Pergamon

0031-9422(95)00825-X

Phytochemistry, Vol. 41, No. 6, pp. 1523–1526, 1996
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0031-9422/96 $15.00 + 0.00

MALONDIALDEHYDE CANNOT BE RELATED TO LIPOPEROXIDATION IN HABITUATED SUGARBEET PLANT CELLS

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(*Received in revised form* 18 *September* 1995)

Key Word Index—*Beta vulgaris*; Chenopodiaceae; lipoperoxidation; habituation; plant tumours; MDA; TBARS; polyamines.

Abstract—Malondialdehyde (TBARS: thiobarbituric acid reactive substances) has been investigated in non-organogenic and organogenic habituated and normal sugarbeet cell lines and compared with lipid hydroperoxides, conjugated diene formation and lipoxygenase activity in order to estimate the reality of lipid peroxidation processes which have been postulated in the habituated non-organogenic cell line. The results presented here exhibit a strong discrepancy between TBARS and the other indices tested. Neither hydroperoxide index, nor conjugated dienes, nor lipoxygenase activity could confirm that the non-organogenic habituated cells studied were submitted to permanent stress due to free radical attacks. Moreover, these results underlined that the use of TBARS to estimate lipid peroxidation must be considered with extreme caution in plants and can lead to misinterpretations.

INTRODUCTION

Habituation is one of the four neoplastic diseases of plants; it occurs spontaneously in plant cell cultures [1]. A habituated non-organogenic (HNO) sugarbeet cell line was used as a model and compared with a normal (N) callus by authors who claimed that peroxidation of polyunsaturated fatty acids would be responsible for the high malondialdehyde (MDA) content measured in this cell line and that a permanent oxidative stress would occur [2, 3]. The extent of lipid peroxidation is considered to be an important parameter for the identification of the oxidative stress [4]; the validity of techniques for the measurement of lipid peroxidation in living tissues is consequently of importance. Among these methods, estimation of thiobarbituric acid (TBA) reactive products is the most widely used. Treatment of a biological sample with TBA under appropriate conditions results in the formation of pink-coloured products with significant absorbance around 530–535 nm, and the results are usually expressed in terms of TBA reactive substances (TBARS). However, when applied to plant materials, many substances like pigments, sucrose, glucose and fructose react with TBA to produce a similar chromogen [5, 6] and this can lead to a misinterpretation of peroxidation processes. As other methods are available for the detection of lipid peroxidation products, we have studied TBARS amounts, lipid hydroperoxides, conjugated diene formation and lipoxygenase activity in N, HNO and organogenic habituated (HO) sugarbeet cell lines. On the basis of the results presented in this report, we propose the reconsideration of the existence of lipoperoxidation processes in the HNO line and we raise objections to the use of the TBA test in plants.

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RESULTS

TBARS

TBARS were significantly higher in the HNO cell line than in the N line during the culture cycle (Fig. 1). Very low levels of TBARS were observed in the HO callus. These levels were significantly lower, except at day 14, than those of N callus and three-fold lower than the amounts determined in the HNO callus throughout the period of culture. TBARS determination revealed no significant variation from one time of culture to another in the three lines. That is, on the basis of similar high amounts of TBARS in the HNO line (only compared with the N line), it was concluded that high lipid peroxidation processes do exist in this habituated callus [2].

Conjugated dienes

Regardless of the day of culture, the determined amounts of conjugated dienes were lower in HO or HNO calli than in the N ones (Fig. 2). A gradation could be underlined from auxin and cytokinin-N requiring callus to HO and HNO calli. This trend indicates that an

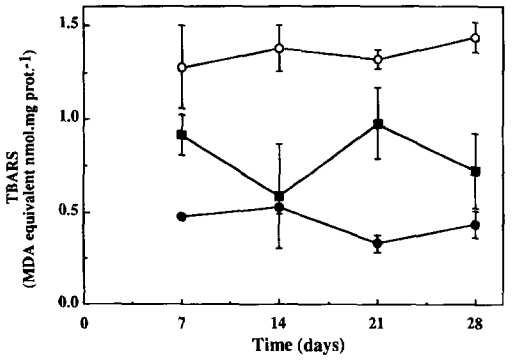

Fig. 1. TBARS estimation during callus culture in normal (■), habituated organogenic (●) and non-organogenic (○) sugarbeet cell lines. Means ± SD ($n > 3$).

