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ARGUMENTS IN FAVOUR OF THE INVOLVEMENT OF POLYAMINES IN FLOWERING INDUCTION OF WINTER RAPE *(Brassica napus* L. var. *oleifera)* DURING VERNALIZATION AND GRAFTING

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Abstract. Polyamines are widely involved in biological processes in plants including growth and development, as well as in the response to stress. However, the interpretation of polyamine participations in the induction of flowering of winter plants, and, hence, the possibility of their exogenous application to accelerate flowering, is ambiguous. Winter plants require prolonged exposure to low temperatures (vernalization) to induce the generative development, thus the changes in the levels of these substances may also result in low-temperature-stress symptoms. The aim of this work was to check, whether the increase of polyamines is actually connected with flowering of winter rape plants (cv. Górczański). To exclude the vernalization effect, vegetative apexes of winter rape were grafted onto generative plants. The content of polyamines: putrescine, spermidine and spermine was measured, using high- performance liquid chromatography, in the apical parts and the youngest leaves of grafted apexes during 1-4 weeks after grafting. For comparison of the changes in the levels of polyamines occurring during vernalization, apexes and the youngest leaves were collected from winter rape after 1-8 weeks of culture at 5°/2° C. In apexes, content of all polyamines increased at 2 week (putrescine) or 2-3 weeks (spermine and spermidine) and in the period of 5-8 weeks of vernalization. For putrescine, the maximum increase was detected during at the 6^{th} week and for spermidine and spermine during the 7^{th} week of cold treatment, i.e when approximately 60-90% of plants were in the generative phase. In leaves, the changes of polyamines composition occurred almost during the same periods as in the apexes. Confirmation of the involvement of polyamines to flowering induction was the increase of these substances observed during generative development of grafted non-vernalized apexes. The registered increase of polyamines concentrations in the first weeks after veranlization (2-3 weeks) was probably connected with the cold-stress response of rape plants.

Key words: grafting, putrescine, spermidine, spermine, vernalization, winter rape

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INTRODUCTION

For winter plants, prolonged chilling (vernalization) is necessary to their flowering in spring. In spite of many studies of plant response to cold stress at the transcriptional and metabolite level [Cook *et al*. 2004, Filek *et al*. 2007, Kim *et al*. 2009], the molecular network controlling vernalization is not yet recognized in terms of physiological events. Difficulties in the precise determining of the mechanism of vernalization is related to the interaction of two physiological processes: resistance to low temperature stress and initiation of generative development.

There is a general agreement on hormones participation in the mechanism of vernalization, although hormonal responses are not universal to all species and are not clearly understood till now. In the last years correlation studies between genes and/or metabolites with cold treatment revealed a prominent role of the polyamine biosynthetic pathway in the plant response to low temperatures [Alcázar *et al*. 2011].

Polyamines (PAs) are present in all compartments of plants and they are involved in a wide range of physiological processes, such as membrane stabilization, enzyme activity modulation, cell division, elongation, replication, transcription and translation [Liu *et al*. 2006, Takahashi and Kakehi 2010]. Their exogenous application can increase the plant's tolerance to a variety of abiotic stresses [Chattopathaday *et al*. 2002, Kusano *et al*. 2008]. Moreover, it has been suggested that polyamines are involved in the flowering process [Bernier *et al*. 2002]. The participation of putrescine (PUT), spermidine (SPD) and spermine (SPM) in this process has been noted in photoperiodic plants [Applewhite *et al*. 2000, Huang *et al*. 2004, Zielińska *et al*. 2006]. Earlier experiments on winter wheat plants provided by Filek *et al.* [2010] indicated the increase of PUT and SPD at the 4^{th} week of vernalization, i.e when cooling was is sufficient to induce generative development. Despite this correlation between the increase of polyamines and the flowering induction of winter wheat, the observed effects should be considered as the cumulative impact of the exposure of plants to low temperature (stress factor) and the initiation of generative development. Separation of both processes can be achieved through grafting of non-generatively-induced plants on to plants which were generatively induced.

