

## Role of Jasmonic Acid and Abscisic Acid Treatments in Alleviating the Adverse Effects of Drought Stress and Regulating Trypsin Inhibitor Production in Soybean Plant.

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**Abstract:** Jasmonic acid and abscisic acid are important intracellular regulators mediating diverse developmental processes, such as seed germination, leaf abscission and senescence. In addition, they are essential mediators in triggering some plant responses to abiotic stresses including drought, salinity and cold. How hormones interact to coordinate these responses which are referred to as signalling crosstalk is an area receives intense interest from plant physiologists. The aim of the present work is to study the possible roles of treatments with jasmonic acid (100 $\mu$ M), abscisic acid (100  $\mu$ M) and their combination (100  $\mu$ M JA + 100  $\mu$ M ABA) on alleviating the harmful effects of drought stress on endogenous phytohormones, polyamines, total soluble protein and protein banding pattern of soybean plant (*Glycine max* L cv. Giza 22). An additional point of interest was to detect the change in trypsin protease inhibitor production in response to treatment with different concentrations of jasmonic acid and abscisic acid using ELISA technique. We found that both jasmonic acid and abscisic acid ameliorate the adverse effects of drought stress on soybean plant, but treatment with jasmonic acid was more efficient. We also revealed that production of trypsin inhibitor in soybean plant could take place via a JA- or ABA-dependending signalling pathway.

**Key words:** Jasmonic acid – abscisic acid - drought stress - hormone– polyamines – protein electrophoresis – JIP – VSP - trypsin inhibitor – ELISA.

### INTRODUCTION

Drought is one of the main environmental factors limiting plant growth and crop production. Water stress causes visible injuries to leaves, induces stomatal closure, leaf rolling and osmotic adjustment. Water stress causes a faster decline in chlorophyll and protein content, altering both the structure and function of membranes (Wang, 1999).

Jasmonic acid (JA) is a naturally occurring growth regulator found in higher plants. Several physiological roles have been described for this compound (or related compound, methyl jasmonate) during plant development and in response to biotic and abiotic stresses by inducing the biosynthesis of defense proteins and protective secondary metabolites (Creelman and Mullet, 1995).

Jasmonates (i.e., JA or its methyl ester) can modulate many physiological events, such as resistance responses to pathogens and insects, pollen development, root growth and senescence (Sasaki *et al.*, 2000). These substances can also activate the expression of several genes, leading to the accumulation of their products, which are referred to as jasmonate-induced proteins (Benedetti *et al.*, 1998). The best studied jasmonate – induced proteins include proteinase inhibitors (Lawrence and Koundal, 2002), thionins (Thi2.1; Vignutelli *et al.*, 1998), vegetative storage proteins (VSPs; Benedetti *et al.*, 1995), lipoxygenases, ribosome – inactivating proteins, enzymes of phenylpropanoid metabolism, and others (Devoto and Turner, 2003).

Plant proteinase inhibitors (PIs) are polypeptides or proteins that occur naturally in a wide range of plants and are considered to be an essential part of plant's natural defense system against herbivores (Zavala *et al.*, 2004).

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Recent development in the field of proteinase inhibitors and the increasing use of these phytochemicals in industry and pharmacy has lead searchers to inquire closely into the exact biological functions (Farmer and Ryan, 1990). These inhibitors are believed to make plants less palatable, even lethal, to insects, thus conferring some selective advantage to the plants (Sasikiran *et al.*, 2002).

Soybean trypsin inhibitor (STI), a serine proteinase inhibitor, has played a key role in the early study of proteinases, having been used as the main substrate in the biochemical and kinetic work that led to the definition of the standard mechanism of action of proteinase inhibitor (Meester *et al.*, 1998 and Lawrence and Koundal, 2002). Evidence for the in planta defective function of trypsin protease inhibitors (TPIs) comes from observations of enhanced herbivore resistance after heterologous TPI expression or the manipulation of signal cascades that activate numerous defense response including TPI production (Zavala *et al.*, 2004).

Absciscic acid (ABA), an apocarotenoid synthesized from cleavage of carotenoids, is involved in the signal transduction pathway regulating several genes that are expressed at specific developmental stages or as a result of an environmental stresses. ABA accumulates in vegetative cells in response to water deficit, salinity, cold temperature, and light variations, was thought to act as a signal for the initiation of acclimation to these stresses (Xiong *et al.*, 2002 and Swiatek *et al.*, 2003).

The present work aims to study the role of jasmonic acid and absciscic acid in alleviating the adverse effects of drought stress and in regulating trypsin inhibitor production in soybean plant.

## MATERIALS AND METHODS

The experimental plant used in the present work is *Glycine max* L. cv Giza 22. The seeds were kindly supplied by Crop Institute, Agriculture Research Centre, Ministry of Agriculture. Absciscic acid, jasmonic acid and trypsin inhibitor were supplied from SIGMA.

### **Extraction, Separation and Estimation of Growth Regulating Substances:**

The method of extraction was essentially similar to that adopted by Shindy and Smith (1975) and described by Hashem (2006).

To estimate the amounts of acidic hormones IAA, ABA and GA<sub>3</sub>, the plant hormone fractions and standard ones were methylated according to Vogel (1975) to be ready for GC analysis. Flame ionization detector was used for identification and determination of acidic hormones using Helwett Packered Gas Chromatography (5890). The chromatography was fitted and equipped with HP-130 mx 0.32 mm x 0.25 mm capillary column coated with methyl silicone. The column oven temperature was programmed at 10°C/min from 200°C (5 min) to 260°C and kept finally to 10 min. Injector and detector temperature were 260 and 300°C, respectively. Gases flow rates were 30, 30, 300 cm/sec for N<sub>2</sub>, H<sub>2</sub> and air, respectively and flow rate inside column was adjusted at 2 ml/min. On the other hand JA was determined according to Kramell (1996) using NUCLEODEX beta-PM, 200 mm, and 4mm ID column, flow rate adjusted at 1ml/min and detected at UV 210 nm. Standards of IAA, GA<sub>3</sub>, ABA and JA were used.

Peak identification was performed by comparing the relative retention time of each peak with those of IAA, GA<sub>3</sub>, ABA and JA standards. Peak area was measured by triangulation and the relative properties of the individual components were therefore obtained at various retention times of samples. On the other hand, cytokinin fractions (zeatin and zeatin riboside) were extracted as previously mentioned for the acidic hormones and were detected by HPLC isocratic UV analyzer, ODS Hyparsil C<sub>18</sub> column, 20 min gradient from 0.1 N acetic acid pH 2.8 to 0.1 N acetic acid in 95% aqueous ethanol; pH 4. The flow rate: 1 ml/min, detection: UV 254 nm, standards of zeatin and zeatin riboside were used and the peak area of the standard was also used in identification and detection of cytokinin in each sample (Müller and Hilgenberg, 1986).

