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An Evaluation of Analytical Methods for the Detection of Secondary Oxidation Products in Dedifferentiated Plant Cultures

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Summary

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A range of techniques have been employed to detect and examine aldehydic products of lipid peroxidation in *D. carota* cultures. Emphasis is placed on the use of HPLC and LC-MS techniques to aid the identification and profiling of aldehydic dinitrophenyl hydrazone (DNPH)-derivatives in dedifferentiated callus tissues. Using these approaches, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) have been identified in cultures of *D. carota*. Using LC-MS, it was found that embryogenic cultures have high levels of HNE and lower levels of MDA compared to non-embryogenic cultures. Future studies will evaluate the role of these lipid peroxidation products in in vitro morphogenesis.

Introduction

Highly reactive free radical species are central to metabolic processes such as photosynthesis and mitochondrial respiration, thus, controlled free radical mechanisms are of considerable importance in biological systems. It has been estimated that 2-5% of free radical activity 'escapes' from this regulated environment and reacts with nearby molecules such as proteins, DNA, RNA and membrane lipids (HALLIWELL & GUTTERIDGE 1989). Thus, studies of secondary

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lipid peroxidation reactions are of considerable importance. Lipid peroxidation proceeds via the formation of conjugated dienes, lipid hydroperoxides and a range of breakdown products are formed including malondialdehyde (MDA) and 4-hydroxyalkenals. 4-Hydroxynonenal (4-HNE), for example, is thought to be formed from the cleavage of omega-6 and omega-3 polyunsaturated fatty acids (PRYOR & PORTER 1990, VAN KUIJK & al 1990). 4-HNE is potentially both cytotoxic and mutagenic in mammalian systems (ESTERBAUER & al. 1991). It also appears to inhibit the Na^+- K^+ - ATPase which may be responsible for secondary oxidative damage following tissue injury (SIEMS & al. 1996). Due to their longevity, toxic secondary products react with primary amine groups to form fluorescent compounds associated with mammalian ageing (HARMAN 1992).

Several methods for the detection of lipid peroxidation products have been developed. These include colorimetric and fluorimetric assays (DRAPER & HADLEY 1990) for thiobarbituric acid reactive substances (TBARS), UV/Visible spectroscopy for conjugated dienes (SMITH & ANDERSON 1987) and commercial kits for the specific detection of MDA and HNE (LPO-586. Bioxytech S.A., Bonneuil/Marne, France) and lipid peroxides (K-Assay LPO-CC. Kamiya Biochemical company, Seattle, WA, USA). In addition methods using HPLC and GC-MS for the analysis of aldehydes (THOMAS & al. 1995) have been explored. More recently, the development of mass spectrometry interfaced with HPLC, allows the separation and identification of less volatile compounds. However, most applications using HPLC require the use of a derivatising agent such as DNPH. This allows the normally volatile aldehydes in the sample to react and form stable, coloured dinitrophenylhydrazone derivatives absorbing at 360 nm. These can be extracted into organic solvent and separated by TLC. Further separation by HPLC allows the identification of individual aldehydic-DNPH components. Separation of aldehydic classes by preparative TLC can aid identification. Alternatively, cyclohexanedione (CHD) may be used with fluorimetric detection, and this approach offers a reproducible and sensitive method (HOLLEY & al. 1993, ESTERBAUER & CHEESEMAN 1990).

The objective of the present study is to develop a definitive method for the extraction, separation and identification of secondary lipid peroxidation products in callus cultures of *D. carota.* Emphasis is given to the detection of malondialdehyde and 4-hydroxynonenal as these aldehydes are implicated in cyto and geno-toxic conditions in animal cells (ESTERBAUER & al. 1988). The wider objective of the research programme is to investigate the role of oxidative stress in in vitro plant culture ageing. To date, little is known about the causative factors which promote the time related loss of plant culture totipotency. As secondary oxidative stress has been implicated in many degenerative conditions in animal cells it may also have a role in in vitro ageing and the control of morphogenesis in plant cells. The development of analytical procedures for detecting lipid peroxidation products will greatly assist the study of this premise.

