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Free Radical Processes in Plant Tissue Cultures: Implications for Plant Biotechnology Programmes

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Summary

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Tissue culture techniques have an important role in the genetic improvement of crop plant species. However, genomic instability, in vitro recalcitrance, and loss of morphogenetic potential are limiting factors affecting plant biotechnology initiatives. The objectives of this review are to evaluate the experimental evidence for the occurrence of free radical processes in plant tissue cultures. Relationships between oxidative status and in vitro plant development will be assessed and the implications that these factors may have for crop plant biotechnology explored. Achieving a better understanding of free radical mechanisms in tissue cultures may have useful applications in crop plant improvement.

Introduction

Tissue culture has a key role in the biotechnological improvement of crop species and the totipotent property of plants (the ability to regenerate whole plants from single cells) is widely exploited. Thus, in vitro manipulations underpin crop improvement initiatives involving genetic manipulations, reproductive technologies, and plant conservation. Tissue culture techniques are also involved in phytosanitary regulation and international germplasm exchange. However, there exist three major limitations to the use of in vitro methods: (1) certain species are unresponsive (recalcitrant) to in vitro manipulation; (2) cultures maintained in the

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dedifferentiated state for extended periods, loose their totipotent capacity and (3) there is an increased propensity for genetic instability in plants maintained in vitro. These factors can severely restrict biotechnology programmes which are dependent upon the maintenance of genetic fidelity and whole plant regeneration. Whilst attempts have been made to elucidate the basis for the deleterious changes generated during in vitro manipulation, these largely describe symptoms rather than underlying causes. It is now widely accepted that free radical processes in animal systems are associated with development as well as cellular and genetic degeneration. Studies of free radical mechanisms in in vitro plant cultures may thus have important implications for plant biotechnology programmes.

Assessments of free radical processes in tissue cultures include direct EPR detection, and measurements of secondary oxidation products and antioxidant status. Table 1 summarises evidence for the involvement of free radical processes in culture responses using these different approaches. Thus, free radical species have been directly detected in *S. tuberosum* using EPR spectroscopy. Preliminary evaluations of EPR spectra suggests that semiquinone and carbon-centred peroxy radicals are formed during cellular dedifferentiation (BAILEY & al 1994).

Several procedures have been used to assess secondary oxidation product formation and malondialdehyde, thiobarbituric acid reactive substances (TBARS), hydroxyalkenals, conjugated dienes, lipid peroxides and fluorescent oxidation products have been detected in a wide range of plant cultures derived from very diverse species (Table 1). Assessments of antioxidant enzymes and sulfhydryl group (SH) status indicate that the formation of secondary oxidation products can be related to dynamic changes in antioxidant capacity. Whilst studies of free radical processes in in vitro plant systems are limited, our own findings confirm those of others (e.g. BIEDINGER & SCHNABEL 1991, CUTLER & al. 1989). Currently, there now exists considerable evidence to support the view that free radicals are a component of in vitro plant development. Interestingly, the application of exogenous antioxidants to plant cultures is used to stimulate morphogenetic responses and ameliorate deleterious oxidative stress (JOY & al. 1988, ISHII 1988).