### **Measurement of Ethylene:**

The method of ethylene determination was essentially similar to that adopted by Kao and Yang (1983). The plant material used for ethylene measurement was 30-days old soybean plants subjected to different treatments and those of control ones.

Soybean leaves were incubated in 14-ml vials containing filter paper moistened with 1 ml of deionised water and sealed with rubber serum caps. The vials were incubated in darkness at 27°C on a rotating shaker (100 rev. /min) for 6 h (Lutts *et al.*, 1996).

For the measurement of ethylene a gas chromatograph (Perkin Elmer) equipped with flame ionization detector was used. The GC was fitted with stainless steel column (8 mm x 1 m) packed with aluminium oxide (alumina Porapak) with 40-60 mesh alumina. The temperature of injector was 80°C and the column works well

with a nitrogen gas at a flow rate of 33 ml/min. It is often necessary to bake the column over night at around 150 to 180°C with a slow flow of carrier gas to condition it for best performance and prevent column contamination with associated gases such as ethane. The temperature of oven and detector were 120°C & 90°C, respectively.

The proper concentrations of ethylene were obtained by comparing their respective peak area in the sample with their corresponding area obtained with the authentic ethylene samples. The peak area and ethylene concentrations were measured automatically by the software attached to the GC.

#### ***Estimation of Polyamines:***

Putrescine, spermine and spermidine were extracted and determined in all tested samples according to Maijala and Eerola (1993), and Ayesh *et al.*(2002) using one dimensional TLC to separate the formed dansylamines. Ten micro litres of standard dansylamine and the sample extract were spotted 2cm from the base of the TLC plates using a Hamilton micro syringe. The plate was developed using chloroform: benzene: triethylamine (6: 4.5: 1) for 17cm highest. The plate was dried at room temperature until the excess of solvent disappeared. The resulting zones were examined and marked under long ultraviolet wavelength (365nm). The marked areas were determined in Microanalysis Centre, Faculty of Science, Cairo University using CS-9000 dual wavelength flying spot scanning densitometer (SHIMADZU) using wavelength 254nm. Standard curve of each dansylamine was used to calculate the concentrations of biogenic amines in the tested samples.

#### ***Estimation of Total Soluble Proteins:***

The total soluble protein concentration was determined Spectrophotometrically using the Bio-Rad protein assay which is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein (Bio Rad Technical Bulletin 1051, 1977).

#### ***Protein Electrophoresis:***

The extracted protein samples were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A solution of 12% SDS-polyacrylamide slab gel was prepared according to the method of Laemmli (1970) as modified by Studier (1973) and equal volumes of proteins extracted were loaded.

Protein samples were mixed with equal volume of sample buffer, and denaturated by heating at 80-90°C for 3-5 minutes followed by immediate cooling on ice and loaded onto the gel. Electrophoresis was carried out at about 80 volts for the stacking gel layer and at about 100 volts for the separating gel layer in 1 X Tris/glycine-SDS-running buffer.

SDS-gels were stained overnight in 200 ml of Commassie brilliant blue R-250 staining solution on shaker. To destain the gel, 200 ml of destaining solution was used. The gel was gently agitated on a shaker for 2 hours. This destaining procedure was repeated several times until the background colour of the gel was removed.

Scanning of the separated protein bands was analysed by the Gel Documentation System (GDS) which comparing polypeptide maps; molecular protein markers, band intensity and molecular weight of each polypeptide in relation to standard markers using gel proanalyzer version 3 MEDIA CYBERNE TICE Imaging Experts Software.

#### ***ELISA Detection:***

JA and ABA-induced accumulation of trypsin inhibitor (TI) in total soluble protein of differently treated soybean leaves was detected using the indirect-enzyme linked immunosorbent assay (indirect ELISA) as mentioned by Regenmortel and Burckard (1980) and Koenig (1981). An ELISA plate was coated with 200µl per well of anti-TI antibody in coating buffer pH 9.6 (1µl/ml), and then the plate was incubated overnight at 4°C. This was followed by washing 5 times (three minutes for each) with washing buffer. Samples with 200µl per well, 2 wells per sample as replicates were added at a dilution of 1:1000 in phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) and incubated at 37°C for 3 hours.

Plates were washed 5 times as mentioned above. Alkaline phosphatase- conjugate (Anti-mouse IgG developed in goat, SIGMA Cat# A-3562) was added at a dilution of 1:30000 in conjugate buffer pH 7.4 (200µl per well) and then incubated at 37°C for 3 hours.

Plates were washed 5 times as mentioned above and then 200µl of the substrate buffer (p-nitrophenyl phosphate 1mg/ml in 10% diethanolamine, pH 9.8) prepared immediately before use was added for each well. The plates were incubated at room temperature for 15-30 minutes or as long as necessary to observe reaction. The reaction was stopped by adding 50µl of 3M NaOH to each well.

The ELISA values were determined at a wavelength 405nm by the Bio-Rad ELISA reader model 3550.

### **Growth Conditions:**

Firstly an experiment was carried out to investigate the effect of drought stress (40% of the water holding capacity) on endogenous phytohormones, polyamines, total soluble protein, and protein banding patterns of soybean plant (*Glycine max* L.cv.Giza 22) and the possible effect of treatment with jasmonic acid (100  $\mu$ M), abscisic acid (100  $\mu$ M) and their combination on alleviating the harmful effect of drought stress.

This experiment was conducted in the research farm, Faculty of Science, Ain Shams University. Uniform sterilized seeds of *Glycine max* L.cv.Giza 22 were sown on 1/6/2004 in 30 cm diameter plastic pots containing equal amounts of homogenous soil (2 clay : 1 sand). Each pot contained 12 seeds. After 10 days, thinning was carried out so as to leave equal number of uniform plants (5 seedlings) at a suitable distance in each pot for experimentation. Plants were exposed to normal daylight and temperature. The water holding capacity in each pot was kept at 80%. Pots were supplied with half strength Hoagland nutrient solution once a week. Two weeks old plants were divided into 4 groups, the plants of the first group were sprayed with water to serve as controls, while the plants of the second groups were sprayed with 100  $\mu$ M JA. The plants of the third group were sprayed with 100 $\mu$ M ABA and the plants of the fourth group were sprayed with a mixture of 100 $\mu$ M JA + 100 $\mu$ M ABA. Plants were sprayed with equal amounts according to their treatment till dripping. One week later, water-sprayed plants were divided into 2 subgroups; the plants of the first subgroup were subjected to 80% water holding capacity of the soil (to serve as a control), whereas plants of the second subgroup and the other three groups were subjected to drought stress (40% of the water holding capacity). This system of watering was continued throughout the experimental period.

