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# INTERACTION BETWEEN METHYL ESTER OF JASMONIC ACID AND BENZYLADENINE DURING THE GROWTH OF EXCISED GREENING COTYLEDONS OF *Cucurbita pepo* L. (zucchini)

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**Summary.** Treatment of excised marrow cotyledons (*Cucurbita pepo* L. (zucchini) growing under photoperiod with methyl ester of jasmonic acid (MeJA) resulted in a decrease of their fresh weight. This effect did not depend on the exogenously applied MeJA concentrations. Furthermore, *in vivo* labelling experiments showed an inhibition of the total protein synthesis. On the other hand, specific quantitative changes were observed in the spectrum of soluble proteins resulting in the formation of abundant jasmonate-induced proteins (JIPs). Benzyladenine (BA) applied together with MeJA neutralized the jasmonate action on the growth of the excised cotyledons, as well as on the *in vivo* protein synthesis. Similar antagonistic interaction between BA and MeJA was observed on the formation of JIPs both when the substances were applied together and in the case of subsequent treatment of cotyledons. The antagonism observed between the two plant growth regulators during the growth and development of excised greening cotyledons can be considered as one possible mechanism in the maintenance of plant cell homeostasis under stress conditions in the earlier stages of germination.

***Key words***: benzyladenine, excised cotyledons, *in vivo* protein synthesis, methyl ester of jasmonic acid, polypeptide pattern

***Abbreviations***: MeJA – methyl ester of jasmonic acid, BA – benzyladenine, PAGE – polyacrylamide gel electrophoresis, SDS – sodium dodecyl sulphate, AAM – aminoacid mixture, PMSF – phenylmethylsulphonylfluoride

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## Introduction

Jasmonates – jasmonic acid (JA), its methyl ester (MeJA) and related compounds represent a novel class of plant growth regulators involved in different physiological processes connected with plant growth and development (Koda, 1992; Sembdner and Parthier, 1993; Creelman and Mullet, 1997). The role of jasmonates in promotion of leaf senescence has been well documented in literature (Ueda and Kato, 1980; Weidhase et al., 1987a; Porat et al., 1993; Ratajczak et al., 1998). On the other hand, it is well known that cytokinins are the major phytohormones with antisenescence activity (Parthier, 1979, 1989). The data on the interaction between jasmonates and cytokinins available in literature are still limited. It has been shown that during the induced senescence of barley leaf segments in the light BA can eliminate to a certain extent the inhibitory effect of MeJA on chlorophyll content and Rubisco activity (Weidhase et al., 1987a), however, the cytokinin cannot prevent the formation of specific polypeptides induced upon MeJA-treatment, so called jasmonate-induced proteins (JIPs) (Weidhase et al., 1987b). Besides, the counteraction of cytokinins to the jasmonate inhibitory effect on the growth of different plants has also been reported (Ueda et al., 1981; Ueda and Kato, 1982).

It is well known that the transition of epigeal cotyledons from reserve organs into photosynthesising cotyledonary leaves is under the positive hormonal control of cytokinins (Kulaeva, 1982). That is why excised cotyledons represent an useful model system for studying the interaction between cytokinins and jasmonates. Our recent results have shown that BA can counteract the MeJA action on protein, RNA synthesis and the activity of nuclear RNA polymerases (Ananieva and Ananiev, 1999) as well as on JIPs accumulation (Ananieva and Ananiev, 1998) in excised marrow cotyledons grown in darkness. On the other hand, we have recently shown that the exposure of etiolated cotyledons excised from dark-grown 4-day-old marrow seedlings under controlled light conditions resulted in a strong increase of chlorophyll content and the net photosynthetic rate up to the 48th of incubation in water, thus suggesting that this biological system is not senescing although being detached from the intact seedling (Ananiev and Ananieva, 2000). The aim of this work was to further analyse the interaction between exogenously applied MeJA and BA in the regulation of protein metabolism in greening excised marrow cotyledons.

## Materials and Methods

### Plant material and cotyledon treatment

Seeds of *Cucurbita pepo* L. (zucchini) cv. Cocozelle were soaked for 4 h in tap water and germinated on a moistened filter paper for 96 h in darkness at 28 °C. After excision

of the embryonic axes, cotyledons were transferred to Petri dishes with distilled water and were kept in darkness for further 24 h in order to decrease endogenous cytokinin content. Then the cotyledons were incubated on distilled water, or aqueous solutions of MeJA (1, 10, 45 or 100 µM), BA (45 µM) or their mixture (45 µM MeJA + 45 µM BA) in the light (photon flux density 120 µmol.$m^{-2}$.$s^{-1}$, 25 °C, humidity 70%, photoperiod 12/12 h) for 1–4 days.

