

INTERACTION BETWEEN FUNGAL ELICITORS AND SOME ACTIVITIES OF *HELIANTHUS ANNUS* L. CALLUS TISSUE

SALWA A. MAKSOUH AND SOAD S. DADOURA

Department of Botany, Faculty of Science, Cairo University, Giza, Egypt

---

Sunflower (*Helianthus annuus* L.) callus culture was treated with different concentrations (1-75 µg glucose equivalents/ml media) of an elicitor preparation heat released from the cell walls of two phytopathogenic fungi (*Alternaria tenuis* and *Verticillium dahlia*) as well as reduced glutathione (GSH) at concentrations in the range (0.01-5.0 millimolar). The PAL activities were induced (50, 15 and 3.6 folds) after two weeks treatment with *Alternaria*, *Verticillium* and GSH respectively. Phenolic content reached a peak 3.9 fold in presence of 0.025 mM glutathione and 1.6, 1.2 folds in elicited cells with 1 and 10 µg glucose equivalents/ml media of *Alternaria* and *Verticillium* elicitors respectively. GSH recorded minor increase in fresh weight (1.3 fold), while addition of both elicitors inhibited the growth of the callus. The synthesis of proteins increased with 1.3, 2.5 and 3.3 folds in the treated callus cells with GSH, *Alternaria* and *Verticillium* elicitors respectively. The extractable PAL activity and protein content reached a peak after 6 hours exposure to GSH as well as elicitors of the two tested fungi at their optimum concentrations, while phenolic content increased gradually up to 48 hours.

---

**Keywords:** *Helianthus annuus*; Interaction of fungal elicitors; *Alternaria tenuis*; *Verticillium dahlia*; Phenylalanine ammonia-lyase

---

## Introduction

L. phenylalanine ammonia-lyase (EC 4.3.1.5) activity is characteristically stimulated by microbial infection leading to the synthesis of lignin-like, wall bound phenolic material and phenylpropanoid-derived phytoalexin antibiotics (Jones, 1984; Dixon et al., 1983). These defense responses can also be induced by elicitors present in fungal cell walls and culture filtrate (Dixon et al., 1983; Darvill and Albersheim, 1984; Robbins et al., 1985).

The hypersensitive reaction is probably the most efficient defense mechanism against pathogenic attack of higher plants. A number of biochemical events take place within the infected tissue. Some of these reactions are the biosynthesis of phytoalexins (Villegas and Brodelius, 1990), induction of a number of enzymes involved in phenylpropanoid pathway (Hahlbrock and Scheel, 1987) and lignification (Goodman et al., 1986).

The extractable activity of phenylalanine ammonia-lyase (PAL) increases in response to fungal infection or exposure of plant cells to elicitor macromolecules isolated from the cell walls of yeast or plant pathogenic fungi (Dixon et al., 1995). The accumulation of phytoalexins in plant in response to infection by pathogenic fungi is believed to be an important factor in disease resistance.

The reduced form of glutathione (GSH) when supplied to suspension cultured cells of bean, (*Phaseolus vulgaris* L.) at concentrations in the range 0.01 to 1.0 millimolar, stimulates transcription of defense genes including the phenylpropanoid biosynthetic enzymes (phenylalanine ammonia lyase and other enzymes). Transcriptional activation of these genes leads to marked accumulation of the transcripts, contributing to a massive change in the overall pattern of protein synthesis which closely resembles that previously observed in response to fungal elicitor (Wingate et al., 1988). In bean, increased PAL activity in response to elicitor has been shown to result from rapid transcriptional activation of PAL genes (Lawton and Lamb, 1987).

It is known that cultivated sunflower is the host for a wide range of fungal pathogens. To elucidate the specificity of an elicitor for sunflower pathogen we used two phytopathogenic fungi, *Alternaria tenuis* and *Verticillium dahlia*. The effect of incubation period and various concentrations of glutathione and elicitor derived from cell walls of the two fungi on phenylalanine ammonia lyase activity, protein and phenolic content as well as fresh weight of *Helianthus annuus* callus culture was studied in this work.

## Materials and Methods

### Culture initiation and maintenance

Five days old sunflower (*Helianthus annuus* L. cv. Miake) seedlings (1.5-2.0 cm. in height) were used for callus initiation. The hypocotyls were cut into sections approximately 3 mm. long, surface sterilized and transferred to Mason Jars containing 30 ml. of modified Murashige and Skoog (1962) medium supplemented with 4 mg. L<sup>-1</sup> Kinetin (Kin) and 0.4 mg.L<sup>-1</sup> naphthyl acetic acid (NAA) (Bevan and Northcote, 1979). Callus normally began to form in the fourth day and was ready for subculturing after 2 weeks in the same medium. The callus cultures were maintained at 25°C under 16 hours light period with white fluorescent lamps (approximately 39 μ E m<sup>-2</sup> S<sup>-1</sup>) and were subcultured every two weeks. Cultures which contained mostly yellow to light green callus and exhibited moderate to rapid growth rates were selected for transfer to fresh media and for subsequent analysis.

### Determination of phenolic content

To determine the content of phenolic compounds, the callus material was dried to constant weight at 50°C. After sifting (0.3 g) dried callus was homogenized with 5 ml of 80% cold methanol and kept at 4°C overnight. The homogenate was then centrifuged at 5000 g for 10 min., the supernatant was collected and the solid residue re-extracted with 5 ml. of 80% cold methanol 3 times for 12h each. The extracts were pooled and methanol was evaporated under vacuum at 30°C, obtaining a constant volume of 5 ml. (Rodriguez et al. 1988).