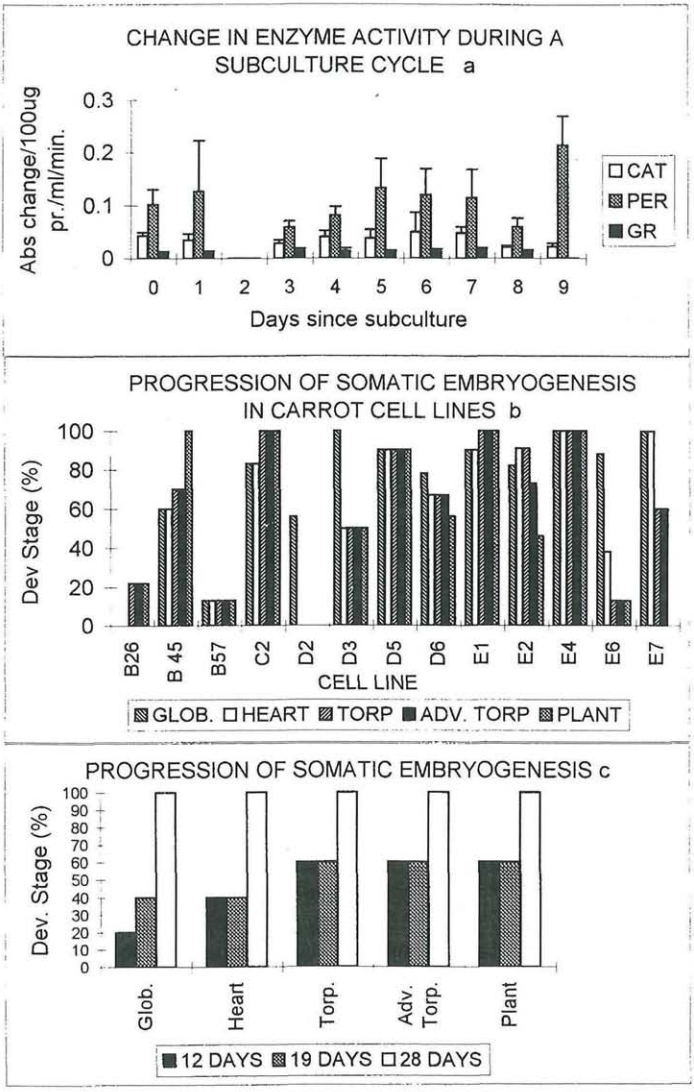
Do free radical processes have a role in vitro plant development?

Our own studies (Table 1) show well-defined trends concerning the pro- and antioxidant status of in vitro plant cultures. There is an overall enhancement in oxidative activity at the onset of a developmental transition. Thus, callus induction in *V. vinifera* stem explants was accompanied by a most pronounced increase in TBARS and aqueous soluble fluorescent oxidation products. Changes in SH status, and catalase activity also occur, though superoxide dismutase (SOD) levels were less influenced by developmental change. Following active dedifferentiation the "peak" in pro- and antioxidant activity declined in the proliferating callus, to levels just above or similar to the original explant (BENSON & ROUBELAKIS-ANGELAKIS

1992, 1994). Similar responses were observed in dedifferentiating *S. tuberosum* explants, where callus induction accompanied a transitory increase in free radical activity (BAILEY & al. 1994). Somatic embryogenesis is proposed as the system of choice for studying in vitro development (ZIMMERMAN 1993). Thus, a differentiated stem explant dedifferentiates on auxin containing callus induction medium. Under these conditions the cells can only proliferate and differentiation is suppressed, however, on transfer to hormone-free medium competent cells undergo somatic embryogenesis Studies involving regenerative pathways demonstrate the same trend. Thus, the hydroxyalkenal content of *D. carota* callus cultures increased on transfer to embryo induction medium and a similar, although less defined increase in malondialdehyde was observed (ROBERTSON & al. 1995).