### 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 phenylmethylsulphonylfluoride (PMSF). The homogenate was then centrifuged at 10 000×g for 30 min. Aliquots of the supernatant were precipitated with ice-cold acetone and SDS-PAGE was performed according to Laemmli (1970). Polypeptides were stained with Coomassie Brilliant Blue R250. 40 µg proteins were charged per slot. Protein content was determined according to Lowry et al. (1951).

### *In vivo* labelling of proteins

The incorporation of [$^{14}$C]-aminoacid mixture (AAM) (Amersham Int. Buckinghamshire, England) in newly synthesized proteins was determined using the filter disc method according to Mans and Novelli (1961) with modifications. Cotyledons from different variants with equal fresh weight were incubated with 185 kBq.$cm^{-3}$ [$^{14}$C]-AAM for 4 h at the end of the respective period of incubation. Cotyledons were homogenized in the extraction buffer and the homogenate was centrifuged for 30 min at 10 000×g. Aliquots of the supernatants (100 µl) were pipetted onto Whatman No1 filter paper discs and precipitated with 10 and 5% TCA. Hydrolysis of aminoacyl-tRNAs was performed in 5% TCA for 20 min at 90 °C and the discs were subsequently washed with cold ethanol, ethanol:ether (3:1) and ether. Radioactivity was counted in a liquid scintillation counter Beckman (USA). For determination of the total uptake of [$^{14}$C]-AAM by the cotyledons 100 µl-aliquots of the supernatants were transferred onto filter discs and the radioactivity was directly counted.

## Results and Discussion

### Effects of MeJA, BA and their mixture on the growth of the excised cotyledons

Treatment of excised marrow cotyledons with MeJA in the light inhibited their growth by 15–22% in the course of the experiment (Fig. 1). Similar data have been reported in literature demonstrating the inhibitory effect of JA and MeJA on the growth of rice

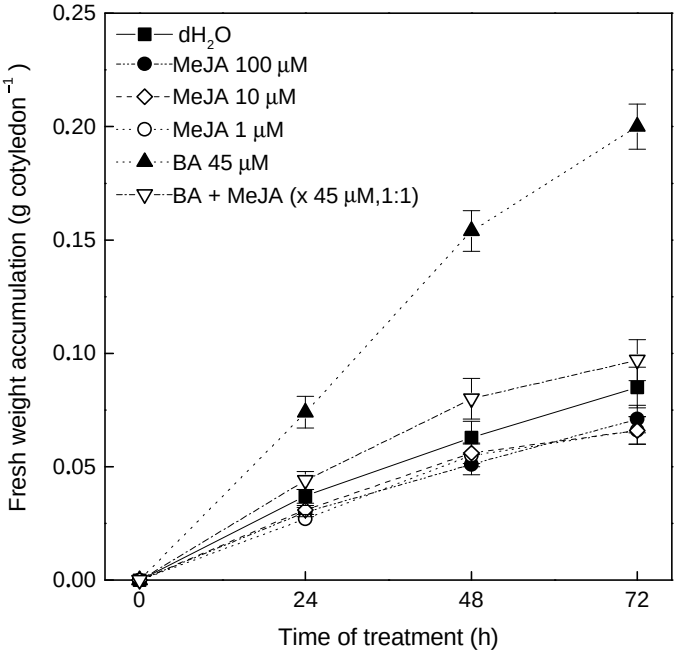


**Fig. 1**. Effects of different concentrations of MeJA (1, 10 and 100 µM), BA (45 µM) and their mixture (45 µM+45 µM) on the growth of excised marrow cotyledons grown in the light. Growth is expressed as the accumulation of fresh weight per cotyledon for different periods of time, referred to the initial value at time "zero" (prior to treatment). Bars indicate SE of the means, obtained from four different experiments.

seedlings (Yamane et al., 1980), wheat seedlings (Dathe et al., 1981), isolated radish cotyledons (Ueda and Kato, 1982). Our result, however, differed from previously obtained data showing that MeJA had no effect on the growth of excised marrow cotyledons in darkness (Ananieva and Ananiev, 1999). Therefore, the growth regulating activity of MeJA in this biological system can be observed only in light conditions. This result suggests different mechanisms of the MeJA action on growth in the presence or absence of light. It should also be pointed out that the MeJA effect on the growth of cotyledons did not depend on the exogenously applied concentrations (1, 10 and 100 µM), (Fig. 1). So, the "dose-effect" response characteristic for the phytohormonal action was not observed. By contrast, the application of BA stimulated strongly the growth of cotyledons. This result confirmed the well-known effect of exogenous cytokinins to increase the fresh weight and the size of isolated cotyledons of different species (Klyachko et al., 1979; Longo et al., 1979; Letham and Palni, 1983). Treatment of cotyledons with the equimolar mixture of MeJA + BA (1:1, x 45 µM) neutralized the individual effects of both substances – the results obtained were close to the control values (Fig. 1).