Phenolic content was determined by the technique of Swain and Hillis (1959), using the Folin Denis reagent and saturated Na<sub>2</sub>CO<sub>3</sub> solution. The intensity of the colour developed was measured at 725 nm. Freshly prepared gallic acid was used as standard.

**PAL extraction and assay:** Typically, PAL activity was extracted from 0.5 g of callus tissue which was homogenized in borate buffer (25mM, pH 8.8) containing 20mM 2-mercaptoethanol, in a 1:10 ratio (g.ml<sup>-1</sup>) using prechilled mortar. The homogenate was centrifuged at 20000 g for 10 min. and the resulting supernatant was used as the enzyme preparation. All the above operations were carried out below 2°C. PAL activity was determined spectrophotometrically by measuring the rate of formation of trans-cinnamic acid (Zucker, 1965). The reaction mixture contained: 33mM sodium borate buffer (pH 8.8), 10mM L-phenylalanine and 0.2 ml of enzyme extract (160 μg protein) in a total volume of 3 ml. The amount of product formed was calculated from the increase in absorbance at 290 nm. One unit of activity is defined as the amount of enzyme required for the formation of 1 μ mole of cinnamate in 1 hour under the standard assay condition. For transformation of A<sub>290</sub> values into trans-cinnamic acid concentration, a molar extinction coefficient of 10800 was used (Tena et al. 1984).

**Protein determination:** Protein was determined by the method of Lowry et al. (1951).

**Culture of *Alternaria tennius* and *Verticillium dahlia*:** The alpha race of both fungus were maintained at 25°C on slants containing solid Doxis medium. The fungus were propagated by transferring spores onto slants every two weeks.

**Isolation of elicitor from *Alternaria tennius* and *Verticillium dahlia* mycelial walls:** (Prouty and Albersheim, 1975). Mycelia from 8 day old liquid cultures were homogenized in Waring blender for 60 sec. using 5 ml of water per g wet weight of mycelia. The homogenate was filtered through a coarse sintered-glass funnel and the residue was homogenized three times more in water, once in a mixture of chloroform and methanol (1:1) and finally in acetone. This preparation, when air dried represents the fraction referred to as mycellial walls. Elicitor was extracted from the mycellial walls by suspending 1 g of walls in 100 ml of H<sub>2</sub>O and autoclaving for 20 min. at 121°C. The autoclaved suspension was filtered through a coarse sintered-glass funnel. The filtrate was then clarified by centrifugation and concentrated to 10 ml under reduced pressure. The carbohydrate content of elicitor preparation was determined by the use of anthrone method (Biermann and McGinnis, 1990).

The two fungal elicitors were applied to sunflower callus cultures to give a final concentrations of 1 - 75 μg glucose equivalents/ml media, and the callus were incubated for two weeks.

## Results

The reduced form of glutathione (GSH), when supplied to callus culture of sunflower (*Helianthus annuus* L.) at concentrations in the range 0.01-5.0 milimolar, stimulates phenylalanine ammonia-lyase (PAL) activity up to maxima at 0.5 mM, then the level declines rapidly. Rapid maxima of phenolic and protein levels were attained at lower dose of glutathione (0.025 mM), followed by gradual decrease. The optimum concentration for fresh weight gain was at 0.25 mM glutathione, whereas sharp decline was attained at 1.0 mM (Fig.1).

PAL activity at low concentration of elicitors derived from cell walls of phytopathogenic fungi (*Alternaria tennius* and *Verticillium dahlia*) increased suddenly up to maxima at 5 and 10 μg glucose equivalent/ml media respectively. Higher doses inhibited the enzyme

