. minuta* achenes during germination and subsequent seedling establishment.

Cytokinin type	Incubation time (h)				
	0	24	48	96	144
Isoprenoid cytokinins					
Free bases	35 (3)	72 (10)	65 (2)	27 (2)	29 (4)
Ribosides	43 (4)	67 (9)	485 (15)	35 (3)	2 (0)
O- and R-O-glucosides	46 (4)	42 (6)	0 (0)	0 (0)	0 (0)
Ribotides	29 (3)	375 (51)	2406 (74)	576 (51)	42 (6)
9-Glucosides	103 (9)	151 (20)	235 (7)	482 (43)	608 (88)
Aromatic cytokinins					
Free bases	870 (77)	32 (4)	52 (2)	9 (1)	13 (2)

Figures in brackets represent the percentage of the overall cytokinin pool.

become toxic (Letham and Palni 1983; Mok and Mok 2001). The free bases are widely considered to be the active forms with conjugation leading to reduced activity. O-glucosides have relatively high biological activity as they are reversibly sequestrated to provide a source of free bases (Letham and Palni 1983; Mok and Mok 2001). The levels of O-glucosides are known to rapidly decrease during developmental processes requiring cytokinins (van Staden and Dimalla 1978). Ribotides are thought to play a central role in the regulation of cytokinin levels as they are readily converted to both the less active ribosides and highly active free base forms (Laloue and Pethe 1982; Palmer et al. 1984). The role that has been proposed for ribotides is storage (Pietraface and Blaydes 1981). Interconversion pathways in an active cytokinin pool are thought to favour ribotide formation, resulting in high ribotide concentrations (Laloue et al. 1981).

As BA was detected at a high concentration in dry *T. minuta* achenes and rapidly decreased upon imbibition to very low levels (Figure 1e), it appears that BA played an important role in the initiation of germination in *T. minuta*. The results suggest that it was actively used as there was no sign of interconversion to other BA conjugates. CBPs have been shown to rapidly accumulate in developing wheat embryos (Fox and Erion 1975; Brinegar et al. 1988). As they have a relatively low binding of isoprenoid cytokinins compared to aromatic ones (Keim et al. 1981), it has been suggested that wheat CBF-1 does not function as a cytokinin receptor in the usual sense of the term. It was hypothesized that CBF-1 may serve as a sequestering protein regulating the access of aromatic cytokinins to the embryo during maturation and germination. These CBPs may function either in signaling of initiation of germination or as proposed by Kamínek et al. (2000) in temporal immobilization of cytokinins bearing an aromatic cytokinin side chain during grain development, thus preventing premature embryo cell division. The identification of BA as a naturally occurring cytokinin in dry *T. minuta* achenes suggests that BA may play a similar role in preventing premature germination.

Although most germination studies have focused on the regulatory role of isoprenoid cytokinins in the cell cycle (del Pozo et al. 2005), exogenous BA does also accelerate DNA synthesis by shortening the starting time of the S-phase of the mitotic cell cycle after imbibition in maize seed axes. Enhanced DNA polymerase activity and protein kinase activity has been recorded (Vasquez-Ramos and Jimenez 1990). It was suggested that BA accelerated the transport of proteins into the nuclei and thus shortened the time to the completion of the first cell division cycle (Herrera-Teigeiro et al. 1999). The present results suggest that serious consideration should be given to the role of aromatic cytokinins in germination of dicotyledonous seeds and its effect on DNA turnover which is essential in triggering and controlling the onset of cell division necessary for the germination process. The presence and role of endogenous BA has been overlooked as it was originally thought to be a synthetic cytokinin. Application of various forms of exogenous cytokinins, including BA, decreased the final germination percentage of *T. minuta* (Taylor et al. 2005), indicating that inhibitory concentrations

were achieved with exogenous application. One can therefore hypothesize that lower levels of endogenous cytokinins are needed for germination and radicle extension. High cytokinin levels are known to inhibit root growth (Hinchee and Rost 1986).

Moreover, some physiological processes (e.g. germination, seedling growth) were thought to be controlled predominantly by cytokinins of the DHZ-type (Letham 1994). Here we show that seedling growth is accompanied by strong accumulation of *c*ZR5MP, cZR and cZ which are present at approximately 20 times higher concentrations than corresponding DHZ and iP cytokinins. The origin and function of these compounds is unclear and warrants further investigation.

In *T. minuta*, the ribotides are the first cytokinins to increase dramatically in concentration after imbibition. At the onset of seedling development (48 h), there was a sharp increase in ribotide concentrations (*c*ZR5MP, DHZR5MP and iPR5MP), suggesting that *de novo* cytokinin biosynthesis may have resumed after radicle emergence. The ribotides are the intermediary products in *de novo* cytokinin biosynthetic pathways eg. *t*ZR5MP is formed in the pathway proposed by Astot et al. (2000) and iP5MP and other nucleotides in the pathway proposed by Kakimoto (2001).

The ribotide levels in *T. minuta* decreased (96–144 h) indicating that the ribotides were utilized and play an active role in seedling development. There are two possible explanations for the absence of the highly active free bases – they do not play a role in germination and seedling establishment of *T. minuta* or they were rapidly utilized and are under tight regulation so that they do not accumulate to high levels. Both O-glucoside and ribotides are readily converted to the free base forms (Palmer et al. 1984; Zazímalová et al. 1999) and are also less susceptible to degradation by cytokinin oxidase (Spíchal et al. 2004) and so may provide the source for the free base conversion in *T. minuta*. The results suggest that DZROG is present in low concentrations in the dry achenes and this is utilized during germination as seen by the decrease in its level upon imbibiton (Figure 1c).

Ribosylation is regarded as a reversible form of conjugation. Although the ribosides do display some biological activity, their primary role is thought to be transportation in the xylem (Saenz et al. 2003). Their increase in concentration 48 h (*c*ZR and iPR) after imbibition in *T. minuta* achenes may be due to the metabolism of the ribotides which are occurring at very high concentrations. Glycosylation of a free base at the 3-, 7- or 9-N position results in metabolically stable conjugates which have greatly reduced biological activity and cannot be converted back to free bases. Thus N-glycosylation is considered as an irreversible detoxification process used as a means of regulating free base levels (Strnad 1997; Holub et al. 1998). As expected, their levels increased in the *T. minuta* achenes over time with the most rapid increase occurring after 48 h once *de novo* cytokinin biosynthesis has probably begun and nutrient mobilization was well underway.

In conclusion, these results suggest that the aromatic cytokinin BA plays an active role in the germination of *T. minuta* achenes while the isoprenoid cytokinin forms and mainly *cis*-zeatins are involved in seedling development as their biosynthesis probably only commences after radicle emergence. The role of aromatic cytokinins in germination bears further investigation. This study should provide a useful baseline for the further investigation of the function of cytokinins in seed germination and seedling establishment.

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