### *In vivo* protein synthesis

Treatment of cotyledons with MeJA for 24 h inhibited *de novo* protein synthesis by 30% (Fig.2). On the other hand, as compared to the initial rate of incorporation measured at time "zero" (cotyledons prior to treatment) no change was observed after 12 h incubation with MeJA unlike the gradual decrease (28%) observed previously in darkness (Ananieva and Ananiev, 1999). This effect was obviously due to the presence of light. Therefore, during the first 12 h of treatment light can eliminate the jasmonate inhibitory effect on protein synthesis. By contrast, incubation of cotyledons with BA stimulated strongly the rate of incorporation of [$^{14}$C]-AAM with a maximum measured at the 12th h (66% compared to the control). The combination of MeJA and BA decreased the cytokinin stimulatory effect to the control values. On the other hand, almost no differences were observed between MeJA, BA and their mixture in comparison with the control when the rates of incorporation were expressed as percentage of total uptake (Tabl. 1). These results indicated that the rates of incorporation changed proportionally to the uptake of the labelled aminoacids by the cotyledon, as shown in Table 1. That is why the changes registered in protein synthesis in the presence of

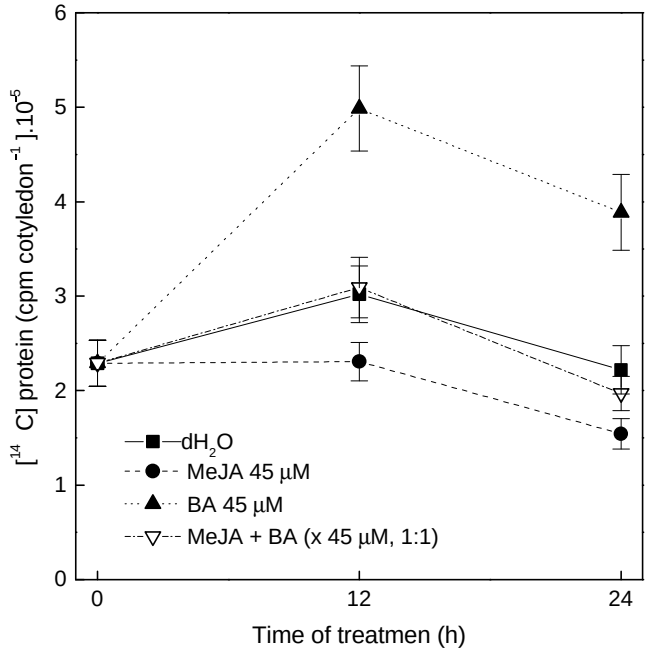


**Fig. 2**. Effects of MeJA (45 µM), BA (45 µM) and their mixture (45 µM + 45 µM) on incorporation of [$^{14}$C]-labelled amino acids in total soluble proteins. Bars indicate SE of the means, obtained from three different experiments.

**Table 1.** Effect of MeJA (45 μM), BA (45 μM) and MeJA + BA (1:1) on the uptake and the incorporation rate of [$^{14}$C]-labelled amino acids into total protein of excised marrow cotyledons.

| Cotyledon treatment | Time of treatment (h) | [$^{14}$C]-AAM uptake (%) | [$^{14}$C]-AAM incorporation (% of uptake) |
|---|---|---|---|
| Water | 12 | 100 | 88 |
| | 24 | 100 | 71 |
| MeJA | 12 | 68 | 85 |
| | 24 | 89 | 72 |
| BA | 12 | 163 | 82 |
| | 24 | 220 | 70 |
| BA + MeJA | 12 | 110 | 83 |
| | 24 | 100 | 78 |

the tested substances could be considered as a consequence of both changed rates of incorporation and of the uptake capacity for labelled aminoacids.