Methods

D. carota culture system: Hypocotyl stem sections of *D. carota* seedlings were placed on MS culture medium containing 1 $mg.l^{-1}$ 2,4-dichlorophenoxyacetic acid and dedifferentiated callus was initiated as described previously (ZIMMERMAN 1993, ROBERTSON & al. 1995). Callus was sub-cultured every two weeks and samples for analysis were harvested mid-cycle. Somatic embryogenic ability was assessed by transfer onto hormone free medium (ZIMMERMAN 1993).

Preparation of DNPH derivatives: DNPH (Aldrich D19,930-3 with 30% water) was dried and purified by butanol recrystallisation. Aldehyde-DNPH standards were prepared according to the method of VOGEL 1989. Malondialdehyde (MDA), as the 1,1,3,3,-tetramethoxypropane, and 4-hydroxynonenal (HNE) as the diethylacetal were hydrolysed prior to formation of DNPH hydrazones. Callus tissues (2.5 g) were disrupted by freezing in liquid nitrogen and the aldehydes extracted in methanol (5 ml). Following centrifugation (4000 rpm, 5 min), DNPH (0.035% (w/v) in 1N HCl; 5 ml) was added to the supernatant and the derivatives treated as described by ESTERBAUER & CHEESEMAN 1990. Solvent was removed in vacuo and the residue dissolved in acetonitrile (500 µl) prior to injection onto the HPLC column.

HPLC Analysis: This was performed using a Spectra Physics IsoChrom pump and integrator (SP4270) coupled to a Severn Analytical SA6500 UV detector set to 360 nm. The column comprised of a FSA Chromatography 25 cm Apex Octodecyl 5µ Spherisorb absorbent. The system was run isocratically using acetonitrile:water at concentrations of 65 - 85% acetonitrile.

LC-MS Analysis: 2,4-Dinitrophenylhydrazones in acetonitrile were analysed on a Finnigan MAT SSQ710C with APCI interface and Hypersil C18 (250x2.0 mm) 5µM column, using either an isocratic (65% acetonitrile in water) or gradient system (A= Water, B= Acetonitrile 75%. Initially 25% A and 75% B but rising to 100% B by 5 minutes). The solvent flow rate was 0.25 ml/min with UV detection at 360 nm and set to 0.01 absorbance units full scale (AUFS). Operating conditions comprised: APCI, negative ion, sheath gas nitrogen at 80 psi, corona ~ 4.5 kV and full scan mode at m/*z* 230-340 in 1.5 seconds. Selected ion monitoring was performed at m/z 230, 234, 317, 335 (±0.15 amu) with a dwell time of 0.25 second/window DEIGHTON & al. 1997.

Results and Discussion

A range of solvent systems (methanol, ethanol, heptane, hexane:isopropanol (3:2), dichloromethane and pH 7.0 potassium phosphate buffer) were tested for their efficacy in extracting aldehydic peroxidation products from callus tissues. Methanol proved to be the optimum extractant as it was practical to use, and produced a compact pellet with a supernatant clear of turbidity. Preliminary HPLC studies, using a simple isocratic system and UV detection were first employed to evaluate tissue extraction efficiency. Thus, the HPLC methanol extracts revealed multicomponent traces. Comparison of these chromatograms showed that an extraction time longer than ten minutes offered no advantage (data not shown). Acetontrile:water ratios of between 65% to 85% gave good separation of the aldehyde-DNPH standards, which eluted in a reproducible manner with clearly separated peaks, emerging in order of increasing chain length. Although MDA and HNE standards eluted at 3.51 minutes and 4.51 minutes respectively, their elution profiles were coincident with acetaldehyde and butyraldehyde when chromatographed in multicomponent systems. Putative identification of the derivatised samples using aldehyde standards revealed the presence of a range of aldehydes in the methanol extracts of *D. carota* callus

(Table 1). However, the presence of many high peaks eluted at the early stage of the chromatographic separation, made the determination of MDA and HNE difficult. Thus, these aldehydes are 'hidden' under considerably larger peaks with similar or the same retention times. For this reason, the purification of solvents by pretreatment with DNPH followed by distillation is strongly recommended to avoid solvent derived peak contamination which may influence resolution (HOLLEY & al. 1993). In addition, pre-separation of samples by TLC can eliminate excess DNPH reagent and contaminating carbonyls (ESTERBAUER & CHEESEMAN 1990). Whilst isocratic HPLC associated with UV detection was useful in the preliminary evaluation of DNPH derivatisation, tissue extraction procedures and the putative identification of aldehydic lipid peroxidation products, this method could not be used to separate MDA and HNE, from other components. The definitive identification of these aldehydes was required, and LC-MS was thus explored.

