

A Comparative Biochemical and Cytological Characterization of Normal and Habituated Sugarbeet Calli

M. CREVECOEUR*, C. KEVERS**, H. GREPPIN* and TH. GASPAR**

*Laboratoire de Physiologie végétale, 3, pl. de l'Université,
CH - 1211 Genève 4, Switzerland, and

**Institut de Botanique B 22, Université de Liège - Sart Tilman,
B - 4000 Liège, Belgium

Abstract. Habituated sugarbeet callus examined by means of a light-microscope is characterized by absence of differentiated tracheary elements and of the reaction with syringaldazine. Habituated tissues also were found to exhibit low guaiacol - and syringaldazine-peroxidase activities, a deficiency of lignin as well as lower cellulose, dry mass and chlorophyll contents as compared to the normal auxin-requiring callus. These histological and cytological features led to consider this habituated callus as a vitrified tissue under stress. The question of a relationship between vitrification and habituation is posed.

The process of habituation in sugarbeet callus was already examined using a comparative analysis of auxin content, auxin protectors, peroxidase pattern and inhibitors between an auxin-nonrequiring and a normal auxin (2,4-D)-requiring cell lines (KEVERS *et al.* 1981). Both tissues contained an equal amount of IAA but habituated callus was typically characterized by a very low enzyme activity in soluble, membrane and wall (ionic and covalent) fractions. Differences were also reported in the calcium-controlled peroxidase secretion by both types of cells in suspension cultures (KEVERS *et al.* 1982, GASPAR *et al.* 1983, PENEL *et al.* 1984) : sugarbeet cells released peroxidases in proportion to their endogenous level. Peroxidase and its release likely play a role in cell wall stiffening and/or lignification (GASPAR *et al.* 1982, EPSTEIN and LAMPORT 1984, MC NEIL *et al.* 1984, TAIZ 1984). Its paucity in habituated cells could be conceivably responsible for their brittle appearance. This leads us to further investigate the habituated callus as to its lignification degree by lignin determination and histological analysis of xylem formation, using syringaldazine as a specific substrate of lignifying cells (GOLDBERG *et al.* 1983). Habituated calli moreover visibly contain less chlorophyll and look more watery than the normal ones. These parameters also have been evaluated here with our former hypothesis in mind that the so-called vitrification process of organized tissues (DEBERGH *et al.* 1981) might result from deficiency of lignification, reduced cell wall pressure and

hyperhydricity (KEVERS *et al.* 1984, KEVERS and GASPAR 1985) and that a comparison may perhaps be established between such vitrified tissues and the habituated cells.

MATERIALS AND METHODS

Experimental conditions for obtaining normal (2,4-D requiring) and habituated (auxin-nonrequiring) calli of sugarbeet (*Beta vulgaris* L. *altissima*) and for maintaining these tissues in stock solid cultures have been reported elsewhere (KEVERS *et al.* 1981). Such calli, when subcultured every four weeks on their respective medium (basal medium without plant growth regulators in the case of the habituated line, but supplemented with 0.1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP in the case of the normal one) were kept unchanged for at least five years. Inocula of such calli have shown constant biochemical and cytological characteristics during at least one year when taken for the present analyses during the exponential phase of growth of each subculture.

Chlorophylls were estimated according to the method of MAC KINNEY (1941) by measuring the absorption at 645, 663 and 652 nm of 80 % (v/v) acetone extracts of fresh materials.

The spectrophotometrical method of JOHNSON *et al.* (1961) as described by ALIBERT and BOUDET (1979) was used for lignin extraction and determination. Lyophilized samples of calli were ground in a mortar to get a dry powder. The soluble substances were extracted first with a solution of NaCl M containing triton X100 (1 : 2, v/v), then with a 1 : 2 (v/v) alcohol-benzene mixture. After alkaline hydrolysis of the residue and solubilization in acetyl bromide, the lignin level was estimated by UV (280 nm) spectrophotometric measurement.

