

6034

Handbook of Phytoalexin Metabolism and Action

edited by

M. Daniel

*The Maharaja Sayajirao University
of Baroda*

Vadodara, Gujarat, India

R. P. Purkayastha

*University of Calcutta
Calcutta, West Bengal, India*

6

Culture Darkening, Cell Aggregate Size, and Phytoalexin Accumulation in Soybean Cell Suspensions Challenged with Biotic Agents

Robert M. Zacharius

R. M. Zacharius and Associates, Science Consultants, Highland, Maryland

William F. Fett

*Eastern Regional Research Center, Agricultural Research Service,
U.S. Department of Agriculture, Philadelphia, Pennsylvania*

Prakash G. Kadkade

Phyton Inc., Ithaca, New York

I. INTRODUCTION

Fett and Zacharius (1982, 1983) demonstrated that bacteria as well as fungal cell wall elicitors can induce the accumulation of the phytoalexin glyceollin in soybean (*Glycine max* L. Merr.) cell suspension cultures. Moreover, the typical hypersensitive response (HR) as defined by rapid host cell death displayed by the intact plant was not a prerequisite for phytoalexin induction in soybean cell cultures. The concentration of glyceollin produced declined with successive culture transfers. Some interesting responses by separate cell lines of the same cultivar to challenge with a biotic agent were observed, suggesting further study.

A subsequent study by Zacharius and Kalan (1990) revealed that soybean cell suspension cultures producing glyceollin when challenged with *Pseudomonas syringae* pv. *glycinea* (Psg) or fungal cell wall elicitor did not undergo an HR but rather darkened with a gradual decline in culture viability. A cell line, Sb-1 (cv. Mandarin), which failed to darken on challenge with Psg, also did not accumulate glyceollin within the cells or media. This Sb-1 culture was found to have rather low levels of constituent isoflavonoid which exhibited a small decline on exposure to biotic agents and produced only trace amounts of glyceollin. The other cell lines of cv. Mandarin having high levels of constituent isoflavonoids, exhibited a dramatic decline

in their isoflavonoids along with accumulation of glyceollin following exposure to biotic agents.

High levels of constitutive isoflavonoids, particularly daidzein, seemed indicative of culture potential for glyceollin production concomitant with a decrease in daidzein, genistein, and coumestrol when either fungal wall elicitors or live bacteria was the stressing agent. However, cell cultures containing very high levels of these isoflavonoids did not accumulate higher levels of the phytoalexin than those with lesser levels. Augmentation of the weakly responsive Sb-1 culture with exogenously supplied isoflavonoids followed by fungal elicitor challenge had little measurable effect on glyceollin production, but resulted in a metabolic breakdown of the exogenously supplied daidzein and genistein.

Thus, on the basis of this study, there appeared to be a link between endogenous levels of cell isoflavonoids, ability for culture darkening, and glyceollin production. From other observations with cv. Mandarin cell suspensions, we noted the larger cell aggregates tended to darken more readily with exposure to fungal wall elicitor than the smaller aggregates. Therefore, our earlier studies were extended to include both smaller and larger cell suspension aggregations of soybean cv. Mandarin and cv. Clark challenged by live bacteria and fungal elicitor. We speculated that large cell aggregates may have a closer relationship to in vivo tissues of an organized structure than single cells or small cell aggregates.

II. METHODOLOGY

A. Suspension Cell Cultures

Calluses of soybean cv. Mandarin cell line Sb-4a were initiated from epicotyl tissue of 7- to 10-day old plants by the procedure described by Fett and Zacharius (1982). Calluses of soybean cv. Clark were also initiated from epicotyl explants on B5 media (Gamborg, 1975) containing 10 mg/liter 2,4-D and 0.21 mg/liter kinetin. A suspension cell culture was developed from friable calluses of each cultivar and grown in 1B5 media on a reciprocating shaker at 150 rpm at 26–27°C under $17.5 \pm 4.5 \mu\text{mol}/\text{m}^2/\text{sec}$ photosynthetic photon flux (PPF).

