Acta Bot. Neerl. 37(3), September 1988, p. 363–370

Peroxidase in variant cell lines of *Haplopappus gracilis*

G. M. M. BREDEMEIJER and H. C. J. BURG

Research Institute Ital, P.O. Box 48, 6700 AA Wageningen, The Netherlands

SUMMARY

A comparative study on growth characteristics, phenolic content, protein content and peroxidase activity revealed differences between a wild type cell suspension culture of *Haplopappus gracilis* and three cell lines, varying in nitrogen metabolism. The variant cell lines showed enhanced growth and protein when compared to the wild type but reduced peroxidase activity, peroxidase isoenzyme polymorphism and phenolic content. Peroxidase activity and growth of *Haplopappus* cells were inversely related. Furthermore, the data indicated that peroxidase activity and phenolic content are not causally related and that the peroxidase is not involved in cell aggregation by polymerization of phenols. On the other hand, peroxidase probably oxidizes phenols to polymeric substances, causing browning during senescence.

Key-words: Haplopappus gracilis, cell culture, growth, peroxidase, phenols.

INTRODUCTION

It has been proposed that peroxidase plays a role in plant growth and differentiation by its involvement in auxin catabolism and assembly of polymers in the cell wall (Gaspar *et al.* 1982, Gaspar *et al.* 1985). Peroxidase activity and growth of plant cells are inversely related, probably as a consequence of the indole acetic acid (IAA) oxidase activity of peroxidase (Chibbar *et al.* 1984) and its ability to decrease the extensibility of cell walls by coupling wall polymer-bound phenols (Fry 1986). Another effect of the action of peroxidase on IAA may be an increase in the biosynthesis of phenols; IAA is known to repress this process (Davies 1972). However, data from the literature on the relation between peroxidase activity and phenol content are contradictory. Shah *et al.* (1978) have reported that tissues with a higher peroxidase activity show a greater accumulation of phenolic compounds. Others, however, have observed an inverse relationship between peroxidase and phenol content in the course of various processes (Druart *et al.* 1982, Gaspar *et al.* 1985).

Changes in peroxidase activity and isoenzyme composition in relation to growth of the wild type cell line of *Haplopappus gracilis* have been described earlier (Bredemeijer & Burg 1986). Several variant cell lines, derived from this wild type (Gilissen *et al.* 1985, Gilissen & Van Staveren 1986), differed in growth characteristics, production of phenols and peroxidase activity, and are, therefore, valuable material for investigations on the inter-relationships between these parameters. The present study reports data on growth characteristics,

Abbreviations: *PCV* = packed cell volume, IAA = Indole acetic acid.

phenol and protein content and peroxidase activity in the *Haplopappus* cell lines varying in nitrogen metabolism.

MATERIALS AND METHODS

Cell cultures and growth characteristics

A wild type (Wt) cell suspension culture of *Haplopappus gracilis* (Nutt) Gray and three cell lines that are variant in nitrogen metabolism (A0, A15 and A54) and originated from the Wt (Gilissen *et al.* 1985) were maintained on B5 medium by subculturing weekly.

The growth of the cell suspension cultures was determined by measuring the increase in the packed cell volume (*PCV*) according to Gilissen *et al.* (1983). For cell size determination, area measurements were obtained from photographs with an image analyser (Kontron Mop 30). For aggregate size measurements, the suspension culture was harvested by passing it through sieves with meshes of 1·0 and 0·5 mm. Thus, three fractions were collected and designed as $<0{\cdot}5$ mm, 0·5–1·0 mm and $>1{\cdot}0$ mm in diameter.

