© Copyright 1989 by The Humana Press Inc.
All rights of any nature whatsoever reserved.
0273-2289/89/2021—0825$03.80

Growth Kinetics and Phenolics Production in *Glycine Max* Cell Suspension Cultures

Effect of Microbial Elicitor, Calcium, Polyamines, and Organic Osmolytes

KALIDAS SHETTY,[1] ROGER A. KORUS,*[,2] AND DON L. CRAWFORD[1]

[1]*Department of Bacteriology and Biochemistry; and* [2]*Department of Chemical Engineering, University of Idaho, Moscow, ID 83843*

ABSTRACT

Glycine max was used as a model plant cell suspension culture to establish relationships among growth kinetics, phenolics production, elicitor action, and peroxidase activity. Timing of elictor addition through monitoring of peroxidase provided an excellent means of optimizing yields of phenolics and reduced the time span during which phenolics were formed, negating the need for a secondary production medium. We have also determined that calcium and other cellular effectors like polyamines and organic osmolytes, when used in conjunction with elicitors, enhance phenolics production. Calcium directly enhanced elicitation, whereas polyamines and other osmolytes such as glycerol and proline extended cell viability. The study also demonstrated potential for enhancing secondary metabolite production by a combination of elicitation, cell viability stabilizers, and by addition of nutrients at the time of elicitation.

Index Entries: Phenolics; *glycine max;* plant cell culture; L-phenylalanine ammonia-lyase; elicitors; calcium, polyamines, and osmolytes.

INTRODUCTION

Plants are important sources of chemicals used in medicine, food flavors and aromas, cosmetics, and agrochemicals. These uses have been

*Author to whom all correspondence and reprint requests should be addressed.

highlighted in several recent articles (*1–3*). Most of these chemicals are secondary metabolites extracted directly from plant tissues. Recently, there has been increased interest in producing chemicals in plant cell culture.

Phenolics are an important example of secondary plant metabolites. They have roles in disease resistance and lignin synthesis (*4*), and phenolics have commercial value as food additives (*5*). Phenolics from *Artemesia capillaris* have been reported to have medicinal value in aiding microcirculation in arteries (*6*).

The use of microbial elicitors is becoming increasingly important in the development of large scale production of plant secondary metabolites through tissue culture (*7–9*). The major objective in the use of elicitors is to manipulate cultured plant cells to increase metabolic flux into specific pathways of secondary product formation (*10*). Natural elicitors are low molecular weight components of the microbial cell wall or extracellular enzymes that induce plants to produce secondary metabolites as a cellular defense mechanism (*11,12*). Enhanced synthesis of secondary metabolites by elicitation has the potential of improving production of many economically important plant products.

In our research we used soybean (*Glycine max*) plant cell suspension cultures as a model system to study production of phenolics. Suspension cell growth kinetics and phenolics production were simultaneously monitored. The time course of activity of the key regulatory enzyme L-phenylalanine ammonia-lyase (PAL) over the entire growth period was established. Our results show that extracellular peroxidase activity can be used as a sensitive indicator of biomass. Enhanced phenolics production by plant cells can be achieved in significantly shortened periods of time by carefully timing the addition of elicitors (microbial cell extracts), compared to previously used growth conditions. We have determined how key cell regulators like calcium, polyamines, and organic osmolytes can be used along with elicitors to extend cell viability and maintain optimum secondary metabolite production following elicitor stress. This is especially important in situations where sudden product formation from elicitation is deleterious to plant cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Suspension cultures of soybean cells (*Glycine max*) were derived from callus generated from soybean hypocotyls (*13*). Soybean seeds were obtained from Wards Natural Science Establishment, Inc., Rochester, NY. The soybean cells were grown in the dark in B-5 medium (*14*) at pH 5.5 with 0.5 ppm of 2,4-D and 0.1 ppm of kinetin at 26°C. Cells were subcultured every 7 d using a 15–20% (v/v) inoculum. The cells grew in clumps of 15–25 cells and sometimes larger aggregates. All experiments were in-

cubated shaken at 125 rpm in 250 mL Erlemeyer flasks containing 100 mL medium. All sampling was done in triplicate with 10 mL samples. The results indicate the cell line used in this work had similar growth rates as other *Glycine max* cell suspensions when using 0.5 ppm 2,4-D (*15,16*).

Cell Dry Weight

Ten mL of actively growing culture was removed every 2 d over the growth period. Cells were removed by filtration onto preweighed Whatman No. 1 filter paper, oven dried for 24–36 h at 70°C, then reweighed to determine the cell dry weight. Cell dry weight values were corrected to g/L of medium. The filtrate was retained and used for determination of peroxidase activity.

Cell Dry Weight to Fresh Weight Correlation

About 70 samples from various stages of the growth cycle were measured for both their dry weight as above and for their fresh weight, which was determined gravimetrically prior to transferring samples to the oven. The correlation of dry weight to wet weight showed that an average of 4.8% of fresh weight is made up of cell dry weight (60% of the estimations fell in the range of 4.6–4.9%).

Peroxidase Activity

Peroxidase activity was determined and correlated to cell dry weight. 4-Amino antipyrine was used as the hydrogen donor in the peroxidase assay (*17*). The procedure involved premixing 1.4 mL of phenol/antipyrine solution with 1.5 mL of 0.0017 *M* hydrogen peroxide, and incubating for 1–2 min to achieve temperature equilibrium and establish the background reaction rate. Then 0.1 mL of extracellular filtrate (enzyme) was added and the increase in absorbance at 510 nm was followed for 4–5 min. Activity was expressed as $\triangle A_{510}$/min.

Phenolic Determination

A 0.5 g (fresh weight) cell sample in 5 mL of 95% ethanol was sonically disrupted for 3–5 min, centrifuged for 30 min at 17,300 × *g*, and the ethanol fraction (supernatant) retained for the phenolic assay. One mL of ethanol extract and 1 mL of 95% ethanol were mixed in 5 mL distilled water. Then, 0.5 mL of 50% Folin-Ciocalteu reagent was added. After 5 min, 1 mL of 5% Na_2CO_3 was added, the reaction mixture was allowed to stand for 60 min, and absorbance was measured at 725 nm. Controls contained only 95% ethanol. A standard curve was developed using various concentrations of gallic acid in 95% ethanol. This assay was a modification of the method described by Chandler and Dodds (*18*), which is similar to the method originally developed by Singleton and Rossi (*19*).