B. Bacterial Cultures

Xanthomonas campestris pv. *glycines* strains XP175 and S-9-8 were grown overnight on nutrient agar at 28°C, suspended in sterile distilled water, washed three times, and resuspended in sterile distilled water to 1.0 OD at 600 nm. Strain XP175 is virulent and causes bacterial pustule disease on cv. Mandarin and cv. Clark while strain S-9-8 is avirulent (Fett, 1984).

C. Fungal Elicitor

A cell-free mycelial elicitor was prepared from *Phytophthora infestans* race 0 by the procedure of Alves et al. (1979) with an added final filtration step through a 0.45- μm Millipore filter before autoclaving. The elicitor preparation contained 6.4 mg dry wt per milliliter distilled water.

D. Interaction of Suspension Cells with Bacteria or Fungal Elicitors

Suspension cultures of each cultivar of soybean were grown and maintained in 60-ml volumes of 1B5 medium in 250-ml Delong flasks and were used 5 days after the last transfer. The contents of several flasks of Sb-4A or Sb-Clark were each aseptically sieved on 100-mesh wire screen to separate large, > 150- μm aggregates and small, < 150- μm aggregates. Aggregates of one size and culture were pooled and 10 ml of loosely packed aggregates was redistributed into flasks with 40 ml of fresh 1B5 media. Fungal elicitor was applied to each flask at 0.75 ml/50 ml of aggregated cell culture and each bacterial strain was added to give an initial concentration of approximately 1×10^7 CFU/ml. Each aggregate size/cultivar/biotic-elicitor interaction and controls were carried out with three replicates. All flasks were shaken at 150 rpm at 26–27°C under $17.5 \pm 4.5 \mu\text{mol}/\text{m}^2/\text{sec}$ PPF.

Cell viability was followed by a dye exclusion test with 0.4% trypan blue (Phillips, 1973).

E. Isoflavonoid Extraction, Identification, and Quantitation

Following 68 hr of interaction, cells plus media were extracted by vortex mixing four times, each with an equal volume of chloroform (Zacharius and Kalan, 1990). The combined extracts were dried at ambient temperature under a stream of nitrogen and taken up in 1.0 ml methanol 0.5 g^{-1} dry weight. Components of the extract were separated qualitatively by thin-layer chromatography (TLC) on Analtech silica gel G plates (250 μm) irrigated with cyclohexane–ethyl acetate (1:1) (Zacharius and Kalan, 1984). Quantitation was performed by high-performance liquid chromatography (HPLC) using a 250-mm C_{18} reverse phase column (Whatman Partisil 10 ODS-325) attached to a Waters Model 6000A solvent delivery system with a Reodyne (70-10) loop injector valve. The column effluent was monitored with a Perkin-Elmer Model LC-55B spectrophotometric detector at 262 nm (genistein and daidzein), 343 nm (coumestrol), and 290 nm (glyceollins). Integrations were made with the Hewlett-Packard 3390A integrator and compared with those of reference compounds (Zacharius and Kalan, 1990).

The column eluant was 40% aqueous acetonitrile containing trifluoroacetic acid (0.4 ml/liter) at a flow rate of 1.5 ml/min. Retention times

(min) were as follows: daidzein, 2.2; genistein, 3.3; formononetin, 4.5; coumestrol, 6.0; glyceollin isomers, 7.8, 8.0; biochanin A, 9.5. The presence of glyceollin, daidzein, genistein, and coumestrol in selected samples was further confirmed by comparison with authentic compounds by mass spectrometry using a Finnegan MAT 311A mass spectrometer.

F. Peroxidase Assay

The peroxidase assay was carried out according to the method of Worthington (1972).

III. RESULTS

The large cell aggregates of both Sb-Clark and Sb-4A dramatically darkened when challenged with either virulent strain XP175 or avirulent strain S-9-8 or the fungal elicitor. On the other hand, these challenges with the small aggregate cultures of either soybean cultivar produced no visual change in color or color intensity (Fig. 1).