## Effects of MeJA, BA and their mixture on the polypeptide profile of soluble proteins

The lowered synthesising capacity for total protein triggered by MeJA in the excised greening marrow cotyledons was accompanied by the accumulation of three abundant polypeptides with relative molecular masses of 97.4, 60 and 43 kDa (Fig. 3, lanes 2 and 6) as already shown by our previous investigations (Ananieva and Ananiev, 1997). Besides, the polypeptide bands with Mr 60 and 43 kDa were proved to be MeJA-inducible whereas the accumulation of 97.4 kDa polypeptide was only strongly stimulated in the presence of MeJA (Ananieva and Ananiev, 1998). On the other hand, the application of BA caused a gradual decrease of total soluble protein content and especially, of a group of polypeptide bands in the region of 20–25 kDa as well as the polypeptides with high molecular masses (Fig. 3, lanes 3 and 7). In addition, BA stimulated the accumulation of two polypeptides – 17 and 18 kDa. Incubation of cotyledons with the mixture of MeJA and BA inhibited strongly the accumulation of both MeJA-inducible polypeptides (60 and 43 kDa), as well as of the high-molecular polypeptide band with Mr 97.4 kDa (Fig. 3, lanes 4 and 8). The resulting polypeptide profile was very similar to the spectrum of soluble proteins obtained after treatment with BA alone (Fig. 3, lanes 3 and 4; lanes 7 and 8). In order to further analyse the cytokinin counteraction on the MeJA-induced changes in the polypeptide profile, cotyledons were subsequently treated with the two plant growth regulators. If cotyledons were incubated with MeJA for 24 h and then transferred to a solution of BA for another 24 h, the ap-

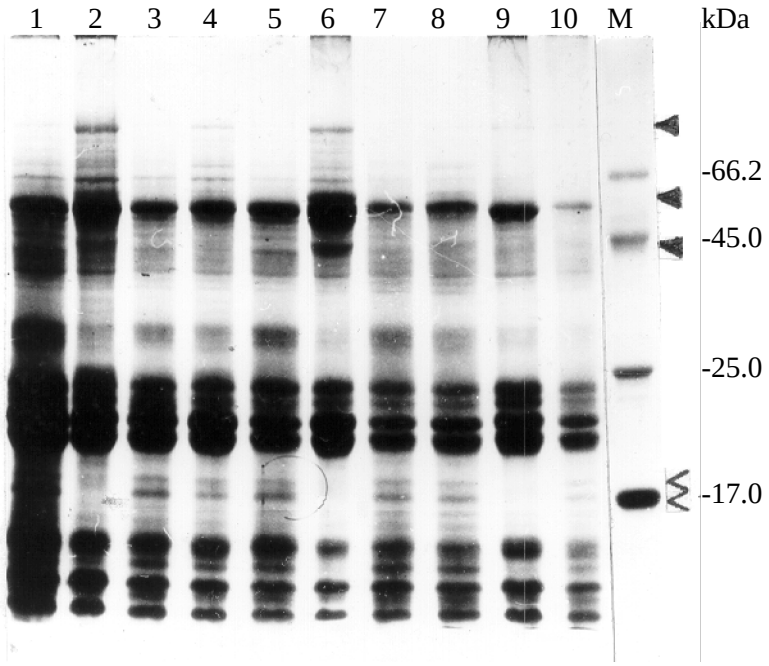


**Fig. 3**. Polypeptide profiles of total soluble proteins extracted from detached marrow cotyledons floated on water, MeJA (45 µM), BA (45 µM) and their mixture (45 µM + 45 µM) in the light. Polypeptides were separated by 12% SDS-PAGE. Key to lane numbers: 24 h incubation on: water (1), MeJA (2), BA (3), BA + MeJA (4); 48 h incubation on: water (5), MeJA (6), BA (7), BA + MeJA (8); treatment with MeJA for 24 h followed by BA for 24 h (9), treatment with BA for 24 h followed by MeJA for 24 h (10). Solid arrowheads indicate the positions of MeJA-affected polypeptides with Mw 97.4, 60 and 43 kDa, respectively. Open arrowheads indicate BA-affected polypeptides. M – molecular weight markers.