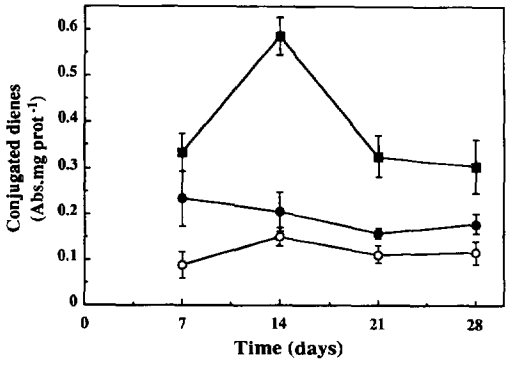

Fig. 2. Conjugated diene amounts during callus culture in normal (■), habituated organogenic (●) and non-organogenic (○) sugarbeet cell lines. Means ± SD ($n > 3$).

inversely proportional relationship may exist between conjugated diene level and habituation progression.

Lipid hydroperoxide estimation

As shown in Fig. 3, lipid hydroperoxide estimation in the three types of calli revealed that the HNO line, proposed to be under permanent stress and permanently submitted to free radical attacks [3], contained lower hydroperoxide amounts than the other two lines. Whatever the day of culture, the HNO line exhibited hydroperoxide levels at least half as much as those detected in the N one. In the former callus, a maximum was observed to day 21, followed by a decrease, at day 28, to levels similar to those determined on the first sampling day. Incidentally, results were quite different in the HO cell line. At days 7 and 14, hydroperoxide levels were significantly higher in this line than in the normal one. Interestingly, N callus, when compared with HO callus, did not demonstrate higher hydroperoxide contents at any day of culture. On the other hand, at the end of culture, N and HO lines did not exhibit a decrease in hydroperoxide index similar to that observed in the HNO line. In these cell lines, this difference could be due

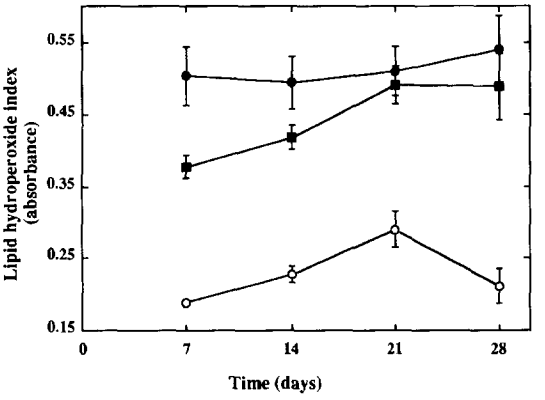

Fig. 3. Lipid hydroperoxide estimation during callus culture in normal (■) , habituated organogenic (●) and non-organogenic (○) sugarbeet cell lines. Means ± SD ($n > 3$).

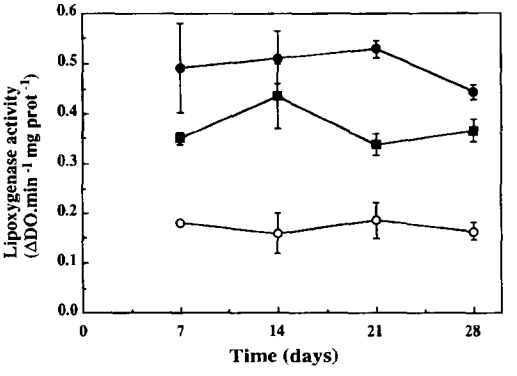

Fig. 4. Lipoxygenase activity during callus culture in normal (■), habituated organogenic (●) and non-organogenic (○) sugarbeet cell lines. Means ± SD ($n > 3$).

to differentiation and maturation processes that involve free radicals. This view may be also supported by the observation that hydroperoxide amounts were higher in the HO line, which is more differentiated and more organized.

Lipoxygenase activity

Lipoxygenase activity determination in the three cell lines (Fig. 4) gave results roughly similar to those obtained with the hydroperoxide index (Fig. 3). In fact, the lipoxygenase activity was significantly lower in HNO callus than in N and HO calli during the whole period of culture. Moreover, the same tendency of decreasing activity from HO to N and HNO calli could be underlined, indicating that a correlation may exist between lipoxygenase activity and maturation and differentiation processes. This concordance in results with the hydroperoxide index is in line with the idea that the production of hydroperoxides is, at least in part, due to lipoxygenase activity.