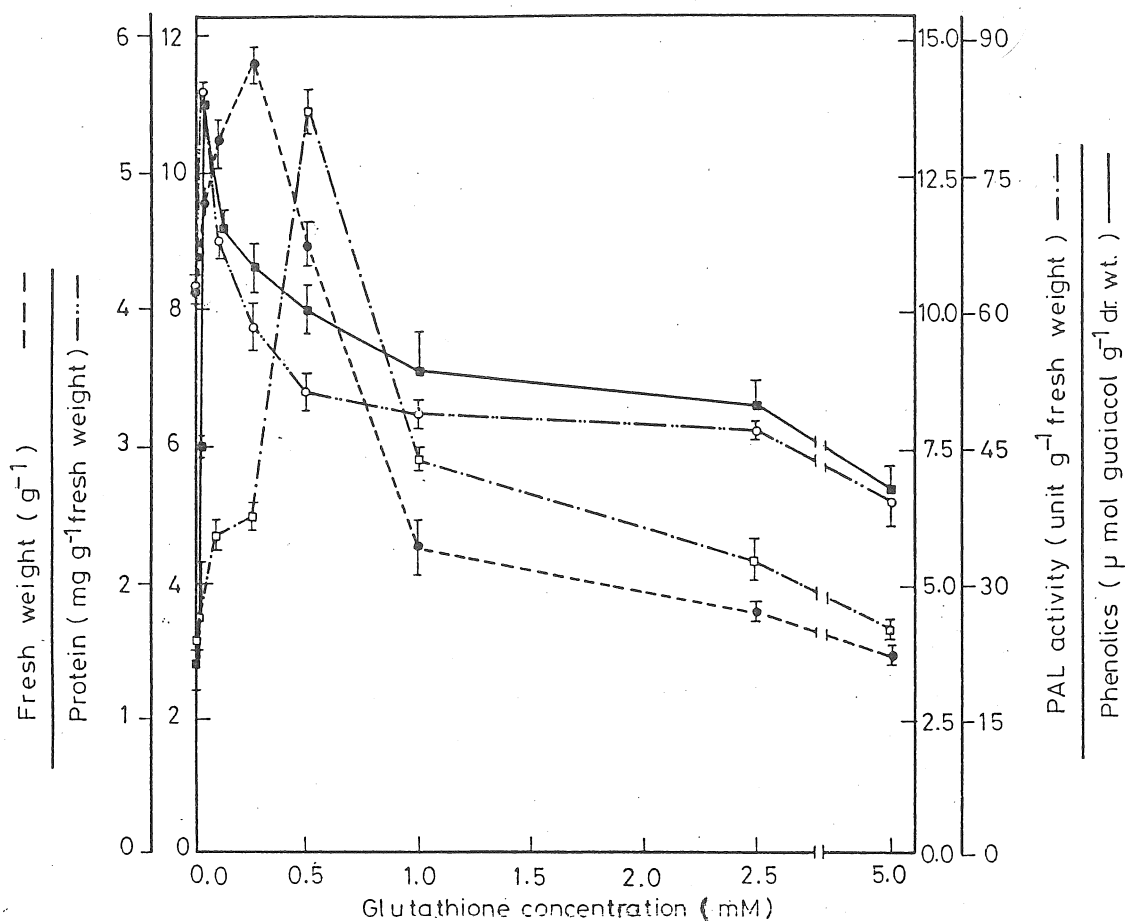


Fig.1. Effect of various concentrations of reduced glutathione on growth measurements (fresh-weight and protein); phenylalanine ammonia-lyase activity and phenolic content of sunflower callus culture. Vertical bars represent  $\pm$  SD (n=4)

activity gradually. The callus growth inversely proportional to elicitor concentrations of both fungi, gradual decrease was attained by increasing elicitor concentrations up to 25  $\mu$ g glucose equivalent/ml media, further increase gave insignificant change. Protein content responded similarly in the presence of elicitor derived from two fungi giving maximum level at 5  $\mu$ g glucose equivalents/ml media. Raising the elicitor concentrations above these maxima gave a sharp and gradual decline in the protein content of callus exposed to *Verticillium* and *Alternaria* elicitor respectively. The highest phenolic accumulation was recorded at 1  $\mu$ g

glucose equivalent in presence of *Alternaria* elicitor, while it attained at 10  $\mu$ g glucose equivalent in case of *Verticillium* elicitor. In both cases further increase in elicitor concentrations were coupled with decrease in phenolic content up to 25  $\mu$ g glucose equivalents, above this concentration insignificant change was obtained (Fig.2,3).

Fig.4 shows a marked increase in extractable activity of PAL up to 6 hours after treatment of callus culture with 0.5 mM glutathione or elicitor derived from either *Alternaria* or *Verticillium* (5 and 10  $\mu$ g glucose equivalent respectively), followed by a rapid loss