Cellulose was isolated after extractions eliminating successively the chlorophylls (acetone 80 %), the proteins (NaOH 0.3 M), the pectins (oxalic acid and ammonium oxalate) and the hemicellulosic substances (NaOH 2.5 M) following MARIGO and BOUDET (1980). All the fractions were discarded by centrifugation at 3000 g for 10 min and the final residue was lyophilized. Cellulose was finally extracted with H_2SO_4 72 % (2 h at 20 °C), then with H_2SO_4 2 % (2 h at 115 °C). The residue was again lyophilized and the cellulose content estimated by mass difference.

Procedures for fractionation and guaiacol peroxidase activity were reported by DARIMONT *et al.* (1977). Syringaldazine-peroxidase activity was measured following GOLDBERG *et al.* (1983). Protein was assayed by the Coomassie blue method (SPECTOR 1978) with bovine serum albumin as a standard.

Cytology. Samples were collected from the cultures and fixed at room temperature in 4 % glutaraldehyde buffered with sodium cacodylate. After thorough washing in distilled water and dehydration through an ethanol series, they were embedded in paraffin. Sections 7 μm thick were cut, then stained with 0.2 % toluidine blue. Free-hand sections from fresh material were also used. 40 μl of 0.1 % solution of syringaldazine in ethanol and 20 μl 0.03 % aqueous H_2O_2 were then added to these sections according to HARKIN and OBST (1973). Controls were performed by incubating sections in medium lacking syringaldazine. Photographs were taken in the following 5 min. All determinations were performed at least three times. Results in Tables 1 and 2 are mean values. Photographs are representative samples of what is commonly seen.

TABLE 1

Biochemical characteristics of normal and habituated calli

Analyses	Normal	Habituated
Water content [%]	95.05	96.11
Dry mass [%]	4.95	3.89
Chlorophyll <i>a</i> [$\mu\text{g g}^{-1}$ (fresh mass)]	4.92	2.50
Chlorophyll <i>b</i> [$\mu\text{g g}^{-1}$ (fresh mass)]	7.88	1.39
Chlorophyll <i>a</i> + <i>b</i>	12.79	3.89
Chlorophyll <i>a/b</i>	0.62	1.79
Lignin [% fresh mass]	0.206	0.048
Lignin [% dry mass]	2.22	0.87
Cellulose [% fresh mass]	3.06	1.24
Cellulose [% dry mass]	33.83	19.04

RESULTS

The habituated callus exhibits a watery and yellowish aspect and is brittle while the normal callus looks greener and more compact. The water content and the dry mass of both calli at the time of biochemical and cytological analyses of the present study are given in Table 1. Habituated calli, per gram fresh mass, contain more water than the normal ones and thus exhibit a lower dry mass.

Greener appearance of normal tissues obviously is attributed to much more chlorophyll of both types *a* and *b* (Table 1). The habituated callus was found to contain three times less total chlorophyll than the normal cell line. It turns out that chlorophyll *a* and chlorophyll *b* are in an inverse ratio in normal and habituated calli (Table 1), which apparently is due to a very low level of chlorophyll *b* in habituated cells.

Lignin as well as cellulose contents are larger in normal cells (Table 1). The lignin level is particularly low in habituated cells.

As already observed (KEVERS *et al.* 1981), guaiacol-peroxidase activity of all four fractions of the habituated callus is somewhat lower (about twice in soluble membrane and wall-ionic fractions, seven times in the wall-covalent one) than in the normal callus (Table 2). Syringaldazine-peroxidase activity is almost inexistent in soluble and membrane fractions of habituated cells;

TABLE 2

Soluble, membrane and wall-bound peroxidase activity measured with guaiacol (10^{-6} mg mg⁻¹ protein) and with syringaldazine ($\Delta A \text{ min}^{-1} \text{ mg}^{-1}$ protein) of normal and habituated sugarbeet calli

Fractions	Normal callus		Habituated callus	
	Guaiacol	Syringaldazine	Guaiacol	Syringaldazine
Soluble	87.7 ± 7.0	0.137 ± 0.008	36.6 ± 5.5	0.001
Membrane	112.7 ± 4.1	0.145 ± 0.018	66.8 ± 4.7	0.012 ± 0.007
Wall-ionic	74.0 ± 7.4	0.056 ± 0.004	43.5 ± 8.3	0.055 ± 0.008
Wall-covalent	141.7 ± 6.9	0.032 ± 0.006	20.1 ± 2.2	0.043 ± 0.008

it is of about the same level in ionically and covalently wall-bound fractions of the two calli (Table 2).

