Peroxidase isoenzymes in normal and habituated calli of sugar beet during transfer from light to darkness

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Abstract

Habituated sugar beet calli have been characterized as having a deficiency in some tetrapyrrole containing compounds. However, peroxidases might be dissociated from the other tetrapyrrole containing compounds. When light-cultured normal and habituated calli were transferred to darkness their peroxidase activity reduced and increased, respectively, indicating that habituation could not strictly be characterized by a deficiency in peroxidase content but rather by a different regulation of its activity. This regulation could be mediated through soluble effectors which act as potential peroxidase inhibitors and/or by a differential expression of the peroxidase isoenzyme patterns which were present in these tissues in both light and darkness. The different peroxidase activity and the nature of acidic and basic peroxidase isoenzymes in normal and habituated tissues could explain the different features of both types of cultures.

Additional key words: *Beta vulgaris*, isoperoxidase, zymographic pattern.

Introduction

Habituated cell lines, *i.e.* auxin- and/or cytokinin-non requiring strains of several species have been characterized by deficiency in tetrapyrrole containing compounds like chlorophylls (Kamínek *et al.* 1981, Crèvecoeur *et al.* 1987, Bisbis *et al.* 1994), cytochromes (Hagège *et al.* 1992a), catalase (Hagège *et al.* 1992a,b) and peroxidases (Kevers *et al.* 1981, Penel *et al.* 1984, Krsnik-Rasol 1991 and Le Dily *et al.* 1993). However, there are some results indicating that the low concentration of peroxidases

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detected in habituated cell lines might be due to the presence of small soluble compounds which mask peroxidase activity. Thus, in a fully habituated (H) cell line of sugar beet where crude extracts developed a very low peroxidase activity, chromatography of the same extract through *Sephadex* revealed a main peroxidase peak as high as in a normal (N) auxin- and cytokinin-dependent line of the same plant (Kevers *et al.* 1981). Furthermore, purified plasma membranes from H and N sugar beet cells showed quite the same specific guaiacol-peroxidase activity and no distinction could be made between their isoperoxidase profiles after isoelectric focusing and revelation by *o*-dianisidine; they *in vitro* degraded indoleacetic acid (IAA) and oxidized NADH indistinctly which means that whether H and N cells should differ by such metabolisms at their plasma membrane level, the regulation should be mediated through soluble effectors rather than through the amount and nature of peroxidases (Hagège *et al.* 1991a). Moreover, H and N calli indeed were characterized by quantitative (Kevers *et al.* 1981) and qualitative (Engelmann *et al.* 1993) differences in the content of phenolic compounds which are potential peroxidase inhibitors. The transfer of light cultured green N and white H sugar beet calli to darkness reduced and increased respectively their peroxidase activity (Kevers *et al.* 1995) indicating that habituation can not be strictly characterized by deficiency in peroxidase content but rather through a different regulation of its activity.

Peroxidase is a well-known enzyme, extensively distributed among several subcellular fractions of plant cells (Gaspar *et al.* 1982). Each peroxidase isoenzyme group plays a different physiological function in plant cell metabolism. Thus, acidic cell wall peroxidases are involved in the oxidative coupling of cell wall phenols in the absence of H_2O_2 and at the expense of IAA and O_2 while soluble basic isoperoxidases of low pI are apparently responsible in the oxidation of polarly transported IAA through the hypocotyl (Ros Barceló *et al.* 1990, Ferrer *et al.* 1991).

On the other hand, the implication of basic cell wall isoperoxidases of high pI in cell wall phenolic cross-linking and IAA catabolism is doubtful. However, the co-localization of these strongly basic isoperoxidases in both cell walls and vacuoles seems to suggest their participation in some type of H_2O_2-dependent oxidation (Perrey *et al.* 1989, Blom *et al.* 1991, Bernal *et al.* 1994, 1995).

The transfer of light cultured green N and white H sugar beet calli to darkness produces modifications in both their peroxidase activity and isoenzyme patterns indicating that exists in both N and H sugar beet calli, a different regulation of this activity.

The subcellular localization of peroxidase and their expression in N and H calli have been studied in callus cultures of *Beta vulgaris* L. ssp. *altissima* in order to understand the different features which exist in both types of cultures.

