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Production of peroxidases by hairy roots of *Brassica napus*

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Abstract

Hairy roots cultures derived from leaf explants of *Brassica napus* L. produced and secreted peroxidases. The enzyme activity in the medium increased with growth but it remained nearly constant in the tissue. The changes in extracellular peroxidase activity seemed to be correlated with the increase in a basic peroxidase of pI: 9.6. Four isoenzymes with pI in the range 8.5–9.6 and a neutral peroxidase of pI 6.3 were the most important peroxidases detected in cell extracts. Ca^{2+} addition at the beginning of the culture stimulated both the excretion of peroxidase to the medium and the enzyme activity in hairy roots but the isoenzyme profiles did not show qualitative changes during the growth cycle for both culture conditions.

Introduction

Plant roots are a unique and varied source of useful chemicals like secondary metabolites and enzymes used by the food and pharmaceutical industries (Flores, 1992; Flores and Curtis, 1992). Horseradish (*Armoracia rusticana* L.) roots contain peroxidases currently used for the commercial production of reagents for clinical purposes and enzyme immunoassays (Krell, 1991; Saitou et al., 1991).

Calluses and suspension cultures of different plant cells produce peroxidases which may be more than those of the plant roots (Shinshi and Noguchi, 1976; Yamada et al., 1987; Moreno et al., 1990; Urmantseva et al., 1991; Gazaryan et al. 1991; Macek et al. 1993). Callus cell heterogeneity and mutation tendency makes continuous selection necessary to maintain a stable cell line. So, horseradish hairy root cultures, obtained by transformation of tissues by inoculation with *Agrobacterium rhizogenes*, have been proposed as an alternative for the production of the enzyme (Parkinson et al., 1990) as well as secondary metabolites (Tanaka et al., 1985; Flores, 1992; Lipp Joao and Brown, 1994).

Hairy root cultures are generally characterized by high biosynthetic capacity and genetic and biochemical stability (Flores and Curtis, 1992; Toivonen and Rosenqvist, 1995). They offer a better prospect for the commercial production of secondary metabolites or enzymes than undifferentiated cell cultures. The ease of handling, the secretion of the enzymes to the medium and the ability of altering growth conditions to increase yields, make hairy root cultures an attractive system for large-scale peroxidase production.

Horseradish, the traditional source of peroxidases, does not grow well in our country. We have studied peroxidase production by turnip (*Brassica napus*) roots, callus and suspension cultures as alternative sources of this enzyme. Here we describe the production of peroxidases by hairy root cultures of turnip, the secretion of the enzyme to the culture medium throughout the growth cycle, both in basal conditions and in the presence of calcium (Penel et al., 1984; Uozomi et al., 1992; Xu and van Huystee, 1993).

Materials and methods

Establishment and maintenance of hairy root cultures

Seeds of *Brassica napus* were obtained from IFFIVE, INTA (Córdoba) and surface sterilized in a 30% solution of domestic bleach for 2 min. They were vacuum filtered and washed with three changes of sterile distilled water followed by soaking for 1 min in 70% ethanol. Seeds were transferred to a 50% solution of domestic bleach containing 50 µl of Tween 80 and were soaked for 15–20 min. Then they were washed three times with sterile distilled water and were placed on basal Murashige and Skoog medium containing 0.9% w/v agar (OXOID). They were incubated for 30 days at 25 ± 2 °C in a dark/ light regime of 16/8 hours under a light intensity of 15 µmoles $m^{-2}s^{-1}$.

Leaves of the resulting seedlings were used to produce hairy roots, obtained by inoculation of leaf explants with *Agrobacterium rhizogenes* strain LBA 9402 (a kind gift of Dr A.M. Giulietti, Fac. de Farmacia y Bioquímica, Universidad de Buenos Aires). Infected explants were transferred to a solid medium (0.8% w/v agar in water) and incubated for four days at 25 ± 2 °C in the dark. The explants were placed on solid Murashige and Skoog medium containing vitamins (Alvarez et al., 1993) and 1 g l^{-1} ampicillin. Fifteen to eighteen days later adventitious roots that appeared on the infected areas were excised and transferred to 125 ml Erlenmeyer flasks containing the same medium (without agar) for 25–30 days. Terminal apices of the growing hairy roots were subcultured to the same medium but without antibiotic and kept in an orbital shaker at 100 rev/min and at 25 ± 2 °C in the dark.