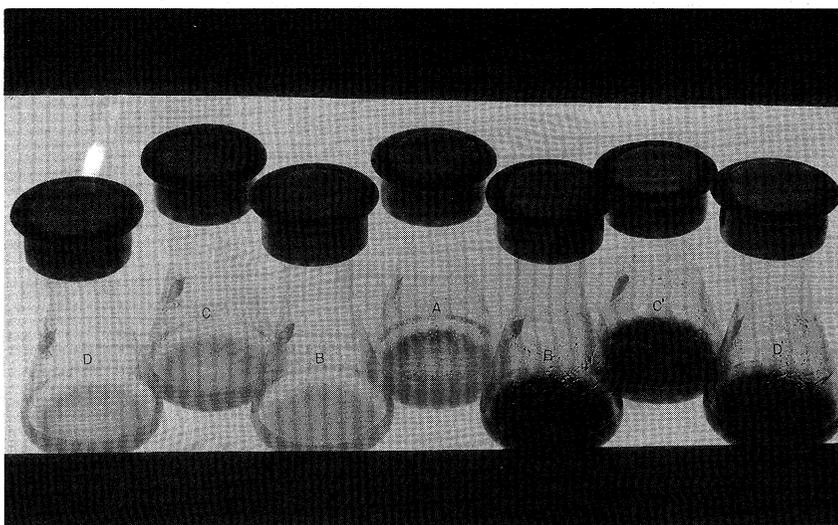


Figure 1 Culture darkening 68 hr after inoculation of small or large cell aggregates of Sb-Clark with *Xanthomonas campestris* pv. *glycines*. Mixed aggregate culture control, A; virulent *X. c.* pv. *glycines* strain XP175 in small (B) or large (B') aggregate cultures; avirulent *X. c.* pv. *glycines* strain S-9-8 in small (C) or large (C') aggregate cultures; fungal elicitor in small (D) or large (D') aggregate cultures.

Both size cell aggregates of Sb-Clark responded to each of the stressing agents producing similar levels of glyceollin with a marked but unequal decrease in the constitutive level of daidzein and genistein. Avirulent strain S-9-8 effected a virtual disappearance of these two isoflavonoids from the cell culture (Table 1).

Both large and small aggregates of Sb-4A when exposed to avirulent strain S-9-8 produced similar levels of glyceollin, whereas the virulent strain XP175 failed to do so with either size cell aggregates. Although the isoflavonoids declined in both size aggregates exposed to strain XP175, S-9-8 caused an almost total loss of daidzein and genistein (Table 1) as in the case of Sb-Clark.

Interaction of the large cell aggregates of either soybean cultivar with the fungal elicitor produced marked darkening, glyceollin accumulation, and a concurrent large decrease in daidzein and genistein. While the latter two phenomena occurred during the interaction with the small aggregates of both cultivars, neither small aggregate cell culture darkened (Fig. 1).

Both aggregate sizes of Sb-4A and the large aggregate culture of Sb-Clark became very viscous and gel-like when exposed to strain S-9-8. This was presumably due to copious production of bacterial exopolysaccharides accompanied by a concurrent increase in CFU per milliliter to 10^{10} . This was not observed in the other interactions. Where the challenged cultures became gel-like, a sharp decline in soybean cell viability occurred by 68 hr.

Table 1 Changes in the Isoflavonoids of Large and Small Cell Aggregates of Sb-Clark and Sb-4A (cv. Mandarin) upon Biotic Stressing ($\mu\text{g/g}$ dry wt)

Treatment	Daidzein		Genistein		Glyceollin	
	Sb-Clark	Sb-4A	Sb-Clark	Sb-4A	Sb-Clark	Sb-4A
Small aggregates						
Control	2560	1573	5080	7343	0	0
Strain XP175 ^a	576	354	940	1565	790	0
Strain S-9-8 ^b	9	50	73	38	908	1395
<i>P. infestans</i> elicitor	1201	236	1180	870	880	975
Large aggregates						
Control	3240	2569	5866	8239	0	0
Strain XP175 ^a	742	1837	515	3585	812	0
Strain S-9-8 ^b	16	40	77	107	867	1140
<i>P. infestans</i> elicitor	1441	385	1109	980	912	1100

^aStrain XP175 = *X. campestris* pv. *glycines* XP175.

