JASMONIC ACID LEVELS AND SEED DEVELOPMENT IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

***Mitko I. Dimitrov*[1], *Anthony A. Donchev*[1], *Kolyo G. Kolev*[2] *and Christo D. Christov*[2]**
[1]*Institute of Biophysics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[2]*Institute of Plant Physiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
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Summary. Using gas–mass chromatography analyses (GS-MS) we have determined the endogenous levels of jasmonic acid (JA) in developing sunflower seeds. The level of JA was higher in the early phases of seed development. We have registered a maximum on day 5 after pollination. Spraying of plants with JA at the time of flowering induced marked changes in protein content. Five days after treatment total protein content decreased by 33% whereas 15 day after flowering it exceeded the control values by 33%. In addition, the results from the SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) revealed qualitative and quantitative changes in the spectra of soluble proteins.

***Key words*:** electrophoresis, jasmonic acid, protein, seed development, sunflower.

INTRODUCTION

Jasmonic acid and its methyl ester belong to the plant growth regulators modulating various developmental processes in plants (Koda, 1992; Wasternack and Hause, 2002; Ananiev et al., 2003; Ananieva and Ananiev, 2003). On the other hand, JA is an essential element in the signal transduction pathways in response to different kinds of abiotic and biotic stress (Sembdner and Parthier, 1993; Wasternack and Parthier, 1997; Wasternack and Hause, 2002). Jasmonates cause dramatic alterations in gene expression. It has been shown that JA promotes pollen maturation and dehiscence

* Corresponding author, e-mail: mitkod@obzor.bio21.bas.bg

during the flowering thus ensuring successful fertilization and seed set (Sanders et al., 2000; Devoto and Turner, 2003).

When applied exogenously jasmonates affect the level of vegetative storage proteins, VSPs) (for reviews see Staswick, 1994; Wasternack and Parthier, 1997). The participation of JA in protein metabolism of reproductive organs is obscure; the level of endogenous JA there remains to be determined more accurately.

Our previous investigations have shown that treatment of sunflower plants with JA during flowering increases the seed yield. In extracts from seeds of treated plants alterations of the proportion of fatty acids in favor of linoleic acid in particular has been registered (Christov et al., 1993).

In the present work, we have determined the level of endogenous JA and JA derivatives as well as the effect of the exogenously applied JA on protein profile of sunflower seeds during the earliest stages of development.

MATERIALS AND METHODS

Sunflower plants of cv. *Peredovic* were grown on alluvial-meadow soil pH 5.6 in the Experimental Field of the Institute of Plant Physiology, Sofia**.** The fertilizer application was done taking into consideration the conditions for optimal root nutrition, such as availability of slightly hydrolyzable nitrogen and mobile phosphorus and potassium (Pandev et al., 1981). Plants were sprayed with 50 :M JA in the middle of flowering.

The analysis of endogenous JA content was carried out with kernels harvested at the end of flowering and by days 5 and 15 after pollination. The extraction medium contained 80% methanol (v/v). The homogenates obtained were filtered through a layer of Celite, frozen, thawed up, centrifuged and fractioned in a medium of acidified ethyl acetate with pH 2.5. The fractions received were subsequently resolved by column chromatography on DEAE-Sephadex A-25 using a discontinuous gradient of acetic acid against 80% methanol. Aliquots of the fractions obtained were tested for inhibitory activity using the method of Tan-ginbozu rice seedlings. Endogenous JA was identified in the fraction eluted by 0.25 N acetic acid, subsequently passed through C_{18} reversed-phase cartridges and purified further by thin-layer chromatography on silicagel GF_{254} plate run in a mixture of n-hexane:ethyl acetate:acetic acid, 6:4:1. Spots containing jasmonates were visualized by anisaldehyde reagent. The endogenous JA derivatives were methylated with ethereal diazomethane and passed through a gas chromatograph (Hewlett-Packard) supplemented with mass-spectrometer (GC-MS).

The protein analyses were carried out with kernels harvested at days 5 and 15 after pollination. The extraction medium contained 2% Triton-X-100 (w/v), 0.15 M KCl and 0.1 M Na-phosphate buffer, pH 6.85. Aliquots of 0.5 g were homogenized

in 7.5 ml of the medium by stirring 30 min at 40°C. The homogenate was clarified by centrifugation for 30 min at 15000 rounds per minute (centrifuge Janetzki K-24) and concentrated up to 0.5 ml using an "Amicon" devise for ultra-filtration, supplied with a membrane DIAFLO^R YM-2. The sample was rinsed with four volumes of deionized water and was kept frozen at –25°C. After thawing the sample was again centrifuged. For removal of Triton-X-100 the protein was precipitated in 20% trichloroacetic acid, rinsed twice with chilled ethanol and dissolved in the appropriate buffer.

Absorbance spectra were registered using a spectrophotometer "SPECORD UV-VIS" (Karl Zeiss, Jena, Germany). For spectral measurements aliquots of the samples were diluted with distilled water in a ratio1:5. Protein concentrations were determined by the absorbance registered at 260 and 280 nm using the relation of Darbre and Clamp (1989).

