J. Agric. Sci. Mansoura Univ., 34 (1): 269 - 277, 2009

ACTIVITIES OF L-ASPARAGINASE FROM *CICER* COTYLEDONS UNDER DIFFERENT TREATMENTS

El-Shora, H. M. and Laila A. U. El-Naqeeb.
Botany Department, Faculty of Science, Mansoura University, Mansoura, Egypt

ABSTRACT

L-asparaginase (EC 3.5.1.1) activity is measured in *Cicer arietinum* cotyledons. Studying the relation between germination time of *Cicer* and L-asparaginase activity throughout 7 days revealed that L-asparaginase activity increased gradually up to the 5th day of germination after which it is declined. Jasmonic acid (JA) induced L-asparaginase activity and 100 µM was the best concentration for the induction. Treatment of cotyledons with 100 µM JA resulted in the increase of the activity increased throughout the experimental period. The results showed that incubating the cotyledons with 100 µM GA_3 for 24 h resulted in the induction of the enzyme activity. However, incubation with 100 µM ABA for 24 h caused a reduction in the enzyme activity. The induction of L-asparaginase by GA_3 was time-dependent throughout the experimental period of 72 h. The results show that very low activity of the enzyme was recorded in cotyledons exposed to dark in absence of L-asparagine. Actinomycin D, cycloheximide and chloramphenicol inhibited the induction of L-asparaginase activity by GA_3 or kinetin in *Cicer* cotyledons.

INTRODUCTION

The cotyledons of plants mainly contain food materials, which are supplied to the embryo during seed germination. The activity of hydrolytic enzymes in cotyledons increase with germination to release the storage food materials for the growing embryo (Bowley and Black, 1994; Biswas *et al*., 1996).

L-asparagine being an important transport form of organic N in a wide range of species (Ireland & Lea, 1999). L-asparagine predominates in the transport of nitrogen in many legumes studied so far and constitutes an important source of reduced nitrogen for developing seeds (Sieciechowicz *et al*., 1988). In developing pea seeds, L-asparagine is initially metabolized in the seed coats (Murray & Cordova-Edwards, 1984) giving rise to ammonia, alanine and glutamine in the endosperm fluid.

There are two known routes for L-asparagine catabolism. The first, catalysed by L-asparagine transaminase, involves the transamination of the amino group to form 2-oxosuccinamic acid and appears to be important in green leaves where it may play a role in photorespiration (Murray *et al*., 1987; Joy, 1988). The second route involves the release of ammonia from the amide group in a reaction catalysed by L-asparaginase.

As the cotyledons start growing, the cotyledons themselves develop a capacity for metabolizing L-asparagine (Atkins *et al*., 1975). Indeed, isolated pea and soybean cotyledons grow in culture with L-asparagine as sole source of nitrogen (Haga & Sodek, 1987).

In legumes, most of the xylem nitrogen is in the form of L-asparagine, which is made in large quantities in the nodule. Following nitrogen fixation, the ammonia produced by the bacteria is assimilated by GS and much of the glutamine is converted to L-asparagine for transport (Sieciechowicz *et al*. 1988c). Although L-asparagine and glutamine are both amides and differ only in chain length, L-asparagine is more soluble and less reactive than glutamine, and has a higher nitrogen/carbon ratio, making it better suited to its role as a transport and storage compound (Sieciechowicz *et al*., 1988c).

In plants, elevated levels of L-asparaginase activity are detected in developing tissues, such as leaves or roots. The activity of the enzyme from leaves undergoes diurnal changes in order to regulate the amounts of free ammonia that can be efficiently reassimilated and further metabolized in pathways dependent on photosynthesis. Particularly high levels of L-asparaginase expression have been observed in developing seeds, where L-asparagine supplies 50–70% of the required nitrogen (Atkins *et al*., 1975).

In developing legume shoots, fruits, and seeds, which receive much of their nitrogen as L-asparagine, L-asparaginase provides a major source of aspartate, and plays the main role in reallocating transported nitrogen, and in metabolism of L-asparagine that may accumulate during stress (Sieciechowicz *et al*., 1988b).