The aim of the presented study was to investigate, whether the polyamine increase was induced in winter rape scions which were stimulated to flower without cooling and to compare these changes with effects occurring in vernalized plants. Analyses were performed on apexes and the youngest leaves of winter rape at different times of their vernalization as well as on the organs isolated from non-vernalized parts of rape, which were grafted onto vernalized, flowering plants. In these experiments we used the winter rape cv. Górczański. The growth and development conditions of this genotype are well recognized and it was indicated that without cooling its flowering is not possible [Filek *et al*. 2007].

MATERIAL AND METHODS

Plant material

Seeds of winter rape (*Brassica napus* L. var. *oleifera*) cv. Górczański were surface – sterilized and placed into pots with peat substrate and sand (2:1; v:v). Plants were grown in a greenhouse under a 16 h photoperiod at 20/17°C (day/night) and 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$

irradiance (PAR). After about 4 weeks of culture, when rape was in the five-leaf rosette stadium, part of plants were transferred to a growth chamber for the low temperature (vernalization) treatment at 5/2°C (day/night) temperature. The second part of plants were grown continuously as a control in the greenhouse at 20/17°C (day/night). Every week, 30 plants were taken from the vernalization (V) and from the control (C) conditions and apical parts (A) and the youngest leaves (L) were collected.

In the second experiment, plants (cv. Górczański) were cultivated under the same conditions as was mentioned above until the end of the vernalization period (8 weeks vernalization). Then, further growth was continued in a greenhouse under 20/17°C (day/night) up to the initiation of the first flower buds. Then, the apical parts of the plants were cut off and replaced by scions from the apical parts (about 2 cm long, with only the two uppermost leaves left) of plants which were growth for 4 weeks (C) under non-vernalized greenhouse conditions. Grafted parts of such plants (W/W, winter/ winter) were covered with parafilm tape and the whole apical part was enclosed in a transparent polyethylene bag to ensure a high humidity. All the leaves appearing on the scions and all the later shoots appearing on the stocks were systematically removed [Filek *et al*. 2003]. Additionally, non-vernalized apical parts of winter rape cv. Górczański were grafted onto flowering plants of spring rape (cv. Młochowski) (W/S, winter/spring), growth continuously at 20/17°C (and 16 h photoperiod), as described above, however, without vernalization. 1, 2, 3 and 4 weeks after grafting, grafted parts (from 25 plants) of both winter and spring grafted rape, were taken for analyses. From these objects, apical parts (A) and the youngest leaves (L) were sampled.

All collected plant material was frozen in liquid nitrogen and stored at -80°C to measure polyamines.

Determination of polyamines

Polyamines were analyzed according to the procedure described by Flores and Galston [1982] and modified by Barbasz *et al*. [2012]. About 0.2 g of fresh tissue was mixed with 2 ml of 5% (w/v) cold perchloric acid and centrifuged at 14 000 x g for 15 minutes to extract polyamines. Polyamines were derivatized with dansyl chloride solution in acetone (5 $mg \cdot ml^{-1}$). After incubation at room temperature (12 h, in darkness) and 0.4 ml of proline application, polyamines were extracted with 2 ml of toluene. Organic phase, containing the investigated substances, was dried (in N_2) and dissolved in 0.3 ml of methanol. Polyamines were used to HPLC analysis from this solution.

High performance liquid chromatography (HPLC) was performed in a system consisting of a UV-detector (L-7400 LaChrom HPLC System MERCK) and linked to a data analysis system (D-7000 HPLC System Manager) used for data acquisition. Content of polyamines was detected at 330 nm. A LiChrospher 100 RP-18 (5 μm) (Merck) column was applied for the separation of the amine derivatives. A water--methanol mixture (40:60; v/v) was used as the eluent in a gradient elution mode at a flow rate of 2 $ml \cdot min^{-1}$. All reagents were of HPLC purity. A standard solution of polyamine at a concentration of 0.1 $mg \cdot ml^{-1}$ was prepared by dissolving the pure compound in water. All solutions were stored in darkness at 4ºC.

Statistical analysis

All measurements were repeated three times. The data were analyzed using SAS, and analysis of variance (ANOVA) was used to compare the differences based on Duncan's multiple range test, taking $P < 0.05$ as significant.

