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HABITUATION IN SUGARBEET PLANT CELLS: PERMANENT STRESS OR ANTIOXIDANT ADAPTATIVE STRATEGY?

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SUMMARY

Habituation is one of the four neoplastic diseases of plants and occurs spontaneously in plant cell cultures. To date, and even if an epigenetic origin has been previously postulated, the fundamental concept that underlies this neoplastic state remains obscure. Recently, a permanent stress hypothesis has been proposed, using habituated nonorganogenic (HNO) sugarbeet cell line (*Beta vulgaris* L. *altissima*) as a model. According to this proposal, the low catalase and peroxidase activities were supposed to be responsible for H_2O_2 accumulation. A supposed lipoxygenase activation would generate LOO° radicals. OH°, produced by the Fenton reaction would be responsible for a lipoperoxidation process, leading to malondialdehyde (MDA) accumulation. In this paper, the elements of this hypothesis have been examined using data previously obtained by several teams, and the permanent stress idea appears less sustainable. Several properties of the habituated nonorganogenic sugarbeet- and some other habituated-cell lines have been described. A more realistic concept emerging from this analysis is that habituated cells exhibit efficient scavenging properties (antioxidant and antilipoperoxidant) against deleterious free radicals produced during cell culture. This thesis is developed in this article.

Key words: antioxidant adaptation; antilipoperoxidant; free radicals; habituation; hyperscavenging capabilities; neoplastic state; peroxidation; sugarbeet.

INTRODUCTION

Habituation is a well-known phenomenon that appears spontaneously in plant cell cultures and is characterized by hormonal autonomy (14). All the biosynthetic systems required for continuous cell growth and division become persistently activated. Habituated cells behave in the same way as true plant tumor cells in that they proliferate actively and continuously on a basic culture medium that does not support the continuous growth of normal cells (2). Habituation is considered a multistep phenomenon. It occurs gradually rather than in a single step, and various degrees of habituation may be found, ranging from cells resembling their normal counterparts to completely dedifferentiated cells. The phenomenon has been considered for a long time as epigenetic (35–37). However, the fundamental cause of the phenomenon is unclear.

THE PERMANENT STRESS HYPOTHESIS

Recently, a "permanent stress" hypothesis has been proposed to explain the habituation phenomenon of sugarbeet (*Beta vulgaris*) callus (29–32). Arbillot et al. (1) compared a nonorganogenic, auxin-, cytokinin-independent callus (N) to another one: nonorganogenic, auxin-, cytokinin-dependent (or habituated) (HNO). They showed that the HNO callus had a higher level of malondialdehyde (MDA) during culture. Furthermore, they showed also that the habituated callus contained more C18:2 fatty acids than its normal counterpart. Because C18:2 is one of the substrates used for lipoxygenase, they postulated that the peroxidation of polyunsaturated fatty acids explains the high MDA content in the HNO callus. Le Dily et al. (31) assumed that a stress was caused by an accumulation of peroxides and used the above finding to support a hypothesis that attempts to explain both the habituation and vitrification (hyperhydricity) phenomena occurring in *in vitro* cultures. According to this view, the low catalase and peroxidase activities would be responsible for H_2O_2 accumulation. The resulting hydrogen peroxide toxicity would be enhanced by lipoxygenase activation, which would generate LOO° radicals. The deleterious effects of H_2O_2 and OH°, generated through the Fenton reaction, would explain the reduced growth, the ultrastructural disturbance, and the necrotic bands observed in this callus. Moreover, these authors postulated that an ammonia stress would occur from the beginning of callus culture and that the nitrogen metabolism, induced by NH_3 would be diverted towards polyamines (PAs) via proline synthesis. Nevertheless, such a proposal should be examined with caution if one considers some properties of habituated cells.