Culture conditions

For all experiments roots were grown for 20 days and then transferred aseptically to 125 ml Erlenmeyer flasks containing 50 ml liquid MS medium containing vitamins (with or without 50 mm $CaCl_2$) using an inoculum size of either 60 or 30 mg of fresh roots, respectively.

Enzyme extraction and peroxidase activity

Complete hairy roots developed in the flasks at each time of sampling were vacuum-filtered and dried between two sheets of filter paper. Roots were homogeneized in a mortar with 10 mM acetate acetic buffer pH 4, containing 1 M NaCl (1 g fresh weight per 10 ml of buffer) at 4 °C. Homogenates were centrifuged at 5000 *g* for 5 min and supernatants were used for enzyme assays. Peroxidase activity was determined with o-dianisidine as substrate in a 1 ml reaction mixture containing: 0.63 mM o-dianisidine, 0.5 mM H_2O_2 and 100 mM acetate acetic buffer pH 5.3 with 2 µl of root extracts or 10–20 µl of medium. Activity was measured following the increase in A_{470nm} for 1 min at 35 °C, produced by o-dianisidine oxidation. One unit of enzyme was arbitrarily defined as that giving an increment of 1.0 in the A_{470nm} under the conditions described.

Protein determination

Proteins were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Analysis of isoperoxidase patterns

Isoenzymes in the medium and homogenates of hairy roots (previously desalted with Sephadex G25 and concentrated when necessary) were identified by polyacrylamide gel electrophoresis (PAGE) (Reisfeld et al., 1962) . The patterns were also analyzed by isoelectric focusing (IEF), in a pH range of 3–10 on polyacrylamide gels using a BioRad Mini IEF system following the manufacturers' instructions. The gels were stained with the benzidine reagent (de Forchetti and Tigier, 1990) to detect peroxidase activity.

Results

As shown in Figure 1, peroxidase activity from extracts of roots in basal medium remained constant with time except for the high value registered on day 3 which is probably due to the stress imposed on the tissue by subculturing. Enzyme activity in the medium increased continuously with time (Figure 2) and was closely associated with growth.

Figures 3 and 4 show, respectively, the changes in peroxidase activity, protein content and fresh weight of the hairy roots and the culture medium with the time when the medium was supplemented with 50 mM $CaCl_2$.

Preliminary studies showed that the presence of calcium in the medium retarded the growth of these hairy root cultures (data not shown). So growth was

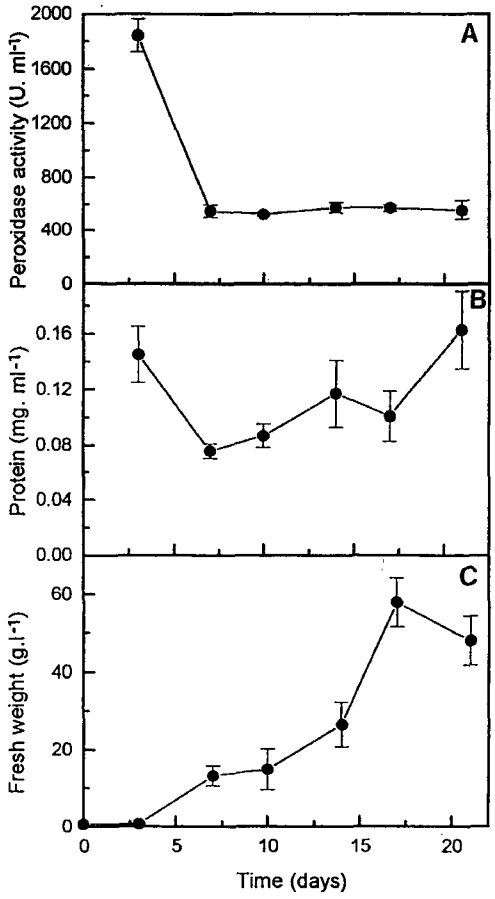

Figure 1. Changes in peroxidase activity (*A*), protein (*B*) and fresh weight (*C*) from root extracts with time of culture in "hairy root" cultures developed in basal medium.