Recently, we have characterised the morphogenetic characteristics of 13 different embryogenic callus cultures of *D. carota* (Fig. 1). These will be used for the study of free radical processes in in vitro ageing. Preliminary findings show that catalase, glutathione reductase and peroxidase activities can vary within a standard culture cycle and they may provide useful markers of in vitro development (Fig. 1a). Maintenance of embryogenic cultures for prolonged periods in the dedifferentiated state can cause a between different clones. The embryogenic pathway is highly complex (Fig. 1c) and future investigations will use these 13 morphogenetically characterised lines (Fig. 1b) to assess, in more detail, the relationships between culture morphogenesis, culture age and oxidative enzyme status. Ascertaining the direct role of antioxidants and free radicals in in vitro ageing and development is likely to be difficult. Free radical activity is associated with stress and it is a contributory factor in culture recalcitrance (Table 1). Clearly, many products of secondary oxidative stress are cytotoxic (ESTERBAUER & al. 1988) and their production and interaction with macromolecules could actually promote genotoxicity and enzymatic dysfunction in culture systems. However, sole consideration of the negative aspects of free radical activity risks over simplification. Oxidative processes may also have a direct role in in vitro development. Morphogenesis is a dynamic process controlled by the application of exogenous, potent, plant growth regulators. These have the capacity to alter primary oxidative metabolism and directly influence hormonal transduction pathways involving activated oxygen species (VAN EMMERIK & al. 1992, GUNSE & ELSTNER 1992). Indeed, the lipid peroxidation product, jasmonic acid can stimulate in vitro plant morphogenesis (RAVNIKAR & GOGALA 1990). Similarly, EARNSHAW & JOHNSON 1985 correlated glutathione status with morphogenetic competence in carrot suspension cultures. GSH levels were higher in proliferating cultures compared to differentiating cultures, and they concluded that development occurs in a more oxidising environment. It is thus essential to consider both positive and negative aspects of in vitro oxidative metabolism in plants.

Recently, HAGEGE 1996 discussed two hypotheses concerning oxidative processes in plant cell culture habituation. Habituation is the condition in which cell cultures spontaneously achieve hormonal autonomy and proliferate in culture



without the need for exogenous plant growth regulators. HAGEGE discusses the "permanent stress" hypothesis proposed by ARBILLOT & al. 1991 and LE DILLY & al. 1993 that states that low levels of catalase and peroxidase in habituated cultures result in the accumulation of activated oxygen species and free radicals. These promote the generation of lipid peroxides that impair cell development. A second "antioxidant adaptive strategy" hypothesis, is favoured by HAGEGE who proposes that cells challenged with a stressful culture environment develop a new "adaptive"

cell population in which free radical scavenging ability is enhanced. This adaptation reduces the capacity of the metabolic pathways that normally generate activated oxygen species and promotes increased free radical scavenging properties in habituated cells. As metabolic and developmental pathways have a dependency on activated oxygen (for example, those involving lipoxygenase) this may explain the frequently observed inability of habituated cultures to differentiate (HAGEGE 1996). These hypotheses give useful "working" models for directing future studies of free radical processes in plant cultures. However, habituated cultures would not be a system of choice in applied biotechnology programmes for which the maintenance of totipotency and genetic stability is the primary concern. For these applications it is more important to determine the role of free radical mechanisms in maintaining genetic fidelity and regenerative capacity. For example, evaluations of non-habituated cell cultures of *O. sativa* provide evidence that loss in embryogenic capacity is associated with enhanced lipid peroxidation, the accumulation of secondary lipid peroxidation products and a decrease in catalase and peroxidase activities (BENSON & al 1992).

Free radical processes in plant cultures: Implications for plant biotechnology

At present there exists no direct evidence to implicate free radicals, activated oxygen species and/or their reaction products as causal agents in either genetic or epigenetic instability in plant cultures. Free radicals are known to mediate genotoxic changes in animal cells (ESTERBAUER & al. 1988) and it may be prudent to explore this possibility in plants. Changes in the pro- and

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Fig. 1. Profiles of catalase, peroxidase and glutathione reductase activities and morphogenetic development in *D.carota* callus cultures