RESULTS

Chilling treatment of the winter rape plants induced their flowering after at least 4 weeks of growth at vernalization temperature (Table 1). The significant increase in the number of plants with flower buds (about 50%) was recorded in the 4th-5th week of cooling. Grafted, non-vernalized apexes were able to form the buds at the 3rd week after grafting of scions on flowering plants, however, those which were inoculated on spring plants, flowered of a higher percentage.

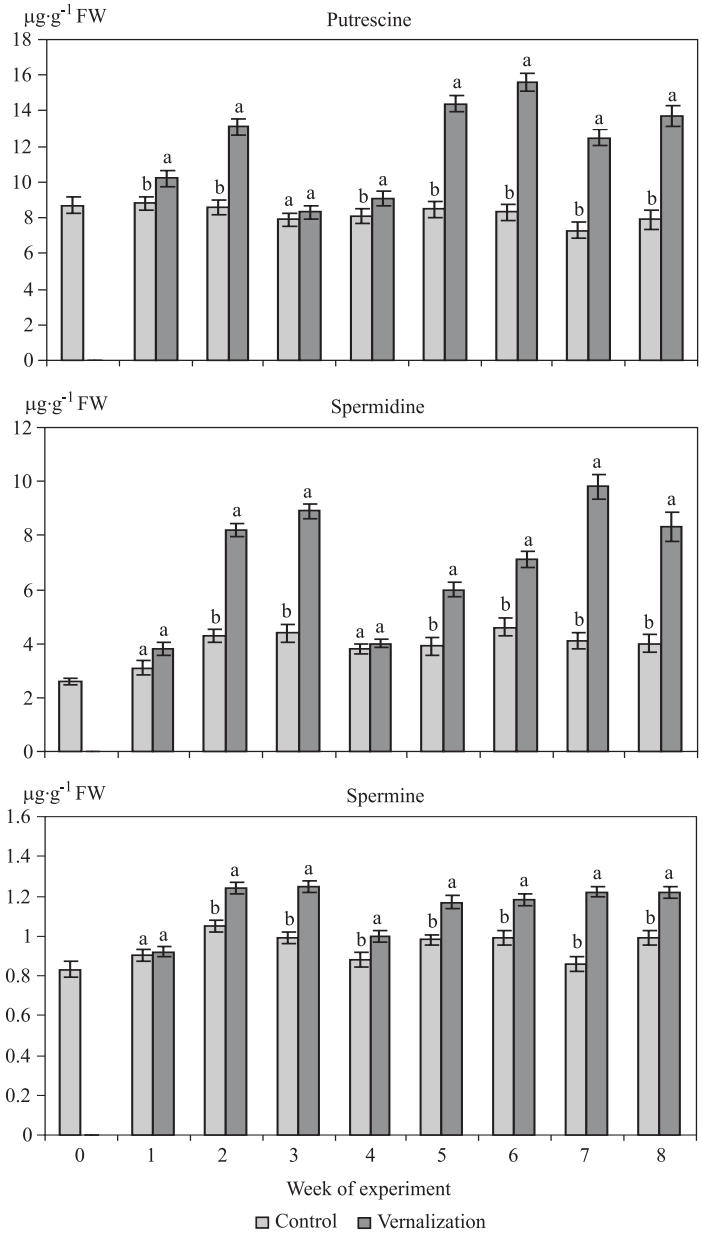
Table 1. The percentage of bud formation on winter rape plants after 1-8 weeks of vernalization and after 1-4 weeks after grafting non-vernalized winter rape apical fragment on flowering, vernalized winter rape (W/W) or non-vernalized, flowering, spring rape (W/S); the term 0 – apical fragments before vernalization or grafting, respectively; data represent the mean values of 30 plants ± SE

Weeks of vernalization (5/2ºC)								
0	1	2	3	4	5	6	7	8
0 ± 0	0 ± 0	0 ± 0	0 ± 0	10 ± 3	48 ± 10	65 ± 12	90 ± 10	100 ± 0
Weeks after grafting of W/W								
0	1	2	3	4				
0 ± 0	0 ± 0	0 ± 0	5 ± 3	76 ± 10				
Weeks after grafting of W/S								
0	1	2	3	4				
0 ± 0	0 ± 0	0 ± 0	10 ± 9	100 ± 0				

The main polyamines detected in both investigated organs of winter rape, i.e. apical parts and the youngest leaves, were free polyamines: putrescine (PUT), spermindine (SPD), and spermine (SPM). Generally, the content of all polyamines was higher in vernalized plants than in the control ones, grown at 20/17°C (Figs. 1, 2). In the control plants, changes in concentrations of all polyamines in the successive weeks of growth were rather small. In the apical parts, the levels of putrescine were similar to the amounts registered in the youngest leaves, whereas those of SPD and especially SPM were significantly lower, in comparison to leaves.