Plants were harvested at random from different treatments at the vegetative stage (36 days after sowing) and were analyzed to determine endogenous phytohormones, polyamines contents, and total soluble proteins. Leaf samples for the detection of protein banding patterns were taken from plants at both the vegetative and flowering stages(50 days after sowing).

A second experiment was carried out to investigate the effect of treatment with different concentrations of JA and ABA on trypsin inhibitor production in soybean plants; in this experiment soybean plants were exposed to normal daylight and temperature. The water holding capacity of soil in each pot was kept at 80% throughout the experimental period. Pots were supplied with half strength Hoagland nutrient solution once a week.

Two weeks old plants were divided into 2 groups; the plants of the first group were sprayed with different concentrations of jasmonic acid (0.0, 25, 100, 400 and 2000 $\mu$ M) while the plants of the second group were sprayed with 0.0, 25, 100, 400 and 2000 $\mu$ M of abscisic acid. Controls were carried out by spraying with tap water. Plants were sprayed with equal amounts according to their treatment till dripping (5ml/plant). Leaf samples were taken after 24, 48 and 72 hours of treatment for extraction of total soluble proteins. The indirect-enzyme linked immunosorbent assay (indirect ELISA) was used to detect the amounts of trypsin inhibitor in the extracted samples.

## **RESULTS AND DISCUSSION**

### ***Role of Jasmonic Acid and Abscisic Acid in Drought Stress Alleviation***

#### ***Effect of Drought Stress on Soybean Plant:***

Drought is one of the most serious world-wide problems for agriculture. Four-tenths of the world's agricultural land lies in arid or semi-arid regions (Collin, 2006). The cellular and molecular responses of plants to environmental stress have been studied intensively (Thomashow, 1999 and Hasegawa *et al.*, 2000). Understanding the cellular machinery to activate adaptive responses is of fundamental importance to biology (Xiong *et al.*, 2002). Recent progress in plant genetic transformation and the availability of potentially useful genes characterized from different sources make it possible to generate stress-tolerant crops using transgenic approaches (Pilon-smits *et al.*, 1995 and Xu *et al.*, 1996).

Phytohormones regulate the protective responses of plants against both biotic and abiotic stresses by means of synergistic or antagonistic actions referred to as signaling crosstalk. A bottle neck in crosstalk research is the quantification of numerous interacting phytohormones and regulators (Schmelz *et al.*, 2003). Data presented in Table (1) demonstrated that IAA and GA3 levels were markedly reduced by 35.70% and 49.36%, respectively in drought stressed soybean plants raised at 40% water holding capacity of soil compared with those of the control (80% WHC).

These results indicate that drought stress appeared to inhibit the biosynthesis of auxins and gibberellins and /or increase their degradation (Poljakoff-Mayber and Lerner, 1993). Furthermore, Shi *et al.* (1994) working on maize seedlings proved that under water stress induced by polyethylene glycol, IAA content declined from 225 to 142ng/g DW, the content of GA3 decreased from 223 to 132 ng/g DW, while ABA content increased from 655 to 1875ng/g DW.

On the other hand, JA and ABA contents (Table 1) were increased by 56.55% and 33.83%, respectively in response to the same treatment (40% WHC) as compared with control values. These results are in a good agreement with those of Lehmann *et al.* (1995) who showed that treatment of *Hordeum vulgare* with sorbitol, mannitol, sucrose, put comma glucose and the neutral desiccation agent polyethylene glycol (PEG) caused an increase in endogenous JA and ABA, similar results were obtained by Xin *et al.* (1997) working on maize plants. Also, Finch-Savage *et al.* (1996) proved that the concentration of JA, MeJA and ABA progressively increased during drying in both cotyledons and axes of whole seeds of *Quercus robur*.

In addition, our results (Table 1) show that, plants grown under drought stress conditions exhibited pronounced increases in the detectable amounts of zeatin, zeatin riboside and total cytokinins over the control value (80% WHC) by 23.35%, 76.23% and 61.58, respectively. Similar results were obtained by El-Meiegy *et al.* (1999) who found that kinetin can partially counteract the adverse effects of drought stress in *Arachis hypogaea* plant.

With regard to ethylene content, it has been found in the present work that this content was increased by 11.59 fold in drought stressed plants (40% WHC) compared with the control value (80% WHC). These results are in a good agreement with those of Mcjeon *et al.* (1982) and Chrominski *et al.* (1988).

Therefore the increase in jasmonic acid, abscisic acid, ethylene and cytokinin contents are adaptive responses and safeguard mechanisms of soybean plants to cope with drought stress.

Polyamines play pivotal roles in plant defense to environmental stresses. They play an important role in maintaining membrane and nucleic acid integrity under most of the stress conditions (Erdei *et al.*, 1996). However, both ionic deficiency and salinity, and osmotic stresses may influence polyamine metabolism in different manners and polyamines may have different and specific functions under these stress conditions (Zhou *et al.*, 1995). In this respect, data recorded in Table 2, illustrated that drought stress has a stimulatory effect on putrescine, spermidine and spermine contents; the magnitude of induction was estimated by 69.85%, 452.95% and 51.78%, respectively as compared with their corresponding control values. Similar results were obtained by Kasukabe *et al.* (2004) working on transgenic *Arabidopsis thaliana* who found that plants exhibited a significant increase in spermidine synthase activity and spermidine content in leaves showing enhanced tolerance to various stresses including drought, salinity, freezing and hyperosmosis. These results strongly suggest an important role for spermidine as a signalling regulator in stress signalling pathways, leading to build-up of stress tolerance mechanisms in plants under stress conditions.

The results reported in the present study emphasized that drought stress caused highly significant decreases in total soluble protein contents (Table 3) in both shoots and roots of the tested plants (the decrease was estimated by 25.657% and 46.392% in shoots and roots, respectively). The highly significant increases in Proline accumulation in both shoots and roots (data are not shown) may suggest that drought stress has a stimulatory effect on protein hydrolysis resulting in proline accumulation and/or inhibited protein synthesis. This conclusion is in a good agreement with that obtained by Singh *et al.* (1994) on *Vigna radiate* and Maharaj and Sudhansha (1995) on pea.