Microscopically (Fig. 1), the habituated callus appears to be entirely composed of two common parenchymatic cell types. One cell type is small ($\pm 50 \mu\text{m}$) and the other one is larger ($\pm 100 \mu\text{m}$). The appearance of cells of a normal callus is illustrated in Fig. 2. In addition to parenchymatic cells, it shows the presence of many tracheary elements with various sorts of secondary wall thickening, namely the reticular type.

Application of syringaldazine or of both syringaldazine and H_2O_2 to sections of freshly hand-cut samples of the habituated callus does not produce any staining. When syringaldazine and H_2O_2 are added to sections of normal callus, a pink staining is obtained which is restricted to the tracheary elements. In the absence of H_2O_2 in the incubation medium, no staining is observed.

DISCUSSION

Data of the present study clearly demonstrate important histological and biochemical differences between the normal and habituated sugarbeet calli. Normal callus contains well differentiated xylem cells with normal secondary wall thickening as generally observed in most calli. Such xylem cells on the way to lignification specifically react with syringaldazine as expected from the work of GOLDBERG *et al.* (1983). Habituated callus does not show any tracheary elements and any reaction with syringaldazine. These observations are substantiated by chemical analyses which indicate a low peroxidase activity and a very poor level of lignin in habituated cells. Such a relationship between tracheary elements differentiation and peroxidase activity has recently been reported by MASUDA *et al.* (1983). The habituated callus also exhibits large parenchymatic cells and a deficient cellulose level. Such anatomical and chemical features when observed in plant organs (GROUT and ASTON 1977, SUTTER and LANGHANS 1979, LESHEM 1983, VIETH *et al.* 1983, BORNMAN and VOGELMANN 1984, KEVERS and GASPARD 1985) characterize the so-called vitrification process. Here too, as formerly hypothesized for entire plantlets (KEVERS *et al.* 1984), one may tentatively speculate about the causal relationship between deficiencies in both cellulose and lignin contents of the habituated callus and its higher water level, which confirms its watery aspect. There is only 1 % difference in water content between the two strains but such a small difference has been found in vitreous tissues of whole plants with a watery aspect. Vitreous plants also generally contain less chlorophyll than normal plants which makes their translucency (HEDEGUS and PHAN 1983, PHAN and LETOUZE 1983, ZIV *et al.* 1983) and presumably lowers their photosynthetic capacity (LESHEM 1983). The level of both chlorophyll *a* and chlorophyll *b* is also particularly low in habituated cells. It is noteworthy that the level of chlorophyll *b* is higher than that of chlorophyll *a*. This is the reverse of what is commonly observed in most other plant tissues but has already been reported in olive callus (LAVEE and MESSER 1969). Why the level of chlorophyll *b* is much higher than that of chlorophyll *a* in normal callus and whether the opposite characterizes the habituated tissues remains unexplained.

The habituated sugarbeet callus thus apparently presents all the characteristics of a vitrified tissue. To our knowledge, histological as well as biochemical

features of watery calli were never considered from the viewpoint of vitrification even though substantial differences with respect to cytological and physiological features between calli are long known (BALL 1969, SHAMINA *et al.* 1978). It has been shown, however, that water stress under certain temperature conditions might prevent xylogenesis, increase the dry matter and the chlorophyll level in calli (BORNMAN and HUBER 1979). As stresses can be considered as factors inducing vitrification (DEBERGH *et al.* 1981, HAKKAART and VERSLUIJS 1983) this could mean that some stresses might be induced by one or some of our chemical and physical culture conditions. The question therefore arises whether all habituated calli exhibit histological and biochemical characteristics similar to those of habituated sugarbeet callus, in other terms, whether a close relationship exists between vitrification and habituation or conversely. Comparative information is lacking. However, we can ascertain that habituated tissues, those of tobacco for instance, are generally poor in peroxidase (BOUCHET *et al.* 1978) which is one of lignifying and/or rigidifying factors of the cell walls (TAIZ 1984).