Carbohydrate Determination

One mL of cell free medium was assayed for sucrose by the anthrone method (*20*). Sucrose depletion was estimated by measuring changes in absorbance of the anthrone color at 540 nm with a standard curve using sucrose as the standard. Total carbohydrate in culture supernatants was expressed as g of carbohydrate/L of medium.

L-Phenylalanine Ammonia-Lyase Activity (PAL) (21)

One gram of cells was suspended in 5 mL of pH 8.8 borate HCl extraction buffer (25 m*M* borate HCl + 2 m*M* sodium bisulfate). The suspension was gently ground with a mortar and pestle, and the resulting mixture was sonically disrupted (1 min; repeated 4–5 times at approximately 0°C). Between sonic disruptions, the probe was cooled with ice for 30 sec. After sonication, the homogenates were centrifuged at $12,100 \times g$ for 20 min, and the supernatant was then used as the enzyme extract. Fifteen μM L-phenylalanine in 100 μM borate buffer (pH 8.8) in a total volume of 2.8 mL was mixed with 0.2 mL of enzyme extract and incubated at 30°C. Controls contained 2.8 mL of 100 μM borate HCl (pH 8.8) and 0.2 mL enzyme extract with no L-phenylalanine. During the first 30 min of incubation, there was a slow decrease in absorbance (290 nm) during stabilization of the reaction mixture. After stabilization, *trans*-cinnamic acid production was followed by measuring the change in absorbance at 290 nm for 60 min. Every assay was done in triplicate and corrected by subtraction of the corresponding control value. PAL activity was reported as milliunits (mu), where 1 mu equalled a 0.001 absorbance change/min/g fresh weight of cells.

Elicitor

One L of *Fusarium solani var pisi* suspension actively growing in potato dextrose medium at 26°C was harvested by filtration after 48–72 hours. The mycelia were centrifuged, resuspended in 50 mL water, and then autoclaved as above after determining fresh weight. A 0.05% (w/v) equivalent of elicitor suspension, after treatment in 5 mL of 100 m*M*, pH 5.2 Na acetate buffer for partial hydrolysis, was added to cell suspension cultures. The concentrations of sterile calcium chloride (2 m*M*), spermidine (5 m*M*), spermine (5 m*M*), proline (5 m*M*), and glycerol (1% w/v) were adjusted within the 5 mL of 100 m*M*, pH 5.2 Na acetate buffer as per treatment requirements. The contents were added to experimental cultures on d 7 of cell growth.

RESULTS AND DISCUSSION

Growth and Phenolic Production

Growth parameters during exponential growth were determined using the equation

$$X = X_o\, e^{\mu max(t - t_o)}$$

where X is dry weight. Growth in batch culture was exponential from d 1 to 6 after a 1-d lag phase (Fig. 1). The lag phase was short because of the high inoculum level (20% v/v). The specific growth rate (μ_{max}) in the exponential phase was 1.12 d^{-1} corresponding to a doubling time of 0.6 d. Before the onset of the stationary phase, the culture entered a declining growth rate period between 6–9 d. This declining growth rate was also reflected in a declining rate of carbohydrate utilization (Fig. 2). The rate of carbohydrate utilization was rapid and approximately linear from the time of inoculation to d 7. After d 7 the rate of carbohydrate utilization shifted from 2.3 g/L-d to 1.0 g/L-d.

Ethanol soluble phenolics were produced in approximately equal amounts during the growth and the stationary phases (Fig. 1). There was little phenolics production in the declining growth phase and after d 12. After an additional 3 d, the phenolics concentration had increased only 15% from the level observed at d 12 (data now shown). Phenolics production did not follow growth, as was previously observed in tobacco (*Nicotiana tabacum*) (22). However, soluble phenolic compounds are secondary metabolites and are expected to be formed during the stationary phase or late exponential phase.

Extracellular Peroxidase and L-Phenylalanine Ammonia Lyase (PAL) Activity During Growth

This study also examined the rate of extracellular peroxidase activity as an indicator of cell growth (Fig. 3). Extracellular peroxidase activities have been shown to closely parallel growth (fresh weight) in peanut (*Arachis hypogaea*) cell cultures (23) and *N. tabacum* (24). In this study, peroxidase activity was correlated with growth. The leveling off of peroxidase activity coincided with the changes in growth kinetics, rate of carbohydrate utilization, and phenolic accumulation. A most interesting observation was that optimum phenolic formation as a result of elicitation was triggered when elicitors were added at the end of 7 d of cell growth. Adding elicitor earlier than 7 d was not feasible because of low cell mass, or beyond 7 d because the enzymes needed to respond to elicitors and turn on phenolic production had reduced activities, as exemplified by the