^bStrain S-9-8 = *X. campestris* pv. *glycines* S-9-8.

There was little change in the soybean cell viability in the other interactions (Table 2).

Following exposure to the stressing agent strain XP175, the larger cell aggregates of both soybean cultivars were found to have higher peroxidase levels than the small aggregates. The changes from the prestressed levels were of similar magnitude for both cell cultivars (Table 3).

Attachment of the bacterial strains to soybean cells of either aggregate size of Sb-Clark or Sb-4A cultures was not observed using phase contrast light microscopy and bacterial-induced clumping of cells of any of the cultures did not occur.

IV. DISCUSSION

In our earlier report (Zacharius and Kalan, 1990), data were presented which appeared to relate soybean suspension culture darkening with glyceollin induction during bacterial or fungal elicitor stress. A high level of cellular daidzein also seemed prerequisite to both phenomena. Concurrent with glyceollin induction, a large decline of the cellular daidzein and genistein usually occurred. In the present study, the small aggregates were able

Table 2 Cell Viability of Sb-Clark and Sb-4A (cv. Mandarin) Cultures and Bacterial Growth after 68 hr Interaction

Cell suspension	Aggregate size	Treatment	CFU/ml	% Viable soybean cells
Clark	Small	Control	0	89
	Large	Control	0	92
	Small	XP175 ^a	2.20×10^8	89
	Large	XP175	1.12×10^9	85
	Small	S-9-8 ^a	2.23×10^8	83
	Large	S-9-8	1.13×10^{10}	0
	Small	Fungal elicitor	0	88
	Large	Fungal elicitor	0	84
Sb-4A	Small	Control	0	85
	Large	Control	0	88
	Small	XP175	8.60×10^7	85
	Large	XP175	6.47×10^8	87
	Small	S-9-8	1.17×10^{10}	45
	Large	S-9-8	1.40×10^{10}	15
	Small	Fungal elicitor	0	85
	Large	Fungal elicitor	0	83

^a*Xanthomonas campestris* pv. *glycines*.

Table 3 Changes in Peroxidase Levels in Cell Aggregates of Sb-Clark and Sb-4A (cv. Mandarin) on Challenge with *Xanthomonas campestris* pv. *glycines* strain XP175

Soybean cultivar	Aggregate size (μm)	Absorbance change at 460 nm/mg protein	
		Suspension	Suspension after XP175
Sb-Clark	< 150	0.60	0.85
	> 150	0.70	2.70
	Whole unsieved culture	0.68	2.50
Sb-4A	< 150	0.65	0.85
	> 150	0.75	2.70
	Whole unsieved culture	0.70	2.50

to accumulate glyceollin without observable darkening. When glyceollin accumulated in either size aggregates, the levels were virtually the same irrespective of whether the aggregates were darkened or not. In addition, in the case of Sb-4A exposed to virulent strain XP175, neither the small undarkened cell aggregates nor the large darkened cell aggregates accumulated glyceollin although the constituent isoflavonoids of the culture underwent a decline. This would suggest that metabolic consumption of these constitutive isoflavonoids need not be linked to glyceollin induction.

The propensity of the soybean culture to darken with biotic stressing was reflected in the size of the cell aggregates of the culture. Large aggregates darkened on exposure to any of the three stressing agents while the small ones did not. Earlier Zacharius and Kalan (1990) had reported that an unsieved cv. Mandarin culture of mixed size aggregates did not show observable darkening on stressing with fungal elicitor but the cultures had a low level of constitutive daidzein and genistein.

