

CHANGES OF ENDOGENOUS POLYAMINES IN EXCISED *CUCURBITA PEPO* L. (ZUCCHINI) COTYLEDONS CULTIVATED IN THE PRESENCE OF BENZYLADENINE AND METHYL JASMONATE

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Received: 23 October 2014 Accepted: 09 January 2015

Summary: In the present work, we studied the changes in the content of polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) in *Cucurbita pepo* L. (zucchini) cotyledons excised from dry seeds and incubated in darkness on solutions of N⁶-benzylaminopurine (BA) or methyl ester of jasmonic acid (MeJA) each applied at a concentration of 0.01mM. Putrescine content was altered most significantly, followed by less considerable amendments of Spd content. No changes of Spm amount in MeJA-treated cotyledons were detected. BA provoked an increase in free polyamines which was accompanied by the ‘disappearance’ of the conjugated forms as well as abundance of bound polyamines. The changes of endogenous polyamines are discussed in terms of the specific action of the tested phytohormones on cell proliferation and confirm the important role of polyamines in cell division processes in excised zucchini cotyledons.

Citation: Todorova D., S. Kamenova, K. Ananieva, 2015. Changes of endogenous polyamines in excised *Cucurbita pepo* L. (zucchini) cotyledons cultivated in the presence of benzyladenine and methyl jasmonate. *Genetics and Plant Physiology*, 5(1): 39–47.

Keywords: Cell division, cotyledons, cytokinin, methyl jasmonate, polyamines.

Abbreviations: BA – N⁶-benzylaminopurine; MeJA – methyl jasmonate; PA – polyamine; Put – putrescine; Spd – spermidine; Spm – spermine, TCA – trichloroacetic acid, TLC – thin layer chromatography.

INTRODUCTION

Polyamines (PAs) are aliphatic low-molecular compounds, usually distributed in both higher and lower plants, which occur as free molecules, but are able to conjugate with other small molecules like phenolic acids or bind to macromolecules like proteins and nucleic acids (Groppa and

Benavides, 2008). Free PAs are considered as biologically active substances, while the conjugated forms have a storage function. However, there is evidence that conjugated and bound PAs also participate actively in major plant cell processes (Bouchereau et al., 1999). PAs are involved in a variety of

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developmental and physiological processes such as cell proliferation and cell death (particularly apoptosis), seed germination, somatic embryogenesis, differentiation and morphogenesis, dormancy breaking of tubers, development and senescence of flowers, leaves and fruits (reviewed by Kusano et al., 2008; Wimalasekera et al., 2011). The PAs universal to all plant species are Put, Spd and Spm (Bouchereau et al., 1999).

Cytokinins (CKs) are phytohormones which play a significant role in plant growth and development, more specifically cell division, differentiation of the photosynthetic apparatus, leaf senescence (Mock, 1994). It was shown that exogenously applied CKs were responsible for the growth of *Cucurbita pepo* L. (zucchini) cotyledons excised from either 4-days-old seedlings (Ananieva and Ananiev, 2001) or from dry seeds (Stoynova-Bakalova et al., 2004, 2008; Stoynova-Bakalova and Petrov, 2006, 2009). Usually PAs content increases after CK application and these alterations depend on plant organs and species as well as CK concentrations applied as was previously reported (Cho 1983; Walker et al., 1988; Sergiev et al., 1995; Legocka and Żarnowska, 1999; Legocka and Żarnowska, 2000; Todorova and Alexieva, 2002; Todorova et al., 2002; Damyanova et al., 2014).

Jasmonates (jasmonic acid-JA, its methyl ester-MeJA and related compounds) are ubiquitously occurring lipid-derived compounds, which possess signaling functions in plant responses to both abiotic and biotic stresses as well as in plant growth and development (Wasternack, 2007). Additionally, there is evidence that MeJA may be involved in

the regulation of cell division in isolated zucchini cotyledons as an antagonist to CK action (Ananieva and Ananiev, 2000; Stoynova-Bakalova et al., 2008).

Relatively little information is available on PAs changes provoked by treatment of plants or excised cotyledons with JA or its methyl ester. Generally, jasmonates affect differently the content and metabolism of PAs in plant tissues depending on of the concentrations of JA or MeJA as well as on plant species, types of tissues, culture methods and other factors (Biondi et al., 2001; Biondi et al., 2003; Haggag and Abd-El-Kareem, 2009; Horbowicz et al., 2011, 2014).