pearance of the MeJA-induced polypeptides was prevented (Fig. 3, lane 9). Besides, in this case the specific for BA two low molecular polypeptide bands were not observed. On the other hand, the incubation with BA for 24 h followed by MeJA for another 24 h resulted in a profile similar to that of the cytokinin variant (Fig. 3, lane 10). Therefore, regardless of the sequence of treatment, BA was able to counteract the MeJA-induced changes in the profile of soluble proteins, including the formation of JIPs. The data on the interaction between jasmonates and cytokinins concerning the polypeptide formation available in literature are quite insufficient. Weidhase et al. (1987b) showed by non-denaturing gel electrophoresis that BA could not prevent or restore the formation of JIPs independent of the sequence of substance application in senescing barley leaf segments treated at continuous light. Only one of the MeJA-induced polypeptide bands was suppressed by BA. By contrast, the result obtained in the present study demonstrated that the cytokinin could counteract the JIP forma-

tion in excised greening, not senescing cotyledons. This may be due to the specificity of the model system used in this study. This result was in accordance with our previous data showing that BA was able to neutralize almost completely the MeJA effect on the formation of JIPs in excised marrow cotyledons growing in darkness (Ananieva and Ananiev, 1999).

It is well documented that MeJA-treatment leads to the induction of the synthesis of specific abundant polypeptides – JIPs in different plant tissues (Weidhase et al., 1987b; Herrmann et al., 1989; Reinbothe et al., 1992). This is an expression of its signalling role during the activation of the signal transduction pathways in response to different kinds of abiotic and biotic stresses (Sembdner and Parthier, 1993; Wasternack and Parthier, 1997). Furthermore, a lot of data have proved that JIPs are not directly involved in the process of natural senescence, but they are rather related to plant defence response under stress conditions (Roloff et al., 1994; Harms et al., 1995; Creelman and Mullet, 1997; Metodiev, 1998). The result showing that BA applied either before or after jasmonate treatment can neutralize the JIP formation points to the possibility of cytokinins both to prevent and eliminate the specific stress mediator effect of jasmonates. Consequently, it could be suggested that the counteraction of BA to the formation of JIPs in the excised greening cotyledons may be one of the possible mechanisms in the maintenance of plant cell tolerance to stress factors.

In conclusion, the results obtained in the present study indicate that MeJA and BA cause specific individual qualitative and quantitative changes in protein metabolism in the model system of excised greening marrow cotyledons. Besides, BA when applied together with MeJA can counteract the jasmonate effects both on physiological and molecular events thus suggesting that cytokinins can play a role of antagonists to jasmonates in the regulation of cotyledon development during the earlier stages of germination.

## References

Ananieva, K. I., E. D. Ananiev, 1997. Comparative study of the effects of methyl jasmonate and abscisic acid on RNA and protein synthesis in excised cotyledons of *Cucurbita pepo* L. (zucchini). Bulg. J. Plant Physiol., 23(3–4), 80–90.

Ananieva, K. I., E. D. Ananiev, 1998. Methyl jasmonate-induced polypeptides in excised cotyledons of *Cucurbita pepo* L. (zucchini) and counteraction by cytokinins. Compt. rend. Acad. bulg. Sci., 51(3–4), 123–126.

Ananieva, K. I., E. D. Ananiev, 1999. Effect of methyl ester of jasmonic acid and benzylaminopurine on growth and protein profile of excised cotyledons of of *Cucurbita pepo* L. (zucchini). Biol. Plant., 42(4), 549–557.

Ananiev, E. D., K. I. Ananieva, 2000. Comparative effects of methyl ester of jasmonic acid, abscisic acid and benzyladenine on chlorophyll content and photosynthesis in excised

cotyledons of *Cucurbita pepo* L. (zucchini). Compt. rend. Acad. bulg. Sci., 53(5), 85–88.

Creelman, R. A., J. E. Mullet, 1997. Biosynthesis and action of jasmonates in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol., 48**,** 355–381.

Dathe, W., H. Ronsch, A. Preiss, W. Scade, G. Sembdner, K. Schreiber, 1981. Endogenous plant hormones of the broad bean, *Vicia faba* L. (-)-jasmonic acid, a plant growth inhibitor in pericarp. Planta, 153, 530–535.

Harms, K., R. Atzorn, A. Brash, H. Kuhn, C. Wasternack, L. Willmitzer, H. Pena-Cortes, 1995. Expression of a flax allene oxide synthase cDNA leads to increased endogenous jasmonic acid (JA) levels in transgenic potato plants but not to a corresponding activation of JA-respoding genes. The Plant Cell, 7, 1645–1654.

Herrmann, G., J. Lehmann, A. Peterson, G. Sembdner, R. Weidhase, B. Parthier, 1989. Species- and tissue-specificity of jasmonate-induced abundant proteins. J. Plant Physiol., 134, 703–709.