Cell darkening of suspension cultures with or without biotic stressing would appear to reflect the level of phenolic compounds present in the cells and medium. Interestingly, small cell aggregates used in this study had lower constitutive isoflavonoid levels than the larger cell aggregates. Siegel and Enns (1979) were able to prevent the discoloration and aggregation of soybean suspension cultures grown in B5 medium with 2,4-D by adsorbing the excess cellular polyphenols with either polyvinylpyrrolidone or bovine serum albumin in the medium. Additionally, Singh et al. (1982) also associated higher levels of polyphenols in cowpea callus (*Vigna unguiculata* L.

Walp. subsp. *unguiculata*) with higher activities of peroxidase and polyphenol oxidase. Moreover, polyphenol content increased with 2,4-D concentration above 1 $\mu\text{g}/\text{ml}$ while supplements of casein hydrolysate and coconut water produced the lowest polyphenol accumulation. In our study, soybean cultures were grown with 1 $\mu\text{g}/\text{ml}$ of 2,4-D without casein hydrolysate and coconut water. Although we did not compare polyphenol levels, increased levels of peroxidase were associated with those cell aggregates which darkened markedly with addition of strain XP175.

Peroxidase levels were found by Verma and Van Huystee (1970) to be 2.5-fold greater in large peanut cell aggregates (2–4 mm) than in small ones (150 μm). It was also found that a cell mass less than 0.5 mm in diameter consists of undifferentiated uniform cells while cellular differentiation appears in large cell aggregates. Working with tobacco suspension cultures, Kuboi and Yamada (1976, 1978) found stimulation of the lignin biosynthetic pathway, during tracheid differentiation, occurs in large aggregates but low activities of shikimate dehydrogenase, cinnamic acid-4-hydroxylase, 5-hydroxyferulic acid-*O*-methyltransferase, and caffeic acid-*O*-methyltransferase inhibit differentiation in small aggregates. Both phenylalanine ammonia-lyase (PAL) and peroxidase exhibited activities in the small aggregates. Hahlbrock et al. (1974) found that the highest specific activity of PAL is associated with single cells and small aggregates, while the specific activities in large aggregates were considerably lower. This would explain finding greater secondary compound production in cultures of small aggregates and viable single cells. Kinnersley and Dougall (1980) succeeded in increasing the anthocyanin yield in *Daucus carota* L. cell cultures by screening for small cell aggregates and subculturing; a consequence of this selection was small aggregates with a lower level of endogenous cytokinin (which reduce anthocyanin yield). Watts et al. (1984) found evidence to suggest that the presence of green, aggregated cells or low-temperature stress contributes to the ability of celery cell suspensions to synthesize secondary compounds. Nevertheless, the level of glyceollin accumulated in the large and small aggregates of soybean described here were quite similar.

Perhaps Apostol et al. (1989) provide in part the most rational explanation of our observations regarding the relationship of aggregate size, darkening, and glyceollin production. They found that elicitor-stimulated plant cell cultures responded with the rapid production of H_2O_2 which was subsequently used by extracellular peroxidases. Exogenous H_2O_2 alone stimulated phytoalexin production in the soybean cell suspension culture, and inhibition of elicitor stimulated glyceollin production was observed upon addition of catalase or other inhibitors of the oxidative burst. For inhibition to occur, the presence of catalase was necessary during elicitor addition.

Montillet and Degousée (1991) have reported much greater glyceollin-eliciting activity in soybean seedlings by two organic hydroperoxides than H_2O_2 . H_2O_2 eliciting efficiency was comparable to the two organic hydroperoxides when tissue catalase activity was suppressed. Of further interest, Graham and Graham (1991) found that deposition of phenolic polymers in soybean cotyledon cell walls is an early and major response to treatment with fungal cell wall glucan. This is accompanied by a rapid and massive increase in activity of a specific group of anionic wall-bound peroxidases.