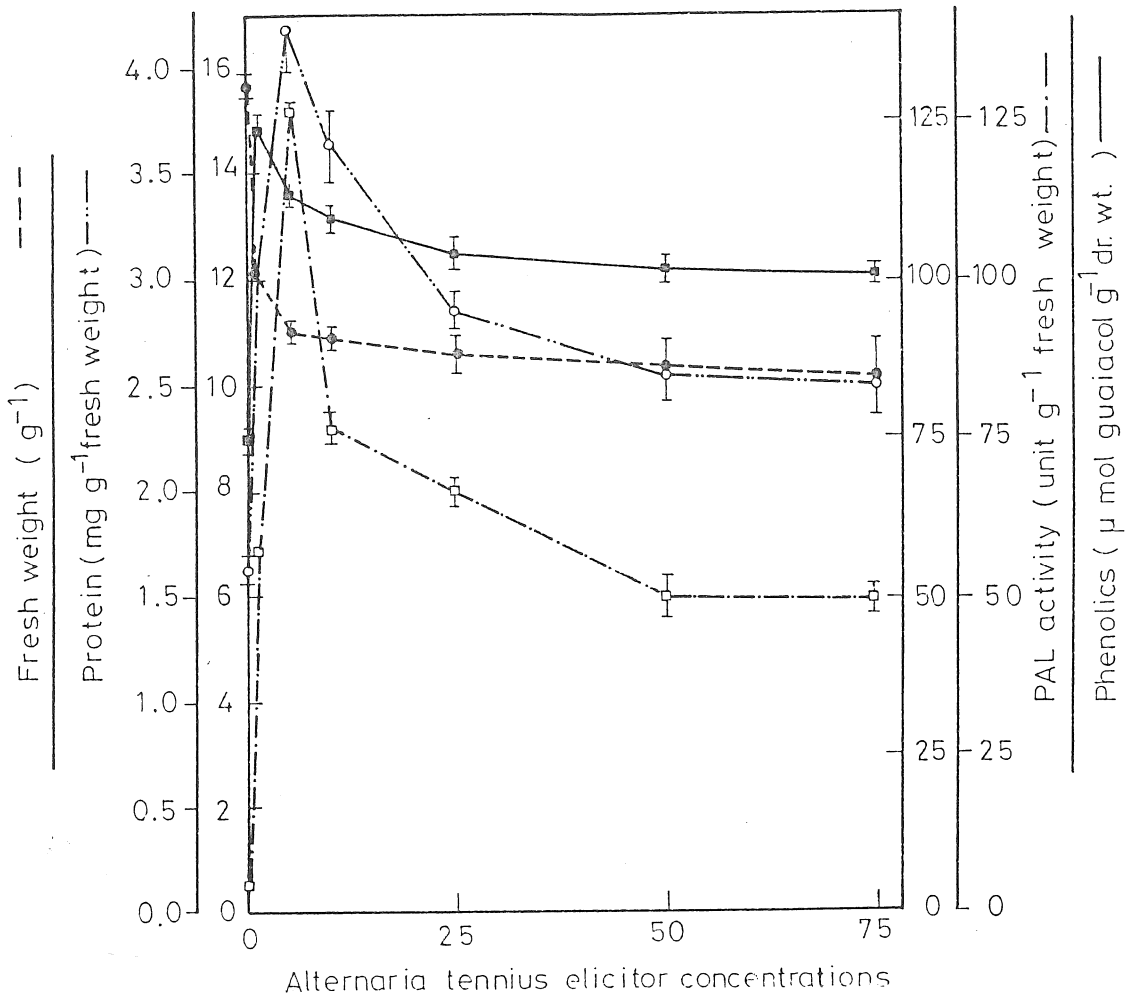


Fig.2. Effect of various concentrations of cell wall *Alternaria tenuis* elicitor on growth measurements (fresh weight and protein), phenylalanine ammonia-lyase activity and phenolic content of sunflower callus culture. Vertical bars represent  $\pm$  SD (n=4)

of activity between 6 and 12 hours. After twelve hours the PAL levels in all cases were almost unchanged, but still higher than untreated callus.

Exposure of sunflower callus culture to elicitor or glutathione at their optimum concentrations gave a gradual increase in phenolic content (Fig.5). Six hours exposure to an elicitor preparations from the two phytopathogenic tested fungi, as well as glutathione, recorded maximum accumulation of proteins in sunflower callus. Further exposure time was coupled with insignificant changes in the protein content (Fig.6).

## Discussion

The present data demonstrate that *Alternaria tenuis* elicitor was the strongest inducer to PAL activity and phenolic synthesis in sunflower callus tissue followed by *Verticillium dahlia* elicitor, whereas minimum induction was attained by glutathione (50, 15 and 3.6 fold maximum increase over control, respectively). This indicates that *Alternaria* elicitor interacted severely with callus tissue resulting in increased PAL activity and consequently phenolic accumulation, which play a prominent role in plant resistance to