#### **Role of Abscisic Acid in Drought Stress Alleviation:**

The phytohormone abscisic acid (ABA) regulates many agronomically important aspects of plant growth and development, including seed maturation, dormancy, stress tolerance, and water relations (Rock, 2000 and Finkelstein *et al.*, 2002). All of these processes are regulated by additional signals, including other phytohormones, stage-specific regulators, and abiotic stresses. Studies of ABA-deficient mutants have shown that ABA is an essential mediator in triggering some plant responses to abiotic stresses, including drought, salinity, and cold (Xiong and Zhu, 2001). Dehydration and low temperatures result in elevated levels of ABA, which, in turn, trigger the synthesis of some proteins responsible for drought or freezing tolerance (Brocard *et al.*, 2002).

Concerning the effect of ABA treatment on endogenous phytohormones of soybean plants, our results (Table 1) clearly demonstrated that drought stressed plants treated with ABA showed a marked increase in their endogenous jasmonic acid and ABA contents as compared with the control plants (80% WHC) or the untreated drought stressed plants. In this respect, ABA is proposed to activate JA biosynthesis, producing higher levels of endogenous JA which, in turn, activate the expression of various sets of genes during water deficit conditions, osmotic stress, wounding and pathogen attack (Reinbothe *et al.*, 1992 and Blechert *et al.*, 1995). Analyzing ABA-deficient tomato and potato plants has demonstrated that the site of action of JA is located downstream of the site of action of ABA (Peña-Cortés *et al.*, 1995). However, the mechanism by which ABA may affect JA biosynthesis is still unknown, possibly by affecting composition of lipid membranes (Peña-Cortés

*et al.*, 1996). Similar to our results Sharma *et al.* (2002) working on sorghum seedlings found that, seedling grown without exogenously supplemented ABA showed a slightly lower level of endogenous ABA in comparison to that of seedlings grown with exogenously supplemented ABA medium.

Concerning the effect of ABA treatment on polyamine contents of soybean plants, our results clearly demonstrated that a marked increase was detected in putrescine contents in response to ABA treatment as compared with the values of control or untreated drought stressed plants. However, the values of spermidine and spermine obtained in response to ABA treatment were lower than that detected in untreated drought stressed soybean plants indicating the inhibitory effect of ABA on spermidine and spermine biosynthesis. In this respect, Perez-Amador *et al.* (2002) detected a transit increase in the level of free putrescine followed the increase in the mRNA level for arginine decarboxylase 2 (ADC2) in ABA-treated *Arabidopsis* plants.

#### **Role of Jasmonic Acid in Drought Stress Alleviation:**

It has been observed in the present work that, the detectable amounts of acidic hormones GA<sub>3</sub> and JA showed marked increase in response to JA treatment. These results are in a good agreement with those of Ranjan and Lewak (1992) who found that JA leads to embryo germination through gibberellin accumulation and alkaline lipase activity. Also, Traw and Bergelson (2003) found that gibberellin and jasmonic acid had a synergistic effect on the induction of trichomes in *Arabidopsis*, suggesting important interactions between these two hormones. Sasaki *et al.* (2001) identified 41 jasmonate-regulated genes (JRGs) in *Arabidopsis* (using cDNA macroarray), whose mRNA levels were changed (elevated or decline), by three fold in response to MeJA. Among the 41-JRGs identified, 5 genes were JA biosynthesis genes. These results suggested the existence of a positive feedback regulatory system for JA biosynthesis and the possibility of cross-talk between JA signaling and other signaling pathways. Similar result was obtained by Bell and Mullet (1993) who proved that AtLOX2 (*Arabidopsis thaliana* lipoxygenase2) mRNA accumulation is rapidly induced in leaves in response to methyl jasmonate. Recently, Wu *et al.* (2004) suggested that TaAOS (*Triticum aestivum* allene oxide synthase) mRNA could be strongly induced by exogenous JA, and the highest level of JA was observed after 10h induction.

In contrast, the amounts of IAA and ABA, according to the present work, were significantly decreased in plants treated with jasmonic acid as compared with untreated plants. Similar results were obtained by Schmelz *et al.* (2003) who proved that corn earthworm (CEW) herbivory on corn seedlings resulted in a significant 3.1-fold increase in JA levels and a 1.3-fold decrease in IAA levels. Lincoln *et al.* (1990) and Tiryaki and Staswick (2002) suggested that jasmonate and auxin might use a similar signalling mechanism. The isolation and characterisation of the *axr1-24* (auxin-resistant mutant) support the hypothesis that JA and auxin might act through a common signalling intermediate (Devoto and Turner, 2003). Anderson *et al.*, (2004) working on *Arabidopsis* found that there are antagonistic interactions between multiple components of ABA and the JA-ethylene signalling pathways modulate defence and stress responsive gene expression in response to biotic and abiotic stresses.

In contrast to our results, Grsic *et al.* (1999) concluded that JA may involve in the up-regulation of three enzymes important for IAA synthesis (tryptophan oxidizing enzyme, which catalyzes the first step in tryptophan-dependent auxin biosynthesis in brassicaceae, nitrilase and myrosinase). In addition, Rakwal and Komatsu (2005) studied the effect of exogenous application of jasmonic acid on rice plant and proved that, cutting or treating leaf sheaths with JA rapidly increased the endogenous level of ABA. Conversely, another study by Buta *et al.* (1996), in which chilling injury in *Cucurbita pepo* fruit could be delayed by application of methyl jasmonate or ABA. MeJA had no consistent effect on endogenous ABA contents.

Concerning the effect of JA treatment on cytokinins contents, it has been found that jasmonic acid treatment caused marked increases in zeatin, zeatin riboside and total cytokinins contents (Table 1). It has been also shown that jasmonic acid induced a stimulatory effect on ethylene contents of soybean plants. Similar results were obtained by Skrzypek *et al.* (2005) who found that MeJA stimulated ethylene production of the first internodes of tulips up to more 5 times at days 1 and days 3 after application. Also, Zhao *et al.* (2004) proved that methyl jasmonate treatment can induce ethylene production, whereas ethylene does not induce jasmonate biosynthesis. During fruit ripening the increase in endogenous jasmonates occurs simultaneously with initial detection of ethylene biosynthesis, indicating that jasmonates may interact with the ethylene signal. To test whether the jasmonate effect requires ethylene action, intact apple fruits were treated with MeJA and an ethylene inhibitor; diazocyclopentadiene (DACP). MeJA alone increased ethylene production, the inhibitor alone inhibited ethylene production, but fruits treated with the combination of DACP and MeJA had the lowest ethylene production indicating that DACP blocked the promotive effect of MeJA on ethylene production (O'Donnell *et al.*, 1996).