L-asparagine levels in the developing soybean embryo are tightly correlated with seed protein content at maturity, suggesting a strict control of L-asparagine biosynthesis and catabolism in these tissues (Hernandez-Sebastia *et al*., 2005). The L-asparaginase levels in legume-developing leaves are regulated by the photoperiod with proteolytic degradation occurring at night (Sieciechowicz *et al*., 1988a). L-asparaginase was first detected in the developing seeds of *Lupinus albus* (Atkins *et al*., 1975).

The aim of the present work to investigate the activity of L-asparaginase from *Cicer arietinum* cotyledons under different treatment.

MATERIALS AND METHODS

Plant material

The experimental plant used in this investigation was *Cicer arietinum* L, (chick-pea, family *Liguminosae*). Pure strain of seeds was obtained from Egyptian Ministry of Agriculture.

Seed germination

Seeds were stored for uniformity; very small and large seeds, and broken seeds were discarded. Seeds were germinated according to (El-Shora & Aprees, 1991). Seeds of the different plants were surface sterilized in 10 % sodium hypochlorite for 10 min, soaked in running tap-water for 24 h, and then germinated between paper towels, moistened with distilled water in sterilized plastic trays. The trays were covered and incubated for 48 h in the dark at 25 ˚C. Seeds with well-grown roots were then supported on plastic bowls containing 0.2 mM calcium chloride. Seeds were grown for a further 48 h in the dark at 25 ˚C. The calcium chloride solution was continuously and vigorously aerated. The cotyledons and roots of 5-day old plants were

excised with a razer blade and kept on ice to be used for extraction immediately.

Assay of enzyme

Sample (0.1 ml) of cell suspension or enzyme solution, 0.9 ml of 0.1 M L-asparagine solution were combined and incubated for 10 min at 37 ˚C. The reaction was stopped by the addition of 0.5 ml of 15% (w/v) trichloroacetic acid. After centrifugation, a 0.1-ml portion of the supernatant fluid was diluted to 8 ml with distilled water and treated with 1.0 ml of Nessler's reagent and 1.0 ml of 2.0 M NaOH. The color reaction was allowed to proceed for 15 min before the OD at 500 nm was determined. The OD was then compared to a standard curve prepared from solutions of ammonium source. One unit (U) of L-asparagine is that amount of enzyme which liberates 1 µmol of ammonia in 1 min at 37 ˚C.

The value in this investigation are the main value ± S.E.

RESULTS AND DISCUSSION

Studying the relation between germination time of *Cicer* and L-asparaginase activity thought out 7 days revealed that L-asparaginase activity increased gradually up to the 5th day of germination after which it is declined (data not shown). Therefore, cotyledons from 5-day old seedlings were used in the future experiments for preparation and purification of L-asparaginase enzyme. The cotyledons of plants contain food materials, which are supplied to the embryo during seed germination. The activity of the enzymes in cotyledons increases with germination to release the storage food materials for the growing embryo (El-shora, 2002).

Examining different concentrations of JA on the enzyme activity showed that t6he enzyme activity increased gradually with increasing concentrations up to100 µM. However, the activity decreased at higher concentrations, probably due to the toxicity of JA. Therefore, 100 µM was chosen for future experiment as the best concentration. The results in the present investigation reveal that L-asparaginase of *Cicer* cotyledons is elevated following exogenous JA application. In support, JA treatment resulted in the elevation of the activity of other plant enzymes such as methyltransferase in *Poaceous* plants (Oikawa *et al*., 2002) and phytase in marrow cotyledons (Mohammed, 2003).

It seems likely that JA treatment leads to specific enrichment of the pre-existing enzyme. The mechanism by which this occurs is unknown, but could involve an activation of L-asparaginase either by posttranslational modification, sesequestration or by failure to target this enzyme for proteolytic degradation.