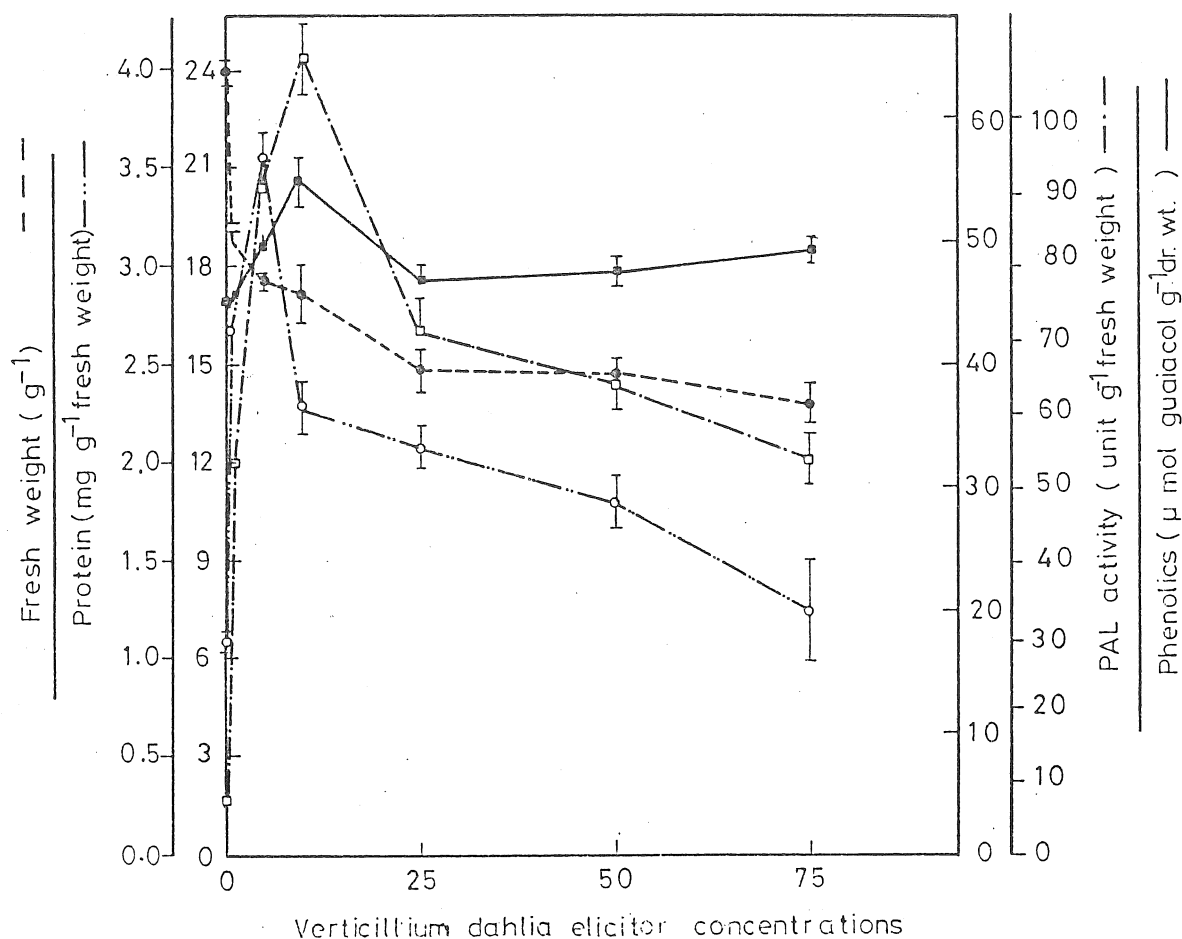


Fig.3. Effect of various concentration of cell wall elicitor derived from *Verticillium dahlia* on growth measurements (fresh weight and protein), phenylalanine ammonia-lyase activity and phenolic content of sunflower callus culture. Vertical bars represent  $\pm$  SD (n=4).

fungal infection. Also it could be concluded that sunflower tissue is more resistant to *Alternaria* than *Verticillium* infection. The rate of increase in PAL activity has also recently been correlated with resistance of alfalfa callus line to infection with the vascular wilt fungus *Verticillium albo atrum* (Latunde-Dada et al., 1987).

In consistent with our results, Hofstra and Klaren (1973) found that the capacity of parasite to induce phenolic acids synthesis by the host determine the severity of the pathogen.

Six hours were the most suitable incubation period of the callus with optimum concentration of glutathione and the two elicitors treatment to attain maximum induction of PAL. In consistent with our results the PAL activity was induced approximately 16 fold within 6

hours of exposure of alfalfa cells to fungal elicitor or yeast extract (Jorin and Dixin, 1990). Also GSH caused a marked increase in extractable PAL activity when supplied to suspension culture cells of bean (*Phaseolus vulgaris* L.) at a concentration in the range 0.01 to 1.0 mM (Wingate et al., 1988). In contrast with our results reduced glutathione was totally inactive as an elicitor in alfalfa cell suspension (Dalkin et al., 1990).

In bean cell suspension cultures PAL has been shown to undergo rapid but transient induction as the result of transcriptional activation of the gene in response to treatment with fungal cell wall elicitor (Dixon, 1986; Dixon and Harrison, 1990), while in alfalfa cell suspension culture the elicitor-induced increase in PAL activity was associated with