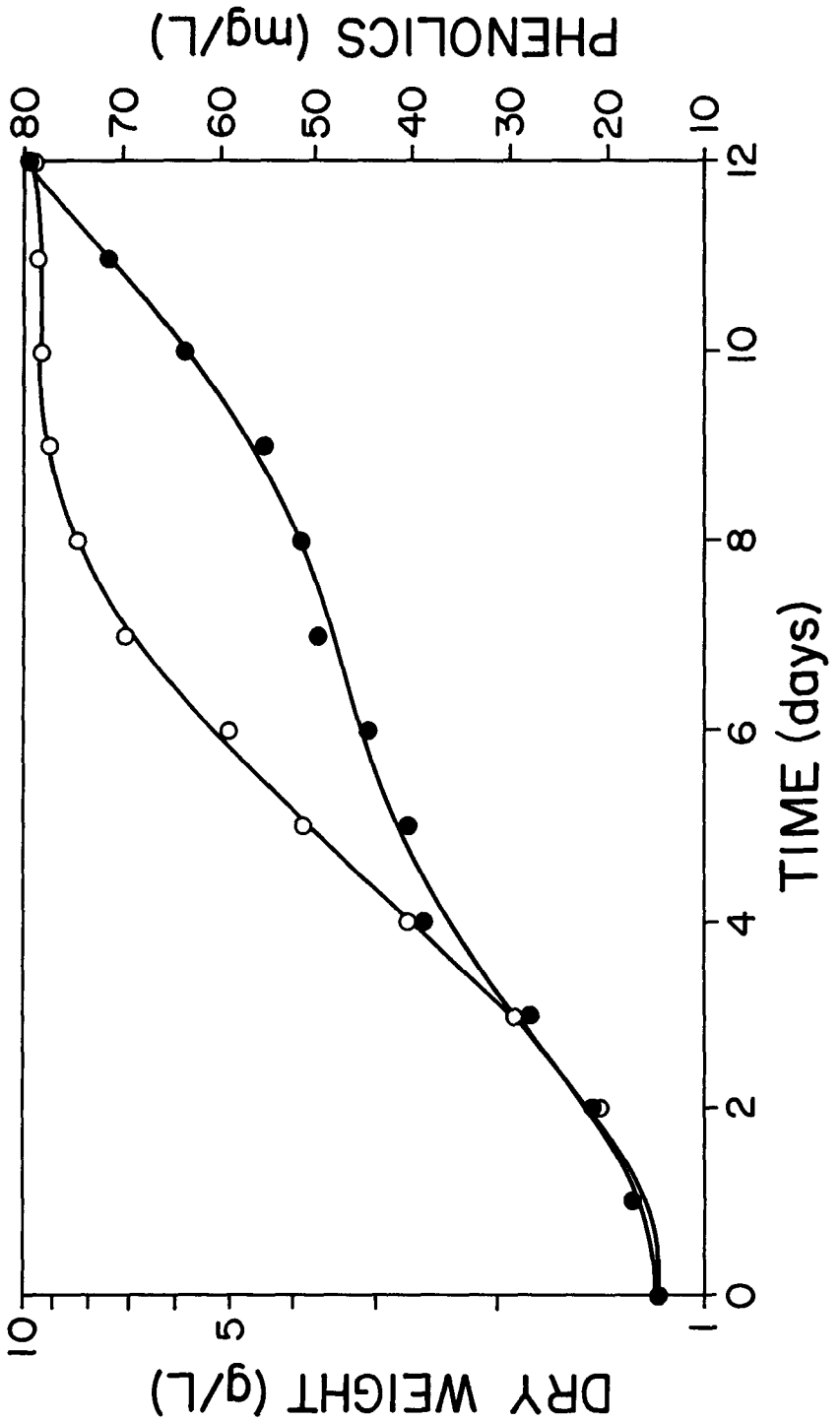

Fig. 1. Dry weight (○) and phenolics production (●) during growth of *Glycine max*.

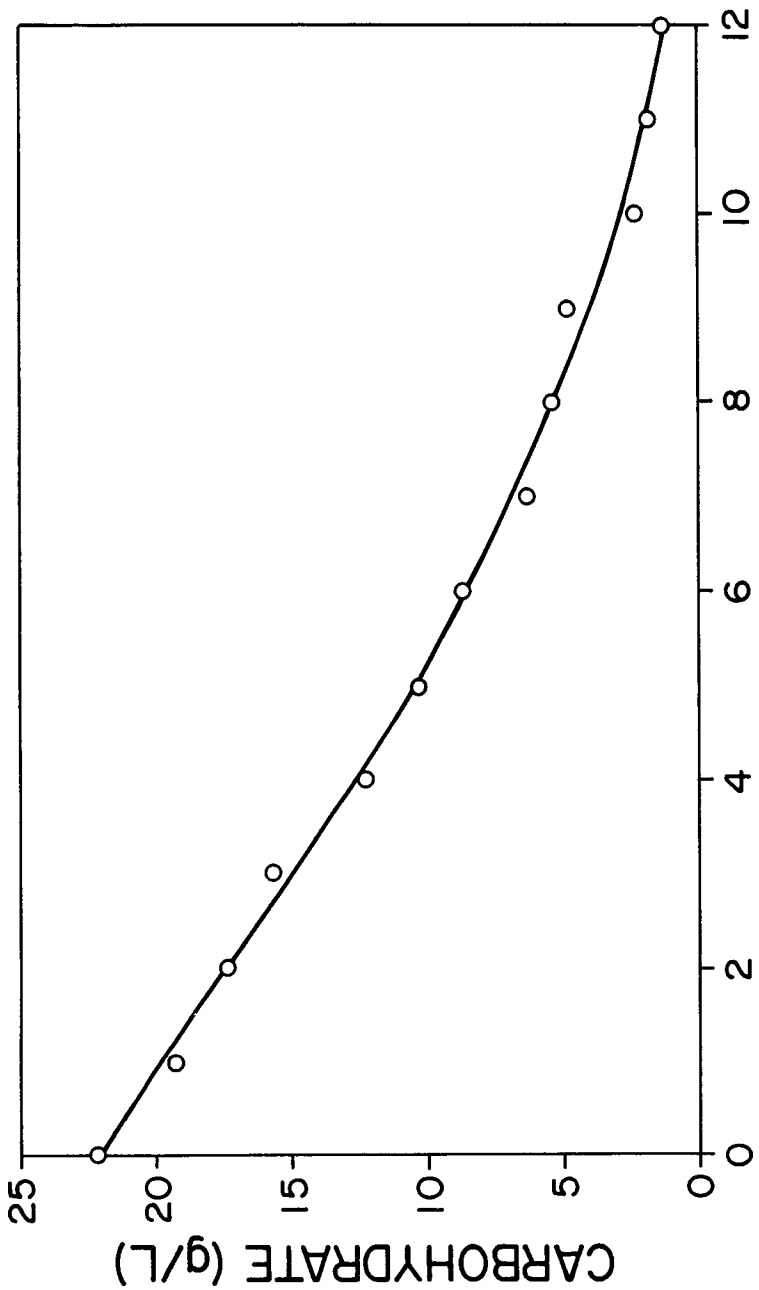

Fig. 2. Carbohydrate concentration during growth of *Glycine max*.

