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EFFECT OF PHENYLMETHYLSULFONYL FLUORIDE – AN INHIBITOR OF PROTEASES, ON THE GROWTH AND POLYPEPTIDE PROFILE OF EXCISED COTYLEDONS OF *CUCURBITA PEPO* L. (ZUCCHINI) AFTER TREATMENT WITH BENZYLADENINE

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Summary. Treatment of excised marrow (*Cucurbita pepo* L., zucchini) cotyledons in darkness with 45 μM BA caused a marked stimulation of the growth accompanied by a strong decrease in cotyledons relative dry weight. PMSF – a well known inhibitor of both thiol- and serine-type proteases, present in the aqueous solutions either alone or with the cytokinin, inhibited significantly the growth of cotyledons and hindered the decrease in cotyledons dry weight in all treatments. SDS-PAGE analysis of the protein profiles showed that PMSF suppressed the gradual decline in the quantity of the 20–2 kDa polypeptide group and the low-molecular-weight polypeptide bands (below 15 kDa) both in the control and BA-treated cotyledons. On the other hand, the polypeptide bands migrating in the higher molecular weight zone of the profiles including the 97.4-kDa polypeptide and the LSU of Rubisco (55 kDa) decreased in quantity in the presence of PMSF. These results suggest that the inhibitory effect of PMSF on the breakdown of storage proteins in the excised marrow cotyledons may be due to an inhibition only of certain PMSF-sensitive proteases taking part in this process during germination.

Key words: benzyladenine, cotyledon, polypeptide profile, storage protein metabolism

Abbreviations: BA – benzyladenine, EDTA – ethylene diamine tetraacetic acid; PAGE – polyacrylamide gel electrophoresis, Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase, SDS – sodiumdodecylsulphate, PMSF – phenylmethylsulfonyl fluoride, RDW – relative dry weight.

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Introduction

Due to their high sensitivity to exogenous cytokinins, excised *Cucurbitaceae* cotyledons have been thoroughly explored for studying the regulatory action of these phytohormones (Kulaeva, 1982; Letham and Palni, 1983; Bewly and Black, 1985, Anania eva and Ananiev, 1999). In dicots cotyledons represent specific reserve organs whose major physiological function is to ensure the development of the growing seedling during germination. Mobilization of stored reserves is an essential event of post-germinative growth (Bewly and Black, 1978; Kermode, 1995). The products of these degradative processes are utilized as both the substrate and the energy source for the growing seedling. The major storage protein in the seeds of Cucurbita sp., the insoluble 11S-type globulin, which comprises more than 90% of total protein content, is hydrolyzed to a limited extent in the first 4 days of germination by globulin-specific proteinases to produce the soluble molecular species – acidic (33 kDa) and basic (22 kDa) polypeptides (Hara et al., 1976; Hara and Matsubara, 1980; Hara et al. 1982). These authors have also shown that during the later stages of germination these polypeptides are further degraded by means of proteases synthesized *de novo*, to a number of small polypeptides with lower molecular weights. Therefore, during seed germination selective proteinase-mediated breakdown of seed storage proteins occurs.

PMSF is known as a compound which can inhibit both thiol- and serine-type proteases in plants (Mikola et al., 1986). It has been proved that PMSF can inhibit the germination of lettuce seeds as well as of seeds of some other plant species (for example, *Cucurbita moschata* D., *Phaseolus vulgaris* L., etc.) by decreasing the growth potential of the embryonic axis (Takeba, 1990). In addition, PMSF was the only inhibitor from several protease inhibitors tested which had an inhibitory effect on the breakdown of the storage peptides (32–39 kDa) in the lettuce seeds during imbibition (Takeba, 1990). Consequently, the inhibition of proteases in the presence of the exogenously applied inhibitor PMSF could result in a suppression of the breakdown of storage proteins.