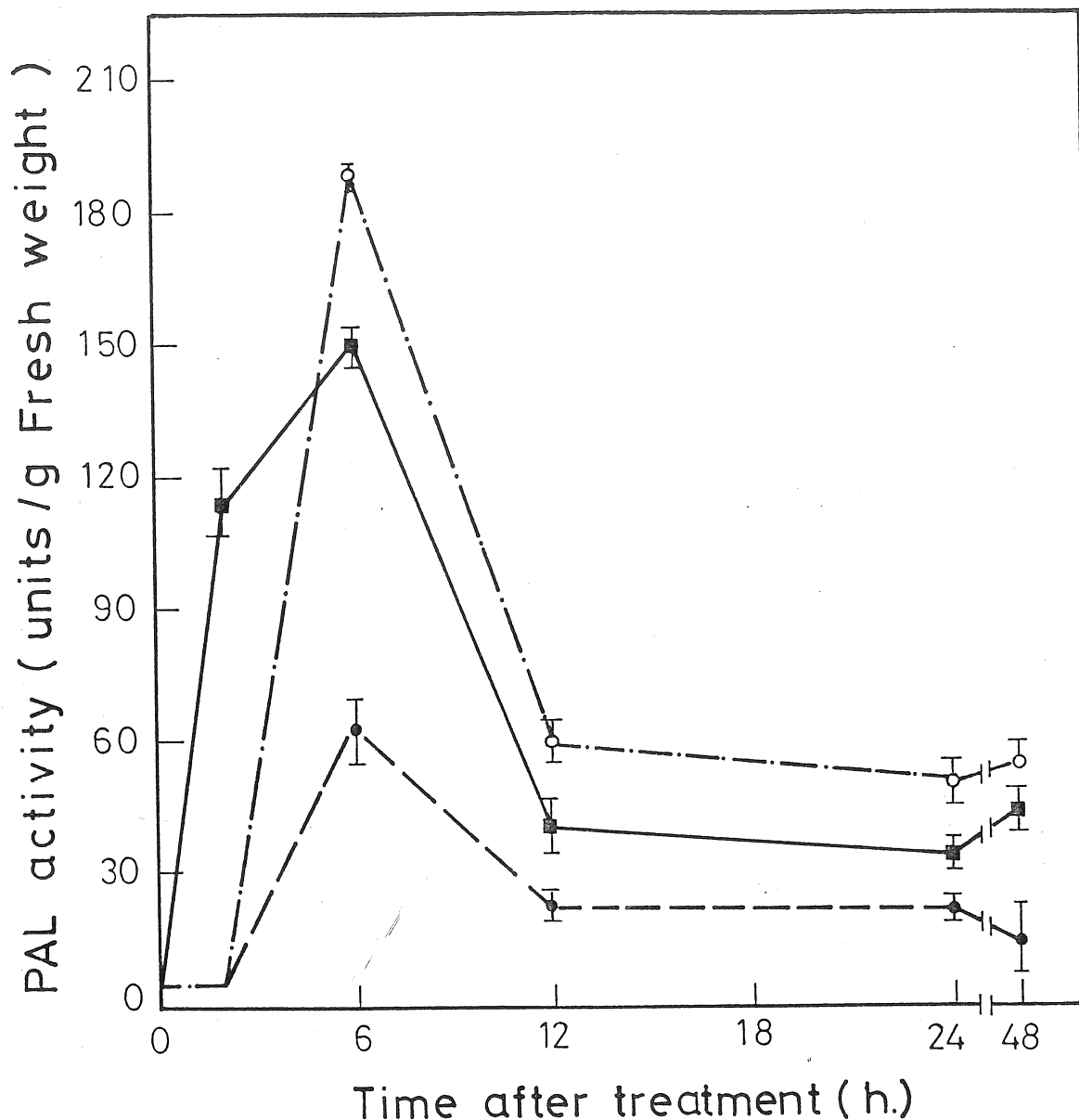


Fig.4. Changes in the extractable activity of phenylalanine ammonia-lyase of sunflower callus culture treated with - .- glutathione 0.5 mM, — *Alternaria tenuis* elicitor (5 µg glucose equivalent/ml media and -- *Verticillium dahlia* (10 µg glucose equivalent/ml media). Vertical bars represent  $\pm$  SD (n=4)

increased translatable PAL mRNA activity in the polysomal fraction (Jorin and Dixon, 1990).

The increase in activity of the key enzyme PAL was a result of de novo synthesis (Callow, 1987). There is also the possibility that hypersensitized cells may then respond to

endogenous elicitors which are the product of the initial reaction, but which then serve to sustain the cell's output after the initial stimulus provided by the fungal elicitor.

Phenolic content peaked 3.9 fold in presence of 0.025 mM GSH, and 1.6, 1.2 fold in elicited cells with *Alternaria* and *Verticillium* elicitors