Nevertheless, an effect of JA at either the transcriptional or translational level cannot be excluded. The ability of JA to induce transcriptional activation of a wide range of genes has been well documented (Wasternalk & Parthier, 1977). JA is also able to exert an effect over translational rates, possibly via induction of ribosome-inactivating proteins,

discriminating between JA-upregulated and constitutive transcripts (Reinbothe *et al*., 1994).

The results present in Fig 1 show the effect of duration time of 100 µM JA treatment on the induction of L-asparaginase activity in *Cicer* cotyledons. In control sample, the activity was increased gradually and reached a maximum after 32 h after which decreased gradually. In treated samples, the activity increased continuously throughout the experimental period.

The effect of GA_3 and ABA either singly or in combination on the induction of L-asparaginase activity in cotyledons of *Cicer* was investigated. Each of the two compounds was tested at 100 µmol. The results are listed in Fig 2 revealed that GA_3 was inducer whereas ABA was inhibitor and reduced GA_3-induced activity when they were tested in combination.

GA_3 induced other plant enzymes such as phytase (Gabard & Jones, 1986), phosphoenolpyrovate carboxylase (Bihzad & El-Shora, 1996), NADH-glutamate synthase (El-Shora, 2001) and isoperoxidase (Perez & Gomez, 1998; El-Shora, 2002). Also, ABA inhibited the activities of other plant enzymes such as (Kumar *et al*., 1987) and phytase (Mohammed, 2003).

The results of this experiment indicate that incubating the cotyledons with 100 µM GA_3 for 24 h resulted in the induction of the enzyme activity with a relative activity of 202.8 ٪. However, incubation with 100 µM ABA caused a reduction in the enzyme activity where only 54.4 ٪ of the activity was detected. Testing the two compounds in combination on the induction of the enzyme revealed that ABA reduced GA_3-induced activity of L-asparaginase.

Since GA_3 proved to be inducer for L-asparaginase in *Cicer* cotyledons, an experiment was carried out to study the relation between the enzyme activity and the time of incubation in presence of 100 µmol GA_3. Samples were taken every 12 h and analyzed for the enzyme activity throughout a period of 72 h. The results in Fig 3 show that the induction of L-asparaginase by GA_3 was time-dependent throughout the experimental period of 72 h.

The effect of light and L-asparagine on L-asparaginase in cotyledons treated with GA_3 was investigated. The results are shown in Fig 4. The results indicate that very low activity of the enzyme was recorded in cotyledons exposed to dark in absence of L-asparagine in dark-treated cotyledons where little induction was observed within the first 36 h followed by continuous decline until the end of the experimental period. Considerable enzyme activity was detected in cotyledons exposed to light in the absence of L-asparagine and the addition of L-asparagine showed an additive effect where continuous remarkable increase of the enzyme activity throughout the experimental period was recorded.

This experiment aimed to test the effect of actinomycin D, cycloheximide and chloramphenicol as inhibitors of protein synthesis on the induction of L-asparaginase activity by GA_3 or kinetin in *Cicer* cotyledons. The results in Fig 5 and 6 show that the three compounds inhibited the enzyme activity when cotyledons were incubated with each of them separately. Cycloheximide was the most potent inhibitor followed by

actinomycin D and chloramphinicol under light treatment in presence or absence of L-asparagine.

When the cotyledons were incubated with 100 µmol GA_3 plus the inhibitors, similar results were obtained , however the relative activities was higher than those calculated in the absence of GA_3 .

On incubating the cotyledons with 100 µmol kinetin, there was a remarkable induction of L-asparaginase activity in presence of light and light + L-asparagine However, the induction of the enzyme by kinetin was lower than that by GA_3. The same phenomenon of enzyme inhibition by the three inhibitors was repeated in presence of kinetin. The calculated relative activity was higher than those calculated in absence of phytohormones, and lower than those calculated in presence of GA_3.

It is observed as a general phenomenon that the inhibitory effect of each particular inhibitor either in presence or absence of the phytohormone was higher in presence of light without L-asparagine. In the presence of L-asparagine the inhibitory effect was declined. Light and L-asparagine seem to regulate L-asparaginase activity in cotyledons treated with GA_3. These results indicate that light-dependent of the action of L-asparagine, might remove a specific block in the genome for L-asparaginase or light and L-asparagine could affect the synthesis of aspartate species of mRNA coding for L-asparaginase.