Analytical methods

Extraction, enzyme assay, and starch gel electrophoresis of peroxidase from cells and culture media were carried out as described previously (Bredemeijer *et al.* 1985; Bredemeijer & Burg 1986). Protein content was measured according to the instructions for the Bio-Rad protein assay, with bovine serum albumin as a standard. Phenolic compounds for quantitative analysis were extracted by stirring 0·5 g of cells at room temperature for 75 min in 10 ml 80% ethanol (Chandler & Dodds 1983). After centrifugation (18,000 ***g***, 30 min), 0·5 ml of supernatant was used to determine the total amount of phenols (Swain & Hillis 1959). The resultant colour after 1 min was measured at 725 nm (Constabel 1968). A calibration curve was made using chlorogenic acid as a standard. Chlorogenic acid is produced in relatively large amounts in *Haplopappus gracilis* callus (Stickland & Sunderland 1972).

RESULTS

Growth and morphological characteristics

The Wt and the three variant cell lines (A lines) of *H. gracilis* differed in growth and morphological characteristics. The A lines showed lower growth rates when compared to the Wt. However, growth in the A lines continued for a longer time so that the final yield of cells, expressed as *PCV* or grams fresh weight, exceeded that of the Wt cells (Fig. 1). On days 8 and 9 the *PCV* of A54 was significantly higher than that of the other cell lines (Student's *t*-test, $P<0{\cdot}02$).

The cell suspensions of all the lines generally consisted of a mixed population of cells of various shapes and sizes, existing either singly, in small groups, or in bigger aggregates (Fig. 2). The cell cultures of A15 and A54 were relatively fine suspensions compared with Wt, while A0 was coarser than Wt (Table 1). The average size of A cells significantly exceeded that of Wt cells (Table 1). In addition, the A lines contained giant cells (>100 μm), whereas the Wt had hardly any giant cells (Fig. 2).

During the 7-days culture cycle, the colour of the Wt was markedly darker (greyish green) than that of the A lines (pale yellow). When the cell suspensions were not sub-

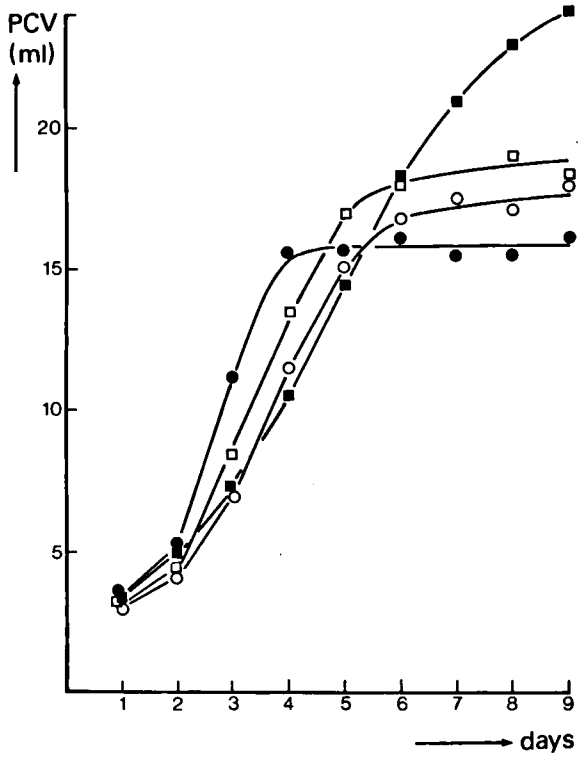

Fig. 1. Growth of cell suspensions as determined by an increase in packed cell volume *PCV* of a wild type (Wt, ●) and three variant cell lines (A0, ○; A15, □ and A54, ■) of *Haplopappus gracilis*. The data are the mean values from at least three independent flasks.

cultured to fresh medium, senescence occurred, resulting in the browning of cultures. Browning took place much earlier and was more intensive in the Wt than in the A lines. In 20-day-old cultures, cells and culture mediums of Wt were almost black; they were brown in the A0 and A15 cultures and pale brown in the A54 culture.

Peroxidase activity and phenol and protein content

When compared to the Wt, the A lines had a very low peroxidase activity and a low content of ethanol-extracted phenols (Table 2). By contrast, protein content was relatively high in the A lines (Table 2). In the most extreme case, the A54 culture, cellular peroxidase activity amounted to only 0·3% of the activity in Wt cells. The A cell lines also differed among themselves in peroxidase activity and phenol content. However, these differences were less pronounced than those between Wt and A lines.