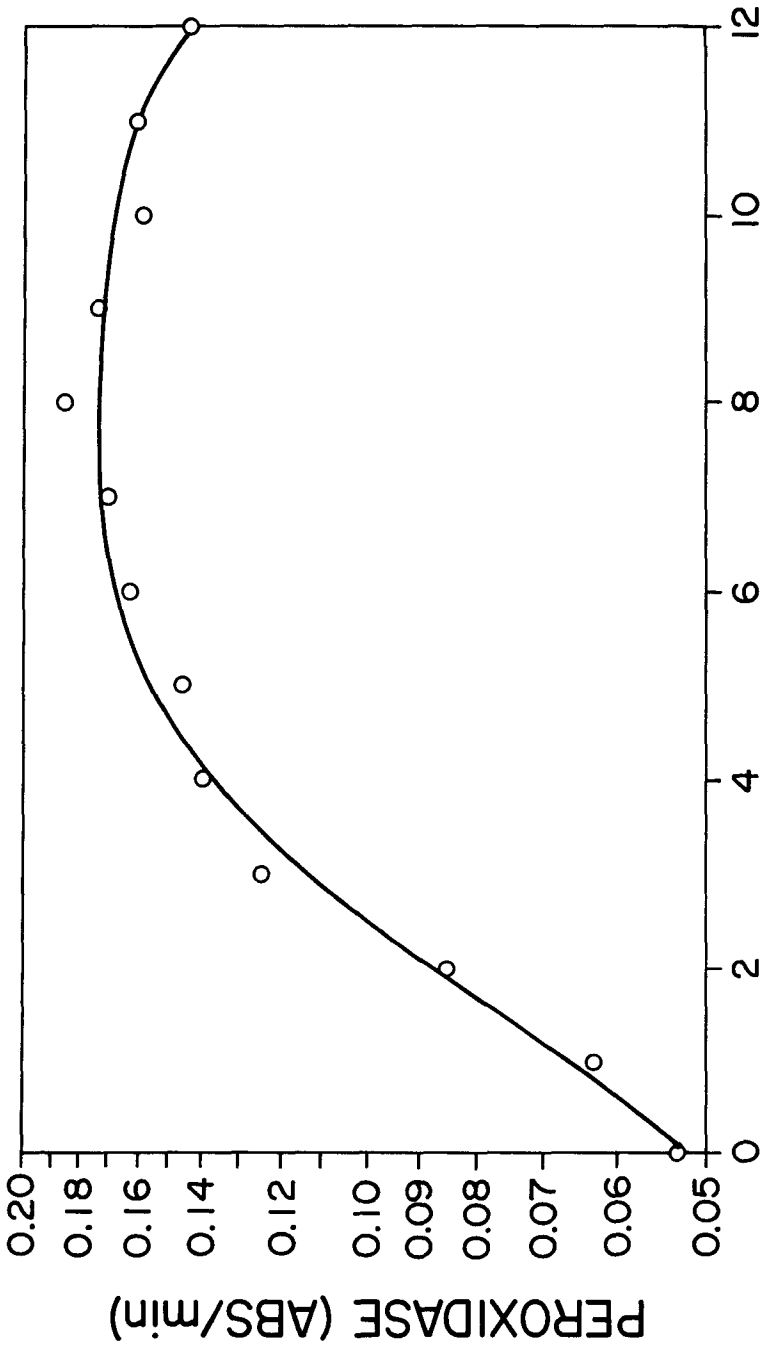

Fig. 3. Peroxidase activity during growth of *Glycine max*.

Table 1
Elicitor Responses as Measured
by Soluble Phenolics, mg/L,
after Addition of Microbial Elicitors

	Time, h		
	0	24	48
Elicitor	61.7	123.1	137.3[a]
Control, no addition	63.4	62.1	67.4

[a] Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

peroxidase activity. Also, with the onset of senescence there was reduced cell division as cells entered stationary pahse.

L-Phenylalanine ammonia-lyase activity was followed over the growth period (Fig. 4). PAL activity increased at the onset of the stationary phase coinciding with the onset of soluble phenolics production. Similar PAL activity has been observed with *A. hypogea* (*25*). PAL is a key enzyme of the phenylpropanoid pathway, and many of these compounds are formed when cell division ceases.

Microbial Cell Extracts as Elicitors in Phenolics Production

Separate growth media and product formation media can be used to obtain higher product concentration in plant cell cultures (*26*). We have circumvented this need and also reduced the time during which the optimum quantity of phenolics are produced by using microbial elicitors (Table 1). Elicitors are biological compounds that trigger production of low molecular weight antimicrobial secondary metabolites in plants. Filtrates of fungal origin or microbial cell wall components have been used as elicitors to enhance secondary metabolite production (*27–29*). In this study, cell extracts of fungus *Fusarium solani var pisi,* when added at the end of exponential growth (7 d), produced optimum phenolics in 8 d, which was comparable to that produced without elicitors in 12–15 d. L-Phenylalanine ammonia lyase activity transiently increased in 6 h following elicitor addition (Fig. 5). Similar observations were made when host specific glucan elicitors of *Phytophthora megasperma* were used (*16,30,31*).

Qualitative characterization of phytoalexins, glyceollin, and other phenylpropanoids has been undertaken to understand plant-pathogen physiological interaction in soybeans (*32*). Our work used only crude cell-wall extracts as elicitors and did not characterize the phenylpropanoids. The objectives were to understand the physiological effect of elicitors phenolics and later to use this model system to understand specific secondary metabolite production in other plant cell culture systems.