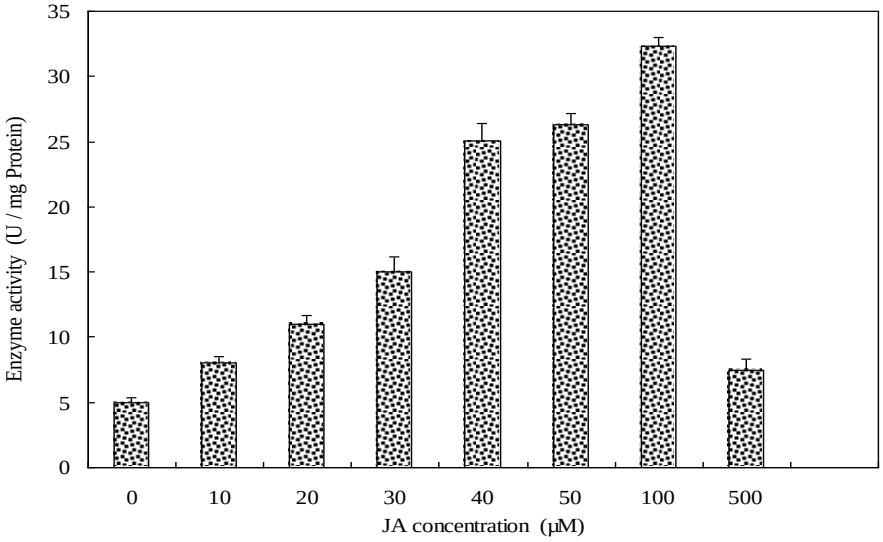

Fig 1: Effect of jasmonic acid (JA) concentration on the induction of L-asparaginase.

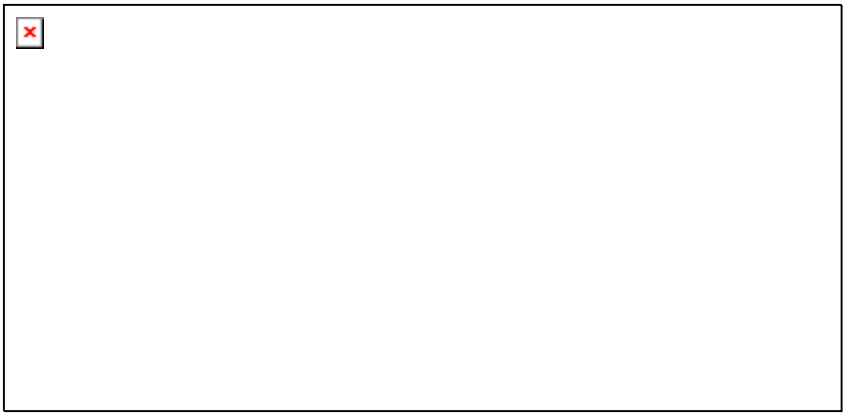

Fig 2: Induction of L-asparaginase activity by GA_3 and its negative reversal by ABA and cycloheximide in cotyledons of light-grown seedlings. Each treatment was for 24 h.

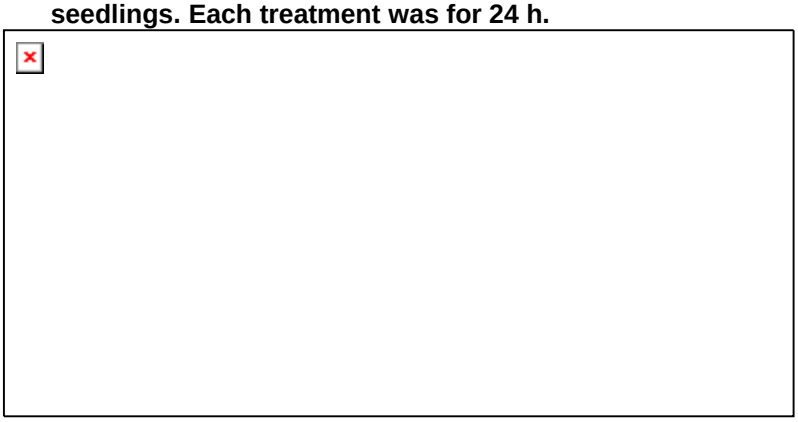

Fig 3 : Time course of development of L-asparaginase activity in response to GA_3.