The aim of the present work was to study the effect of PMSF on the growth of excised marrow (*C. pepo* L., zucchini) cotyledons and the changes in the polypeptide spectrum of their total soluble proteins after treatment with BA.

Materials and Methods

Plant material and growth conditions

Seedlings of *Cucurbita pepo* L. (zucchini), cv. Cocozelle, v. Tripolis were grown for 96 h in darkness at 28 °C. After excision of the embryonic axes, cotyledons were transferred to Petri dishes with distilled water for another 24 h in order to reduce the endogen-

ous cytokinins levels. After that the cotyledons were incubated on distilled water (control) or aqueous solutions of BA applied at a concentration of 45 μM in the presence (1 mM) or absence of PMSF. The solutions containing the inhibitor were changed every 12h in order to hinder its possible degradation in water. All experiments were carried out in darkness at 37 °C. Measurements were done at 24, 48 and 72 h of treatment.

Fresh and dry mass analyses

Fresh mass accumulation was expressed as an increase in fresh weight per cotyledon measured at 24, 48 and 72 h of treatment, referred to the initial value at time zero (fresh weight per cotyledon prior to treatment).

The dry weight per cotyledon was measured after drying at 105 °C for 5 h. The relative dry weight was calculated as (dry weight/fresh weight)×100%.

Protein extraction and SDS-PAGE

Frozen cotyledons were ground with mortar and pestle in extraction buffer containing 50 mM HEPES-NaOH, pH 8.0, 330 mM sorbitol, 2 mM KNO_3, 2 mM EDTA, 1 mM $MnCl_2$, 0,5 mM K_2HPO_4, 20 mM NaCl, 2 mM PMSF. The homogenate was then centrifuged at 10 000 g for 30 min. An aliquot of the supernatant was mixed with an equal volume of sample buffer containing 80 mM TRIS-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% β-ME and 5 mM EDTA. The samples were heated for 5 min in boiling water. Proteins were separated by SDS-PAGE on 12% gels according to Laemmli (1970). Each lane was loaded with 40 μg of protein and the gels were run at 150 V. Polypeptides were stained with Coomassie Brilliant Blue R-250. Protein content was determined according to Lowry et al. (1951).

Results

Changes in fresh weight and relative dry weight

Treatment of excised marrow cotyledons grown for 72 h in darkness with 45 μM BA stimulated strongly the growth of cotyledons (Fig. 1). This result is in accordance with the well known effect of exogenously applied cytokinins to increase the fresh weight and the size of excised cotyledons of different species (Longo et al., 1979; Letham,1971; Bewly and Black, 1985). PMSF, present in the aqueous solutions either alone or with BA, decreased the growth of cotyledons by approximately 40–45% as compared to controls over the 72 h treatment. In contrast to the gradual increase in fresh weight, the relative dry weight of excised cotyledons grown in darkness showed a tendency to decrease (Fig. 2). BA decreased cotyledons dry weight by approximately 40% as compared to controls over the whole period of treatment. In contrast to the