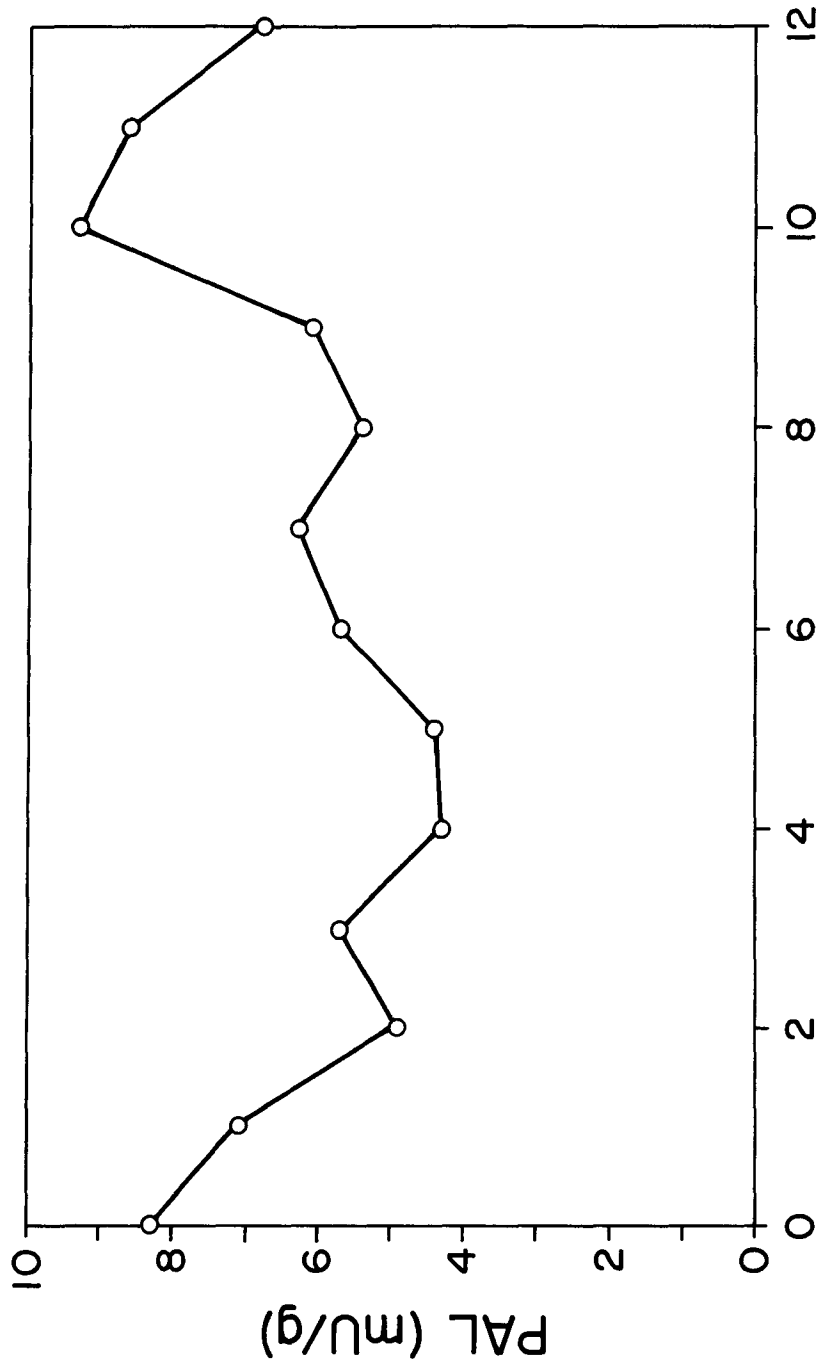

Fig. 4. L-Phenylalanine ammonium lyase activity during growth of *Glycine max*.

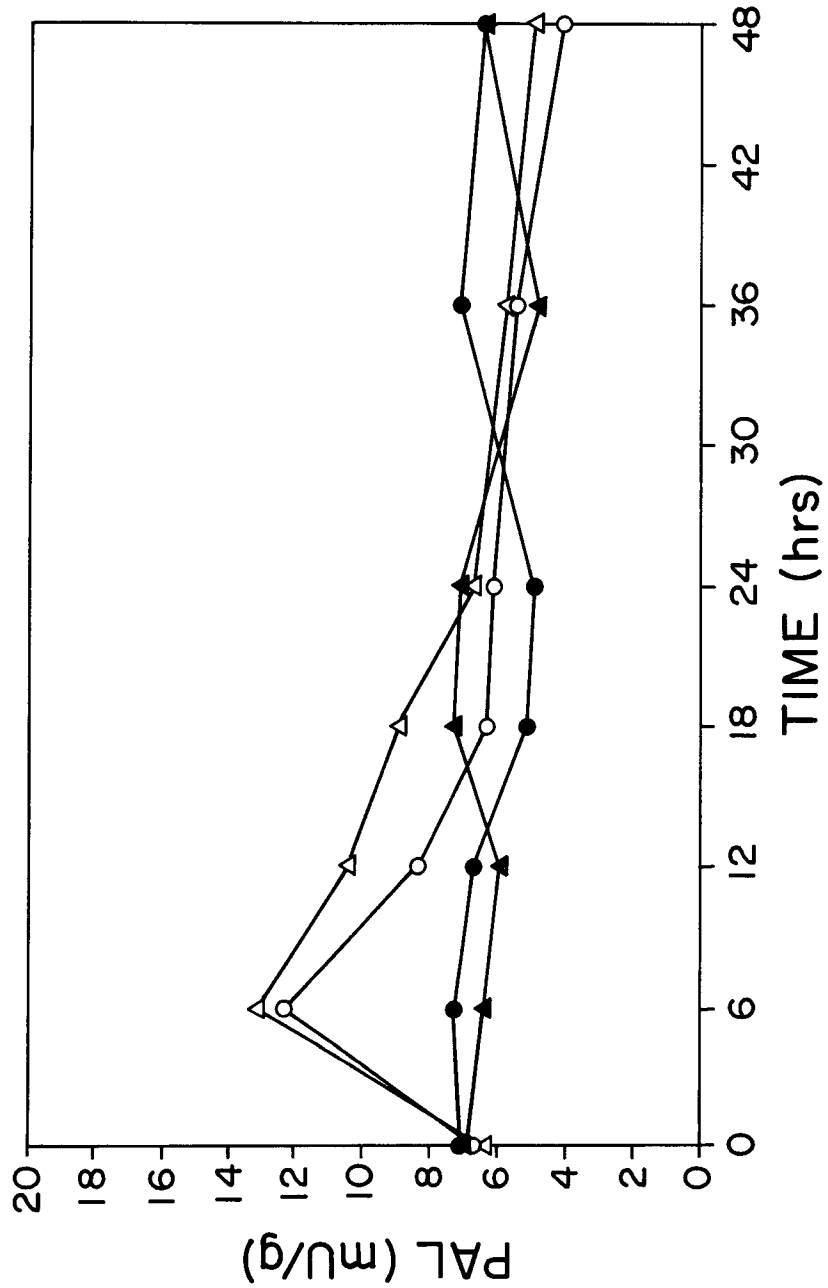

Fig. 5. L-phenylalanine ammonium lyase activity in *Glycine max* following addition of (a) elicitor+calcium (△), (b) elicitor only (○), (c) no addition (▲), and (d) calcium only (●). The additions were made on d 7 of the growth cycle.

Table 2
Elicitor Responses as Measured
by Soluble Phenolics, mg/L,
after Addition of Microbial Elicitors:
Effect of Calcium, Ca^{++}

	Time, h		
	0	24	48
Elicitor	63.4	117.4	139.8
Ca^{++}	61.3	63.4	64.6
Elicitor + Ca^{++}	59.7	123.7	149.8[a]
Control, no addition	58.7	61.3	62.7

[a] Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

ROLE OF CALCIUM, POLYAMINES, AND OSMOLYTES IN CELLULAR PRODUCTION OF PHENOLICS

Calcium

Calcium increased activity of PAL and concomitantly increased total phenolics when added along with the elicitor (Table 2). The transient increase in PAL was similar to what was observed by elicitor alone through overall activity was higher (Fig. 5). Similar enhancement of elicitor action in soybean by calcium has been studied in phytoalexin synthesis (*33*). In that study β-glucan elicitor of fungus *Phytophthora megasperma* induced phenylpropanoid phytoalexin accumulation with transient increases in PAL activity. Changes in calcium concentration affected the elicitor mediated phytoalexin accumulation, and by removal of all calcium with chelating agents, elicitor mediated enzyme induction, and phytoalexin production were eliminated. Calcium is known to have a wide role in plants by affecting metabolism through modulation of the protein calmodulin (*34*).