SOME CYTOLOGICAL, ULTRASTRUCTURAL, AND BIOCHEMICAL CHARACTERISTICS OF THE SUGARBEET NONORGANOGENIC CELL LINE

For a better understanding of the problem, several results, obtained in the past on this material should be recalled: the habituated nonorganogenic sugarbeet callus arose in the form of a small clump on the surface of the habituated organogenic one, and a monoclonal origin was proposed (15). When this small clump was placed on a medium identical to the one used for habituated organogenic callus and subcultured, it gave rise to the habituated line cells that have

been used subsequently. The cytological, ultrastructural, and biochemical characteristics of these cells have been compared to the characteristics of normal callus cells arising from the same plant (13,15). A microscopic examination showed that habituated cells were smaller than the normal ones (25 to 200 μ instead of 250 to 650 μ). They tended to be spherical somewhat like protoplasts, which was due to an incomplete development of the cell wall (15,22). This result was confirmed by the withered appearance of dead cells. No differentiation in the form of lignified treachery elements occurred. Two other abnormal features were pseudo-cell budding and protrusion of cytoplasm through the cell wall. Habituated cells were unable to differentiate and to expand normally. Scanning electron micrographs from these cells exhibited an abnormal parietal architecture consisting of some cell wall polysaccharides laid down in loose parallel strands in an amorphous matrix (22). Cellulose and lignin quantitations carried out on the cell wall revealed an absence of lignin, a deficit of ferulic acid (a lignin monomer precursor) and of cellulose (5,19,22). Examination by electron microscopy showed very large and polylobed nuclei, irregular in shape, with deep invaginations, containing several nucleoli (2 to 6) and micronuclei. They were also characterized by an abundant cytoplasm, multiple vacuoles, and juvenile (or unfunctional) chloroplasts. Also the cells were polyploid and aneuploid (17).

On the basis of a monoclonal origin, hormone independence, and cytological and ultrastructural alterations described above, it was assumed that the cells from habituated callus had undergone a modification of their genetic potential that lead to a neoplastic transformation analogous to the transformation suffered by animal and human tumor cells. On these bases, the phenomenon must be considered more the results of point mutations than epigenetic changes. As free-radicals have often been implicated in such neoformation, the question arises if some physiological processes, such as ethylene production, would depend on molecular and radical species or if any protective systems, which ensure protection against free radicals, would have undergone modifications during this transformation (15).

The biochemical results obtained (13,15,18,20–23,25) have shown that the cells of the habituated line were characterized by: (a) a very low production of ethylene, not very different from experimental noise, (b) a rather important increased superoxide dismutase (SOD) activity in the first half of culture cycle, followed by a decrease to nearly the same activity of the normal callus, (c) a very low catalase activity throughout the whole culture cycle, weak peroxidase activities, (d) a high glutathione reductase (GR) activity, (e) a high content of spermine and spermidine that are considered as having scavenging properties against free radicals (7), and (f) low levels in lipid hydroperoxides, but high amount of TBARS. It has been assumed that the strong scavenging properties for active oxygen species of the habituated cells could explain the very low level of ethylene emission, the incomplete development of the cell wall, and the meristematic characteristics of these lines. Under these conditions, electron transport would be seriously inhibited, thus greatly reducing photosynthesis and respiration. A great number of free radical-dependent biochemical pathways may have difficulty in being achieved in these strong reduced conditions (15,23). Recent results have also shown low lipoperoxidase activity and low conjugated diene production (4).

It has also been stated that this sugarbeet habituated cell line exhibits a high amount of auxin protectors and phenolic compounds (benzoic acid derivatives) (9,13). Auxin protectors (Prs) were first described in *Pharbitis nil* by Stonier and Yoneda (46) and were considered as a class of plant growth substances able to inhibit IAA oxidation. Based on gel filtration studies, three Prs were found to be Pr-A (MS>20 000 daltons), Pr-I (approximately 8000 daltons), and Pr-II (approximately 2000 daltons). Prs have been found in a number of other dicotyledonous plants. Prs act as antioxidants and play an important role in the redox regulation, particularly in relation to the juvenile state (43). They were found at the apex of growing plants, with mature stem tissue responding to a wound stimulus, with stem callus *in vitro* and with crown gall development (40,41,42). They were identified as phenolic redox regulators (*o*-dihydroxyphenolic compounds), which were a prerequisite for cell division. The synthesis of large quantities of protectors by tumor tissues may maintain a reduced state in such tissues and thereby inhibit differentiation and lignification and, therefore, account for anaplasia (44). Prs reduce H_2O_2 and prevent its formation. In this manner, Prs exercise an effect similar to that of catalase (45). The Prs act not only as an antioxidant but also as an oxidant under certain circumstances. Protectors, oxidized by H_2O_2 act then as an electron acceptor in the peroxidase catalyzed oxidation of NADH. Because of their reversible redox role, these protectors would be of utmost importance in determining whether a cell divides or differentiates (43).