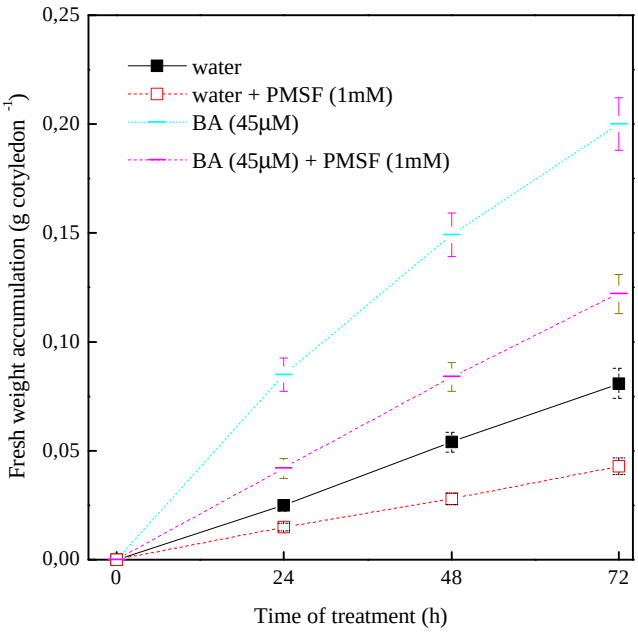

Fig. 1. Effect of 45 µM BA applied with or without PMSF (1 mM) on the growth of excised marrow cotyledons in darkness. Growth is expressed as the accumulation of fresh weight per cotyledon for different periods of time, referred to the initial value at time "zero" (fresh weight per cotyledon prior to treatment). Bars indicate SE of the means, obtained from three different experiments.

effect produced on fresh mass accumulation, PMSF maintained the relative dry weight of excised cotyledons at a higher level as compared to controls. Therefore, PMSF hinders the decrease in cotyledons dry weight. It should be pointed out that this effect of the protease inhibitor was most strongly expressed when cotyledons were floated on a BA + PMSF solution (40% higher RDW at 72 h as compared to 20% RDW for cotyledons floated on a water + PMSF solution).

Changes in the polypeptide profiles of total soluble protein

The polypeptide profiles of soluble proteins of excised cotyledons treated with BA in the presence or absence of PMSF are presented in Fig. 3. BA caused a gradual decrease in soluble protein content as judged by the decreased intensity of all bands in the polypeptide spectrum compared with controls, and especially, in the quantity of the polypeptides migrating in the middle (20–25 kDa) and low-molecular-weight zones (below 15 kDa), (Fig. 3, lanes 3 compared to 1 and lane 7 compared to 5).

The effect of PMSF on the changes in the polypeptide pattern of soluble proteins triggered by BA and especially on the amounts of the 20–25 kDa polypeptide group

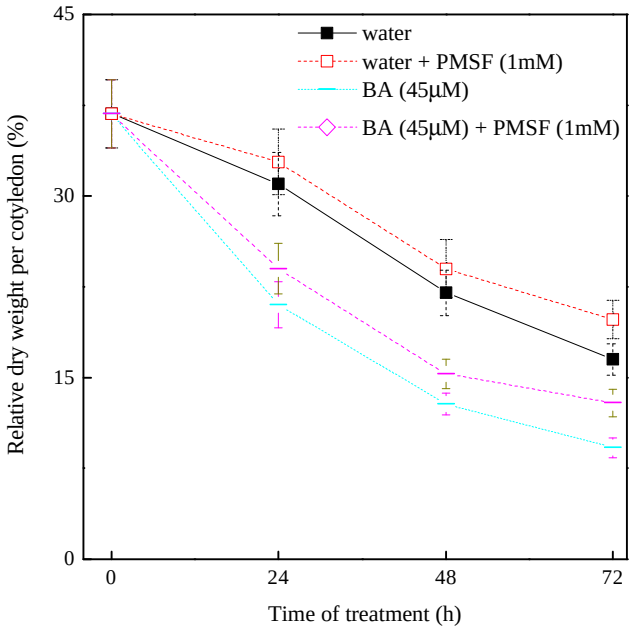

Fig. 2. Effect of 45 µM BA applied with or without PMSF (1 mM) on the relative dry weight of excised marrow cotyledons in darkness. The relative dry weight was calculated as (dry weight/fresh weight)×100%. Bars indicate SE of the means, obtained from three different experiments.

and the low-molecular-weight two polypeptide bands (below 15 kDa) was investigated next. The results showed that PMSF hindered the gradual decline in the quantity of both polypeptide groups in the profiles of the control cotyledons as well as after treatment with BA (Fig. 3, lanes 2, 4 as compared to 1,3 – at 48 h; and lanes 6, 8 as compared to 5, 7 – at 72 h). On the other hand, the polypeptide bands migrating in the higher molecular zone of the profiles including the 97.4-kDa polypeptide and the LSU of Rubisco (55 kDa) decreased in quantity in the presence of PMSF in all treatments.