Materials and methods

Plant material: Two nonorganogenic callus cultures of *Beta vulgaris* L. ssp. *altissima* have been used for these studies. The auxin- and cytokinin-requiring N callus is chlorophyllous, compact and contains large cells and well differentiated tracheary elements (Crèvecoeur *et al.* 1987). The auxin- and cytokinin-independent callus, so called fully H, appears watery, whitish, very friable and is composed of little differentiated protoplast-like cells which separate easily, in part as a consequence of a deficient cell wall (low lignin and cellulose content) (Le Dily *et al.* 1993) and also due to high pectin esterification (Liners *et al.* 1994).

Experimental conditions for obtaining N and H calli of sugar beet (*Beta vulgaris* L. ssp. *altissima*) and for maintaining these tissues in stock solid cultures have been reported elsewhere (Kevers *et al.* 1981). In order to have the same conditions until 28 d, calli were maintained in the same medium without subculture.

Tissue homogenization, subcellular fractionation and estimation of peroxidase activity: About 2 g fresh mass callus were frozen and dried at -80 °C and homogenized at 4 °C in 5 cm^3 of 0.25 M saccharose, 1 mM magnesium acetate and 5 mM Tris-HCl buffer, pH 7.2. The homogenate was immediately squeezed through one layer of cellulose gauze (0.2 mm mesh) and centrifuged at 1000 *g* for 15 min. Soluble and cell wall fractions were prepared as described previously (Ros Barceló *et al.* 1989). Ionically-bound cell wall peroxidases were removed from the purified cell wall pellet according to the method of Ros Barceló *et al.* (1987).

All peroxidase fractions were dialysed overnight against 50 mM Tris-HCl buffer, pH 7.2.

Peroxidase activity was estimated at 25 °C with guaiacol as substrate according to Ferrer *et al.* (1990). Kinetic analyses of peroxidase in N and H calli in both conditions (light and darkness) were carried out in 1 cm^3 of final reaction volume in 0.1 M Tris-acetate buffer, pH 6.0 (only in H calli in the light the pH was changed to 5.0). Enzyme activities were expressed in pkat which was defined as the amount of protein that oxidized 1 pmol of substrate into product per s.

In order to determine the kinetic constants for H_2O_2 16 pkat of peroxidase were added to the reaction media which contain an optimal concentration of guaiacol and different concentrations of H_2O_2 (0.25 - 3 mM, in N and H calli in the darkness; and 0.25 - 5 mM, in N and H calli in the light). Guaiacol oxidation rate was estimated by measuring changes in absorbance during the first minute of the reaction and apparent Km and Vm values were calculated by Lineweaver-Burk plots.

Isoelectric focusing and staining of peroxidase isoenzymes: Gels were performed with *Servalyt ampholytes* (pH 3 - 10) according to the instructions of the manufacturer. Peroxidase activity was revealed by using *o*-dianisidine and H_2O_2 (Penel *et al.* 1990).

Results and discussion

Peroxidase activity in light: Peroxidase activity in H callus was lower than in N callus in both studied fractions (Table 1). Also cell wall peroxidase activity in H callus was lower than in N cells. This fact can be explained if we take into account the different features in both types of cultures (Crèvecoeur *et al.* 1987). The H callus shows a very poor level of lignin and a lack of acidic peroxidase isoenzymes which are more efficient in the oxidation of lignin precursors than strongly basic isoperoxidases (Ferrer *et al.* 1992) which are the only isoenzymes present in this tissue. Only, at 21 d of culture, the level of

Table 1. Peroxidase activity [pkat g^{-1}(f.m.)] or [%] of soluble and cell wall fractions of normal and habituated sugar beet calli in light and darkness during cultivation.