SDS-PAGE was performed according to Fling and Gregerson (1986). A laboratory build apparatus with gel-plate dimensions of 83/79/1 mm was used. Separating gels contained gradient of acrylamide of 8-25% or 12-25% (w/v). The sampling conditions were according to Piccioni et al. (1982). The sample buffer contained lithium dodecyl sulfate (LDS) instead of sodium dodecyl sulfate (SDS). 15 мl of samples with 0.1-0.5 mg ml^{-1} protein were loaded on the gel slots. Electrophoresis was run using a LKB 1809 Power Supply. The protein bands were stained with Coomassie R-250 "Bio-Rad" and scanned on a "Shimadzu CS-930" apparatus using a regime of transition light at 580 nm.

RESULTS

Endogenous JA was identified in developing seeds of *Helianthus annuus* L. 5 days after flowering using the established procedures (Grabner et al., 1976; Dathe et al., 1989). At the early stage of purification we used the Tan-ginbozu rice seedlings bioassay for identification of the JA inhibitory activity which was registered in the fraction eluted by 0.25 N acetic acid (data not shown). The methylated derivative of JA was analyzed by GC-MS. The mass spectra of ionization states obtained from the 0.25 N acetic acid fraction resolved (2) and that of methyl di-2-epijasmonate as a standard (1) are shown in Fig.1. Library algorithm calculations revealed higher than 88% identity between the spectrum of putative endogenous JA and that of the standard. The gas chromatograms relative to plant materials harvested at the end of flowering (1), on day 5 after pollination (2) and on day 15 after pollination (3) are shown in Fig.2. The peak areas are indicative for the level of endogenous JA. At the end of flowering it was relatively low in comparison with later stages of seed development (Fig.2, 1). The maximum value of endogenous JA content was registered on day 5after pollination (Fig.2). During the next stage of seed development JA levels decreased (Fig.2, 3). These results confirm previously reported data showing high lev-

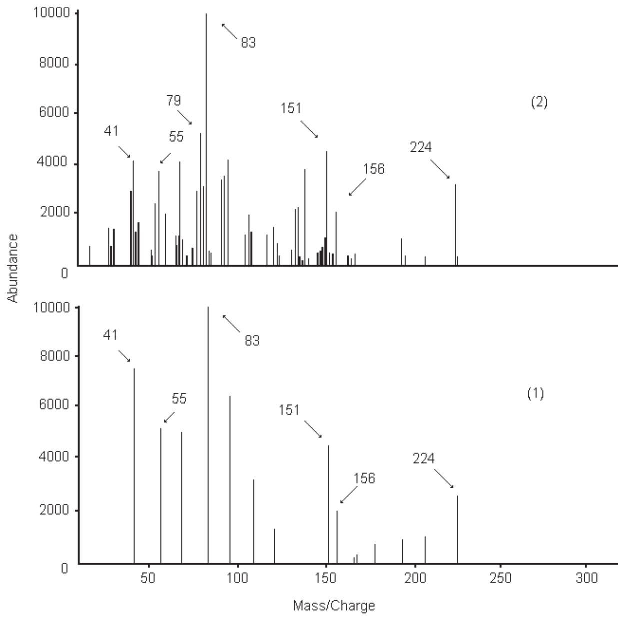

Fig.1. Comparison of mass spectra of JA-standard (1) and endogenous JA isolated from seeds of *Helianthus annuus L* (2). Both samples were methylated prior to GC-MC.

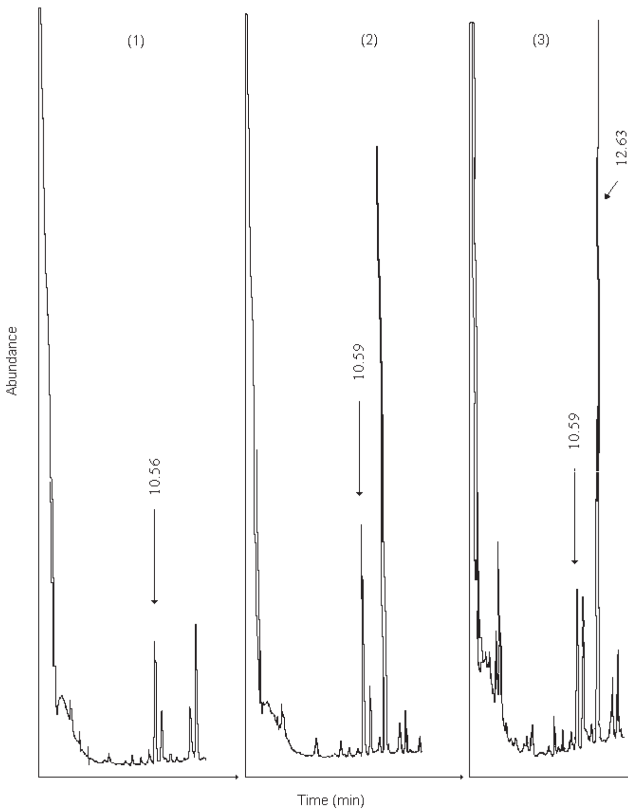

Fig.2. Gas chromatography of JA-derivative compounds isolated from seeds of *Helianthus annuus L,* harvested as follows: (1) at the end of flowering; (2) 5 days after pollination; (3) 15 days after pollination.

els of JA in the pericarp, hilum and testa tissues of soybean during seed development (Creelman and Mullet, 1995; Lopez et al., 1987).