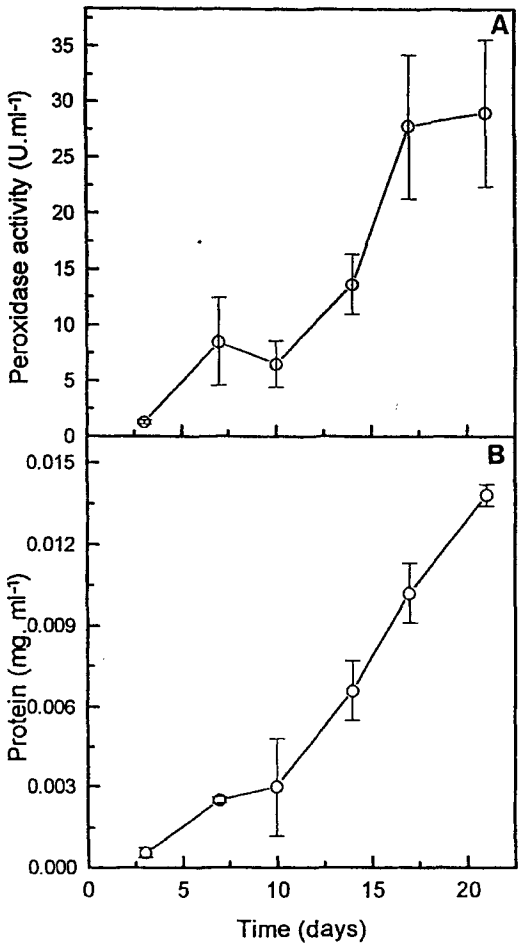

Figure 2. Increase in peroxidase activity (*A*) and protein content (*B*) of the culture basal medium with time of culture. Each point is the mean of at least three replicates and is shown with the standard error of the mean.

initiated with 60 mg of fresh roots per 50 ml medium instead of 30 mg per 50 ml used previously.

The fresh weight increased 10 times after 21 days of growth in the basal medium but only five times when hairy roots were exposed to 50 mM Ca^{2+}.

During the first days of growth both cultures showed similar changes in tissue peroxidase activities: the high levels detected at the beginning decreased to a minimum registered on day 6 (basal medium, Figure 1) or day 8 (Ca^{2+} supplemented medium, Figure 3). Then enzyme activities remained constant in the basal conditions but increased with time in the Ca^{2+} treatment. Extracellular peroxidase activity, detected in the medium, increased in tandem with the increase in fresh weight through the growth cycle for both cultures (Figures 2 and 4).

Protein content and specific peroxidase activity of Ca^{+2} supplemented medium were higher than those determined in the basal medium after 21 days of growth (Table 1). Nevertheless a direct effect of calcium on the enzyme activity was discarded because the addition of Ca^{+2} to the reaction mixture did not enhance the reaction rate of basal medium peroxidases.

No differences were detected in the pattern of isoenzyme bands in IEF or PAGE of crude extracts from treated and control roots, nor in both culture filtrates (Figures 5 and 6). The increase in extracellular peroxidase activity in the Ca^{2+} containing medium seemed to be correlated with an increment in a basic peroxidase (pI 9.6), the most prominent activity detected in the filtrate.

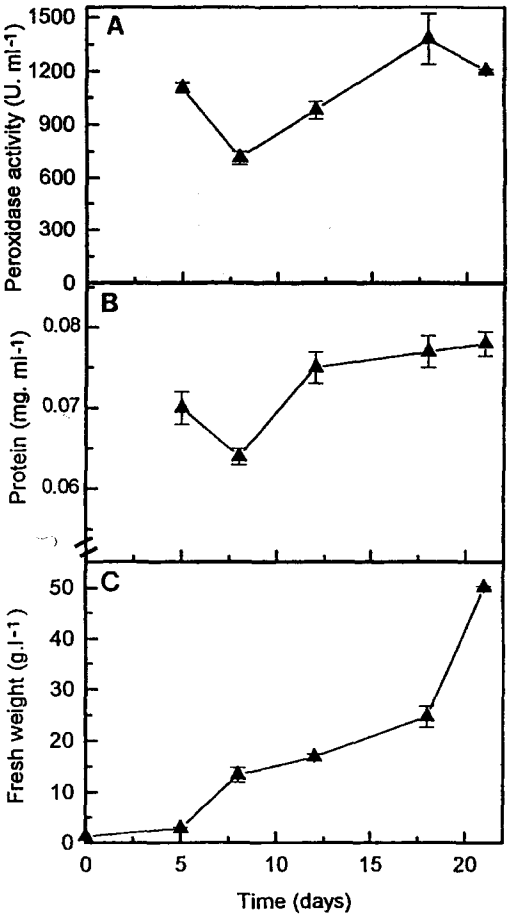

Figure 3. Changes in peroxidase activity (*A*) , protein (*B*) and fresh weight (*C*) of hairy root with the time of culture in medium supplemented with 50 mM $CaCl_2$