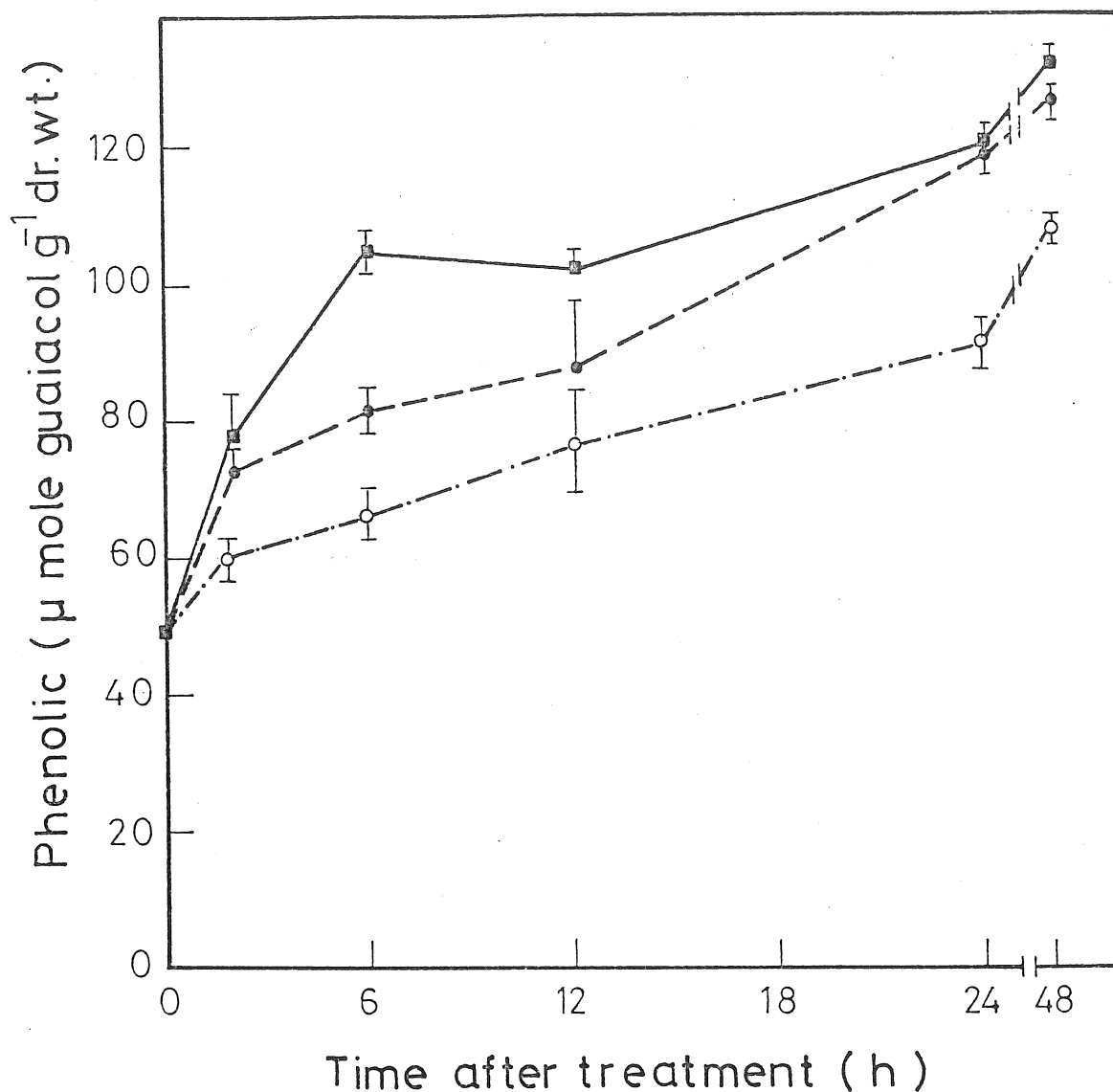


Fig.5. Changes in phenolic content of sunflower callus culture treated with - · - glutathione (0.5 mM),— *Alternaria tenuis* elicitor (5 µg glucose equivalent/ml media) and - - - *Verticillium dahlia* (10 µg glucose equivalent/ml media). Vertical bars represent  $\pm$  SD (n=4)

respectively, while treatment of the three inducers at their optimum concentrations increased phenolic content gradually up to 48 hours. Elicitor induced a rapid accumulation of phenolic material into the hemicellulose fraction of the bean cell walls. This fraction is known to be a site of ferulate acylation of the wall (Markwalder and Neukom, 1976). Also treatment of cell suspension cultures of bean with

an elicitor of phytopathogenic fungus (*Colletorichum lindemuthianum*) resulted in increase in phenolic material bound to the cellulosic and hemicellulosic fractions of the wall (Bolwell et al., 1985), a process that may be an antifungal defense response (Swain, 1977).

Except glutathione which recorded minor increase in fresh weight, addition of both

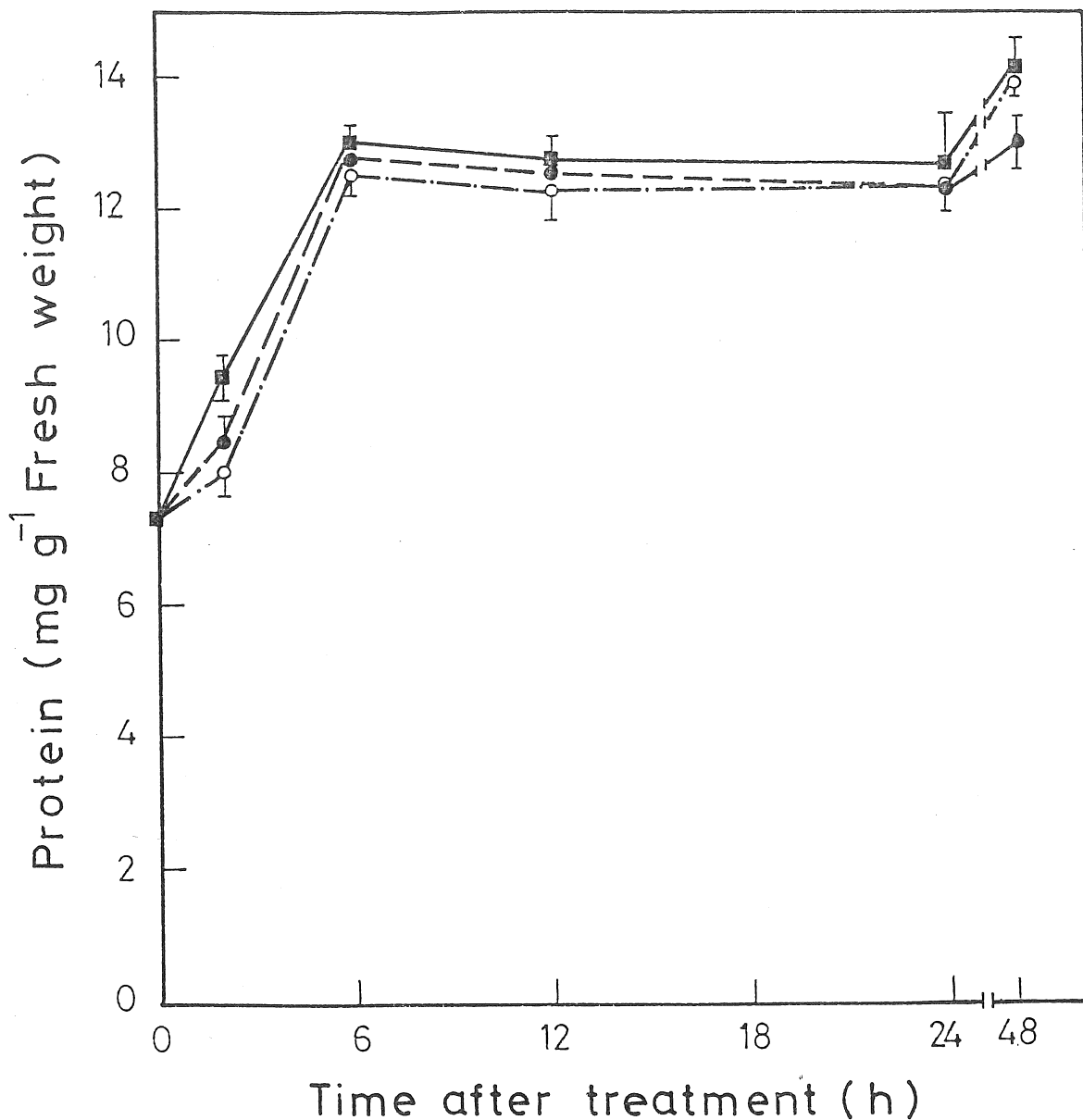


Fig.6. Changes in protein content of sunflower callus culture treated with - · - · glutathione (0.5 mM),— *Alternaria tenuis* elicitor (5 µg glucose equivalent/ml media) and - - - *Verticillium dahlia* (10 µg glucose equivalent/ml media). Vertical bars represent SD (n=4)