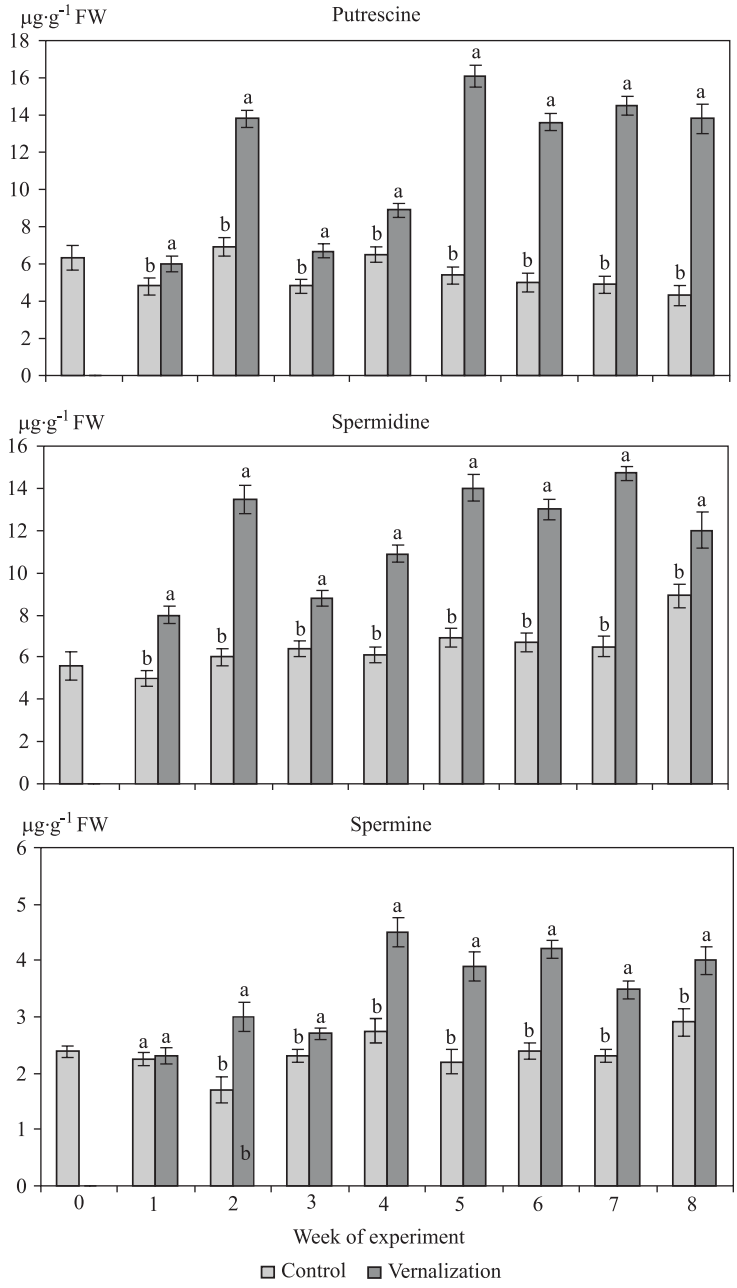
In vernalized plants, the maximum concentration of all analyzed polyamines occurred in both investigated organs at the 2nd weeks of the chilling treatment. After this time, the level of polyamines decreased and raised again at the 4th week (leaves) or at the 5th week (apical parts) of vernalization. High levels of SPD and SPM, which were observed in the 2nd week of cooling, lasted up to the 3rd week of vernalization in the apical parts. Increased concentrations of polyamines, which occurred in the intermediate period of chilling, remained high until the 8th week of the low temperature treatment. This rise was at about 6 $\mu g \cdot g^{-1}$ of the fresh weight (FW) for PUT and SPD and about 0.5