Table 1. HPLC/UV detection, separation and putative identification of DNPH-derivatives of C1-C9 aldehydes in methanol extracts of embryogenic and non-embryogenic callus cultures of *D. carota*

Aldehyde (as DNPH derivative)	Embryogenic culture	Non-embryogenic culture
acetaldehyde	+	+
propanal	+	+
butanal	+	+
pentanal	+	+
hexanal	+	+
heptanal	+	+
octanal	-	+
nonanal	-	+

The HPLC system comprised an acetonitrile/water solvent (85:15) applied at a flow rate of 1ml min^{-1}. DNPH derivatives of aldehydes detected in the methanol extracts of callus were identified using co-chromatography of the DNPH derivatives of standard aldehydes. DNPH derivatives of malondialdehye and 4-hydroxynonenal standards could be separated using this system, however, these could not be separated in plant extracts as they co-eluted with acetaldeyhde and butanal.

LC-MS profiles were obtained from methanol-extracted DNPH-derivatised samples of varying embryogenic ability and selected ion monitoring (SIM) revealed MDA (m/z=234, retention time = 5.30 minutes) and HNE (m/z=335, retention time = 16.40 minutes) DNPH derivatives. Whilst the co-elution of MDA and HNE with acetaldehyde and butyraldehyde respectively did not prevent their individual identification by LC-MS, the application of a gradient program effectively separated these components. Figures 1a and 1b present data obtained from the integrals of these two peaks. In general the callus culture line with a high level of HNE had a correspondingly low level of MDA and vice versa. However, in one case, the embryogenic Line E(1) had elevated levels of both MDA and HNE. Several factors may influence the production and distribution of HNE in callus tissues and it is likely our sample preparation procedure would allow for detection of 'free HNE' only. Further refinement of extraction procedures will be necessary

to detect bound HNE, which for example, may be associated with proteins and SH-groups. In our future studies we will investigate this possibility.

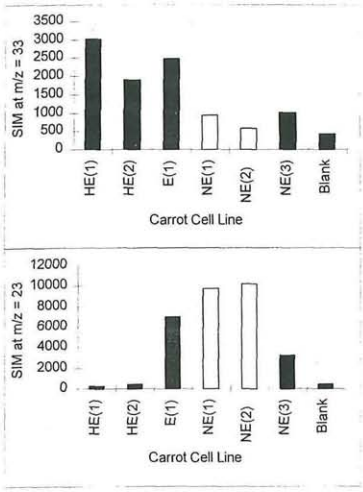

Fig. 1a and b. Integrals of peaks from LC-MS SIM for HNE (m/z=335) and MDA (m/z=234) respectively. All lines represent DNPH derivatives prepared from 2.5g of callus. Results are means of duplicate runs. Bars with the same shading indicate data from different samples of the same line. HE = Highly Embryogenic, E = Embryogenic, NE = Non-Embryogenic.

The development of methodology evaluating oxidative status in plant systems is of considerable importance. For example, many studies of secondary oxidation products employ the widely used TBARS assay for MDA. Though a useful indicator of changes in oxidative status in model systems the indiscriminate use of this assay has been criticised owing to the high reactivity of the reagent with other compounds. Some workers suggest its application, to plant systems in particular, must be considered very carefully (HAGEGE 1995, CHERIF & al. 1996). Our previous studies (ROBERTSON & al. 1995, BENSON & al. 1992) used the TBARS method and a commercial kit (LPO-586 Bioxytech S.A., Bonneuil/Marne, France) to evaluate lipid peroxidation products in plant tissue cultures. However, the LC-MS approach presented in this study offers many advantages in that MDA and HNE are identified using MS.