Time [d]	Fractions	Light normal [pkat g^{-1}]	[%]	habituated [pkat g^{-1}]	[%]	Dark normal [pkat g^{-1}]	[%]	habituated [pkat g^{-1}]	[%]
7	soluble	36.064	80.77	33.936	87.14	18.144	70.43	32.000	94.41
	wall	7.106	15.91	4.088	10.49	6.824	26.49	1.376	4.03
14	soluble	45.976	82.33	33.040	86.36	55.216	82.40	32.795	87.86
	wall	7.952	14.24	4.288	11.20	9.488	14.15	3.780	10.12
21	soluble	31.920	85.25	37.744	84.90	30.408	82.83	44.688	88.41
	wall	4.328	11.55	5.732	12.89	4.972	13.54	4.440	8.78
28	soluble	33.824	79.58	29.064	82.81	32.564	80.98	38.248	91.48
	wall	7.091	16.68	5.397	15.37	6.168	15.23	2.584	6.18

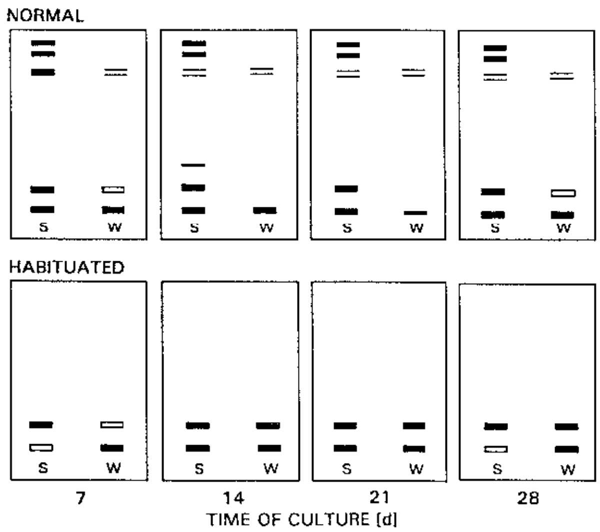

Fig. 1. Isoenzyme patterns (using *o*-dianisidine as substrate) of soluble (*S*) and cell wall (*W*) peroxidases of normal and habituated sugar beet calli in light at different days of culture.

peroxidase in both soluble and cell wall fractions in H callus was slightly greater coinciding with the maximum growth of the culture. The decrease in non-particulate peroxidase activity after 21 d of culture coincided with the retardation of growth of this callus and the appearance of brownish zones of necrosis described by Le Dily *et al.* (1993). This low peroxidase activity could be due to phenolic compounds which are produced during browning and could act as inhibitors of this activity (Kevers *et al.* 1981).

On the other hand, the highest level of soluble peroxidase in N callus was found at 14 d coinciding with the beginning of exponential growth phase.

Soluble and cell wall peroxidase fractions showed only slight differences in the zymographic pattern of N callus during growth culture.

Peroxidase isoenzyme patterns of cell wall fractions in N callus were similar at 7 and 28 d old of culture. However, at 14 and 21 d, there was a loss of one strongly basic isoenzyme that corresponded with a fall in the percentage of total cell wall activity. Soluble peroxidase fractions of N and H calli showed two strongly basic isoperoxidases whereas acidic isoperoxidases were only present in N callus cultures (Fig. 1).

The co-localization of basic isoperoxidases of high pI in soluble (bound to the tonoplast membrane) and cell wall-bound fractions has been already described by Ferrer *et al.* (1992) and Bernal *et al.* (1993) in lupin hypocotyls and *Capsicum annuum* leaves, respectively, as well as a differential reactivity of cell wall localized isoenzymes in both oxidase and peroxidase reactions by using coniferyl alcohol, NADH and IAA as substrates. Thus, acidic isoperoxidases are much more important in coniferyl alcohol oxidation in the presence of H_2O_2 and in both IAA and NADH oxidation in its absence than basic isoperoxidases of high pI (Ferrer *et al.* 1992).

In fact, we have found an efficiency (Kcat/Km ratio) of H_2O_2 as substrate slightly greater in N than H callus in light. This difference is markedly higher in dark (Table 2); it can be ascribed to the zymographic patterns (Fig. 1) since only N callus shows the presence of an acidic isoenzyme group which is more efficient in the oxidation of substrates in the presence of H_2O_2.

Table 2. Km, Kcat and efficiency in the oxidation of H_2O_2 by *Beta vulgaris* calli (normal or habituated) peroxidases.