Analysis of our observations in light of the above suggests that the large aggregates of Sb-Clark and Sb-4A responded to biotic stress by severely darkening and at the same time a burst of H_2O_2 induced (as indicated by fourfold increase in peroxidase level) the glyceollin production. The small aggregates of both cultivars did not discolor because of a limited H_2O_2 burst indicated by only a slight increase in peroxidase level, but these aggregates presumably contained high activities of the early phytoalexin pathway enzymes, allowing for glyceollin production. The sharp decline in isoflavonoids in soybean suspension cultures treated with biotic elicitors (Zacharius and Kalan, 1990) almost certainly can be explained by the oxidative burst occurring during elicitation. Culture darkening and the level of destruction of the measured isoflavonoids following elicitation probably reflects firstly on the intensity of the oxidative burst and secondly on the induced level of the peroxidases present in the culture.

Strain S-9-8 is avirulent and strain XP175 is virulent on both cv. Clark and cv. Mandarin leaves. Fett (1984), however, found moderate glyceollin accumulation in leaves of cv. Clark inoculated with virulent strain XP175 and a weak hypersensitive response with no glyceollin accumulation following inoculation with avirulent strain S-9-8. Growth of strain S-9-8 was restricted in leaves cv. Clark 24 hr after inoculation when compared to growth of strain XP175. The leaf observations with cv. Clark were inconsistent with those described here for the cell cultures. Leaves of Sb-4A (cv. Mandarin) were not treated with either strain.

The level of glyceollin which accumulated in the inoculated large and small aggregate cultures did not appear to determine the bacterial cell populations attained (Table 2). Strain S-9-8 grew to much higher levels than strain XP175 in both the large and small aggregate Sb-4A cultures, while strain S-9-8 but not strain XP175 induced accumulation of appreciable levels of glyceollin. Both aggregate size cultures of Sb-Clark stressed with either strain of *X. campestris* yielded similar amounts of glyceollin (Table 1), yet strain S-9-8 grew 200 times greater in the large cell aggregations than in the small aggregates. Strain S-9-8 attained higher populations than did strain XP175 under all cell culture conditions except for the small aggregate sized Sb-Clark. Evidence offered here indicates little or no control by gly-

ceollin on the growth of *X. campestris* in the cell suspension cultures. The percentage of viable soybean cells remaining after 68 hr exposure to the *X. campestris* agrees well with the bacterial populations attained.

Observations with phase microscopy did not indicate any attachment of strains S-9-8 or XP175 to Sb-Clark suspension cells of either aggregate type. This is of interest in view of the findings of Jones and Fett (1984) that strain S-9-8 is immobilized by electron-dense material in leaf intercellular spaces of cv. Clark while strain XP175 is not.

V. EPILOG

The use of plant cell suspension cultures to study the interaction of plants with biotic elicitors such as bacteria may lead to results that do not accurately reflect responses of the intact plant. This is evidenced by the result for glyceollin induction, bacterial growth, and bacterial immobilization presented here and in our earlier studies of the interaction of soybean cells with plant pathogenic bacteria (Fett, 1984; Fett and Zacharius, 1982, 1983). Careful considerations need to be given to the age of the cell suspension culture, the culture medium, and cell aggregate size distribution. The fact that plant cells in suspension cultures are continually bathed in large volumes of liquid whereas leaf intercellular spaces (where leaf-spotting bacteria reside and grow) are initially deficient in free water may preclude certain important interactions such as prolonged bacterial cell-plant cell contact from taking place. However, response of cell suspension culture to bacterial inoculation can accurately reflect certain in planta phenomena during plant-bacterial interactions as demonstrated by recent studies of Orlandi et al. (1992).

Future research should be directed at determining optimal cell suspension culture conditions and cell aggregate sizes to be used in order to more closely mimic in planta phenomena.