Polyamines

The polyamines spermidine and spermine, when added along with calcium and elicitors, stabilized the transient increase in PAL activity following elicitation (Fig. 6). This also minimally increased the amount of phenolics formed compared to using only calcium and elicitors (Tables 3 and 4). Also, the quality of phenolics formed may be affected by polyamines. Muhitch and Fletcher (*35*) observed that spermidine not only extended the life span of mature nondividing cells but also increased yields of phenolics and gave a wider assortment of phenolics. Polyamines are cationic at cellular pH and arise from polybasic amino acids arginine and lysine. Polyamines increase in plants following application of plant

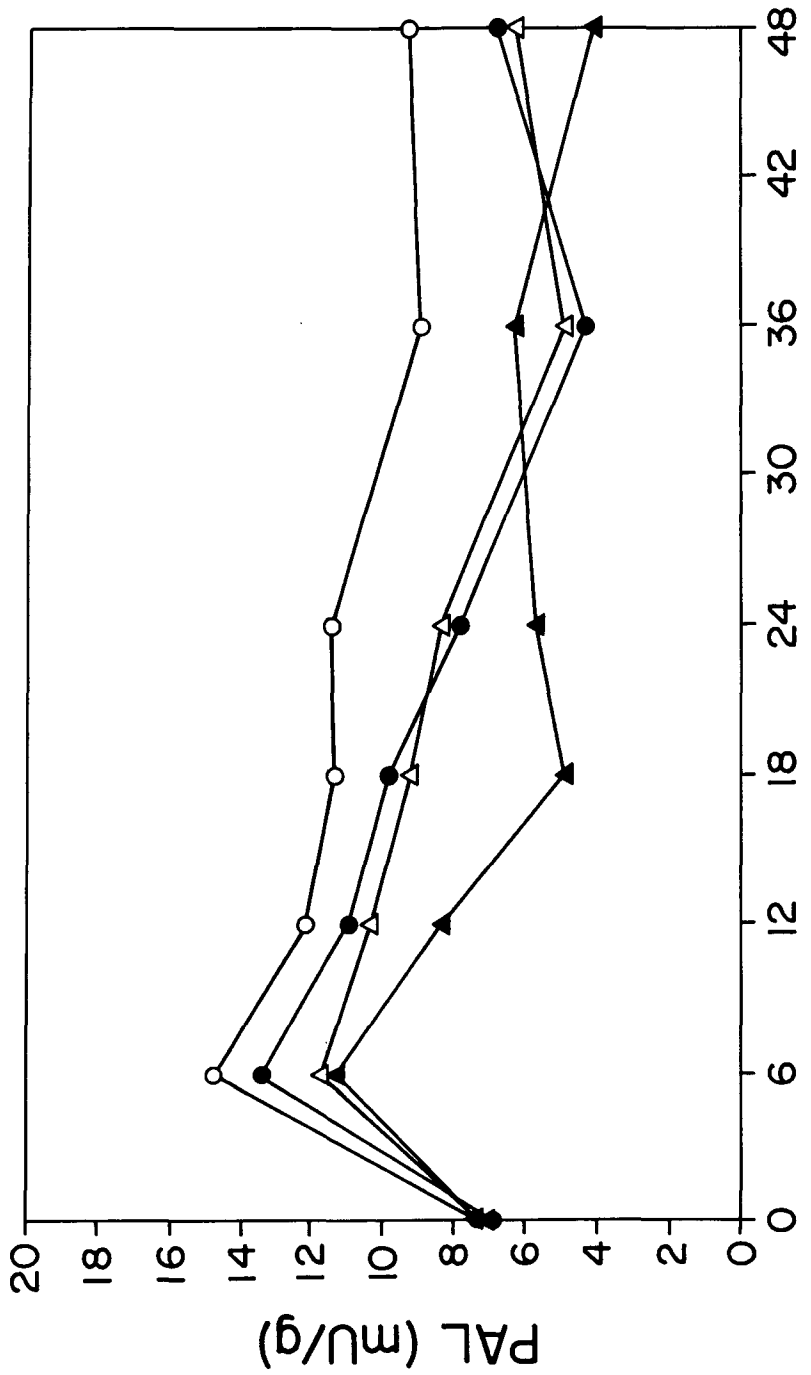

Fig. 6. L-Phenylalanine ammonium lyase activity in *Glycine max* cell suspensions following addition of (a) elicitor + calcium + spermine (○), (b) elicitor + calcium (●), (c) elicitor + spermine (△), and (d) elicitor only (▲).

Table 3
Elicitor Responses as Measured
by Soluble Phenolics, mg/L,
After Addition of Microbial Elicitors:
Effect of Calcium (2 m*M*) and Spermidine (5 m*M*)

	Time, h		
	0	24	48
Elicitor + Ca^{++} + spermidine	63.7	127.8	158.6[a]
Elicitor + Ca^{++}	61.8	118.7	143.6
Elicitor + spermidine	63.9	116.3	141.3
Elicitor	64.6	123.6	133.7
Control + Ca^{++} + spermidine	59.2	63.4	62.7
Control + Ca^{++}	67.3	64.6	65.3
Control + spermidine	58.6	61.4	63.6
Control	63.7	61.6	62.3

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 4
Elicitor Responses as Measured
by Soluble Phenolics, mg/L,
After Addition of Microbial Elicitors:
Effect of Calcium (2 m*M*) and Spermine (5 m*M*)

	Time, h		
	0	24	48
Elicitor + Ca^{++} + spermine	72.3	123.7	158.6[a]
Elicitor + Ca^{++}	69.6	124.2	143.3
Elicitor + spermine	63.7	118.7	140.8
Elicitor	68.7	120.3	131.4
Control + Ca^{++} + spermine	63.6	70.4	69.8
Control + Ca^{++}	64.8	67.3	68.1
Control + spermine	61.2	62.8	70.3
Control	69.1	67.4	62.8

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

hormones (*36*) and perhaps serve as second messengers similar to cAMP. Polyamines seem to stabilize cells against lysis and tetramines appeared more effective than di- or triamines (*37*). In general, polyamines regulate physical and chemical properties in membranes and thereby alter permeability (*37*). In plant response against plant pathogens, polyamine changes are observed (*38*). Phenolic compounds form conjugates with polyamines in the surrounding hypersensitive infected zone, and when polyamines

Table 5
Elicitor Responses as Measured
by Soluble Phenolics, mg/L,
After Addition of Microbial Elicitors:
Effect of Calcium (2 m*M*) and Proline (5 m*M*)

	Time, h		
	0	24	48
Elicitor + Ca^{++} + proline	71.4	117.3	158.1[a]
Elicitor + Ca^{++}	63.8	119.7	141.6
Elicitor + proline	61.3	118.8	147.3
Elicitor	63.7	121.7	138.8
Control + Ca^{++} + proline	69.8	63.8	64.3
Control + Ca^{++}	63.9	65.6	67.8
Control + proline	69.8	69.3	68.4
Control	70.6	71.4	72.3

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

are added exogenously the infection is reduced (*39*). Therefore, enhanced phenolics production and stabilization of PAL through use of polyamines during elicitation in *Glycine max* cell suspensions provides a potential to increase secondary metabolite production in other systems.