**Table 1:** Effect of jasmonic acid, abscisic acid and their combination on acidic hormones, cytokinins and ethylene contents of soybean plant (*Glycine max.* L.cv. Giza 22) subjected to drought stress at vegetative stage.

Treatment	Acidic hormones ( $\mu\text{g } 100\text{g}^{-1}$ f.wt.)				Cytokinins ( $\mu\text{g } 100\text{g}^{-1}$ f.wt.)			Ethylene n mole $\text{g}^{-1}$ $\text{h}^{-1}$
	IAA	GA3	JA	ABA	Zeatin	Zeatin riboside	Total cytokinins	
80% W.H.C (control)	104.05	121.74	69.43	17.96	71.11	185.56	256.66	0.24
40% W.H.C.(drought-stressed plant)	66.91	61.66	108.69	24.04	87.71	327.01	414.72	2.769
40% W.H.C + 100 $\mu\text{M}$ JA	6.78	330.68	314.21	11.6	407.04	491.12	898.16	6.43
40% W.H.C. + 100 $\mu\text{M}$ ABA	2.67	57.71	176.97	94.47	59.24	164.8	224.04	2.804
40% W.H.C. + 100 $\mu\text{M}$ JA + 100 $\mu\text{M}$ ABA	3.35	129.67	218.3	55.17	259.48	426.66	686.15	3.98

Earlier reports point to an involvement of jasmonates in altering cellular titres of free putrescine, spermidine and spermine. According to our results putrescine content detected in soybean plants increased in response to jasmonic acid treatment, whereas spermidine and spermine contents were markedly decreased in plant treated with jasmonic acid compared with the untreated drought-stressed plants (Table 2).

**Table 2:** Effect of jasmonic acid, abscisic acid and their combination on polyamine contents of soybean plant (*Glycine max.* L.cv. Giza 22) subjected to drought stress at vegetative stage. Values listed are expressed as mg 100g-1dry weight.

Treatment	Putrescine	Spermidine	Spermine	Total polyamines
80 % WHC (control)	13.830	8.650	11.530	34.010
40 % WHC (drought-stressed plant)	23.490	47.830	17.500	88.820
40 % + 100 $\mu\text{M}$ JA	119.630	18.070	11.840	149.540
40% + 100 $\mu\text{M}$ ABA	43.219	17.379	12.27	72.868
40% + 100 $\mu\text{M}$ JA + 100 $\mu\text{M}$ ABA	129.452	15.693	10.784	155.929

Perez-Amador *et al*(2002) have studied the expression of genes involved in polyamine biosynthesis in response to mechanical wounding and methyl jasmonate treatment in *Arabidopsis* and proved that arginine decarboxylase 2 (ADC2) is the only gene of polyamine biosynthesis involved in the wounding response mediated by JA. They also detect a transient increase in the level of free putrescine followed the increase in the mRNA level for ADC2, and observed a decrease in the level of free spermine, coincident with the increase in putrescine.

In whole rice plants, MeJA induced the accumulation of putrescine and spermine while spermidine levels decreased (Lee *et al.*, 1996), whereas in tobacco cell culture levels of putrescine increased while spermidine and spermine did not change (Imanishi *et al.*, 1998).

The altered levels of free polyamines occurred in response to treatment with jasmonate may be due to changes in the activities and/or transcript levels of the main biosynthetic enzymes, arginine decarboxylase, ornithine decarboxylase and S-adenosylmethionine decarboxylase (Imanishi *et al.*, 1998 and Mader, 1999). Nevertheless, a complete picture of the effects of jasmonates on polyamine metabolism is still lacking, and the data are as yet partly contradictory.

#### **Effect of Treatment with Ja plus ABA in Drought Stress Alleviation:**

It has long been obvious that hormones do not function in discrete pathways, but rather exhibit extensive cross-talk and signal integration with each other and with environmental and developmental signalling pathways (Gray, 2004).

Phytohormones regulate the protective responses of plants against both biotic and abiotic stresses by means of synergistic or antagonistic actions (Schmelz *et al.*, 2003).

Results obtained from the current study reveal that, when soybean plants treated with JA plus ABA they affect plant metabolism synergistically or antagonistically. Their antagonistic effect appears in the detected amounts of endogenous GA3, ABA and total cytokinins contents. Whereas, they act synergistically to increase putrescine content, and to decrease spermidine and spermine contents (Table 2).

**Table 3:** Effect of jasmonic acid, abscisic acid and their combination on total soluble protein contents of soybean plants (*Glycine max.* L.cv. Giza 22) at vegetative stage. Values listed are expressed as mg  $\text{g}^{-1}$  fresh weight. Each value is a mean of 3 samples.

Treatment	Shoot	Root
80 % WHC (control)	18.350	11.558
40% WHC (drought-stressed plant)	13.642**	6.196**
40 % + 100 $\mu\text{M}$ JA	17.06**	5.796**
40% + 100 $\mu\text{M}$ ABA	13.872**	2.906**
40% + 100 $\mu\text{M}$ JA + 100 $\mu\text{M}$ ABA	15.66**	5.11**
LSD at 5%	0.0634	0.0152
LSD at 1%	0.096	0.0231

Finally, we concluded that ABA treatment caused a partial alleviation to the harmful effect of drought stress (40%WHC) on soybean plants, whereas jasmonic acid caused a complete recovery to most of the drastic effects of water deficit on soybean plants. However, the values of the detected metabolic activities in response to treatment with JA plus ABA were in most cases between their values in plants treated with JA or ABA alone. The values obtained were closer to those of JA alone than to ABA indicating the predomination of JA effect.