In all cell lines a considerable proportion of cellular peroxidase was released into the culture medium. On day 7, after subculture and depending on the cell lines, 50–90% of the total peroxidase activity of the cultures was detected in the media (Table 2). The proportion of peroxidase released by Wt and A15 was similar but that released by A0 and A54 was clearly higher. In absolute amounts, however, the maximum peroxidase activity was detected in the Wt medium. The cells of A lines released relatively little of the total protein amount into the culture medium, as compared to Wt cells.

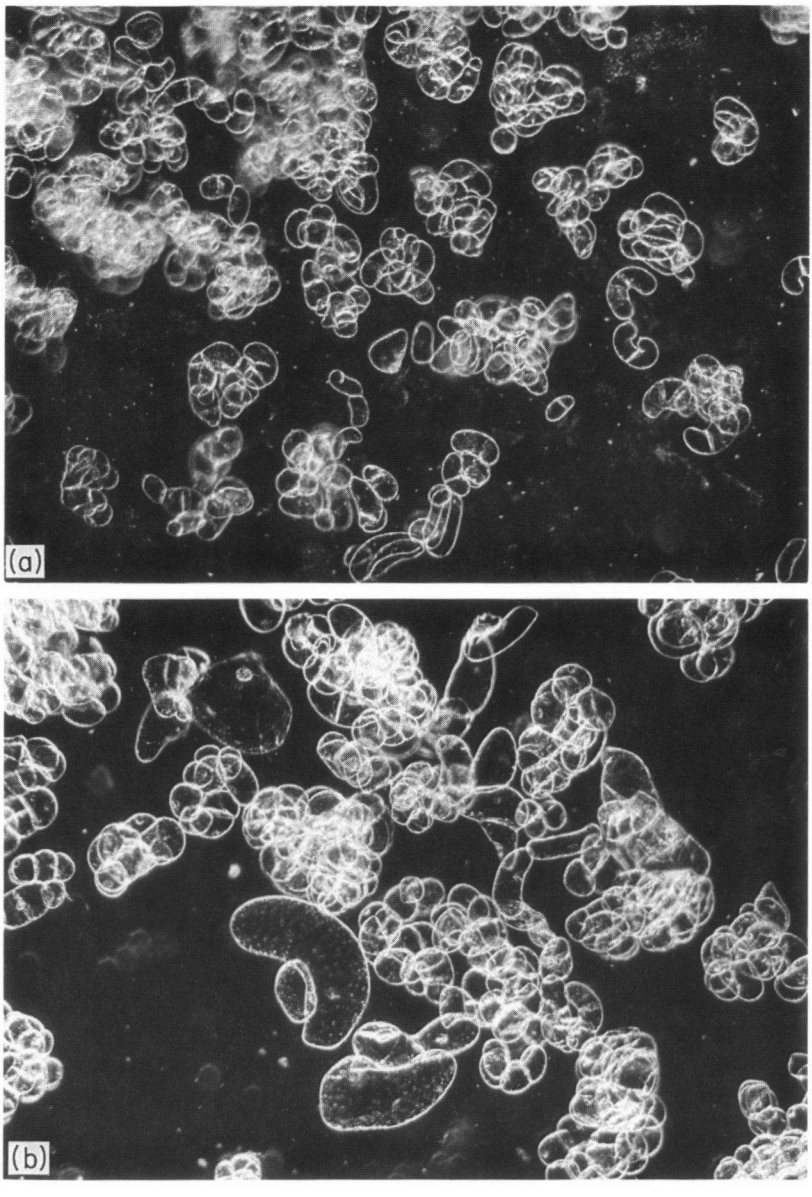

Fig. 2. Representative cells from Wt (a) and A54 (b) *Haplopappus gracilis* cultures grown for 7 days.