When tumor plant cells were cultured on low mineral media, it has been demonstrated that endogenously synthesized Prs rapidly leaked from the tissue, and were rapidly oxidized into quinones (44). The surrounding medium turned a deep brown or a reddish purple, while the control medium remained clear. Since *o*-quinone are themselves strong oxidants, the oxidative processes in the system would be accelerated and no longer inhibited. Moreover, the presence of quinone would inhibit cell division and cause cell death. Thus, high concentrations of Prs and related *o*-dihydroxyphenols favor the high electronegativity (reducing power) associated with the juvenile state and these high concentrations are, in fact, causally related to that morphogenetic state (44,45). Such a system based on phenolquinone balance may act both as antioxidant or oxidant (tendencies to donate or to accept electrons).

Permanent Stress Hypothesis or Antioxidant Adaptive Strategy?

The permanent stress hypothesis of habituation (and vitrification) is based on lipid peroxidation and ammonia accumulation (29–32). In plants, lipid peroxidation may, at least, have two origins: (a) enzymatic, due to lipoxygenase activity; and (b) nonenzymatic (autocatalytic), due to activated oxygen species (26). Polyunsaturated fatty acids are the main targets for lipoperoxidation, which leads to the formation of unstable hydroperoxides, cleaved in conjugated dienes, giving rise to TBA-reactive substances (TBARS) such as MDA and 4-hydroxynonenal.

It is true that the HNO line exhibited higher TBARS content (1,15). Among the indicators of oxidative damage, the most abused and misunderstood is the TBA-test. It is frequently used because it is easy to measure, however, it does not distinguish between different reaction components. Many workers have erroneously assumed that the reaction product is indicative of the pre-existing or *in vivo* levels of lipid peroxides in the tissues, whereas, in fact, TBA-reactants are produced as a result of exposure of the tissue homogenates to oxygen during the period of reaction and may not be a true index of *in vivo* generation of lipid peroxides (39). The simplicity of the test has led

many scientists to use it as an index of peroxidation without understanding exactly what it can measure (27). Nevertheless, the question remains as to what extent these high TBARS amounts are indicative of any lipid peroxidation in the habituated cell line.

My own opinion is that these results should be considered in two ways: (a) the levels measured in the different cell lines; and (b) the evolution of the TBARS content, in each line, throughout the duration of culture. The absolute values of TBARS content in these very different cell lines (normal and habituated nonorganogenic), per se, might not be considered as a lipid peroxidation index (11), but as representative of the specific content of each cell line in aldehydic compounds and other interfering substances in the TBA reaction (8,24). If a lipid peroxidation index is required, it would be advisable to consider the evolution of TBARS content during the culture, in each cell line, taking the first measurement (Day 3 or 7, for example) as an endogenous control, and to correlate it with other indicators of peroxidation. Taking these remarks into consideration, we can say that the evolution of free and bound TBARS was rather similar to the two lines studied. HNO cells did not exhibit any drastic increase in TBARS that could be correlated to any peroxidation. Moreover, no relation, in the sense of any peroxidation, can be found between hydroperoxide index, conjugated dienes, lipoxygenase activity, and TBARS content. On the other hand, it has been reported that membranes of the HNO line were mainly constituted of C18:2 fatty acids (1). MDA can only be produced from fatty acids having more than two double bounds. Thus, C18:2 may not be proposed as a MDA source (11). If MDA constitutes the main compound estimated by TBA reaction in the HNO line, its origin might not be found in lipid peroxidation.