Table 1. Effect of $CaCl_2$ supplementation on cell mass, protein content and peroxidase specific activity of the culture medium after 21 days of growth.

Treatment	Cell mass ($g\ l^{-1}$)	Px. specific activity in the medium ($U\ mg^{-1}$)	Protein content in the medium ($mg\ ml^{-1}$)
Basal	57.9	1750	0.016
$CaCl_2$ 50 mM	50.1	5044	0.045

Four bands of high pI (in the pH range 8.5–9.6) and one of a near neutral isoform (pI 6.3) were the most important isoperoxidases detected in cell extracts. All of them increased their activity with the Ca^{2+} treatment.

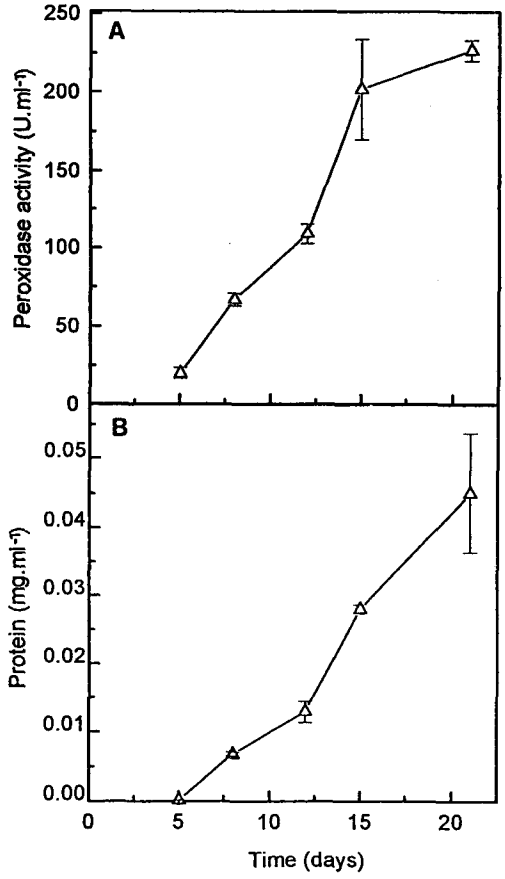

Figure 4. Peroxidase activity (*A*) and protein content (*B*) in the medium of hairy root cultures supplemented with 50 mM $CaCl_2$ along the growth cycle.

Discussion

Turnip hairy root cultures grew well in hormone-free liquid medium of Murashige and Skoog enriched with vitamins under the conditions described. They produce intra and extracellular peroxidases with PAGE and IEF isozyme patterns that did not show qualitative changes through the growth cycle.

Hairy roots developed faster and had better yields on a fresh weight basis in the basal medium than in that containing 50 mM calcium chloride. Nevertheless, peroxidase activities were higher in the latter. Uozomi et al., (1992) reported that treatments with 50 mM Na^+, K^+, Mg^{2+}, and Ca^{2+} promoted peroxidase excretion from horseradish hairy roots and their effects were proportional to the ionic strength and did not depend of the type of cation. They found $CaCl_2$ and $MgCl_2$ to be the most effective agents for enzyme secretion.