Peroxidase isoenzymes

The peroxidase isoenzyme patterns of all four cell lines differed both qualitatively and quantitatively (Fig. 3). When compared to Wt, the A lines had a lower number and a lower activity of peroxidase isoenzymes. The most extreme case was the isoenzyme pattern of A54 cells which showed only two weak bands. Except peroxidase isoenzyme C1 that was specific for the Wt, each of the peroxidase isoenzymes detected in the Wt occurred at least in one of the A lines.

Table 1. Aggregate size distribution and average cell size in 7-day-old Wt and A lines of *Haplopappus gracilis*. Data on aggregate size are the mean values from three flasks. For cell size determination 500 cells (250 per experiment), occurring singly or in small aggregates, were measured

	Cell aggregation Percentage fresh weight of tissue in total sample			Cell size Area (μm^2)	
Cell line	<0·5 mm	0·5–1·0 mm	>1 mm	Exp. 1	Exp. 2
Wt	74	19	7	1550	1690
A0	61	29	10	2780	2480
A15	70	28	2	3950	4130
A54	89	10	1	3300	3240

Table 2. Peroxidase activity, phenolic content and protein content of 7-day-old Wt and A lines of *Haplopappus gracilis*. Data are averages of two independent experiments (two flasks were pooled in each experiment)

	Cells			Culture medium	
Cell line	Peroxidase (Units/g fr w)	Phenolics (mg/g fr w)	Protein (mg/g fr w)	Percentage of total peroxidase	Percentage of total protein
Wt	281	5·1	1·1	51	20
A0	5	0·9	1·7	72	6
A15	14	0·3	1·9	53	2
A54	1	0·1	1·9	90	3

Comparison of the cellular peroxidase isoenzyme patterns with those of the culture medium (Fig. 3) demonstrates that release of peroxidase isoenzymes by plant cells is a selective process. The release of the three groups of peroxidase isoenzymes, i.e. A, B and C (Fig. 3) was different. In all cell lines used, the release of group A was relatively weak or even absent, while release of group C was relatively strong. In contrast to the A0 medium, the culture medium of the A15 cell line did not contain peroxidase isoenzymes of group A, while cells of both lines contained approximately the same amount of these isoenzymes. Thus, the results indicate that the cell lines differ not only in peroxidase activity and isoenzyme composition but also in the release of peroxidase.

DISCUSSION

The present study on the inter-relationships between peroxidase, phenol content and growth characteristics of *Haplopappus gracilis* cells confirms the view that peroxidase activity and growth are inversely correlated. Compared to that Wt cell line (high peroxidase activity), the variant A lines (low peroxidase activity) grew slightly more slowly

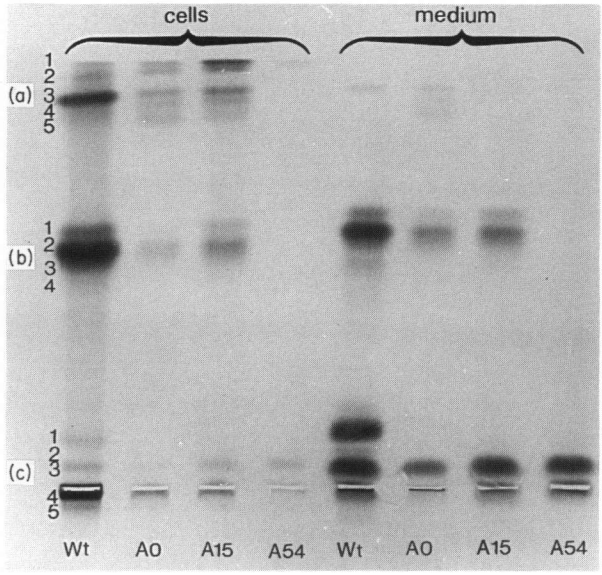

Fig. 3. Starch gel electrophoresis of peroxidase isoenzymes of cells and culture media of 7-day-old cultures of the Wt and A lines of *Haplopappus gracilis*.

during the exponential growth phase. However, the ultimate yield of cells was greater because growth continued for a longer time, especially in the peroxidase-poor A54 line. The increased yield in the A lines resulted from a considerable increase in average cell size, and, in A54, probably also in an increase in the number of cells. The latter is most likely because the yield of cells (*PCV*) in A54 exceeded that of A15, while cell size in A54 was less. Chibbar *et al.* (1984) have observed, by comparing two closely related carrot cell lines, that the number of cells was twofold greater in the cell culture that synthesized and released ten times less peroxidase.