The aim of this study was to examine the effects of exogenously applied BA or MeJA on PAs quantity in *Cucurbita pepo* L. (zucchini) cotyledons excised from dry seeds and grown in complete darkness.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *Cucurbita pepo* L. (zucchini), cv. Cocozelle var. Tripolis were stripped of their seed coats. Cotyledons excised from the embryonic axes of the seed were placed in covered Petri dishes on filter paper soaked with distilled water, supplemented with either MeJA (Sigma Chemical Co., St. Louis, MO, USA) or BA (N⁶-benzylaminopurine) (Serva, Heidelberg, Germany), each applied at a concentration of 0.01 mM. Cotyledons were cultivated in the dark at 25°C. The analyses were performed on 6-day-old cotyledons.

Analysis of PAs content

The direct dansylation method was used to determine free Put, Spd and Spm

levels (Smith and Best, 1977). Conjugated PAs were measured in acid-hydrolysed supernatants as TCA-soluble bound PAs, and bound PAs in acid-hydrolysed pellets as TCA-insoluble bound polyamines, according to the method of Torrigiani et al. (1989). The three fractions of PAs were separated using TLC precoated plates of Silicagel G 60 (Merck) in a cyclohexane:ethylacetate (3:2, v/v) solvent system. Spots visualized under UV light were scraped off and eluted in 2 ml anhydrous acetone. Fluorescence was measured (excitation 360 nm, emission 505.5 nm) and the results were compared with dansylated PA standards (Sigma) on the same plate.

Statistical analyses

All experiments were repeated three times with three to five replications. The results presented are means with standard error (\pm SE).

RESULTS

The comparative analysis of the content of Put, Spd and Spm in the control excised cotyledons showed that their free forms predominated over the conjugated and bound forms (Figs. 1-3).

MeJA provoked a substantial increase of endogenous free Put (by 59%) as compared to control (Fig. 1A). Incubation of cotyledons on BA alone also led to an increase of this fraction, but to a lesser extent (by 30%). However, MeJA slightly increased conjugated Put (by 17%), (Fig. 1B). Only traces of conjugated Put were detected after the application of BA. On the other hand, BA increased significantly the content of bound Put (by 54%) whereas in the MeJA-treated cotyledons its content

remained close to the control value (Fig. 1C).

The application of MeJA caused a slight decrease of endogenous free Spd (by 13%) as compared to control (Fig. 2A). On the contrary, BA increased significantly free Spd (by 41%). Similar to conjugated Put, there was no conjugated Spd detected

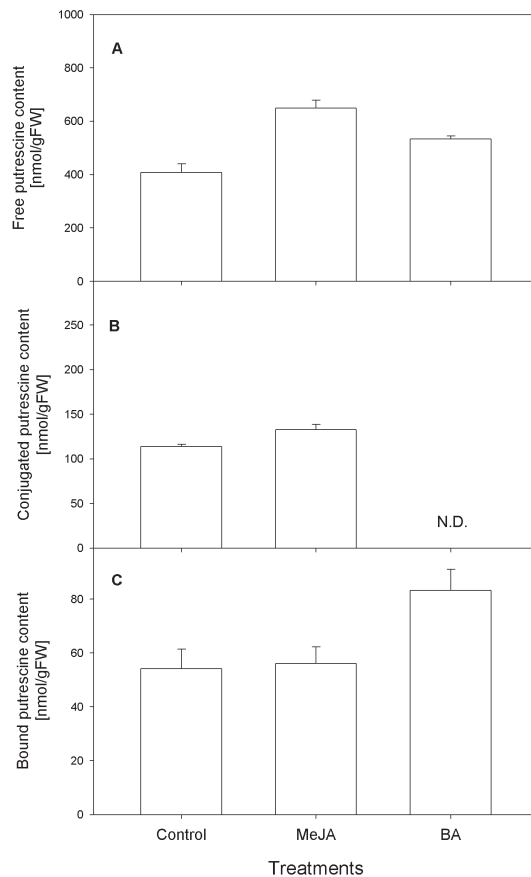


Figure 1. Content of free (A), conjugated (B) and bound (C) putrescine in zucchini cotyledons (mean values \pm SE). Cotyledons were excised from the embryonic axes of dry *Cucurbita pepo* L. (zucchini) seeds and placed in covered Petri dishes on filter paper soaked with distilled water supplemented with either MeJA or BA, each applied at a concentration of 0.01 mM. Cotyledons were cultivated in the dark at 25°C. The analyses were performed on 6-day-old cotyledons. N.D. – not detected.

after BA treatment (Fig. 2B). On the other hand, this fraction was augmented by 34% in cotyledons incubated on MeJA solution. Approximately 50% augmentation of bound Spd was detected after MeJA treatment (Fig. 2C). The application of BA yielded a comparable amount of bound Spd.