Another argument developed by Le Dily et al. (31) to sustain H_2O_2 accumulation was the reported decrease in catalase and peroxidase activities in HNO callus (19,25). They supposed that OH° generation would occur through the Fenton reaction, leading to subsequent lipoperoxidation and membrane damage, and that these deleterious effects would be enhanced by lipoxygenase activation that generates LOO°. HNO line exhibited a lower lipoxygenase activity and fewer lipid hyperperoxides, when compared to the N line. A simpler explanation would be that neither catalase nor peroxidase activities were high because of the absence of the substrate, H_2O_2. As a matter of fact, the presence of H_2O_2 and O_2°- (and redox properties) has been investigated in the sugar beet nonorganogenic sugarbeet cell line using chemiluminescence (3). Chemiluminescence was lower in HNO cells when compared to N ones. It was concluded that HNO cells contained less hydrogen peroxides and less O_2°- than normal ones. These results are in line with our proposal of a higher protection against activated oxygen species and free radicals in the HNO line (15,23,25). The antilipoperoxidant potential of the HNO callus was also investigated (18). HNO cells inhibited 76% of an autooxidation cycle of linoleic acid initiated by γ-rays (versus 42% for the N callus).

Oxidative stresses have been related to free radical generation, C_2H_4 production, differentiation, and lignification (34,47). If we consider that inhibition of C_2H_4 production was recognized as a characteristic of habituated callus (21,28,38), which is also considered to be dedifferentiated and juvenile, it is surprising to propose the production of hydrogen peroxides and the generation of free radicals through the Fenton reaction in this line.

As previously indicated, Prs and phenolic compounds have been found in higher levels in the habituated cell line and their role in preventing H_2O_2 formation has been emphasized. Interestingly, Carriè et al. (3) have reported that HNO cells were either able to reduce ferricyanide more efficiently or to produce less hydrogen peroxide than normal cells. This result is in line with the role proposed by Stonier and his collaborators for Prs (able to act both as antioxidant or oxidant). As a matter of fact, and as reported by Stonier and Yang (44), after 28 d in cultures, agar turned brown at the same time that necrosis appeared in some parts of the callus. If these necroses were due to a permanent free radical attack, the further subculturing of this callus would be seriously compromised and the reversal of the cell death could not be stopped because the oxidation goes on (as postulated) after subculturing. Thus, as proposed by Stonier et al. (42), it is quite possible that necrosis is due to phenolic compounds released in the surrounding medium and subsequently oxidized into *o*-quinones by atmospheric oxygen. External quinone accumulation could lead to the existence of an important oxidant pool, able to counteract, suppress, and reverse the antioxidant properties of endogenous phenolic compounds, and to kill juvenile cells in a short time without allowing them to undertake maturation processes. The phenomenon of external release could be increased in this line because of the exhaustion of nutrients in the culture medium, this line being more heterotrophic than their normal counterpart [which can be classed as phytomixotrophic, according to William et al. (48)]. Thus, we are probably dealing with a chemical switch: reduction leading to the juvenile state and cell division, whereas oxidation leads to maturation, differentiation, and senescence or to a more rapid cell death if the oxidant is too strong. The same substances (antioxidants), which favor one class of reactions (reductant) at the same time, inhibit a second class of reactions (oxidations).

Phenolic compounds are chain-breaking antioxidants. Indirect antioxidant functions are also the replenishment of reduced glutathione GSH, from glutathione disulfite (GSSG) by the flavoprotein GSSG reductase and the transport and elimination of the reactive compounds. HNO lines exhibit a higher glutathione reductase activity, with roughly similar content of GSH and GSSG, associated with higher monodehydroascorbate, dihydroascorbate reductase, and ascorbate peroxidase activities than its normal counterpart (23). The dedifferentiated state of the habituated cells could also be considered as an indirect proof of the antioxidant properties of these cells. It has been stated that the loss of lipid peroxidation is proportional to the degree of dedifferentiation in hepatic cells. Possible reasons for the decline in lipid peroxidation are the low activity of the enzymes of the monooxygenase microsomal chain (6). The decreased activity of cytochrome *P450*, *P420*, and *b5* has been observed in habituated sugarbeet cells (25). For Dianzani (6), the change in the lipid composition of membranes, with marked decrease in polyunsaturated fatty acids [analogous results for HNO line (1)] and the increased rigidity of the membrane (which prevents to some extent the influx of oxygen inside the membranes) are arguments in favor of a decreased lipid peroxidation in tumors.