Organic Osmolytes

Organic osmolytes are cytoplasmic components serving as osmoregulators and perhaps working primarily as protein stabilizers (*40*). The organic osmolyte proline used in this study did not stabilize the transient increase in PAL activity following elicitor and calcium addition. However, proline gave an increase in phenolics produced comparable to adding elicitor and calcium alone (Table 5). The organic osmolyte glycerol stabilized the transient increase in PAL activity following elicitor addition (Fig. 7). The addition of glycerol also resulted in corresponding increase in phenolics (Table 6). Whether osmolytes act to maintain membrane structure or if there is protein interaction to protect against toxic secondary metabolites is not known, but they do extend metabolic productivity of plant cell suspension cultures.

Addition of Sucrose and a Nitrogen Source During Elicitation for Phenolics Production

Phenolics production in *Glycine* max cell suspensions was highest when elicitor was added with a cell viability stabilizer, sucrose and potassium nitrate (Table 7). It has not been established whether this effect was

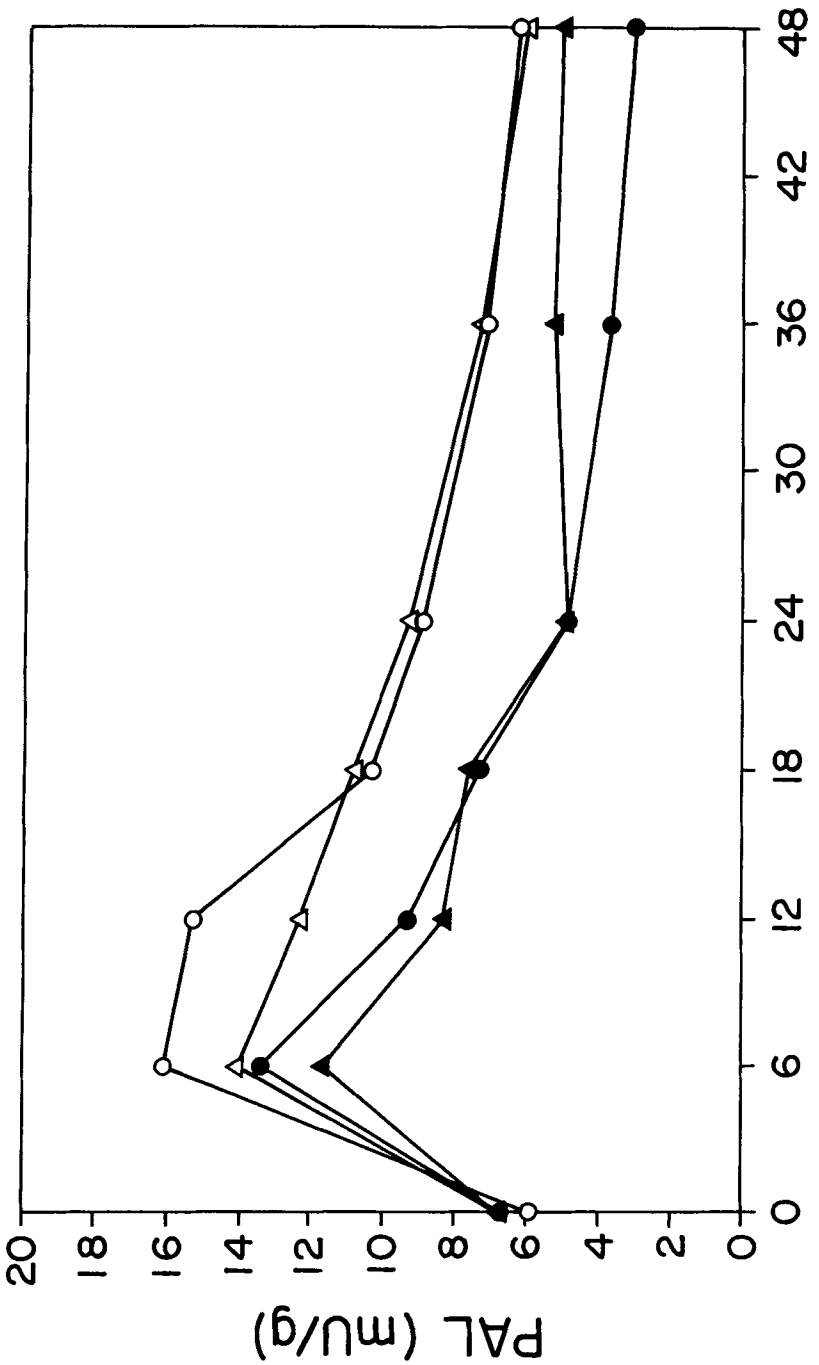

Fig. 7. L-Phenylalanine ammonium lyase activity in *Glycine max* cell suspensions following addition of (a) elicitor+calcium+glycerol (○), (b) elicitor+calcium (●), (c) elicitor+glycerol (△), and (d) elicitor only(▲).