		Km [mM]	Kcat [mol kat^{-1} s^{-1}]	Kcat/Km
Light	normal	0.290	1.021	3.520
	habituated	0.520	1.800	3.461
Dark	normal	0.185	1.017	5.497
	habituated	0.590	0.760	1.288

Peroxidase activity in darkness: Changes in physiological and biochemical parameters have frequently been shown to take place during the transition of both N and H calli from light to darkness (Kevers *et al.* 1995). Thus, Kevers *et al.* (1995) have found that the transfer of light-cultured green N and white H sugar beet calli to darkness reduced the growth of N callus and improved growth and delayed necrosis in the H callus. Moreover, a significant increase of total peroxidase activity, which did not occur in N callus, was induced by darkness in the H callus. We have found changes in soluble and cell wall-bound peroxidase activity and in their isoenzyme patterns (Table 1 and Fig. 2) after the transition of N and H calli from light to darkness. In fact, during the whole growth process, peroxidase activity (expressed in percentage) in soluble fraction of H callus is always higher than in N ones. These results are in accordance with the above statement, and could be explained by the lack of necrosis and browning of the H callus due to reduced stress in darkness (Kevers *et al.* 1995). This avoids the appearance of phenolic compounds that could act as inhibitors of peroxidase activity.

As shown several times (Hagège *et al.* 1991b, Le Dily *et al.* 1993, Kevers *et al.* 1995) the N callus, under usual light conditions, presented a typical growth curve with exponential and stationary phases. Transfer of the N callus to darkness induced a long lag period before regrowth. This feature observed in the N callus during the dark growth process could be explained if we take into account its isoenzyme patterns progress. Thus, as can be seen in Fig. 2, during the time of culture there is a differential expression of an isoperoxidase group in soluble fractions which show a pI near to neutral pH. These isoenzymes could be responsible for the decrease of growth because they can play a role as IAA-oxidases and modulate the auxin levels in the culture. These results could explain the retardation of growth until 21 d due to the presence of this isoenzyme group and its increase from 21 d which is in agreement with the disappearance of neutral isoperoxidases in the soluble fraction.

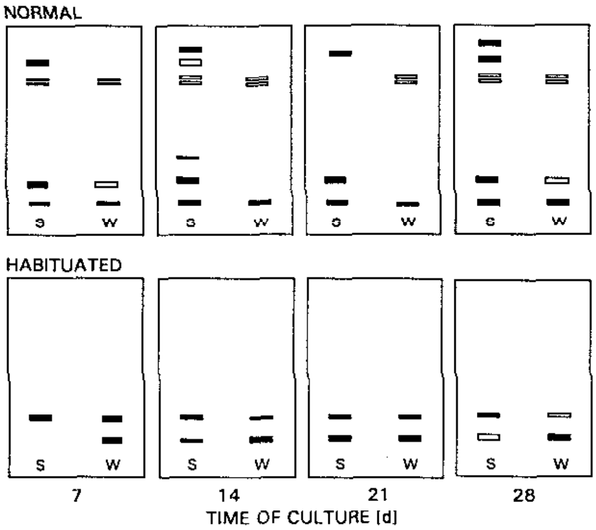

Fig. 2. Isoenzyme patterns of soluble (*S*) and cell wall (*W*) peroxidases of normal and habituated sugar beet calli in darkness at different days of culture.

Finally, the modification of both cell wall-bound peroxidase activity and isoenzyme patterns during the transition from light to darkness in N callus is very little as compared with that occurred in H ones (Table 1 and Fig. 2). In fact, the decrease of cell wall peroxidase activity in H callus is due to a disappearance and low intensity of some cell wall isoperoxidases during the dark-growth progress. These results can also be seen in a different efficiency in guaiacol oxidation when H_2O_2 is used as a substrate which is markedly greater in N callus that in H one (Table 2). Thus, H tissues showed a deficiency of lignin as well as lower cellulose content (Crèvecoeur *et al.* 1987) because these calli had not got acidic isoperoxidases which are directly involved in the lignification process.

In conclusion, the transfer of light cultured N and H calli to darkness produces modifications in both their peroxidase activity and peroxidase isoenzyme patterns indicating that habituation cannot be strictly characterized by a deficiency in peroxidase content but rather by a different regulation of its activity and a differential expression of peroxidase isoenzymes in these types of cultures in both conditions (light and darkness).

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