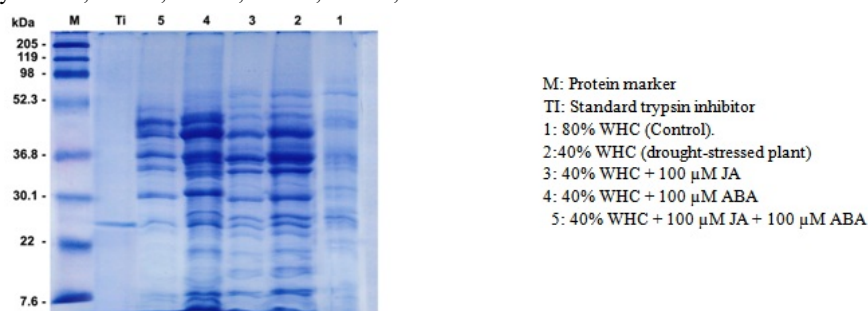
**-Effect of Drought Stress, Jasmonic Acid and / or Abscisic Acid on Protein Banding Patterns of Soybean Plant (*Glycine max L.cv. Giza 22*):**

Various processes of plant development or of plant responses to biotic and abiotic stresses exhibit induction of gene expression via jasmonate alone or in concerted manner with other hormones, such as abscisic acid (ABA) (Wasternack *et al.*, 1998).

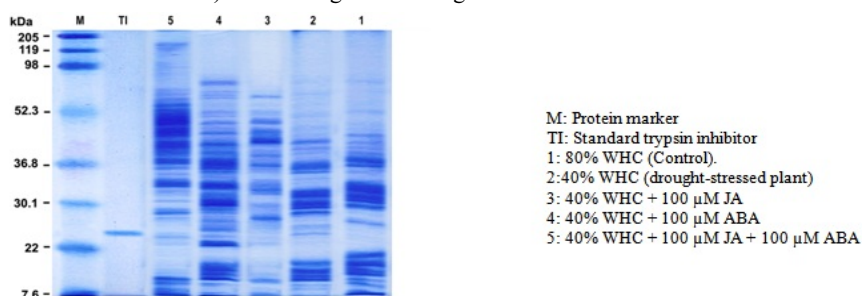
Reinbothe *et al.* (1994) proved that whether released endogenously upon pathogen attack or stress treatment or applied exogenously, jasmonates induce numerous plant defence and stress proteins and simultaneously lowered or even shut down the expression of many genes. Jasmonate-induced protein (JIP) genes are rapidly transcriptionally activated, and the resulting JIP mRNAs are preferentially translated compared to most pre-existing mRNAs (Reinbothe *et al.*, 1994).

The present work is an attempt to distinguish the role of both plant growth regulators (JA and ABA) and to highlight the similarities and differences between them in plant stress response and gene expression; determined by the changes in one dimensional SDS-PAGE

According to the results obtained from the present work (Plates 1&2 and Table 4), using one dimensional SDS-PAGE, 6 JIPs were detected at vegetative and flowering stages of plant growth; their molecular weights were estimated by 38.85, 32.91, 27.25, 12.78, 11.85, and 9.81kDa.



**Plate 1:** Effect of drought stress, jasmonic acid and abscisic acid on protein banding patterns of soybean plant (*Glycine max. L.cv. Giza 22*) at the vegetative stage.



**Plate 2:** Effect of drought stress, jasmonic acid and abscisic acid on protein banding patterns of soybean plant (*Glycine max. L.cv. Giza 22*) at the flowering stage.

Low molecular weights JIPs are suggested to be proteinase inhibitors. In this respect, many authors proved that jasmonic acid and its methyl ester (MeJA) induced the synthesis of inhibitor I and II proteins and mRNA in a manner similar to wounding (Farmer *et al.*, 1992 and Doares *et al.* 1995) working on tomato plants.

The JIP with molecular weights 27.25 kDa is most probably vegetative storage protein (VSP). In this respect, Mason and Mullet (1990) pointed to the expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding and jasmonic acid treatment. The gene VSPA and VSPB encode VSP $\alpha$  and VSP $\beta$ , vacuole-localized polypeptides of 28 and 31 kDa, respectively. In addition, Franceschi and Grimes (1991) found that low levels of atmospheric MeJA induced accumulation of three VSPs in soybeans with molecular masses of 27, 29 and 94kDa (vsp27, vsp29 and vsp94, respectively).

**Table 4:** Relative area (%) of each protein band of leaves of soybean plants exposed to drought stress, jasmonic acid and/or abscisic acid treatments.

(A) at the vegetative stage .						
40% WHC + 100 µM JA + 100 µM ABA	40% WHC+ 100 µM ABA	40% WHC+ 100 µM JA	40% WHC (drought stressed plant)	80% WHC (Control)	TI	Mol.wt. (kDa)
	2.37	4.04	4.07	5.78		63.29
		4.16	3.31	4.83		51.78
6.46	4.6	5.86	5.18	5.31		49.61
						48.09
15.41	5.11			6.58		46.26
				6.42		44.25
13.03	15.25	10.09	9.84			42.58
5.36		5.3	5.18			39.16
				9.65		37.98
13.38	7.17	8.94	7.79			36.76
	5.69	6.19	6.74	5.46		35.66
8.51	4.3	7.43	8.3	6.07		34.4
4.25		4.97				33.66
	3.78					32.77
3.45	2.09	3.17	3.33	5.61		32.02
	5.37			4.59		30.83
9.32	3.15	7.1	7.72			29.85
	3.68			5.3		27.44
		5.36	4.79	6.9		26.30
4.36	4.9	2.92	4.27	5.86		25.40
2.97	2.28	2.53	3.15		100	24.50
	1.92					23.04
	2.05			4.49		22.08
		2.14	2.29	2.45		21.36
			3.26			20.55
			1.84			19.57
	1.92					18.37
						16.34
	1.74	2.96	3.58			15.27
	1.91		2.59			13.41
		2.65				12.78
	2.27					11.85
	1.62					10.65
			3.34			10.25
		2.94				9.81
4.44	3.48	3.38	4.15	4.58		8.72
				2.75		8.24
2.69	3.36					7.89
6.36	7.86	7.72	7.55	2.76		2.28
14	25	20	20	18		Total Band

The other JIPs of molecular weights 38.85 and 32.91 kDa are probably not proteinase inhibitor proteins because no plant proteinase inhibitors are known to be larger than about 25 kDa (Farmer *et al.*, 1992). These proteins may be vegetative storage proteins or may possibly be enzymes associated with the signal pathways for induction of the proteinase inhibitors or may be proteins involved in other defence responses.