Table 6
Elicitor Responses as Measured
by Soluble Phenolics, mg/L,
After Addition of Microbial Elicitors:
Effect of Calcium (2 m*M*) and Glycerol (1% w/v)

	Time, h		
	0	24	48
Elicitor + Ca^{++} + glycerol	53.4	123.7	160.4[a]
Elicitor + Ca^{++}	54.3	119.3	143.3
Elicitor + glycerol	59.8	129.6	153.3
Elicitor	57.4	123.8	141.7
Control + Ca^{++} + glycerol	61.8	63.8	67.4
Control + Ca^{++}	59.6	61.7	62.2
Control + glycerol	57.6	59.3	58.4
Control	63.4	61.8	67.6

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 7
Elicitor Responses as Measured
by Soluble Phenolics, mg/L,
After Addition of Microbial Elicitors:
Effect of Calcium, Polyamines/Osmolytes,
and Sucrose + KNO_3

	Time, h	
	0	48
Control	57.8	64.7
Sucrose + KNO_3	68.4	63.4
Elicitor + (sucrose + KNO_3)	63.4	154.7
Elicitor + Ca^{++} + (sucrose + KNO_3)	59.3	159.8
Elicitor + Ca^{++} + (sucrose + KNO_3) + spermine	63.3	160.4
Elicitor + Ca^{++} + (sucrose + KNO_3) + spermidine	59.5	171.3[a]
Elicitor + Ca^{++} + (sucrose + KNO_3) + proline	65.4	159.7
Elicitor + Ca^{++} + (sucrose + KNO_3) + glycerol	59.6	174.6[a]

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

owing to osmotic variability and stabilization or conversion of added sucrose to phenolics. Muhitch and Fletcher (35) have shown that sucrose, when added along with spermidine during cell senescence in scarlet rose cultures, extends cell viability and increases the types of phenolics produced. Therefore, this could provide a means of increasing the yield of secondary metabolites in cell cultures during elicitation.

REFERENCES

1. Stafford, A., Morris, P., and Fowler, M. N. (1986), *Enzym. Microl. Tech.* **8,** pp. 575–587.
2. Lee, S. C. and Scott, A. I. (1979), *Dev. Ind. Microb.* **20,** 381.
3. Zenk, M. H. (1978), *Frontiers of Plant Tissue Culture,* Thorpe, T. A., ed., International Association for Plant Tissue Culture, Calgary, Alberta, Canada, pp. 1–13.
4. Vance, C. P., Kirk, T. K., and Sherwood, R. T. (1980), *Ann. Rev. Phytopath.* **18,** 259.
5. Maga, J. (1978), *Crit. Rev. Fd. Sci. Nutr.* **10,** 323.
6. Koo, A., Int. Symposium of Chinese Medicinal Materials Research, June 1984, Hong Kong.
7. Dougall, D. K. (1986), *N. Z. J. Technol.* **2,** 71.
8. Kurz, W. G. W. (1986), *N. Z. J. Technol.* **2,** 77.
9. Eilert, U., Constabel, F., and Kurz, W. G. W. (1986), *J. Plant Physiol.* **126,** 11.
10. Dixon, R. A. and Bolwell, G. P. (1986), *Secondary Metabolism in Plant Cell Cultures,* Morris, P., Scragg, A. H., Stafford, A., and Fowler, M. N., eds., Cambridge University Press, chapter 11, pp. 89–102.
11. Darvill, A. G. and Albersheim, P. (1984), *Ann. Rev. Plant Physiol.* **35,** 243.
12. Amin, M., Kurosaki, F., and Nishi, A. (1986), *J. Gen. Microbiol.* **132,** 771.
13. Gamborg, O. L. (1981), *Plant Tissue Culture Methods,* Gamborg, O. L. and Wetter, L. R., eds., National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, pp. 1–9.
14. Gamborg, O. L., Miller, R. A., and Ojima, K. (1968), *Exp. Cell Res.* **50,** 151.
15. Kubek, D. J. and Shuler, M. L. (1980), *J. Nat. Prod.* **43,** 1987.
16. Hille, A., Purwin, C., and Ebel, J. (1982), *Plant Cell Rpts.* **1,** 123.
17. Decker, A. L. (1977), *Worthington Enzymes, Enzyme Reagents and Related Biochemicals,* Worthington Biochemical, Freehold, NJ, pp. 66–67.
18. Chandler, S. F. and Dodds, J. H. (1983), *Plant Cell Rpts.* **2,** 105.
19. Singleton, V. L. and Rossi, J. A. (1965), *Am. J. Enol. Vitic.* **163,** 144.
20. Morris, D. L. (1948), *Science* **107,** 254.
21. Zucker, M. (1967), *Plant Physiol.* **44,** 912.
22. Sahai, O. P. and Shuler, M. L. (1984), *Botech. Bioeng.* **26,** 111.
23. Krell, H. W., Artmann, M., and Seidel, H. (1986), Abstr. VI Int. Cong. Plant Tissue and Cell Culture, Minneapolis, MN.
24. Mader, M. and Walter, C. (1986), *Planta* **169,** 273.
25. Rolfs, C. H., Schon, H., Steffens, M., and Kindl, H. (1987), *Planta* **172,** 238.
26. Zenk, M. H., El-Shagi, H., Arens, H., Stockigt, J., Weiler, E. W., and Deus, B. (1977), *Plant Tissue Culture and its Biotechnological Application,* Barz, W., Reinhard, E., and Zenk, M. H., eds., Springer Verlag, Berlin, pp. 27–43.
27. DiCosmo, F. and Misawa, M. (1985), *Biotech.* **3,** 318.
28. Eilert, U., Kurz, W. G. N., and Constabel, F. (1985), *J. Plant Physiol.* **119,** 65.
29. Eilert, U. (1987), *Cell Culture and Somatic Cell Genetics of Plants,* vol. 4, Vasil, I. K., and Constabel, F. eds., Academic, NY.
30. Hahlbrock, K., Schroder, J., and Vieregge, J. (1980), *Advances in Biochem. ogy/Bioengineering,* Springer Verlag, Berlin, pp. 000.
31. Kochs, G., Welle, R., and Grisebach, H. (1987), *Planta* **171,** 519.

32. Ebel, J. (1986), *Ann. Rev. Phytopathol.* **24,** 235.
33. Margarita, R., Stab, J., and Ebel, J. (1986), Molecular and Cellular Aspects of Calcium in Plant Development, Trewavas, A. J., ed., NATO ASI Series A: Life Sciences, **104,** 331.
34. Anderson, J. M. and Cormier, M. J. (1978), *Biochem. Biophys. Res. Commun.* **84,** 595.
35. Muhitch, M. J. and Fletcher, J. S. (1985), *Plant Physiol.* **78,** 25.
36. Bagni, N., Torrigianni, P., and Barbieri, P. (1981), *Med. Biol.* **59,** 403.
37. Altman, A., Kaur-sawhney, R., and Galston, A. W. (1977), *Plant Physiol.* **60,** 570.
38. Greenland, A. J. and Lewis, D. H. (1984), *New Phytol.* **96,** 283.
39. Martin-Tanguy, J., Martin, C., Gallet, M., and Vernoy, R. (1976), *C. R. Acad. Sci.* (Paris) **282,** 2231.
40. Schobert, B. (1977), *J. Theor. Biol.* **68,** 17.