REFERENCES

- Alves, L. M., Heisler, E. G., Kissinger, J. C., Patterson, J. M., and Kalan, E. B. (1979). Effects of controlled atmosphere on production of sesquiterpenoid stress metabolites by white potato tuber. Possible involvement of cyanide-resistant respiration. *Plant Physiol.* 63:359-362.
- Apostol, I., Heinstejn, P. F., and Low, P. S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiol.* 90:109-116.
- Fett, W. F. (1984). Accumulation of isoflavonoids and isoflavone glucosides after inoculation of soybean leaves with *Xanthomonas campestris* pv. *glycines* and

- pv. *campestris* and a study of their role in resistance. *Physiol. Plant Pathol.* 24: 303–320.
- Fett, W. F., and Zacharius, R. M. (1982). Bacterially induced glyceollin production in soybean cell suspension cultures. *Plant Sci. Lett.* 24:303–309.
- Fett, W. F., and Zacharius, R. M. (1983). Bacterial growth and phytoalexin elicitation in soybean cell suspension cultures inoculated with *Pseudomonas syringae* pathovars. *Physiol. Plant Pathol.* 22:151–172.
- Gamborg, O. L. (1975). Callus and cell culture. *Plant Tissue Culture Methods* (O. L. Gamborg and L. R. Wetter, eds.), National Research Council of Canada, Saskatoon.
- Graham, M. Y., and Graham, T. L. (1991). Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. *Plant Physiol.* 97: 1445–1455.
- Hahlbrock, K., Ebel, J., and Oaks, A. (1974). Determination of specific growth stages of plant cell suspension cultures by monitoring conductivity changes in the medium. *Planta* 118:75–84.
- Jones, S. B., and Fett, W. F. (1985). Fate of *Xanthomonas campestris* infiltrated into soybean leaves: an ultrastructural study. *Phytopathology* 45:733–741.
- Kinnersley, A. M., and Dougall, D. K. (1980). Increase in anthocyanin yield from wild carrot cell cultures by a selection system based on cell aggregate size. *Planta* 149:200–204.
- Kuboi, T., and Yamada, Y. (1976). Caffeic acid-O-methyl transferase in a suspension of cell aggregates of tobacco. *Phytochemistry* 15:397–400.
- Kuboi, T., and Yamada, Y. (1978). Regulation of the enzyme activities related to lignin synthesis in cell aggregates of tobacco cell culture. *Biochim. Biophys. Acta* 542:181–190.
- Montillet, J. L., and Degoussé, N. (1991). Hydroperoxydes induce glyceollin accumulation in soybean. *Plant Physiol. Biochem.* 29:689–694.
- Orlandi, E. W., Hutcheson, S. W., and Baker, C. J. (1992). Early physiological responses associated with race-specific recognition in soybean leaf tissue and cell suspension treated with *Pseudomonas syringae* pv. *glycinea*. *Physiol. Mol. Plant Pathol.* 40:173–180.
- Phillips, J. H. (1973). Dye exclusion tests for cell viability. *Tissue Culture Methods and Applications* (F. F. Kruse, Jr., and M. K. Patterson, Jr., eds.), Academic Press, New York, pp. 406–408.
- Siegel, N. R., and Enns, R. K. (1979). Soluble polyvinylpyrrolidone and bovine serum albumin adsorb polyphenols from soybean suspension cultures. *Plant Physiol.* 63:206–208.
- Singh, B. D., Rao, G. S. R. L., and Singh, R. P. (1982). Polyphenol accumulation in callus cultures of cowpea (*Vigna sinensis*). *Indian J. Exp. Biol.* 20:387–389.
- Verma, D. P. S., and van Huystee, R. B. (1970). Cellular differentiation and peroxidase isozymes in cell culture of peanut cotyledons. *Can. J. Bot.* 48:429–431.
- Watts, M. J., Galpin, I. J., and Collin, H. A. (1984). The effect of growth regulators, light and temperature on flavour production in celery tissue cultures. *N. Phytol.* 98:583–591.

- Worthington Biochemical Corporation (1972). *Enzymes and Enzyme Reagents*, Freehold, NJ, pp. 43-44.
- Zacharius, R. M., and Kalan, E. B. (1984). Biotransformation of the potato stress metabolite, solavetivone, by cell suspension cultures of two solanaceous and three non-solanaceous species. *Plant Cell Rep.* 3:189-192.
- Zacharius, R. M., and Kalan, E. B. (1990). Isoflavonoid changes in soybean cell suspensions when challenged with intact bacteria or fungal elicitors. *J. Plant Physiol.* 135:732-736.