Acknowledgement

One of the authors (C. K.) acknowledges with gratitude the postdoctoral fellowship from the Belgian I.R.S.I.A institution.

REFERENCES

- ALBERT, G., BOUDRY, A.: La lignification chez le peuplier. I. Mise au point d'une méthode de dosage et d'analyse monomérique des lignines. — *Physiol. vég.* **17** : 67–74, 1979.
- BALL, E.: Histology of mixed callus. — *Bull. Torrey bot. Club* **96** : 52–59, 1969.
- BORNMAN, C. H., HUBER, W.: *Nicotiana tabacum* callus studies. IX. Development in stressed explants. — *Biochim. Physiol. Pflanzen* **174** : 345–356, 1979.
- BORNMAN, C. H., VOGELMANN, T. C.: Effect of rigidity of gel medium on benzyladenine-induced bud formation and vitrification *in vitro* in *Picea abies*. — *Physiol. Plant.* **61** : 505–512, 1984.
- BOUCHET, M., GASPAS, TH., THORPE, T. A.: Soluble and cell-wall peroxidases and auxin destruction in normal and habituated tobacco callus. — *In Vitro* **14** : 819–823, 1978.
- DARIMONT, E., PENEL, C., AUDERSET, G., GREPPIN, H., GASPAS, TH.: Peroxydases de haut poids moléculaire identifiées à des peroxydases membranaires chez la lentille. — *Arch. Int. Physiol. Biochim.* **85** : 497–507, 1977.
- DEBERGH, P., HARBAOUI, Y., LEMEUR, R.: Mass propagation of globe artichoke (*Cynara scolymus*): evaluation of different hypotheses to overcome vitrification with special reference to water potential. — *Physiol. Plant.* **53** : 181–187, 1981.
- EPSTEIN, L., LAMPORT, D. T. A.: An intramolecular linkage involving isodityrosine in extension. — *Phytochemistry* **23** : 1241–1246, 1984.
- GASPAS, TH., KEVERS, C., PENEL, C., GREPPIN, H.: Auxin control of calcium-mediated peroxidase secretion by auxin-dependent and auxin-independent sugarbeet cells. — *Phytochemistry* **22** : 2657–2660, 1983.
- GASPAS, TH., PENEL, C., THORPE, T., GREPPIN, H.: Peroxidases 1970–1980. A Survey of their Biochemical and Physiological Roles in Higher Plants. — Univ. de Genève — Centre de Botanique Publ., Genève 1982.
- GOLDBERG, R., CATTESSON, A. M., CZANINSKI, Y.: Some properties of syringaldazineoxidase, a peroxidase specifically involved in the lignification processes. — *Z. Pflanzenphysiol.* **110** : 267–279, 1983.
- GROUT, B. W. W., ASTON, M. J.: Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. — *Hort. Res.* **17** : 1–7, 1977.
- HAKKAART, F. A., VERSLUIJS, J. M. A.: Some factors affecting glassiness in carnation meristem tip cultures. — *Neth. J. Plant Pathol.* **89** : 47–53, 1983.
- HARKIN, J. M., OBST, J. R.: Lignification in trees: indication of exclusive peroxidase participation. — *Science* **180** : 296–298, 1973.