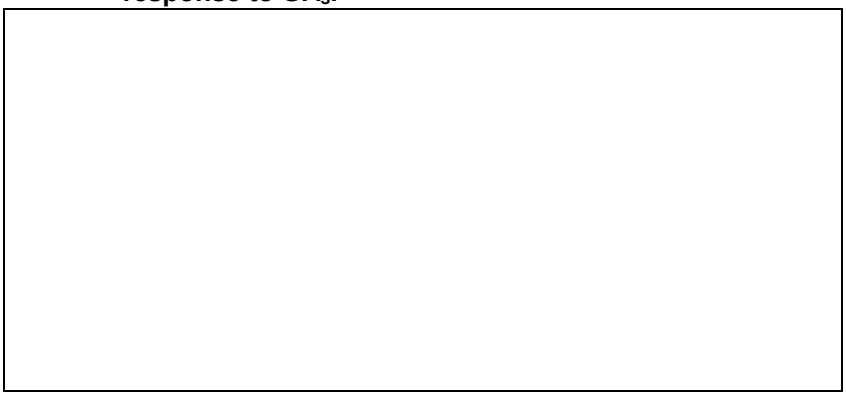

Fig 4: Effect of light and L-asparagine on L-asparaginase activity in GA_3-treated cotyledons.

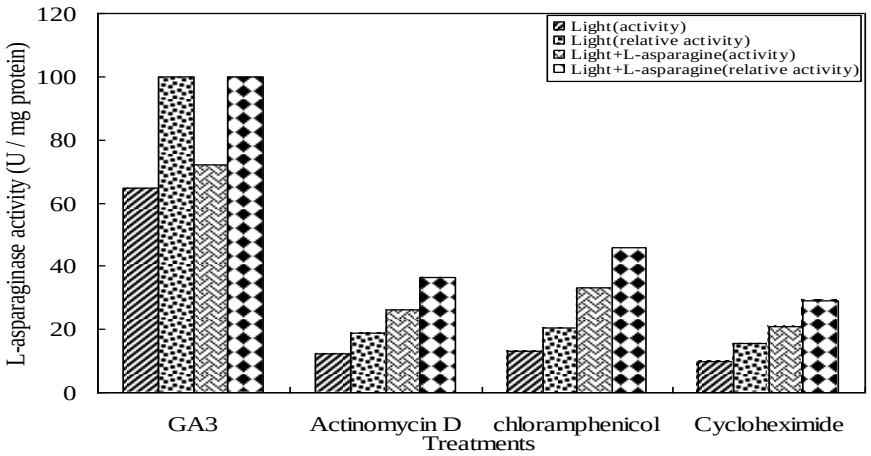

Fig 5 : Effect of actinomycin D, cycloheximide and chloramphenicol on L-asparaginase activity in presence of GA_3.