Discussion

It is well known that the degradation of storage proteins is part of mobilization of stored reserves as an essential event during the post-germinative growth (Bewly and Black, 1978). Generally, the accumulation of fresh mass in the excised cotyledons grown in darkness is due mainly to the processes of mobilization of storage reserves accompanied by an increased water uptake (Bewly and Black, 1978; Letham and Palni, 1983). It should be pointed out that the dominating storage reserves in the seeds

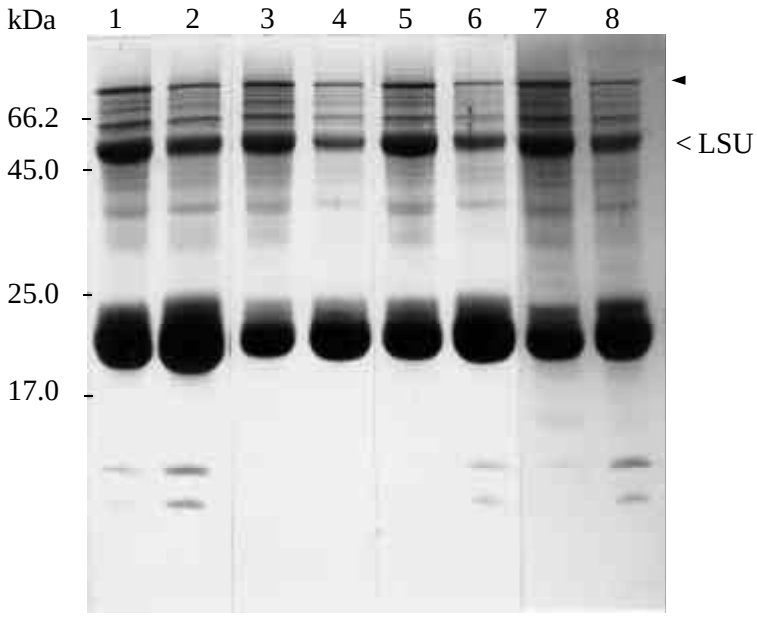

Fig. 3. Polypeptide profiles of total soluble protein extracted from excised marrow cotyledons floated on water or BA (45 μM) with or without PMSF (1 mM) in darkness. Polypeptides were separated by 12% SDS-PAGE. Key to lane numbers: 48 h incubation on: water (1), water + 1 mM PMSF (2), BA (3), BA + 1 mM PMSF (4); 72 h incubation on: water (5), water +1 mM PMSF (6), BA (7), BA + 1 mM PMSF (8). The solid arrowhead indicates the position of 97.4-kDa polypeptide band. LSU – the large subunit of Rubisco (55 kDa). Molecular mass markers are shown on the left.

of Cucurbita sp. are lipids and proteins. Besides, the active catabolism of the storage substances in dicots results in a decrease in the dry weight fraction of the cotyledons which is a normal event during the earlier stages of germination (Bewly and Black, 1985). The products of these degradative processes are utilized for anabolic processes and energy sources. In addition, in our specific assay system of excised cotyledons, leakage of some low-molecular products (for example, amino acids) in the medium could also occur.

If PMSF is applied to the medium, an inhibition of certain PMSF-sensitive proteases occurs which results in a suppression of the breakdown of storage proteins. Our results showed that PMSF inhibited the growth of excised cotyledons in darkness (Fig. 1) and hindered the decrease in cotyledons dry weight (Fig. 2) which can be accounted for by the down-regulation of the breakdown of storage proteins.