It is not yet known whether peroxidase activity influences the yield of cells. Chibbar *et al.* (1984) assumed that an increase in peroxidase synthesis may be one of the factors for slow growth because peroxidase is also able to function as an IAA oxidase. In addition, it is possible that peroxidase-catalysed coupling of phenols, bound to wall polymers (extensin and polysaccharides), can tighten the structure of the cell wall matrix, resulting in decreased cell extensibility (Fry 1986). Finally, the products of peroxidase-catalysed oxidation of free phenols may inhibit growth by interaction with proteins and hence inhibition of metabolic pathways (Kefeli & Kutacek 1977). The fact that only cell line A54, with the lowest peroxidase activity and lowest phenol content, exhibited a significant increase in growth compared to Wt is in agreement with the view that peroxidase may reduce growth by oxidation of phenols.

The peroxidase-catalysed oxidation of phenols may not only play a role in growth of cell cultures but also in cell aggregation. According to King (1976), an increase in oxidation and polymerization of phenols by peroxidase may be one of the factors that contributes to an increased aggregation of the cells. From the data presented in this paper, it is evident that, in *Haplopappus gracilis* cell cultures, cell aggregation is not dependent on peroxidase activity and the content of ethanol-extracted phenols. The A0 culture was

coarser than Wt in spite of its much lower peroxidase activity and phenol content. This result, however, does not rule out the possibility that wall-bound phenols and wall peroxidase play a role in cell aggregation.

The peroxidase-rich Wt cells showed a great accumulation of phenolic compounds while the peroxidase-poor A cells contained few phenolic compounds, as reported for other tissues (Shah *et al.* 1978). It seems unlikely, however, that peroxidase in the *Haplopappus* culture regulates the phenol biosynthesis, since in the Wt culture the accumulation of phenols during the culture cycle preceded the maximum rate of peroxidase development (data not shown).

The enhanced yield of cells and protein synthesis, together with a decreased peroxidase activity and phenol content in the A lines, illustrates the lack of importance of peroxidase and phenols for the synthesizing cell itself, a characteristic feature of secondary metabolic enzymes and secondary products (Luckner 1984). According to Davies (1972), cell division and growth can take place even in the absence of phenol synthesis. Whether this also applies to peroxidase is unknown. However, the present results show that the *Haplopappus* cells can divide, grow and synthesize protein in spite of a very low peroxidase activity. It seems likely, therefore, that peroxidase in *Haplopappus* is mainly, if not at all, involved in secondary metabolism, for example in lignification processes (Fukuda & Komamine 1982), modification and breakdown of secondary plant products (Berlin & Barz 1975, Hösel *et al.* 1975) and oxidation of phenols to brown polymeric substances (Sheen 1974, Plumbley *et al.* 1981). The fact that the A cell cultures became brown later during senescence and to a much lesser extent, provides evidence that peroxidase may be involved in browning of the *Haplopappus* cultures.

ACKNOWLEDGEMENTS

The authors thank Dr K. Sree Ramulu for reading the manuscript and Dr L. J. W. Gilissen and M. J. van Staveren for supplying the various cell lines.

REFERENCES

Berlin, J. & Barz, W. (1975): Oxidative decarboxylation of para-hydroxybenzoic acids by peroxidases under *in vivo* and *in vitro* conditions. *Z. Naturforsch.* **30C:** 650–658.

Bredemeijer, G.M.M. & Burg, H.C.J. (1986): Peroxidase in suspension cultures of *Haplopappus gracilis*. *Acta Bot. Neerl.* **35:** 153–159.

—,—, Sree Ramulu, K. & Dijkhuis, P. (1985): Release of peroxidase by cultured potato cells. *Acta Bot. Neerl.* **34:** 325–335.