elicitors inhibited growth of the callus. This inhibition may be attributed to the phenolic accumulation. In consistent with our results growth (dry weight) in suspension culture of *Dioscorea deltoidea* cells were decreased after addition of various fungal mycelia (Callow, 1987).

In sunflower callus culture protein content increased to a maxima at 0.02 mM glutathione as well as 5 µg glucose equivalent elicitor of both tested fungi, followed by gradual decrease. Six hours exposure either to elicitor prepared from two fungi or to glutathione recorded maximum protein accumulation.



GSH caused a major change in the pattern of protein synthesis compared to that in untreated cells of bean (*Phaseolus vulgaris* L.) (Wingate et al., 1988). Thus, GSH markedly stimulate the synthesis of a large number of polypeptides including sets of PAL. The effects of GSH on the pattern of protein synthesis closely resembles that observed following treatment of equivalent cells with fungal elicitor (Wingate et al., 1988). However, the close resemblance between the effects of exogenous GSH and fungal elicitor does not necessarily imply physiological role in the elicitor action (Wingate et al., 1988).

### References

1. Bevan, M., Northcote, D.: *Planta* 147, 77 (1979)
2. Biermann, C. J., McGinnis, G. D. (Eds): *Analysis of Carbohydrates By GLC and MS*, CRC Press, Boca Raton, Ann Arbor, Boston 1990
3. Bolwell, G. P., Robbins, M. P., Dixon, R. A.: *Eur. J. Biochem* 148, 571 (1985)
4. Callow, J. A. (Ed.): *Advances In Botanical Research*, Vol. 13, Academic Press, London, Orlando, San Diego, New York, Austin, Boston, Sydney, Tokyo, Toronto 1987
5. Dalkin, K., Edwards, R., Edington, B., Dixon, R.A.: *Plant Physiol.* 92, 440 (1990)
6. Darvill, A. G., and Albersheim, P.: *Annu. Rev. Plant Physiol.* 35, 243 (1984)
7. Dixon, R.A., Dey, P.M., Lamb, C.J.: *Adv. Enzymol* 53, 1 (1983)
8. Dixon, R. A.: *Biol. Rev.* 61, 239 (1986)
9. Dixon, R. A., Harrison, M.J.: *Adv. Gent.* 28, 166 (1990)
10. Dixon, R. A., Harrison, M. J., Paiva, N. L.: *Physiol. Plant.* 93, 385 (1995)
11. Goodman, R. N., Király, Z., Wood, K. P.: *The Biochemistry and Physiology of Plant Disease*-University of Missouri Press, Columbia, ISBN 0-8262-0349-3, 1986
12. Hahlbrock, K., Scheel, D. (1987). Biochemical responses of plant pathogens. In: Chet, I.(Ed.) *Innovative Approaches to Plant Disease Control*, pp 227-254 Wiley and Sons, New York, ISBN 0-471-80962-4, 1987
13. Hofstra, J. J., Klaren, C. H.: *Proc. Eur. Weed Res. Coun. Symp. Parasitic Weeds*, pp 247-265, 1973
14. Jones, D. H.: *Phytochemistry* 23, 1349 (1984)
15. Jorin, J., Dixon, R.A.: *Plant Physiol.* 92, 447 (1990)
16. Latunde-Dada, A. O., Dixon, R.M., Lucas, J. A.: *Physiol. Mol. Plant Pathol.* 31, 15 (1987)
17. Lawton, M. A., Lamb, C. J.: *Mol. Cell. Biol.* 7, 335 (1987)
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: *J. Biol. Chem.* 193, 265 (1951)
19. Markwalder, H. U., Neukom, U.: *Phytochemistry* 15, 836 (1976)
20. Murashige, T., Skoog, F.: *Physiol. Plant.* 15, 473 (1962)
21. Prouty, A. J. A., Albersheim, P.: *Plant Physiol.* 56, 286 (1975)
22. Robbins, M. P., Bolwell, G. P., Dixon, R. A. : *Eur. J. Biochem.* 148, 563 (1985)
23. Rodriguez, A., Canal, M. J., Tamés, R. S.: *Physiol.* 73, 92 (1988)
24. Swain, T., Hillis, W. E.: *J. Sci. Food Agri.* 10, 63 (1959)
25. Swain, T.: *Annu. Rev. Plant. Physiol.* 28, 479 (1977)
26. Tena, M., López-Valbuena, R., Jorrín, J.: *Physiol. Plant.* 60, 159 (1984)
27. Villegas, M., Brodelius, P. E.: *Physiol. Plant.* 78, 414 (1990)
28. Wingate, V. P. M., Lawton, M. A., Lamb, C. J.: *Plant. Physiol.* 87, 206 (1988)
29. Zucker, M.: *Plant Physiol.* 40, 779 (1965)

Accepted : 13.02.1996