Klyachko, N. L., E. D. Ananiev, O. N. Kulaeva, 1979. Effect of 6-benzylaminopurine and abscissa acid on protein synthesis in isolated pumpkin cotyledons. Physiol.Veg., 17, 607–617.

Koda, Y., 1992. The role of jasmonic acid and related compounds in the regulation of plant development. Int. Rev. Cytol., 135, 155–199.

Kulaeva, O. N., 1982. Hormonal Regulation of Plant Physiological Processes at the Level of RNA and Protein Synthesis. Nauka, Moscow.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685.

Letham, D. S., L. M. S. Palni, 1983. The biosynthesis and metabolism of cytokinins. Ann. Rev. Plant Physiol., 34, 163–197.

Longo, G., M. Pedretti, G. Rossi, C. Longo, 1979. Effect of benzyladenine on the development of plastids and microbodies in excised watermelon cotyledons. Planta, 145, 209–217.

Lowry, H. O., N. J. Rosebrough, A. L. Farr, R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.

Mans, R. J., C. D. Novelli, 1961. Measurement of the incorporation of radioactive aminoacids into protein by a filter paper disk method. Arch. Biochem. Biophys., 94, p. 4.

Metodiev, M., 1998. Hornet venom induces abundant expression of specific polypeptides in young barley leaves. J. Exp. Bot., 449, 27–32.

Parthier, B., 1979. The role of phytohormones (cytokinins) in chloroplast development. Biochem. Biophys. Pflanz., 174, 173–214.

Parthier, B., 1989. Hormone-induced alterations in plant gene expression. Biochem. Biophys. Pflanz., 185, 289–314.

Porat, R., A. Borochov, A. H. Halevy, 1993. Enhancement of petunia and dendrobium flower senescence by jasmonic acid methyl ester is via the promotion of ethylene production. Plant Growth Regul., 13, 297–301.

Ratajczak, R., I. Feussner, B. Hause, A. Bohm, B. Parthier, C. Wasternack, 1998. Alteration of V-type $H^+$-ATPase during methyljasmonate-induced senescence in barley (*Hordeum vulgare* L. cv. Salome). J. Plant Physiol., 152, 199–206.

Reinbothe, S., A. Machmudova, C. Wasternack, C. Reinbothe, B. Parthier, 1992. Jasmonate-induced proteins in cotton: immunological relationship to the respective barley proteins and homology of transcripts to late embryogenesis abundant (Lea) mRNAs. J. Plant Growth Regul., 11**,** 7–14.

Roloff, A., B. Parthier, C. Wasternack, 1994. Relationship between degradation of ribulose-bisphosphate carboxylase/oxygenase and synthesis of an abundant protein of 23 kDa of barley leaves (*Hordeum vulgare* cv. Salome) induced by jasmonates. J. Plant Physiol., 143, 39–46.

Sembdner, G., B. Parthier, 1993. The biochemistry and the physiological and molecular actions of jasmonates. Ann. Rev. Plant Physiol. Plant Mol. Biol., 44, 569–589.

Ueda, J., J. Kato, 1980. Isolation and identification of a senescence-promoting substance from wormwood (*Arthemisia absinthium* L.). Plant Phys., 66, 246–249.

Ueda, J., J. Kato, H. Yamane, N. Takahashi, 1981. Inhibitory effect of methyl jasmonate and its related compounds on kinetin-induced retardation of oat leaf senescence. Physiol. Plant., 52, 305–309.

Ueda, J., J. Kato, 1982. Inhibition of cytokinin-induced plant growth by jasmonic acid and its methyl ester. Physiol. Plant., 54, 249–252.

Wasternack, C., B. Parthier, 1997. Jasmonate-signalled plant gene expression. Trends Plant Sci., 2, 302–307.

Weidhase, R. A., J. Lehmann, H. Kramell, G. Sembdner, B. Parthier, 1987a. Degradation of ribulose-1,5-biphosphate carboxylase and chlorophyll in senescing barley leaf segments triggered by jasmonic acid methylester, and counteraction by cytokinin. Physiol. Plant., 669, 161–166.

Weidhase, R. A., H. Kramell, J. Lehmann, W. Liebisch, W. Lerbs, B. Parthier, 1987b. Methyljasmonate-induced changes in the polypeptide pattern of senescing barley leaf segments. Plant Sci., 51, 177–186.

Yamane, H., J. Sugawara, Y. Suzuki, E. Shimamura, N. Takahashi, 1980. Synthesis of jasmonic acid related compounds and their structure-activity relationships on the growth of rice seedlings. Agric. Biol. Chem., 44, 2857–64.