The polypeptides with molecular weights 27.25 and 11.85 kDa were also found to be induced in response to ABA; indicating that there is a similarities in ABA- and JA-induced gene expression, suggesting overlapping functions for these plant growth regulators. In addition, the promoter elements of many genes regulated by abscisic acid (ABA) are similar to those found in JIP genes. They contain cis-regulatory regions that are closely related or even identical to the G-box motif (Williams *et al.*, 1992). G-box thus seems to be essential to confer both jasmonic acid and ABA-regulated transcriptional control. However, there are an increasing number of reports demonstrating distinctions between the responses to JA and ABA (Hildmann *et al.*, 1992 and Moons *et al.*, 1997). The present study also highlighted this distinction between JA- and ABA-induced proteins as there are about 3 JIPs were seems to be specific to JA (M.wt. 38.85, 12.78 and 9.81 kDa) and non of them detected in ABA-treated plants. Moreover, a number of proteins were proved to be induced only in response to ABA treatment (M.wt. 49.82, 23.04, 18.37, 15.08 and 10.66 kDa).

Three high molecular weight proteins were newly synthesized at the flowering stage only in drought stressed plants treated with JA+ABA (148.17, 96.85 and 55.72 kDa). Indicating that JA or ABA alone was insufficient to induce these proteins but they must work synergistically to produce these proteins under drought stress conditions. These proteins might be involved in plant adaptation to drought stress.

**Table 4:** (B) at the flowering stage.

40% WHC + 100 µM JA + 100 µM ABA	40% WHC+ 100 µM ABA	40% WHC+ 100 µM JA	40% WHC (drought stressed plant)	80% WHC (Control)	TI	Mol.wt. (kDa)
2.42						148.17
2.16						96.85
	3.37			4.3		76.79
2.05	1.75					68.32
3.08	2.08	3.25	3.81			64.95
3.38	1.98					60.95
	2.09	3.31	2.91			57.11
4.05						55.72
4.56	2.32	3.9	3.13	4.09		51.76
	2.01					49.82
5.3	2.88	6.15		2.61		48.1
5.12	2.78	5.17	3.89	3.01		46.08
4.94	2.55			3.29		44.54
		8.33	5.07	3.63		42.65
6.8	3.63		3.65	4.02		41.27
4.1	3.48	4.61	3.03	3.1		39.79
		3.69				38.85
4.67	4.04			6.22		37.53
2.97	3.26	5.38	7.49	4.96		36.41
2.31	3.43	3.99	4.25	3.11		35.18
3.27	3.01	4.33	3.44	4.96		34.01
3.77	6.33	5.11				32.91
2.32	2.81	4.41	9.23	6.4		31.98
		3.81		5.84		31.19
2.19	5.45	4.77	5.82			29.98
2.64	3.11		3.11	4.86		29.25
2.7	2.38		4.37			28.26
2.06	2.08	6.34				27.25
				5.52		26.39
	3.88	3.38	2.92			25.49
1.77	2.29	2.65	2.83		100	24.50
2						23.71
2.38	4.99		2.25			22.88
2.05	2.1			6.99		19.2
	3.14	2.98	5.49	7.55		17.89
	3.92		5.61			16.57
						15.08
						14.61
2.36	4.18	3.32		4.48		13.83
						13.45
3.74	2.3		5.55	3.11		12.3
		5.1				11.85
2.76	2.54					10.87
			2.58	3.34		10.5
3.32	1.89		3.36			9.26
2.75	1.94	3.66	6.21	4.62		7.45
31	33	23	23	22		Total Band

Moreover, a protein band with molecular weight 60.95 kDa was detected in drought stressed plants treated with ABA and show an over expression in drought stressed plants treated with JA plus ABA. The JIP60 was previously identified by Reinbothe *et al.* (1994) as a defence protein and a potent regulator of protein synthesis in stressed plant tissues.

Our data (Plates 1&2 and Table 4A&B) also shows that water deficit induced *de novo* synthesis of three polypeptides with molecular weights 19.57, 14.61 and 10.25 kDa. It has been hypothesized that the induced proteins may be related to the activation of metabolic pathways which allows the maintenance of the cellular turgor or of the integrity of cellular and sub-cellular structures (Grillo *et al.*, 1994). In addition, the diversity of the reported proteins indicates the complexity of the water stress response phenomenon in plants. Vilardell *et al.* (1990) and Close *et al.* (1993) identified a dehydrin of molecular weight about 20 kDa in maize plants. Such dehydrin may be the 19.57 kDa band that detected in the present work. Dehydrins are predicted to act as stabilizers (chaperone-like) prevent or reduce the denaturation of other cellular macromolecules under dehydration conditions (Campbell *et al.*, 1998).

Concerning the effect of drought stress, treatment with JA and /or ABA on trypsin inhibitor production (M.wt. 24.5 kDa), it was found that trypsin inhibitor was induced in untreated plants subjected to drought stress or treated with JA and/or ABA at the vegetative and flowering stages. Sanchez *et al.* (2004) working on amaranth found that trypsin inhibitor activity was increased by exposure to diverse treatments, particularly water stress, salt stress, insect herbivory and treatment with exogenous methyl jasmonate (MeJA) or abscisic acid (ABA) which also induced trypsin inhibitor activity accumulation.

**- Effect of Jasmonic Acid and Abscisic Acid on Regulating Trypsin Inhibitor Production in Soybean Plant:**

In order to protect themselves against insects and other herbivores, plants have evolved a variety of defence mechanisms. Induction of defences in plants involves complex signalling pathways, and usually requires rapid changes in gene expression (Agrawal *et al.*, 1999).

Serine proteinase inhibitors have been described in many plant species, and are universal throughout the plant kingdom, with trypsin inhibitors being the most common type (Ryan, 1990 and Zavala *et al.*, 2004). Similar to other inducible herbivore defense systems, trypsin inhibitor (TI) appears to be regulated via the octadecanoid pathway (Farmer and Ryan, 1991). The results obtained from the present study (Table 5) proved that, jasmonic acid plays a key regulator role in trypsin inhibitor production in soybean plants indicated by the increase in foliar trypsin inhibitor contents in JA-treated plants as compared with untreated control ones.

**Table 5:** The ELISA values of trypsin inhibitor determination in soybean leaves treated with different concentrations of jasmonic acid after 24, 48 and 72 hours of treatment. Each value is a mean of 3 replicates.