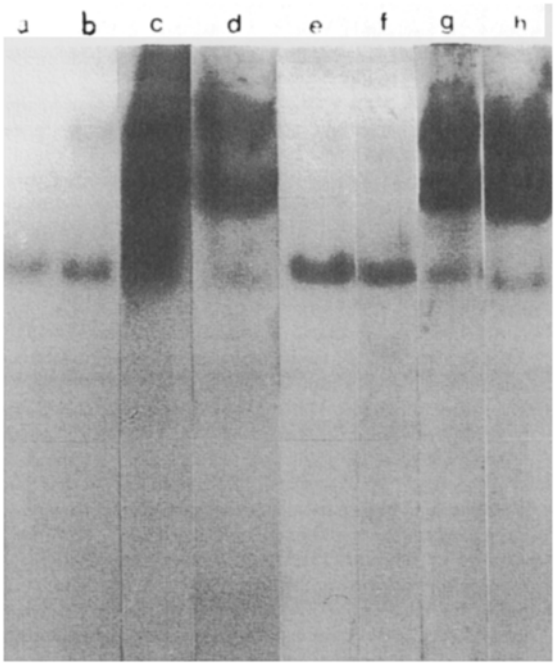

Figure 5. Peroxidase zymograms obtained by cationic PAGE from hairy roots and culture medium a) and b): culture filtrates from days 3 and 21 of growth in basal medium; c) and d): hairy root extracts from those cultures. e) and f): culture filtrates from day 3 and 21 of growth in Ca^{2+} supplemented medium: g) and h): extracts of the hairy roots developed in Ca^{2+} supplemented medium.

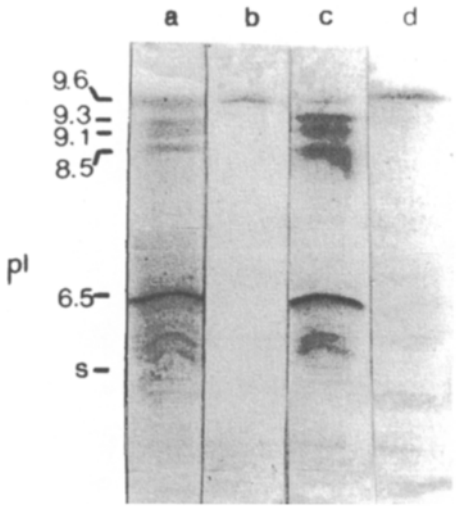

Figure 6. IEF patterns of peroxidases from hairy roots and culture medium. s) Corresponds to the sample loading position. Basal medium: a) hairy root extracts, b) culture filtrate. Ca^{2+} supplemented medium: c) hairy root extracts, d) culture filtrate.

Moreover, Ca^{2+} supplementation at the beginning of a culture was most effective for peroxidase excretion without loss of final cell mass compared with addition during the culture.

Turnip hairy roots secreted peroxidases to the culture medium during the growth cycle. Contrary to what has been described for horseradish hairy roots , in our cultures the enzyme levels increased with time. This is an important feature for the commercial production of the enzyme because extraction and purification would be simplified.

The isozyme profiles of the medium did not show any significant increase in those isoforms detected in cell extracts.

Both, intra and extracellular peroxidase activities, increased with calcium treatment although increase in biomass was limited in the supplemented medium.

The idea that Ca^{2+} controls peroxidase release (Kevers et al., 1982; Penel, 1986; Hu et al., 1987) was reexamined by Xu and van Huystee (1993 a, b). They found that cationic peroxidase secretion by cell suspension cultures of peanut is Ca^{2+} - dependent meanwhile release of the anionic isozyme is not Ca^{2+} -regulated. Whatever the mechanism involved in the process, calcium treatment would be advantageous to increase the release of peroxidases from *Brassica napus* hairy roots into the culture medium. The availability of peroxidases in culture filtrates would not only simplify their separation and purification, but would also allow the conservation of biomass to be re-used in repeated batch culture for the commercial production of the enzyme.

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References

Alvarez MA, Nigra HM & Giulietti AM (1993) Solasodine production by *Solanum eleagnifolium* Cav. *in vitro* cultures: Influence of plant growth regulators, age and inoculum size. Large scale production. Nat. Prod. Lett. 3 (1): 9–19

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

De Forchetti SM & Tigier HA (1990) Indole-3-acetic acid oxidase and syringaldazine oxidase of peroxidase isozymes in soybean root nodules. Physiol. Plant. 79: 327–330

Flores HE (1992) Plant roots as chemical factories. Chemistry & Industry: 374–377

Flores HE & Curtis WR (1992) Approaches to understanding and manipulating the biosynthetic potential of plant roots. Ann. N. Y. Acad. Sci. 665: 188–209