- HEDEGUS, P., PHAN, C. T.: Actions de phénols sur les malformations observées chez les porte-greffes de pommiers M-26 et 0-3 cultivés *in vitro*. — *Rev. can. Biol. exp.* **42** : 33–38, 1983.
- JOHNSON, D. B., MOORE, W. E., ZANK, L. C.: The spectrophotometric determination of lignins in small wood samples. — *TAPPI (Technical Association of the Pulp and Paper Industry)* **44** : 793–798, 1961.
- KEVERS, C., COUMANS, M., COUMANS-GILLÈS, M. F., GASPAR, TH.: Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. — *Physiol. Plant.* **61** : 69–74, 1984.
- KEVERS, C., COUMANS, M., DE GREEF, W., HOFINGER, M., GASPAR, TH.: Habituation in sugarbeet callus: auxin content, auxin protectors, peroxidase pattern and inhibitors. — *Physiol. Plant.* **51** : 281–286, 1981.
- KEVERS, C., GASPAR, TH.: Soluble, membrane and wall peroxidases, phenylalanine ammonia-lyase, and lignin changes in relation to vitrification of carnation tissues cultured *in vitro*. — *J. Plant Physiol.* **118** : 41–48, 1985.
- KEVERS, C., STICHER, L., PENEL, C., GREPPIN, H., GASPAR, TH.: Calcium-controlled peroxidase secretion by sugarbeet cell suspension in relation to habituation. — *Plant Growth Regulation* **1** : 61–66, 1982.
- LAVEE, S., MESSER, G.: The effect of growth-regulating substances and light on olive callus growth *in vitro*. — *J. exp. Bot.* **20** : 604–614, 1969.
- LESEM, B.: Growth of carnation meristems *in vitro*: anatomical structure of abnormal plantlets and the effect of agar concentration in the medium on their formation. — *Ann. Bot.* **52** : 413–415, 1983.
- MAC KINNEY, G.: Absorption of light by chlorophyll solutions. — *J. biol. Chem.* **140** : 315–322, 1941.
- MARIGO, G., BOUDET, A. M.: Relations polyphénols croissance: lignification et limitation de croissance chez *Lycopersicon esculentum*. — *Physiol. Plant.* **49** : 425–430, 1980.
- MASUDA, H., FUKUDA, H., KOMAMIME, A.: Changes in peroxidase isoenzyme patterns during tracheary element differentiation in a culture of single cells isolated from the mesophyll of *Zinnia elegans*. — *Z. Pflanzenphysiol.* **112** : 417–426, 1983.
- MC NEIL, M., DARVILL, A. G., FRY, S. C., ALBERSHEIM, P.: Structure and function of the primary cell walls of plants. — *Annu. Rev. Biochem.* **53** : 625–663, 1984.
- PENEL, C., STICHER, L., KEVERS, C., GASPAR, TH., GREPPIN, H.: Calcium-controlled peroxidase secretion by sugarbeet cells: effect of ionophores in relation to organogenesis. — *Biochem. Physiol. Pflanzen* **179** : 173–180, 1984.
- PHAN, C. T., LETOUZE, R.: A comparative study of chlorophyll, phenolic and protein contents and of hydroxycinnamate: CoA ligase activity of normal and vitreous plants (*Prunus avium* L.) obtained *in vitro*. — *Plant. Sci. Lett.* **31** : 323–327, 1983.
- SHAMINA, Z. B., SCHEUNERT, E. U., KOBLITZ, H.: Studies on barley calluses cultured *in vitro*: II. Cytological and morphological features of barley callus cultures. — *Plant Sci. Lett.* **13** : 177–184, 1978.
- SPECTOR, T.: Refinement of the Coomassie blue method of protein quantitation. — *Anal. Biochem.* **86** : 142–146, 1978.
- SUTTER, E., LANGHANS, R. W.: Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. — *J. amer. Soc. hort. Sci.* **104** : 493–496, 1979.
- TAIZ, L.: Plant cell expansion: regulation of cell wall mechanical properties. — *Annu. Rev. Plant Physiol.* **35** : 585–657, 1984.
- VIETH, J., MORISSET, C., LAMOND, M.: Histologie de plantules vitreuses de *Pyrus malus* cv. M26 et de *Pelargonium peltatum* cv. Chester Frank. issues de la culture *in vitro* (Etude préliminaire). — *Rev. can. Biol. exp.* **42** : 29–32, 1983.
- ZIV, M., MEIR, G., HALEVY, A. H.: Factors influencing the production of hardened glaucous carnation plantlets *in vitro*. — *Plant Cell Tissue Organ Culture* **2** : 55–63, 1983.

Figures at the end of the issue.