Chandler, S.F. & Dodds, J.H. (1983): The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasodine in callus cultures of *Solanum laciniatum*. *Plant Cell Repo.* **2:** 205–208.

Chibbar, R.N., Cella, R., Albani, D. & Van Huystee R.B. (1984): The growth of and peroxidase synthesis by two carrot cell lines. *J. Exp. Bot.* **35:** 1846–1852.

Constabel, F. (1968): Gerbstoffproduktion der Calluskulturen von *Juniperus communis* L. *Planta*, **79:** 58–64.

Davies, M.E. (1972): Polyphenol synthesis in cell suspension cultures of Paul's Scarlet rose. *Planta*, **104:** 50–65.

Druart, Ph, Kevers, Cl., Boxus, Ph. & Gaspar, Th. (1982): *In vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. *Z. Pflanzenphysiol.* **108:** 429–436.

Fry, S.C. (1986): Cross-linking of matrix polymers in the growing cell walls of Angiosperms. *Ann. Rev. Plant Physiol.* **37:** 165–186.

Fukuda, H. & Komamine, A. (1982): Lignin synthesis and its related enzymes as markers of tracheary-element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. *Planta*, **155:** 423–430.

Gaspar, Th., Penel, C., Castillo, F.J. & Greppin, H. (1985): A two-step control of basic and acidic peroxidases and its significance for growth and development. *Physiol. Plant.* **64:** 418–423.

—, —, Thorpe, T. & Greppin, H. (1982): *Peroxidases 1970–1980.* Université de Genève, Centre de Botanique, Genève.

Gilissen, L.J.W., Barneix, A.T., Van Staveren, M. & Breteler, H. (1985): Variant cell lines of *Haplopappus gracilis* with disturbed activities of nitrate reductase and nitrite reductase. *Plant Physiol.* **78:** 658–660.

—, Hänisch Ten Cate, Ch. & Keen, B. (1983): A rapid method of determining growth characteristics of plant cell populations in batch suspension culture. *Plant Cell Repo.* **2:** 232–235.

— & Van Staveren, M.J. (1986): Zinc-resistent cell lines of *Haplopappus gracilis. J. Plant Physiol.* **125:** 95–103.

Hösel, W., Frey, G. & Barz, W. (1975): Ueber den Abbau von Flavonolen durch pflanzliche Peroxidasen. *Phytochemistry,* **14:** 417–422.

Kefeli, V.I. & Kutacek, M. (1977): Phenolic substances and their possible role in plant growth regulation. In: Pilot, P. E. (ed.): *Plant Growth Regulation.* 181–188. Springer-Verlag, Berlin.

King, P.J. (1976): Studies on the growth in culture of plant cells. XX. Utilization of 2,4-dichlorophenoxyacetic acid by steady state cell cultures of *Acer pseudoplatanus* L. *J. Exp. Bot.* **27:** 1053–1072.

Luckner, M. (1984): *Secondary Metabolism in Microorganisms, Plants and Animals.* Springer-Verlag, Berlin.

Plumbley, R.A., Hughes, P.A. & Marriott, J. (1981): Studies on peroxidases and vascular discoloration in Cassava root tissue. *J. Sci. Food Agric.* **32:** 723–731.

Shah, R.R., Subbaiah, K.V. & Metha, A.R. (1978): Carbohydrate influence on polyphenol accumulation in Cassia and Datura tissues cultured *in vitro. Biol. Plant.* **20:** 5–13.

Sheen, S.J. (1974): Polyphenol oxidation by leaf peroxidases in *Nicotiana. Bot. Gaz.* **135:** 155–161.

Stickland, R.G. & Sunderland, N. (1972): Production of anthocyanins, flavonols, and chlorogenic acids by cultured callus tissues of *Haplopappus gracilis. Ann. Bot.* **36:** 443–457.

Swain, T. & Hillis, W.E. (1959): The phenolic constituents of *Prunus domestica.* I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **10:** 63–68.