DISCUSSION

From the results obtained by the TBA test, it is tempting to conclude that the HNO cell line is submitted to lipoperoxidation processes, but the results presented here indicate that there is no correlation between hydroperoxide index, conjugated dienes, lipoxygenase activity and TBARS content. Thus, the absolute values of TBARS content in the different cell lines, *per se*, cannot be considered as a lipid peroxidation index as previously proposed [7], but is representative of the specific content of each cell line in aldehydic compounds and other interfering substances in the TBA reaction [5, 6]. Claiming the existence of lipoperoxidation processes in plant cell lines and particularly in our case in the HNO cell line [2, 3] on the sole basis of TBARS (apparent MDA) estimation, can lead to a misinterpretation of results. It has already been underlined that, among the indicators of oxidative damage, the most abused and misunderstood was the TBA test. It was frequently used because of the ease of measurement; however, it did not distinguish between different reaction components. Many workers have erroneously assumed that the reaction product is indicative of the pre-existing or *in vitro* levels of lipid peroxides in the tissues. In fact, TBA reactants are produced as a result of tissue homogenates exposure to oxygen during the period of reaction and are not an index of *in vivo* generation of lipid peroxides [8]. The simplicity of the test has led many scientists to use it as an index of peroxidation without understanding exactly what it can measure [9], and this unreliability in lipid oxidation has already been underlined [10].

TBARS results presented here 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. If TBARS could be used as a lipid peroxidation index, it would be necessary to consider the evolution of TBARS content during the culture, in each cell line, taking (for example) the first measurement (day 7 in this case) as an endogenous control. Taking these remarks into consideration, we can say that the evolution of TBARS was rather similar in the three lines studied. HNO cells did not exhibit any drastic increase in TBARS, which could be correlated to any peroxidation. On the other hand, it has been reported that the membranes of the HNO line were mainly constituted with C18: 2 fatty acids [2]. MDA can only be produced from fatty acids having more than two double bounds [7]. Thus, C18: 2 may not be proposed, in plants, as a MDA source. If MDA constitutes the main compound estimated by TBA reaction in the HNO line, its origin might not be found only in lipid peroxidation. It has been demonstrated that both spermidine and spermine can give rise to MDA and that this compound could play an important role in growth control [11, 12]. Interestingly, HNO spermidine and spermine estimations were in line with the TBARS level measured in the present work [13]. This leads us to suspect a possible origin of TBARS from polyamine degradation by polyamine oxidase or diamine oxidase as proposed by Quash *et al.* [12]. This biochemical pathway is under examination and a role for MDA-like compounds in cell growth remains to be established. Moreover, high TBARS contents could not be considered as a characteristic of habituation since the HO line exhibited lower amounts than the N line.

The other lipoperoxidation index measured in this study associated with other results previously obtained lead us to conclude that the proposal of a permanent oxidative stress [3] due to lipoperoxidation processes could be the opposite of the truth. On the one hand, the previous authors have postulated that $OH^{\bullet}$ generation would occur through the Fenton reaction, in the HNO line, leading to subsequent lipoperoxidation and membrane damage. These deleterious effects would be enhanced by lipoxygenase activation, which generate $LOO^{\bullet}$. The results presented here invalidate this analysis since the HNO line exhibited a lower lipoxygenase activity and fewer lipid hydroperoxides than the N line. On the other hand, the presence of H_2O_2 and O_2 (and redox properties) has been investigated in HNO cell line using chemiluminescence. It was concluded that HNO cells contained less hydrogen peroxide and less $O_2^{\bullet -}$ than normal ones [14]. In the same line, HNO cells exhibited a higher glutathione reductase activity, with a roughly similar content of GSH and GSSG, associated with higher monodehydroascorbate, dihydroascorbate reductase and ascorbate peroxidase activity than its normal counterpart [15]. The anti-lipoperoxidant potential of the HNO callus was also investigated. HNO cells inhibited 76% of an auto-oxidation cycle of linoleic acid initiated by γ-rays (versus 42% for the N callus) [16]. The de-differentiated state of the habituated cells could also be considered as an indirect proof of the antioxidant properties of these cells, since it has been stated that the loss of lipid peroxidation is proportional to the degree of de-differentiation in hepatoma cells [17].

The results presented in this paper underline that the use of MDA (TBA test) as a lipoperoxidation index must be considered with extreme caution, particularly in plants, in order to avoid interpretations that overestimate the capacities of this method.