In a recent review, Le Dily et al. (30) considered that habituation is due to the accumulation of H_2O_2 and the generation of HO° in ammonia stressed and lipoperoxidated cell lines, which are hyperprotected against free radical and oxidative damages. Accordingly, the growth is limited by high levels in Pas, which also give a proliferative advantage to the cells. Numerous mutations would occur giving rise to "cancerous" cells (with permanent GTP binding to small G protein that mimic growth factor action) in which the synthesis of growth substances would be due to altered nitrogen metabolism and

activation of the HMP pathway. The data could equally well, if not better, be interpreted in terms of an adaptive antioxidant strategy.

Conclusion

It appears that the induction of the habituation phenomenon and the habituated state itself have become mixed up. The role of free radicals and of active states of oxygen can be of crucial importance in the origin of the phenomenon. Recently, Levine et al. (33), studying plant hypersensitive disease resistance response, have demonstrated that the treatment of cells with hydrogen peroxide causes their cellular death. The oxidative burst is transient and begins to decline after 40 to 50 min. It is followed by an increase in the extent of cell death. A short pulse of H_2O_2 is sufficient to activate the hypersensitive cell death potentialized by catalase inhibition. H_2O_2 would have a dual role: on the one hand, from the oxidative bursts, it would act as a local signal that would trigger hypersensitive death and, on the other hand, it would act as a diffusible signal that would induce cellular protectant genes to block the programmed cell death. Mutations in genes that condition the biochemical pathways of the diffusible signal could explain the occurrence of uncontrolled phenotypes.

The fact that habituation is considered as a plant tumor is well established (2). Because of numerous analogies between habituation and animal tumors, and the new insight brought by the discovery and involvement of proto-oncogenes in neoplastic growth, a hypothesis based on point mutations occurring on analogous plant genes has been proposed (12,15,16). To explain some of the abnormal cytological and biochemical features observed in the habituated nonorganogenic cell line, it has been proposed that free radical attacks could occur due to the abnormal conditions offered to plant cells in culture. Under these conditions of stress, cells may die or survive. According to Le Chatelier's principle: a stable system under stress will move in the direction that tends to minimize the stress. Consequently, habituated nonorganogenic cell lines may have undertaken an adaptative strategy to protect themselves against deleterious free radicals produced by *in vitro* techniques. Free radicals could be involved in the genesis of the phenomenon. H_2O_2 could appear and, according to Levine et al. (33), trigger hypersensitive death in some cells and induce cellular protectant genes to block the programmed cell death in others. Mutations on "hot spots" in genes that condition the biochemical pathways could explain the occurrence of these uncontrolled phenotypes. The genesis of the phenomenon is probably difficult to study because it would randomly occur in a single cell, giving it a proliferative advantage over the others, and it is quite probable that there is not only one habituation but several habituations. The onset of the phenomenon would be adaptative in nature. The cells react to an unfriendly hostile environment with clonal development of a new cell population resistant to the noxious environment (10). One of the best strategies to obtain good protection could be to increase the synthesis of free radical scavengers and to limit the biochemical pathways able to generate oxygenated reactive species. Such a strategy is suggested for animal tumor cell lines, which exhibit low rate of lipid peroxidation (6,10). For habituated cells, this adaptation may lead to an increase in the free radical scavenging properties of the cell (high spermidine, spermine, phenolic compounds, etc.) and to a decrease in free radical producing pathways (C_2H_4 production, auxin catabolism, peroxidase activity, photosynthesis, etc.).

Thus, a role for free radicals in plant tissues cultured *in vitro* might be suggested. Although free radicals are often presented as harmful for the cell, they are unavoidable and necessary intermediates in numerous biochemical pathways in the living cell. The intracellular free radical concentration is physiologically controlled by radical scavengers (enzymatic or nonenzymatic) and by spatial restrictions and limitations in their intracellular diffusion. Under stress conditions, the generation of free radicals has been demonstrated and was reported to lead to senescence processes. Another scenario could be proposed: submitted to free radical attacks, escapes could exist via mutations allowing hyperscavenging capabilities to the cell. As a result, free radical-dependent metabolic pathways would be limited to background activities, turning the individual cell towards an "eternal juvenility." Unfortunately, this rejuvenation appears to be incompatible with the organized life of multicellular organisms.

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