Gazaryan IG, Urmansteva VV, Veryovkin AN & Fechina VA (1991) Peroxidase preparation from *Medicago sativa* L-1 cell culture. In: Lobarzewski J, Greppin H, Penel C & Gaspar Th (Eds) Biochemical, Molecular and Physiological Aspects of Plant Peroxidases (pp 505–506). University of Geneva, Switzerland

Hu C, Lee D, Chibbar RN & van Huystee RB (1987) Ca^{2+} and peroxidase derived from cultured peanut cells. Physiol. Plant.70: 99–102

Kevers C, Sticher L, Penel C, Greppin H & Gaspar Th (1982) Calcium-controlled peroxidase secretion by sugarbeet cell suspension in relation to habituation. Plant Growth Regul.1: 61–65

Krell HW (1991) Peroxidase: an important enzyme for diagnostic test kits. In: Lobarzewski J, Greppin H, Penel C & Gaspar Th (Eds) Biochemical, Molecular and Physiological Aspects of Plant Peroxidases (pp 469–478). University of Geneva, Switzerland

Lipp Joao KH & Brown TA (1994) Long-term stability of root cultures of tomato transformed with *Agrobacterium rhizogenes* R1601. J. Exp. Bot. 45: 641–647

Macek T, Dobransky T, Pospíšilová R, Vaněk T & Králová B (1993) Peroxidases from *in vitro* cultures of different plant species. In: Welinder KG, Rasmussen SK, Penel C & Greppin H (eds) Plant Peroxidases: Biochemistry and Physiology (pp 451–455). University of Geneva

Moreno OA, Vazquez-Duhalt R & Nolasco H (1990) Extracellular accumulation of high specific-activity peroxidase by cell suspension cultures of cowpea. Plant Cell Rep. 9: 147–150

Parkinson M, Cotter T & Dix PJ (1990) Peroxidase production by cell suspension and hairy root cultures of horseradish (*Armoracia rusticana*). Plant Sci. 66: 271–277

Penel C, Sticher L, Kevers C, Gaspar Th & Greppin H (1984) Calcium-controlled peroxidase secretion by sugarbeet cells: Effect of ionophores in relation to organogenesis. Biochem. Physiol. Pflanzen 179: 173–180

Penel C (1986) The role of calcium in the control of peroxidase activity. In: Greppin H, Penel C & Gaspar Th (eds) Molecular and Physiological Aspects of Plant Peroxidases (pp 155–164). University of Geneva

Reisfeld RA, Lewis VT & Williams DE (1962) Disc- Electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195: 281–283

Saitou T, Kamada H & Harada H (1991) Isoperoxidase in hairy roots and regenerated plants of horseradish (*Armoracia lapathifolia*). Plant Sci. 75: 195–201

Shinshi H & Noguchi M (1976) Comparison of isoperoxidase patterns in tobacco cell cultures and in the intact plant. Phytochem. 15: 556–557

Tanaka N, Hayakawa M, Mano Y, Ohkawa H & Matsui Ch (1985) Infection of turnip and radish storage roots with *Agrobacterium rhizogenes*. Plant Cell Rep. 4: 74–77

Toivoneen L & Rosenqvist H (1995) Establishment and growth characteristics of *Glycyrriza glabra* hairy root cultures Plant Cell Tiss. Org. Cult. 41: 249–258

Uozomi N, Kato Y, Nakashimada Y & Kobayashi T (1992) Excretion of peroxidase from horseradish root in combination with ion supplementation. Appl. Microbiol. Biotechnol. 37: 560–565

Urmansteva V, Volkova L, Lipski A & Chaianova S (1991) Peroxidase production by the cell suspension of *Medicago sativa*. In: Lobarzewski J, Greppin H, Penel C & Gaspar Th (eds) Biochemical, Molecular and Physiological Aspects of Plant Peroxidases (pp 501–504). University of Geneva, Geneva

Yamada Y, Kobayashi S, Watanabe K & Hayashi U (1987) Production of horseradish peroxidase by plant cell culture. J. Chem. Biotechnol. 38: 31–39

Xu Y & van Huystee RB (1993 a) Association of calcium and calmodulin to peroxidase secretion and activation. J. Plant Physiol. 141: 141–146

Xu Y & van Huystee RB (1993 b) Effect of calcium, its inhibitors, and heavy metals on the growth cycle of peanut cell aggregates. Plant Cell Tiss. Org. Cult. 32: 319–328