Samples	Jasmonic acid concentration (µM)				
	0	25	100	400	2000
24 h	0.534(100%)	1.605(301%)	1.829(343%)	1.633(306%)	1.478(277%)
48 h	0.368(100%)	0.779(212%)	1.184(322%)	0.561(152%)	0.503(137%)
72 h	0.345(100%)	0.672(195%)	0.914(265%)	0.547(159%)	0.481(139%)

Similar results were obtained by Delano-Frier *et al.* (2004) working on amaranth (*Amaranthus hypochondriacus*) who found that foliar trypsin and alpha-amylase inhibitors accumulate by treatment with exogenous jasmonic acid in controlled laboratory conditions. Also, exogenous application of JA significantly increased trypsin inhibitor activity in wild mustard (*Brassica kaber*) (Cipollini and Sipe, 2001).

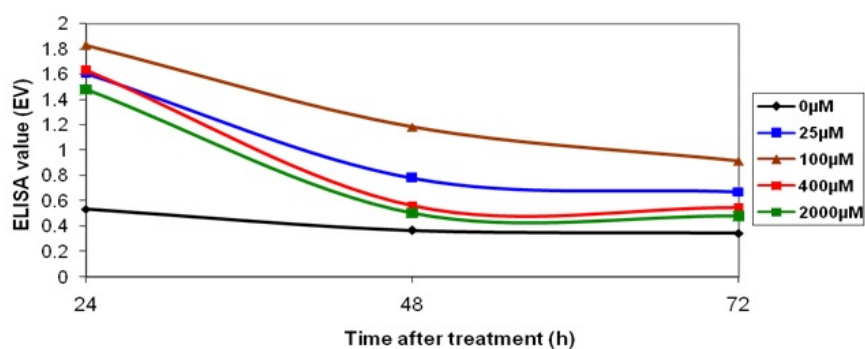
In addition, *Populus tremuloides* trypsin inhibitor (PtTI) was found to be induced by wounding and herbivory, permitting rapid adaptive responses to herbivore pressure. The response appears to be mediated by an octadecanoid-based signaling pathway, as methyl jasmonate treatments induced the trypsin inhibitors (Haruta *et al.*, 2001).

The results obtained in the present work demonstrated that all the applied concentrations of jasmonic acid increased trypsin inhibitor production in soybean leaves as compared with untreated control values. The maximum trypsin inhibitor production was observed after 24 h of treatment and decreased by time; indicating that the trypsin inhibitor production reached the highest values after 24 h of treatment or less in JA- treated plants.

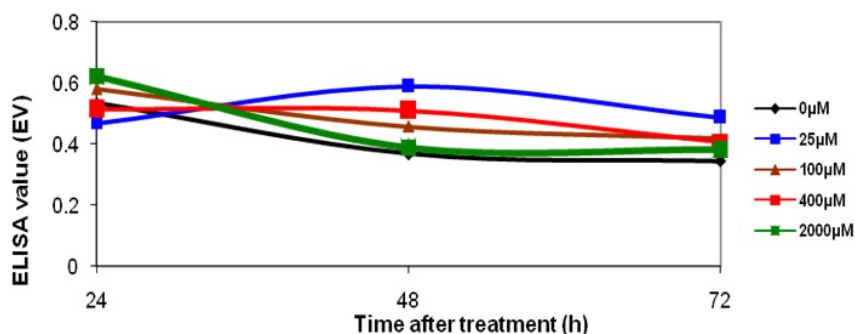
Foliar spraying treatment with different concentrations of ABA (Table 6) caused an accumulation of trypsin inhibitor in soybean leaves as compared with untreated control plants indicating that ABA has a positive effect on trypsin inhibitor production. This increase could be detected after 24h of treatment with 100 and 2000µM ABA and after 48h of spraying with 25 and 400µM ABA. However, the maximum trypsin inhibitor production was in almost all cases detected after 48h of treatment as compared with the corresponding control value; suggesting that after 48h of ABA application trypsin inhibitor reached its peak value then decreased by time in most concentrations.

**Table 6:** The ELISA values of trypsin inhibitor determination in soybean leaves treated with different concentrations of abscisic acid after 24, 48 and 72 hours of treatment. Each value is a mean of 3 replicates.

Samples	Abscisic acid concentration (µM)				
	0	25	100	400	2000
24 h	0.534(100%)	0.468(88%)	0.582(109%)	0.515(96%)	0.622(116%)
48 h	0.368(100%)	0.590(160%)	0.458(124%)	0.510(139%)	0.389(106%)
72 h	0.345(100%)	0.488(141%)	0.418(121%)	0.407(118%)	0.383(111%)



**Fig. 1:** Effect of different concentrations of jasmonic acid on trypsin inhibitor contents of soybean leaves after 24, 48 and 72h of treatment.



**Fig. 2:** Effect of different concentrations of abscisic acid on trypsin inhibitor contents of soybean leaves after 24, 48 and 72h of treatment.

The role of ABA in plant disease resistance has been suggested by many authors (Audenaert *et al.*, 2002 and Mauch-Mani and Mauch, 2005). Penã-Cortés *et al.* (1995) proved that jasmonic acid and abscisic acid are able to induce chymotrypsin/trypsin proteinase inhibitor II gene expression without any mechanical wounding. They initiate Pin2 mRNA accumulation in the directly treated leaves and in untreated leaves (systemic) that are located distal to the treated ones. Also, Sanchez *et al.* (2004) found that trypsin inhibitor activity was increased by exposure to diverse treatments; water stress, salt stress, insect herbivory and treatment with exogenous methyl jasmonate or abscisic acid. In contrast, Casaretto *et al.* (2004) working on barley leaves found that following ABA treatment, chymotrypsin inhibitory activity increased up to 51% over the untreated plants, while trypsin inhibitory activity remained virtually unchanged.

Interestingly, the results obtained in the present investigation clearly demonstrated that soybean leaves sprayed with jasmonic acid have higher trypsin inhibitor contents as compared with those treated with the same concentrations of ABA; indicating that jasmonic acid is more effective in trypsin inhibitor induction than ABA. In this respect, the proteinase inhibitor induction in *Nicotiana attenuata* leaves by different wounding treatments feeding by *Manduca sexta* larvae, methyl jasmonate treatment, and mechanical wounding was reported by Van Dam *et al.* (2001), who observed that the response to methyl jasmonate was stronger and longer lasting than mechanical wounding.

We also found that trypsin inhibitor is a constitutively-produced protein in soybean leaves; it could have a role in regulating the endogenous proteases during plant growth and development. Similar results were obtained by Sasikiran *et al.* (2002) working on sweet potato.

In conclusion, our data suggested that different regulatory mechanisms may participate in the induction of trypsin proteinase inhibitor in soybean plants. It is proposed to be a JA- or ABA-depending signalling pathway.

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