μg·g^{-1} FW for SPM in comparison to control in apical parts, and at about 9 μg·g^{-1} FW for PUT and SPD and 2.5 μg·g^{-1} FW for SPM, in leaves (Figs. 1, 2).



different letters indicate significant ($P \leq 0.05$) difference between temperature treatment

Fig. 1. Concentration of putrescine, spermidine and spermine in the apical parts of winter rape plants before vernalization (0, stage of 5-leaves rosette) and after 1-8 weeks of culture at 5/2°C (day/night) – vernalization or 20/17°C (day/night) – control



different letters indicate significant ($P \leq 0.05$) difference between temperature treatment

Fig. 2. Concentration of putrescine, spermidine and spermine in the youngest leaves of winter rape plants before vernalization (0, stage of 5-leaves rosette) and after 1-8 weeks of culture at 5/2°C (day/night) – vernalization or 20/17°C (day/night) – control

In the youngest leaves, developed at the apical parts of scions, the levels of all polyamines were higher than in the apical parts and increased in the following weeks of culture, irrespective of the kind of plants (winter W, spring S) onto which they were grafted (Fig. 3 A, L). Generally, differences between the levels of polyamines in W/W and W/S were very small and usually negligible. For PUT, SPD and SPM, maximum of its concentration appeared at the 4th week of culture (in both W/W and W/S kinds of grafting) (Fig. 3 A, L). In the apical part of scions, the content of PUT fluctuated with maxima at the 1st and the 4th week of culture (Fig. 3). The levels of SPD and SPM in apical parts increased continuously after grafting, especially at the 3rd week (both polyamines) and at the 4th week of growth (mainly SPD). In the last week of culture the increase in the level of polyamines, in comparison to the control, was approximately about 6 $\mu g \cdot g^{-1}$ FW for PUT and SPD and approximately about 1 $\mu g \cdot g^{-1}$ FW for SPM in apical parts, and approximately about 11-7 $\mu g \cdot g^{-1}$ FW for PUT and SPD, respectively, and 2 $\mu g \cdot g^{-1}$ FW for SPM in leaves (Fig. 3).

Grafting of apical parts of vegetative plants on the plants which were in the vegetative phase (both Młochowski and Górczański, without vernalization), did not result in significant changes of polyamines content during 4 weeks of growth after this process (data not shown).

DISCUSSION

It is known that polyamines can participate in plant response to low temperature stress. Gao *et al*. [2009] postulated that PUT, and the increase of its concentration, can be treated as an indicator of chilling damage in seedling tissues. It was suggested that PUT can be bound to antioxidant enzymes such as superoxide dismutase or can be conjugated with small antioxidant molecules allowing them to permeate to sites of action of oxidative stress within cells [Bouchereau *et al*. 1999]. Thus, the increase of polyamines levels, which were detected in the present experiment, in the first two weeks of vernalization in both apical parts and the youngest leaves, is consistent with the result of other authors [Gao *et al*. 2009, Alcázar *et al*. 2011] and can be recognized as a result of adaptation of winter rape plants to low temperature.

A decrease in the PUT concentrations in the apical parts at the 3rd week of cooling might be attributed to its metabolic conversion to SPD and SPM. In fact, levels of both polyamines increased slightly in this time of vernalization. After the 4th week, when the levels of all polyamines decreased, the formation of buds was microscopically recorded at approximately 10% of the plants. The appearance of buds, even in such a small number of plants indicates the initiation of the generative development during this cooling period. The re-increase of polyamines content (particularly of PUT and SPD) in apical parts and leaves, occurred during the 5th week of cooling, when about 50% of plants developed the buds. These results are in good agreement with the observations of Huang *et al*. [2004] for day – neutral *Polianthes tuberose* plants. They registered the decrease of polyamines (PUT and SPM) during the initial phase of floral initiation and then a rise, especially of the level of SPD.