EXPERIMENTAL

Plant material and cultures. Experimental conditions for obtaining N, HO, and HNO calli of sugar beet (*Beta vulgaris* L. *altissima*) and for maintaining these tissues in solid stock cultures under light (16 hr photoperiod of Sylvania Grolux fluorescent light providing 17 $W m^{-2}$, 25°) have been reported elsewhere [18]. Calli were subcultured every 2 weeks on their respective solid medium (basal medium without plant growth regulators for habituated lines, but supplemented with 0.1 $mg l^{-1}$ 2,4-D and 0.1 $mg l^{-1}$ BAP for normal lines). For experiments beyond day 14, the cells were left on the same medium without subculturing.

TBARS. TBARS were estimated according to ref. [19]. Fresh callus (500 mg) was homogenized using 1 ml 10% (w/v) TCA, 50 μl 0.05% butylated hydroxytoluene

(Sigma). The mixt. was centrifuged (4000 *g*, 10 min, 4°). The pellet was washed (× 2) with 5 ml Me_2CO and removed after centrifugation (4000 g, 10 min, 4°). The new pellet obtained was used for the TBA reaction and incubated with 3 ml H_3PO_4 1% (v/v), 1 ml TBA (Sigma) 0.6% (p/v) at 100°, 30 min, then cooled on ice. *n*-BuOH (3 ml) was added. After homogenization and centrifugation (4000 *g*, 10 min, 4°), 1 ml of butanolic supernatant was used directly for spectrophotometric determination. *A* was defined according to the following calculation:

$$DO_2 - ((WL_1 - WL_2).DO_3 + (WL_2 - WL_3)\cdot DO_1)/(WL_1 - WL_3)$$

Wavelengths (WL) were defined as follows: $WL_1 = 565$ nm, $WL_2 = 534$ nm and $WL_3 = 510$ nm, DO_1, DO_2, DO_3, being the corresponding absorbances. MDA prepd from 1,1,3,3-tetraethoxypropane (Aldrich) was used as standard. Results were expressed in nmol (MDA equivalent).mg prot^{-1}.

Conjugated dienes. Tissues (500 mg) were pestled in liquid N_2, then ground in 3 ml MeOH, 100 mg EDTA, 3 ml $CHCl_3$ and 3 ml of a soln containing 5 mM EDTA and 1% NaCl [20]. The mixt. was centrifuged (4°, 10 mn, 400 *g*) in glass tubes. The chloroformic phase was evapd under N_2. The residue was dissolved in 500 μl $CHCl_3$. 50 μl were again dried under N_2 and then dissolved in absolute EtOH. *A* was read at 234 nm in a black quartz curve He (Ref: 108. 002B-QS) in a Shimadzu UV-160 A spectrophotometer. Results were expressed in *A* mg prot^{-1}. Proteins were determined according to ref. [21], using serum albumin as standard.

Lipid hydroperoxides. These were quantitatively analysed using the method of ref. [22]. Lipids from 500 mg of tissues were extracted according to ref. [23]. The $CHCl_3$ soln (2ml) was added to 5 ml EtOH, 0.2 ml HCl 1 M and 0.1 ml ammonium ferrous sulphate (1% w/v). After a further 30 sec, 1 ml ammonium thiocyanate (20% w/v) was added and the *A* at 480 nm was read 3 min later.

Lipoxygenase activity. Tissues (500 mg) were pestled in liquid N_2 then ground in 0.1 M KPi buffer (pH 7.5) containing 10 mM $Na_2S_2O_5$, 1 mM EDTA (Sigma) and 1% insoluble PVP (Sigma). Homogenates were centrifuged (4°, 25 min, 20 000 *g*) and supernatants were saved for determining LOX activity. LOX activity was assayed spectrophotometrically using linoleic acid as substrate, according to ref. [24]. The assay soln, freshly prepd (substrate instability) contained 0.25 mM linoleic acid (Sigma) and 0.25% Tween 20 in 0.2 M citrate–phosphate buffer (pH 7). The reaction mixt. contained 2.4 ml assay soln and 0.1 ml plant extract. *A* was read at 234 nm. Activity was expressed in DO·min^{-1}·mg prot^{-1}.

Acknowledgements—This work has been supported by 'Région Bretagne', C.U.B. (Communauté Urbaine de Brest) and M.E.S.R. (Plan Etat-Région).

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