The finding that MDA and HNE are present in plant tissue cultures has important implications in that they have been associated with a range of toxicological phenomena in animal cells. Thus, lipid peroxidation may contribute to culture stress and the decline in totipotency (BENSON & al. 1992). The apparent differential distribution of HNE and MDA in cultures with different morphogenetic competencies is most interesting and their possible role in in vitro development must be considered. The study presented here comprises our preliminary investigations using this methodology. Further detailed, profiling of aldehydes in a wider range of cultures exhibiting different morphogenetic potentials is now required. Our further studies will thus explore the role of secondary oxidation products in plant tissue culture responses. The development of the above analytical procedures presented will greatly assist this aim.

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References

BENSON E.E., LYNCH P.T. & JONES J. 1992. Variation in free-radical damage in rice cell suspensions with different embryogenic potentials. - Planta 188: 296-305.

CHERIF M., NODET P. & HAGEGE D. 1996. Malondialdehyde cannot be related to lipid peroxidation in habituated sugarbeet plant cells. - Phytochemistry 41(6): 1523-1526.

DEIGHTON N., MAGILL W.J., BREMNER D.H. & BENSON E.E. 1997. LC-MS Determination of malondialdehyde and 4-hydroxynonen-2-al derivatives in plant tissues cultures. - Free Radical Res. Comm. in press.

DRAPER H.H. & HADLEY M. 1990. Malondialdehyde determination as index of lipid peroxidation. - In: PACKER L. & GLAZER A.N. (eds.), Methods in enzymology 186: 421-431

ESTERBAUER H. & CHEESEMAN K.H. 1990. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. - In: PACKER L. & GLAZER A. N. (eds.), Methods in enzymology 186: 407 - 421.

— , SCHAUR R.J. & ZOLLNER H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. - Free Rad. Biol. Med. 11: 81-128.

— , ZOLLNER H. & SCHAUR R.J. 1988. Hydroxyalkenals: Cytotoxic products of lipid peroxidation. - ISI Atlas of Science: Biochemistry 0898-3753: pp. 311-318.

HAGEGE D. 1995. Habituation in sugarbeet plant cells: permanent stress or antioxidant adaptive strategy? - In vitro Cell Dev. Biol. 32: 1-5.

HALLIWELL B. & GUTTERIDGE G.M.C. 1989. Free radicals in biology and medicine, 2nd Edition. - Oxford University Press, Oxford.

HARMAN D. 1992. Free radical theory of ageing. - In: Free radicals and ageing. Edited by Emerit and Chance. Birkhauser, Verlag. Basel.

HOLLEY A.E., WALKER M.K., CHEESEMAN K.H. & SLATER T.F. 1993. Measurement of *n*-alkanals and hydroxyalkenals in biological samples. - Free Radical Biology & Medicine 15: 281-289.

PRYOR W.A. & PORTER N.A. 1990. Suggested mechanisms for the production of 4-Hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. - Free Radical Biology and Medicine 8: 541-543

ROBERTSON L., MAGILL W.J., BENSON E.E., BREMNER D.H. & BUULTJENS E.J. 1995. Oxidative stress in the plant tissue culture environment. - Biochemical Society Transactions 23: 263S.

SIEMS W.G., HAPNER S.J. & VANKUIJK F.J.G.M. 1996. 4-Hydroxynonenal inhibites Na+- K+-ATPase. - Free Radical Biology and Medicine 20: 215-233.

SMITH V.C. & ANDERSON R.E. 1987. Methods for determination of lipid peroxidation in biological samples. - Free Radical Biology and Medicine 3: 341-344.

THOMAS M.J., ROBISON T.W., SAMUEL M. & FORMAN H.J. 1995. Detecting and identifying volatile aldehydes as dinitrophenylhydrazones using gas chromatography mass spectrometry. - Free Radical Biology and Medicine 18 (3): 553-557.

VAN KUIJK F.J.G.M., HOLTE L.L. & DRATZ E.A. 1990. 4-Hydroxyhexenal: A lipid peroxidation product derived from oxidised docosa-hexanoic acid. - Biochim. Biophys. Acta 1043: 116-118.

VOGEL A.I. 1989. Vogel's Textbook of Organic Chemistry. 5th Edition. Rev. by FURNISS B.S., HANNAFORD A.J., SMITH P.W.G. & TATCHELL A.R. - Longman Scientific & Technical. England.

ZIMMERMAN J.L. 1993. Somatic embryogenesis: A model for early development in higher plants. - The Plant Cell. 5: 1411-1423.