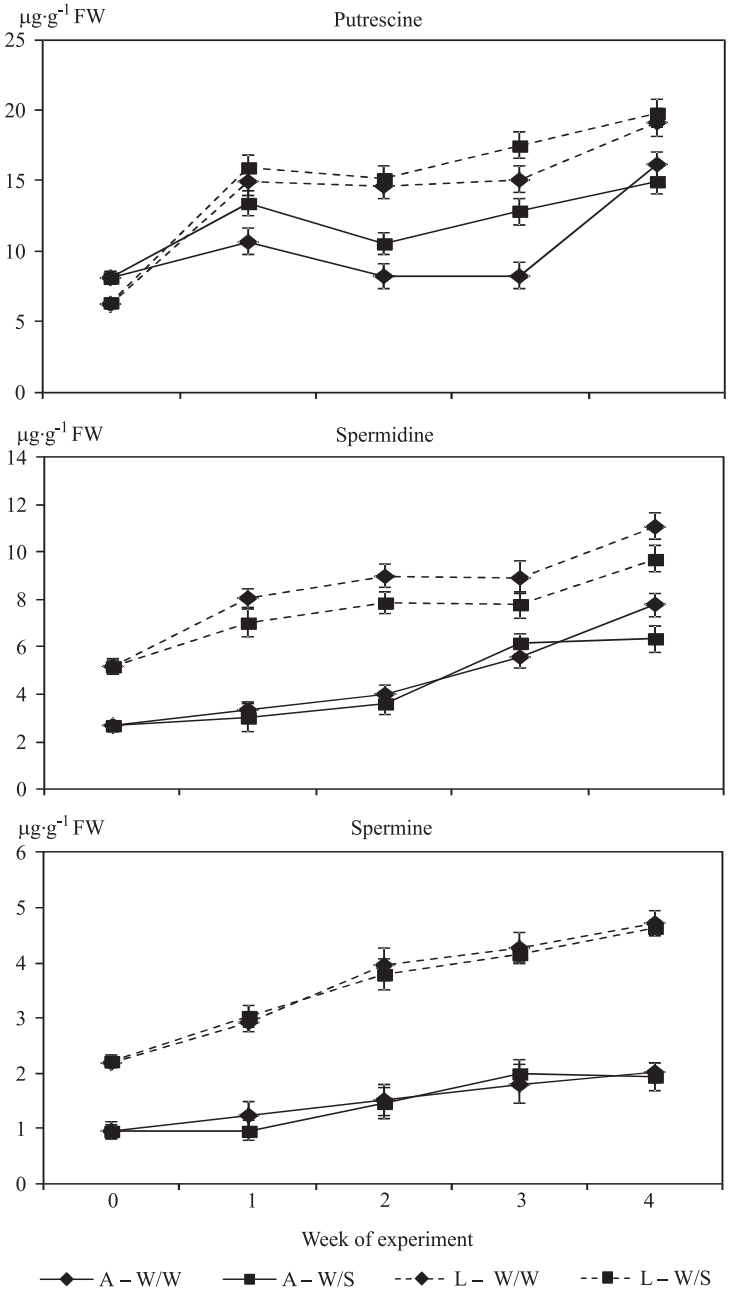


Fig. 3. Polyamine concentration detected in apical parts (A) and the youngest leaves (L) of non-vernalized apical fragments of winter rape before grafting (0) and after 1, 2, 3, 4 weeks after grafting on vernalized, flowering winter rape (W/W) and non-vernalized, flowering spring rape (W/S)

The significant and prolonged increase of all polyamines after the 5th week of cooling, to the end of vernalization, when 100% of apexes were in the phase of bud formation (Table 1), indicates that polyamines might be involved in the generative development of winter rape, as it was observed in winter wheat [Filek *et al*. 2010]. Confirmation the involvement of the analyzed substances in the induction of flowering of plants represent the experiments with grafted plants. In this case the increase of polyamines appeared during the formation of buds in non-vernalized plants. Generally, the sequences of changes of particular polyamines in grafted plants were similar to those observed in vernalized ones.

The increase in the contents of all tested polyamines in the period of buds setting, detected in both: the apical parts and the youngest leaves, regardless of whether stimulation of the generative development occurred through vernalization or grafting, confirms the involvement of these compounds in the induction of generative development in winter rape. Such results led us conclude that the possibility to stimulate the development of flowering of rape plants by exogenous application of these compounds represents an interesting hypothesis. In the photoperiodic plants, promotion of flowering by exogenous application was already demonstrated [Bregoli *et al*. 2002, Torrigiani *et al*. 2004, Liu *et al*. 2006]. For winter plants, especially for the agronomically important rape plant, the obtained results, provide information about the doses of polyamines, which may stimulate generative development.