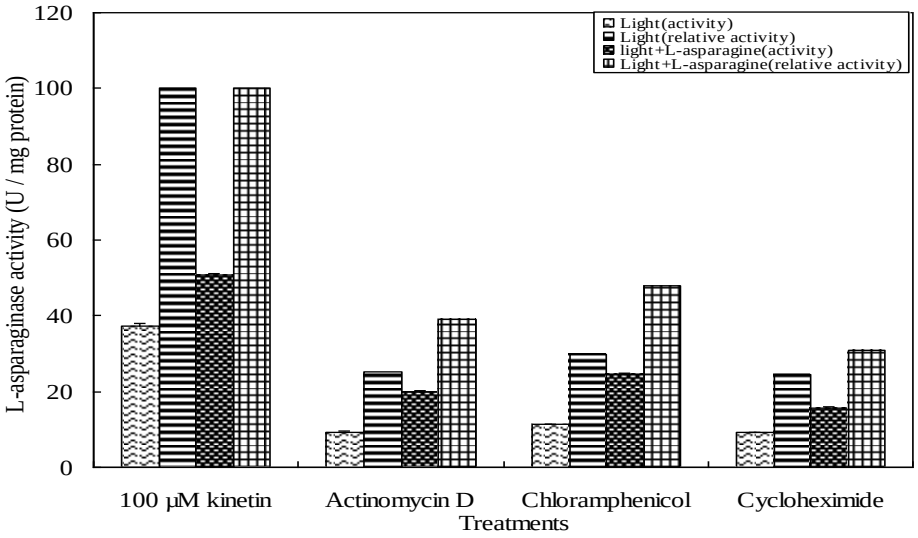

Fig 6 : Effect of actinomycin D, cycloheximide and chloramphenicol on L-asparaginase activity in presence of kinetin.

REFERENCES

Atkins, C. A., Pate, J. S. and Sharkey, P.J. (1975). Asparagine metabolism key to the nitrogen nutrition of developing legume seeds. *Plant Physiol.* 56: 807-812.

Bihzad, M. A. and El-shora, H. M. (1996). Phosphoenol pyruvate carboxylase from *Rumex dentatus a* C_3-plant. *Plant Physiol*. 149: 669-676.

Biswas, T. K., Promo, M. and Biswas, B. (1996). Purification of acid phosphatase from germinating seeds of *Vigna sinensis*. *Phytochem*. 41: 1457-1458.

Bowley, J. D. and Black, M. (1994). Seed physiology, Development and Germination. Second Edition. Plenum Press. New York and London, pp 241-250.

El-Shora, H, M. (2001). Effect of growth regulators and group-modifiers on NADH-glutamate synthase of marrow cotyledons. Biological Sci. 1: 597-602.

El-Shora, H, M. (2002). Properties of phenyl alanine ammonia lyase from *marrow* cotyledons. *Plant sci*. 162: 1-7.

El-Shora, H. M. and ApRees, T. (1991). Intracellular location of NADP-linked malic enzymes in C_3 plants. *Planta*. 185: 362-367.

Gabard, K. A. and Jones, R. L. (1986). Localization of phytase and acid phosphatase isoenzymes in aleurone layers of barley. *Physiologia Plantarum*. 67: 182-192.

Haga, K. I. and Sodek, L. (1987). Utilization of nitrogen sources by immature soybean cotyledons in culture. *Ann. Bot*. 9: 597-601.

Hernandez-Sebastia, C., Marsolais, F., Saravitz, C., Dewey, R.E. and Huber, S.C. (2005). Free amino acid profiles suggest a possible role for asparagine in the control of storage-product accumulation in developing seeds of low- and high-protein soybean lines. *J. Exp. Bot*. 56: 1951-1963.

Ireland, R.J. and Lea, P.J. (1999). The enzymes of glutamine, glutamate, asparagine, and aspartate metabolism. In: Singh BK (ed) Plant Amino Acids: Biochem. and Biotechnol. Dekker, New York, pp 49-109.

Joy, K. W. (1988). Ammonia, glutamine and asparagine: a carbon-nitrogen interface. *Can. J. Bot*. 66: 2103-2109.

Kumar, V., Basra, S. and Malik, C. B. (1987). Enzymes of nonphotosynthetic C_4-dicarboxylic acid metabolism in germinating grains of wheat. *Biochem. Physiol. Pflanzen*. 182: 261-262.

Mohammed, R. A. (2003). Studies on plant hydrolases. M. SC. Thesis, Botany Dept., Faculty of Sci., Mansoura Univ. Egypt.