M. CREVECOEUR ET AL.
BIOCHEMISTRY AND CYTOLOGY OF SUGARBEET CALLI

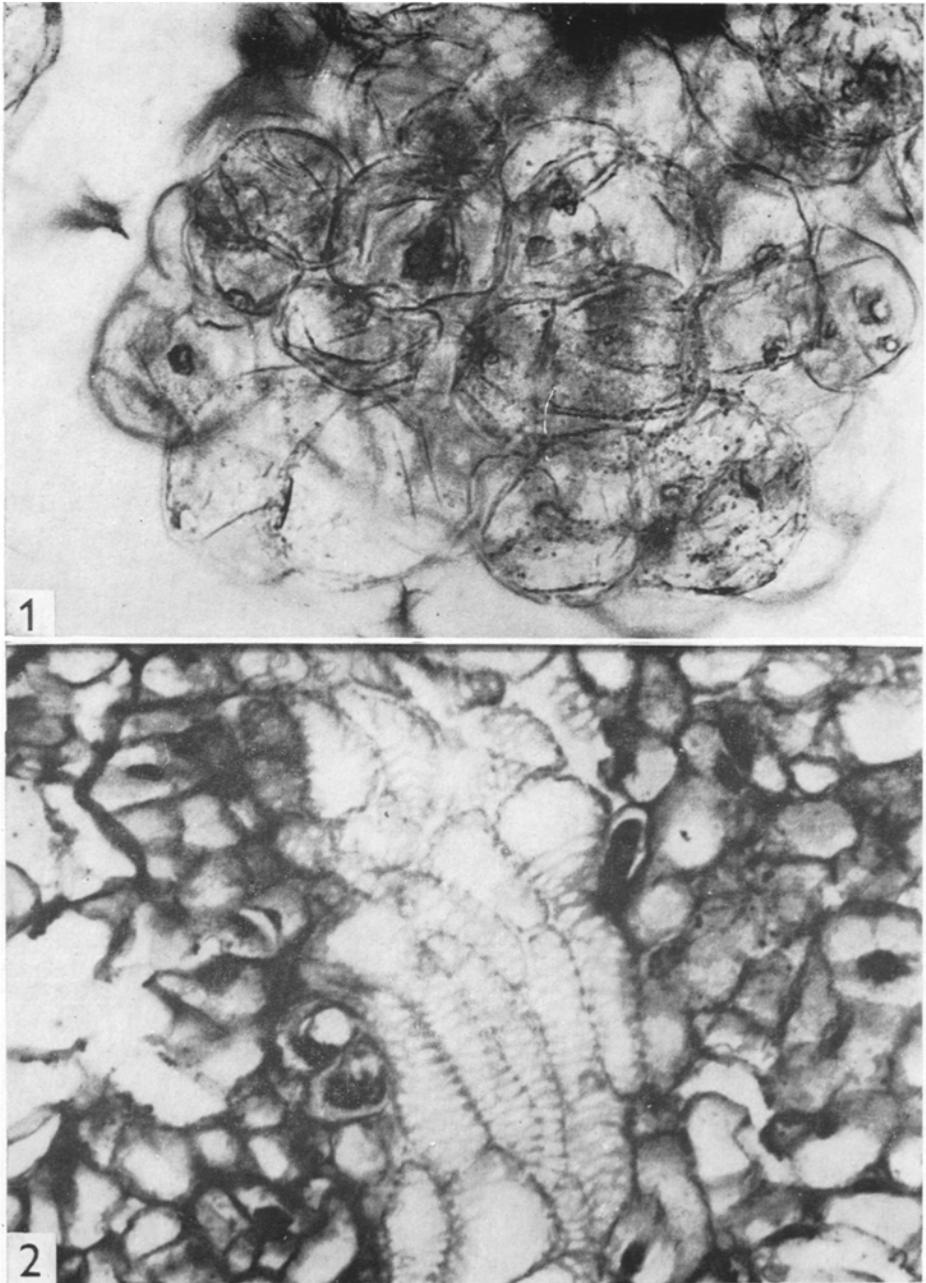


Fig. 1. Appearance of parenchymatic cells in the habituated callus after coloration with toluidine blue. 1850 \times .

Fig. 2. Sections through a normal callus showing xylem cells with lignin thickening. Toluidine blue coloration. 925 \times .