CONCLUSIONS

In the presented studies we suggest that changes in polyamine contents may be involved in the stimulation of flowering in winter plants which need low temperature treatment to the generative development. The increase of all free polyamines (putrescine, spermidine, spermine) was observed in the phase of bud formation in both the apexes and the youngest leaves of plants subjected to vernalization as well as in non-vernalized apical parts, which were grafted on generative rape plants. Flowering induction of grafted winter plants caused similar changes of polyamines concentration irrespective of the genotype on which there were placed (winter and spring). Grafting of vegetative apical parts on generative induced plants allowed to eliminate the effect connected with the changes of polyamines concentration caused by vernalization. The increase of the content of these substances, associated with the low temperature treatment, occurred in the first weeks of vernalization.

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UDZIAŁ POLIAMIN W INDUKCJI KWITNIENIA ROŚLIN RZEPAKU OZIMEGO *(Brassica napus* L. var. *oleifera)* PODDANYCH WERNALIZACJI I SZCZEPIENIU

Streszczenie. Poliaminy są związkami zaangażowanymi w wiele procesów zarówno wzrostowych, jak i rozwojowych, a także związanych z reakcją roślin na działanie

czynników stresowych. Rola poliamin w indukcji kwitnienia roślin ozimych, a tym samym możliwość zastosowania ich egzogennego podania w celu przyspieszenia rozwoju pąków kwiatowych, nie jest do końca wyjaśniona. Rozwój generatywny roślin ozimych uwarunkowany jest występowaniem okresu obniżonej temperatury. Obserwowane zmiany poziomu poliamin mogą być zatem wynikiem stresogennego działania chłodu. W pracy przedstawiono wyniki badań dynamiki zmian zawartości tych substancji podczas procesu wernalizacji rzepaku ozimego (cv. Górczański) i określono korelacje zmian z okresem indukcji kwitnienia. W celu całkowitego wykluczenia efektu działania niskiej temperatury wykonano eksperymenty, w których rośliny zostały zaindukowane do kwitnienia poprzez szczepienie części wierzchołkowych, będących w fazie wegetatywnej, na roślinach generatywnych. Stężenie endogennych poliamin: putrescyny, spermidyny i sperminy, rejestrowane przy użyciu metody wysokosprawnej chromatografii cieczowej, badano w wierzchołkowych częściach roślin oraz w najmłodszych liściach. Materiał pobierano w tygodniowych odstępach podczas 8-tygodniowej wernalizacji (5°/2° C) oraz w okresie 4 tygodni po szczepieniu. W wierzchołkowych częściach roślin wernalizowanych zawartość wszystkich poliamin wzrosła między 2. a 3. oraz 5. a 8. tygodniem wernalizacji – w stosunku do kontroli (20°C). Największy wzrost stężenia putrescyny obserwowano w 6. tygodniu wernalizacji, podczas gdy spermidyny i sperminy w 7. tygodniu, to jest tym okresie, w którym około 60-90% roślin zawiązało pąki kwiatowe. Również w wegetatywnych wierzchołkach szczepionych na roślinach generatywnych wzrost stężenia badanych poliamin wystąpił w terminie, w którym 80-100% roślin zostało zainicjowanych do kwitnienia. Kierunek zmian stężenia poliamin w najmłodszych liściach (zarówno w wernalizowanych, jak i szczepionych) był zbliżony do obserwowanego w wierzchołkach. Uzyskane wyniki wskazują na zaangażowanie poliamin w indukcję kwitnienia roślin ozimych, co wiąże się z możliwością stymulacji rozwoju generatywnego tych roślin poprzez egzogenną aplikację poliamin. Wzrost stężenia wszystkich poliamin w pierwszych tygodniach chłodzenia może być efektem adaptacji roślin do niskiej temperatury.

Słowa kluczowe: putrescyna, rzepak ozimy, spermidyna, spermina, szczepienie, wernalizacja

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