Similarly to free Spd, BA provoked

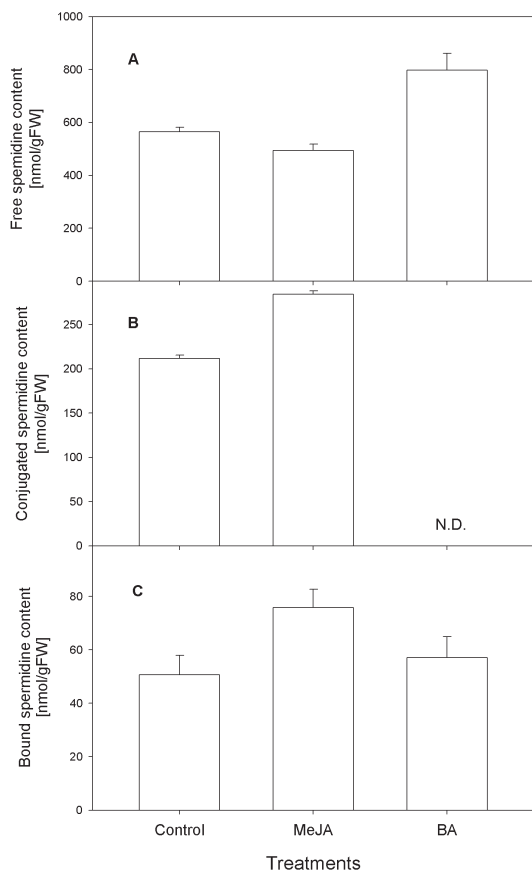


Figure 2. Content of free (A), conjugated (B) and bound (C) spermidine in zucchini cotyledons (mean values \pm SE). Cotyledons were excised from the embryonic axes of dry *Cucurbita pepo* L. (zucchini) seeds and placed in covered Petri dishes on filter paper soaked with distilled water supplemented with either MeJA or BA, each applied at a concentration of 0.01 mM. Cotyledons were cultivated in the dark at 25°C. The analyses were performed on 6-day-old cotyledons. N.D. – not detected.

a substantial increase of endogenous free Spm (by almost 40%) as compared to control (Fig. 3A). The alterations of this fraction were not significant in MeJA-incubated cotyledons, the amount remaining near to the control value. The application of MeJA alone did not change noticeably conjugated Spm (Fig. 3B). However, BA totally reduced TCA-soluble

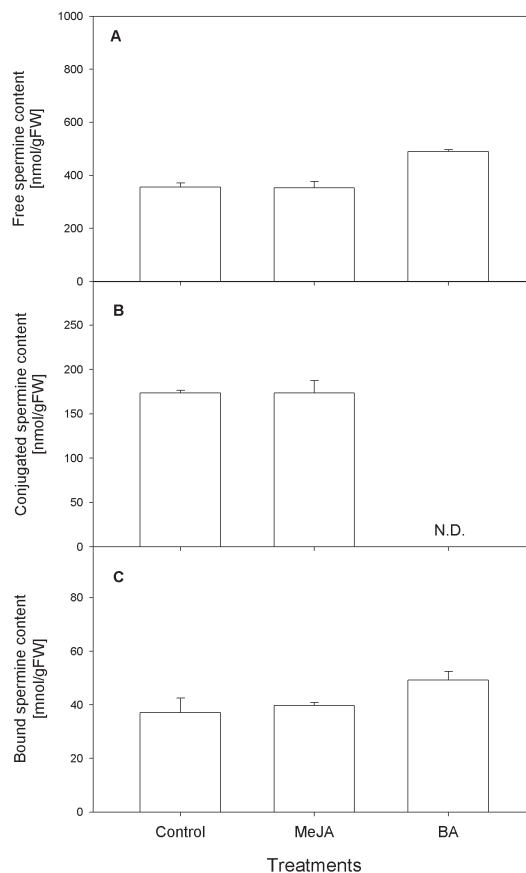


Figure 3. Content of free (A), conjugated (B) and bound (C) spermine in zucchini cotyledons (mean values \pm SE). Cotyledons were excised from the embryonic axes of dry *Cucurbita pepo* L. (zucchini) seeds and placed in covered Petri dishes on filter paper soaked with distilled water supplemented with either MeJA or BA, each applied at a concentration of 0.01 mM. Cotyledons were cultivated in the dark at 25°C. The analyses were performed on 6-day-old cotyledons. N.D. – not detected.

bound Spm. No significant changes in the content of bound Spm were detected in MeJA-treated cotyledons whereas the addition of BA into the medium increased bound Spm by 33% (Fig. 3C).