LEGEND: (a) Changes in catalase (CAT) peroxidase (PER) and Glutathione (GR) activities (on the basis of protein, (pr)) measured during a sub-culture cycle of callus originally derived from hypocotyl explants of *D. carota* cultivar Early Nantes (culture age = 15 months). Evaluations were performed on replicates of 3-9 callus extracts and error terms are standard deviations (data is not available for time = 2 days). Catalase and peroxidase assays were performed as described previously (BENSON & al. 1992), glutathione reductase was measured using the method of GOLDBERG & SPOONER 1983. (b) Variation in embryogenic capabilities of 13 different clonal callus cultures of *D.carota*: cultures B26, D3, E6 are from cultivar (cv) Autumn King, cultures B45 and B57 are from cv Golden King, cultures C2 D2, E1,2 and 4 are from cv Early Nantes, D6 and E7 are from cv Chanteney Red Core and D5 from cv Saint Valery. Culture ages comprise 15 months (E1, E2, E4, E6, E7); 19 months (B45, B26, B57); 20 months (D2,D3,D5 D6) and 32 months (C2). Morphogenetic evaluations (as % colonies exhibiting developmental progressions) were performed 28 days after transfer to hormone free embryo-induction medium. A total of 10 different callus colonies were evaluated for each cv (c) Progression of somatic embryogenesis of culture E4 (Early Nantes) evaluated during a 28 day embryo-induction cycle following transfer to hormone free medium. Progression in embryo development is characterised: as globular and heart morphology (early stages) torpedo [torp] (mid stage) and advanced [adv.] torpedo and plant (late stage). Carrot cultures were maintained and manipulated as described by ROBERTSON & al. 1995 and ZIMMERMAN 1993.

antioxidant status of plant cultures, particularly those which are dedifferentiated may be related to totipotent capacity (Table 1, Fig. 1). Manipulation of the oxidative status of plant cultures may provide a useful means of maintaining morphogenetic competence or overcoming recalcitrance and this may best be explored in relation to the application of exogenous hormones and cell signalling pathways (GUNSE & ELSTNER 1992).

Table 1.Summary of experimental evidence implicating the involvement of free radical and antioxidant mechanisms in plant culture responses.

Species/culture system	Evidence	Reference
Helianthus annuus protoplasts	generation of ethane negatively correlated with protoplast division	BIEDINGER & SCHNABEL 1991
Cereal protoplasts	accumulation of peroxidation products and a decrease in antioxidants correlated with protoplast recalcitrance	CUTLER & al. 1989
Vitis vinifera callogenesis	catalase, SH, TBARS and fluorescent peroxidation products increased at onset of dedifferentiation, SOD decreased	BENSON & ROUBELAKIS-ANGELAKIS 1994
Vitis vinifera micropropagation	fluorescent peroxidation products increased during micropropagation, SOD activity did not change	BENSON & ROUBELAKIS-ANGELAKIS 1992
Oryza sativa cell competence	lipid peroxidation higher in cell lines which had lost or were losing embryogenic competence, differences in SOD activity between lines not observed, peroxidase and catalase activity higher in competent cells	BENSON & al.1992
Solanum tuberosum callus induction	EPR spectroscopy signals assigned to enhanced free radical activity at onset of callogenesis	BAILEY & al. 1994
Daucus carota callus proliferation, somatic embryo induction	Detection of TBARS, malondialdehyde, hydroxyalkenals in proliferating callus, enhancement of HNE levels during early somatic embryo induction	ROBERTSON & al. 1995
Beta vulgaris habituated cells	Hypothesis for in vitro habituation proposed: cells undergo a hyperantioxidant scavenging which reduces pro-oxidant activity	HAGEGE 1996

Future studies and cautionary points

Substantial correlative evidence supports the premise that free radical mechanisms have a role in in vitro plant development. Future studies must utilise stringent experimental strategies and employ more discerning methods for the analysis of free radical products. Meaningful investigations can only be carried out on well characterised cultures and parallel analyses of pro- and antioxidants must be performed. Oxidative status and morphogenetic capacity may be interrelated and both may be influenced by subculture cycle, culture age and genotype (Fig.1). We strongly caution the use of tissue cultures as "models" for the study of stress

responses without prior knowledge of the culture's developmental history. Habituation, culture age and competence greatly influence the oxidative status of cultures and may confound experimental interpretations. Finally, the application of transformation technologies offers immense potential for the study of antioxidant systems in plants (BADIANI & al. 1996). However, if transgenic in vitro systems are to be utilised it may be useful to be aware of the interactions between culture competence, age and oxidative status.

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