Murray, D. R. and Cordova-Edwards, M. (1984). Amino acid and amide metabolism in the hulls and seeds of developing fruits of garden pea, *Pisum sativum*. II. Asparagine. *New Phytol*. 97: 253-260.

Murray, A.J.S., Blackwell, R.D., Joy, K.W. and Lea, P.J. (1987). Photorespiratory N donors, aminotransferase and photosynthesis in mutant of barely deficient in serine: glyoxylate aminotransferase activity. *Planta*. 172: 106-113.

Oikawa, A., Ishihara, A. and Iwamura, H. (2002). Induction of HDM- BOA-Glc accumulation and DIMBOA-Glc 4-*O*-methyltransferase by jasmonic acid in poaceous plants. *Phytochem*. 61:331–337.

Perez, F. and Gomez, M. (1998). Gibberellic acid stimulation of isperoxidase from pedicel of grape. *Phytochem.* 48: 411-414.

Reinbothe, S., Mollenhauser, B. and Reinbothe, C. (1994). The regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. *The Plant Cell*. 6: 1197-1209.

Sieciechowicz, K., Joy, K.W. and Ireland, R.J. (1988a). Diurnal changes in asparaginase activity in pea leaves. I. The requirement for light for increased activity. *J. Exp. Bot*. 39: 695-706.

Sieciechowicz, K., Ireland, R.J. and Joy, K.W. (1988b). Diurnal changes in asparaginase activity in pea leaves. II. Regulation of activity. *J. Exp. Bot*. 39: 707-721.

Sieciechowicz, K., Joy, K.W. and Ireland, R.J. (1988c). The metabolism of asparagine in plants. *Phytochem*. 27: 663-671.

Wasternalk, C. and Parthier, B. (1997). Jasmonate-signalled plant gene expression. *Trends in Plant Sci*. 2: 302-307.

نشاط انزيم الاسباراجينيز في فلقات نبات الحمص تحت معاملات مختلفة

حامد محمد الشوري و ليلي علي يوسف النقيب.
قسم النبات- كلية العلوم- جامعة المنصورة.

تم في هذا البحث قياس نشاط انزيم الأسباراجينيز((EC. 5.3.1.1) في فلقات نبات الحمص تحت معاملات فسيولوجية مختلفة. أظهرت النتائج من دراسة العلاقة بين وقت الانبات والنشاط الانزيمي علي مدي سبعة أيام ان النشاط الانزيمي قد اذداد تدريجيا الي ان وصل الي ذروته في اليوم الخامس من الانبات والذي بعده انخفض النشاط الانزيمي. بدراسة تأثير تركيزات مختلفة من حمض الجاسمونيك تراوحت بين ١٠٠-٥٠٠ ميكرومول أظهرت النتائج ان حمض الجاسمونيك قد حفز النشاط الانزيمي وكان التركيز ١٠٠ ميكرومول أفضل التركيزات التي اختبرت.

أوضحت النتائج أن معاملة فلقات نبات الحمص بواسطة ١٠٠ ميكرومول من حمض الجاسمونيك ادي الي زيادة النشاط الانزيمي خلال فترة ٥ أيام إذا ما قورنت بالفلقات الغير معاملة من نفس الحمض وفي نفس الفترة. أوضحت النتائج أن معاملة الفلقات بواسطة ١٠٠ ميكرومول حمض الجبريليك لمدة ٢٤ ساعة قد ادت إلي زيادة تحفيز النشاط الانزيمي بينما معاملة الفلقات بحمض الابسيسيك لنفس الفترة الزمنية أدي الي انخفاض النشاط الانزيمي وقد بينت النتائج أن تحفيز النشاط الانزيمي بحمض الجبريليك كان معتمدا علي الفترة الزمنية للتجربة.

أظهرت النتائج أن الفلقات التي عوملت بحمض الجبريليك أو الكينيتين في وجود اكتينوميسين والسيكلوهيكسيميد والكلورامفينيكول أدت الي قلة النشاط الانزيمي اذا ما قورنت بالفلقات التي عمولت بحمض الجبريليك والكينيتين في غياب المركبات الثلاثة.