Spraying the plants with 50 μM JA resulted in distinctive variations in protein content of developing seeds. Fig.3 shows the UV-absorption spectra of protein extracts at different stages of seed development. JA applied at the end of flowering induced substantial changes in protein content which were time-dependent. Protein content measured on day 5 after JA treatment was approximately 33% lower when compared with the control. Fifteen days after spraying, however, protein content increased and even exceeded the control values by 33%. In addition, some small decrease in protein levels of the respective two controls (5- and 15-day-old control plants) was also registered. The spectral picture was typical for protein solutions with predominant presence of tyrosil residues (main and additional maxima at 277 nm and 284 nm, respectively), (Herskovits, 1967).

The results from the SDS-PAGE analyses are summarized in Table1. Three of the components designated as *a, d* and *j* dominated in the protein sample of the 5-day JA-treated plants and were suppressed or fully absent in the other three samples (the two controls and the 15-day JA-treated plants). Similar distinctions were visible for the 15-day JA-treated protein sample whose dominant fractions *c* and *e* were suppressed in the other three samples (the two controls and the 5-day JA-treated plants). On the other hand, the fractions *b* and *f* occurring in the JA-untreated control samples disappeared after JA treatment.

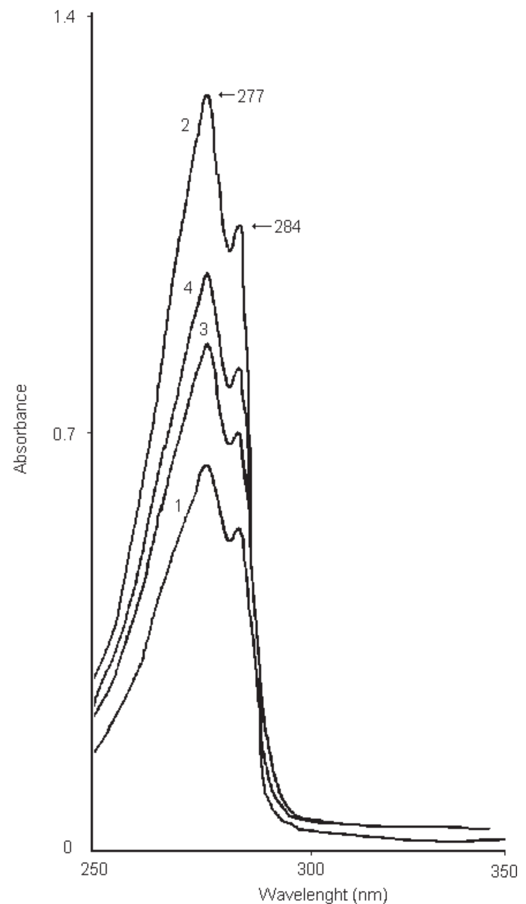

Fig.3. UV-absorption spectra of protein solutions extracted from seeds of *Helianthus annuus L,* harvested as follows: (1) and (2) - on days 5 and 15 after spraying with JA, respectively; (4) and (3) - controls.

Table 1. Molecular weights (M.W.) and relative contents of some protein components resolved by SDS-electrophoresis. M.W. of the protein components were determined using “Pharmacia” low molecular weight calibration kit: 94, 67, 43, 30, 20.1 and 14.4 kDa.

Symbol	M.W. (kDa)	Relative content (%)
a	70.5	11
b	54.8	6
c	49.3	8
d	44.7	6
e	38.4	8
f	12.2	7
j	10.2	21

DISCUSSION

It is known that the level of endogenous JA in plant tissues varies as a function of type of tissues, stage of development and external stimuli. The highest levels were registered in flowers and reproductive tissues (for reviews see Creelman and Mullet, 1995). In the present study, we showed that the level of endogenous JA in developing sunflower seeds is higher during the early stage of seed developing. The maximum value was registered on day 5 after pollination. Furthermore, a significant reduction in JA levels was registered 15 days after pollination. The period studied overlaps the stage of intensive synthesis of seed storage reserves. The exogenous JA affects protein profiles in young seeds. A decrease in the relative protein amount accompanied by the appearance of specific fractions with molecular mass of 70.5, 44.7 and 10.2 kDa were found to occur 5 days 5 days after JA treatment. Wilen et al. (1991) have found that seed storage proteins, napin and cruceferin, could be expressed in *Brassica napus* embryo cultures as a result of JA treatment. The JA responsive polypeptide with molecular mass of 44.7 kDa is very similar to the major jasmonate-inducible polypeptide in *C. pepo* (zucchini) cotyledons with molecular mass of 43.0 kDa (Ananieva and Ananiev, 1999). Further experiments are needed to characterize these JA-responsive polypeptides in sunflower.

In memory of Dr. Anthony A. Donchev whose untimely death was a great loss for our scientific team.

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