Based on the data of Hara et al. (1976), Hara and Matsubara (1980), concerning the gradual degradation of globulins in the seeds of Cucurbita sp. it can be assumed that the 20–25 kDa polypeptide group and the polypeptide fractions with lower molec-

ular weights obtained after resolving total soluble protein by means of SDS-PAGE, represent the products of proteolytic degradation of the seed storage proteins. So, the analysis of the polypeptide profiles upon treatment with BA revealed its specific effect on the dynamics of protein degradation processes. It is evident that BA causes stimulation of protein breakdown reflected by the gradual decrease in the content of 20–25 kDa polypeptide group with duration of treatment. This result is in accordance with the well known effect of endogenous cytokinins to regulate storage proteins mobilization in cotyledons of different dicots through proteolytic activity stimulation (Gepstein and Ilan, 1979, 1980; Munoz et al., 1990). That is why the effect of PMSF on the relative dry weight was most strongly expressed in the presence of the exogenously applied cytokinin (BA + PMSF).

Furthermore, it is well documented that cytokinins promote storage reserves mobilization in cotyledons isolated from different plants (Kagawa et al., 1973; Longo et al., 1979) via stimulation of the activity of various enzymes (Gepstein and Ilan, 1979; Howard and Witham, 1983; Chen and Leisner, 1985). The well known cytokinin effect to increase both the size and fresh weight of excised cotyledons has been proved to be based on the enhanced water uptake capacity of the cells, due to an increased production of reducing sugars thus leading to enhanced cell osmotic potential, accompanied by cell wall loosening (Tsui et al., 1980).

The effect of PMSF to suppress or delay the breakdown of the 20–25 kDa polypeptide group in the excised marrow cotyledons is obviously due to its inhibitory action on peptidases participating in the breakdown of storage proteins. That is why this effect was observed also in the profiles of the control cotyledons. This result confirms our assumption that the affected polypeptides represent the products of proteolytic degradation of storage proteins. The result showing that the degradation of this polypeptide group upon treatment of cotyledons with BA was stimulated in the absence of PMSF is supportive of the role of cytokinins to induce or stimulate proteolytic enzymes involved in storage proteins degradation. Similar data showing that the incorporation of PMSF in the nutrient media with either IAA or BA inhibited the disappearance of a polypeptide group in the region 20–25 kDa as evidenced by SDS-PAGE and Western blot analyses of total soluble proteins from excised melon cotyledons (*Cucumis melo* L.) cultured *in vitro* were obtained by Leshem et al. (1994). The authors showed also that 10^{-5} M BA in the medium enhanced the disappearance of this polypeptide group.

Our results showed also that in the presence of PMSF the polypeptide bands migrating in the higher molecular zone of the profiles including the 97.4-kDa polypeptide and the LSU of Rubisco (55 kDa) decreased in quantity in all treatments. These results suggest that PMSF can inhibit only part of the proteases in the cells, while others obviously insensitive to this inhibitor, remain active. Similar data showing that PMSF inhibited the activities of two types of peptidases (a carboxypeptidase and BAPAase) during imbibition of lettuce seeds, but had no effect on the activity of another endo-

peptidase (an acid protease) were obtained by Takeba (1990). Therefore, the inhibitory effect of PMSF on the breakdown of storage proteins in the excised marrow cotyledons may be due to its effect on certain PMSF-sensitive peptidases participating in the hydrolysis of storage proteins.

In conclusion, our results indicate that the presence of PMSF – a well known inhibitor of both thiol- and serine-type proteases, either alone or in combination with BA, inhibited fresh mass accumulation and hindered the decrease in dry weight of excised marrow cotyledons in darkness. In addition, PMSF suppressed the breakdown of the 20–25 kDa polypeptide group, but could not affect the gradual decrease in the quantity of the 97.4-kDa polypeptide band and the LSU of Rubisco (55 kDa) suggesting that the inhibitory effect of PMSF on the breakdown of storage proteins in the excised marrow cotyledons may be due to an inhibition of only certain PMSF-sensitive proteases taking part in this process during germination.

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