DISCUSSION

Polyamines are involved in a variety of developmental processes including plant cell growth and development (Kusano et al., 2008; Wimalasekera et al., 2011). For example, PAs levels were found to be high in proliferating tissues of tobacco and tomato ovaries, and dividing protoplasts of *Vigna aconitifolia* as reviewed by Egea-Cortines and Mizrahi (1991). In the present work, we focused on the changes in PAs content at the end of cell proliferation in excised zucchini cotyledons since on day 6 the cell division processes had been finished and further growth of cotyledons incubated on water was mainly due to cell enlargement as reported by Stoyanova-Bakalova (2007). Besides, at this stage cell division in cotyledons incubated on BA had finished 2-3 days earlier whereas those cultivated on MeJA were still dividing (Stoyanova-Bakalova et al., 2008). The potential of CKs to stimulate proliferation of plant cells is very well known (Letham, 1971). On the other hand, the inhibitory effect of MeJA on cell proliferation has also been identified (Stoyanova-Bakalova et al., 2008).

We found that free PAs (Figs. 1-3) were increased which was accompanied by the “disappearance” of the conjugated forms in cotyledons incubated on BA solution. Most probably, these alterations could be a result of the conversion of conjugated PAs into free forms since

conjugation of polyamines has been considered as a mechanism regulating the free PAs pool (Martin-Tanguy, 2001). The abundance of bound PAs after BA application (Figs. 1-3B) could result from the specific function of this kind of PAs to bind covalently to macromolecules and stabilize the cellular constituents (Martin-Tanguy, 2001) after termination of the cell division processes.

In the present study, we found that Put pool rose substantially in the cotyledons subjected to MeJA treatment and this was due mainly to free and conjugated forms (Fig. 1A, B). This effect could be considered as a consequence of stress events as reported previously in other studies (Todorova et al., 2007; Zapata et al., 2008). Moreover, Ananieva et al. (2004) showed that MeJA treatment down-regulated endogenous levels of CKs and their interconversion between active and inactive forms in cotyledons of zucchini seedlings, and this could affect normal cell division processes. In order to overcome the inhibition of cell division provoked by MeJA plant cells need to maintain high levels of PAs. In view of the fact that according to Galston (1983) PAs are described as ‘second messengers of cytokinin action’, accumulation of Put found in our model system was required to compensate the decreased CK content and Put probably affected directly cell proliferation. Besides, Put might act as a stress protector and/or signaling molecule for activating certain adaptation mechanisms as previously proposed by Groppa et al. (2008), which could be another possible explanation of Put augmentation.

Jasmonates are endogenous signals involved not only in the regulation of

various biological processes, but also in plant responses to both biotic and abiotic stresses, as well (Ueda and Kato 1982, Wasternack 2007, Kolaksazov et al., 2013). Exogenously applied jasmonates also provoke stress-related plant responses (Parthier, 1990). Our results showed that treatment with MeJA strongly enhanced both conjugated and bound Spd levels, the accumulation of bound Spd in MeJA-treated cotyledons being even higher than that of the conjugated fraction (Fig. 2B, C). Since Spd was shown to play a significant role in plant stress adaptation reactions (Kasukabe et al. 2004), our results suggest a similar role of these Spd fractions in excised zucchini cotyledons cultivated in the presence of MeJA, which could act as a stressor inhibiting cell division in our model system.

Recently published results have shown that MeJA treatment affects differently the content of biogenic amines in seedlings of several vegetable species (Horbowicz et al., 2014). These authors showed that while Put level increased in tomato leaves, in tissues of radish and onion a very strong decline in Put and Spd contents was observed. On the other hand, MeJA had no effect on levels of Put in leaves and epicotyls of maize and tomato hypocotyls (Horbowicz et al., 2014). The present study confirms the differential impact of MeJA on PAs content in MeJA-treated cotyledons showing that Put was altered most significantly, followed by less considerable amendments of Spd content whereas no changes were detected in Spm amounts (Fig. 1-3). The lack of Spm alterations in MeJA-incubated cotyledons could be due to some 'lag' period of the plant response to the elicitor applied since Spm is tetraamine, which is synthesized at

the last step of PAs biosynthetic pathway (Groppa and Benavides, 2008; Kusano et al., 2008).

CONCLUSION

Limited information is available about the alterations of PAs quantity provoked by treatment of plants with exogenous inhibitors or stimulators of cell division. Our study presents new information on the possible role of PAs in cell proliferation of excised zucchini cotyledons incubated in darkness on solutions of either MeJA or BA. The changes provoked by the tested phytohormones confirm the involvement of endogenous PAs in cell division processes.

ACKNOWLEDGEMENTS

The Operational Programme Human Resources Development (2007–2013), the European Social Fund and the Ministry of Education and Science of Bulgaria (Grant № BG051PO001-3.3.06-0025) are thanked for financial support.

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