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The Role of Cellular Antioxidants (Glutathione and Ascorbic Acid) in the Growth and Development of Wild Carrot Suspension Cultures

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THE ROLE OF CELLULAR ANTIOXIDANTS (GLUTATHIONE AND ASCORBIC ACID) IN THE GROWTH AND DEVELOPMENT OF WILD CARROT SUSPENSION CULTURES

A thesis submitted by

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SUMMARY

In this study, the hypothesis that antioxidant levels are important in determining whether cells differentiate or divide proliferatively was tested in wild carrot suspension cultures. This culture system served as a good test system because proliferative growth could be separated from development. The antioxidant, glutathione, was the primary focus; however, ascorbic acid and vitamin E were used to verify the antioxidant function of glutathione.

Determinations of endogenous glutathione (GSH), glutathione disulfide (GSSG), ascorbic acid (AA) and dehydroascorbic acid (DHA) in proliferating and developing wild carrot cultures showed that lower levels of GSH and AA were associated with developing cultures. The GSSG and DHA levels did not account for the changes in the levels of antioxidants between proliferating and developing cultures.

The GSH concentrations in developing cultures were changed by adding buthionine sulfoximine (BSO, a glutathione biosynthesis inhibitor) and GSH. Addition of BSO lowered GSH levels and significantly enhanced embryogenesis, whereas addition of GSH increased endogenous GSH levels and significantly inhibited embryogenesis. Addition of AA and vitamin E to developing cultures also inhibited embryogenesis. These results indicate the importance of the GSH concentration to wild carrot somatic embryogenesis and the antioxidant function of GSH.

Studies were designed to test an observed auxin (2,4-dichlorophenoxyacetic acid, 2,4-D)-antioxidant association in wild carrot cultures. Two fractions (embryo and less developed) were obtained by screening developed cultures which originated from cultures grown in the presence of ^{14}C-2,4-D. The embryo fraction had a lower concentration of ^{14}C than the less developed fraction, supporting the association, since the two fractions showed a similar relationship

with respect to GSH and AA concentrations. Determinations of GSH and AA levels of cells grown in various concentrations of 2,4-D showed the association, decreases in the 2,4-D concentration correlated with decreases in GSH and AA concentrations.

The existence of a respiratory pathway involving GSSG reductase, DHA reductase, and AA oxidase was investigated to test whether inhibition of AA oxidase by 2,4-D could explain the auxin-antioxidant association. The reductases were active and in line with the antioxidant hypothesis, high GSSG reductase activity was observed in the proliferative cultures. AA oxidase activity could not be detected, indicating that this pathway does not offer an explanation for the observed association.

INTRODUCTION

The capability to clone trees with desirable characteristics and reproduce them in large, cost effective numbers using tissue culture methods is of major interest to the pulp and paper industry. At The Institute of Paper Chemistry (IPC) research has been directed at inducing somatic embryogenesis in suspension cultures of conifers, principally loblolly pine, and thus far the attempts have not been successful.

One approach to obtaining somatic embryos in conifers is to understand a plant system which readily undergoes somatic embryogenesis and then apply the knowledge gained to solve the problems associated with conifer suspension cultures. This approach has been used at IPC, and this thesis represents a contribution to the understanding of wild carrot somatic embryogenesis with the intention that the knowledge gained will be applied to the conifer problem.

Several studies in plant and animal systems indicate that cellular antioxidants act to determine whether a cell population grows proliferatively or differentiates and develops.[1-3] Glutathione (GSH) and ascorbic acid (AA) have been shown to function as cellular antioxidants because they are capable of inhibiting the oxidation of other substrates. Through this action, it has also been suggested that these compounds act to regulate the cellular redox potential.[4-7] Along this line, Barron[8] proposed that in the reduced state antioxidants act as poisoning agents against oxidation.

Large amounts of GSH and AA have been associated with young undifferentiated tissue when compared to older developed tissue.[9,10] As a result, young undifferentiated tissue has been characterized as being in a more reduced state.

These studies uphold a hypothesis which proposes that cell growth and development is affected by antioxidant levels and that lower levels create a more oxidizing environment which is a prerequisite for development.[1] Included in this hypothesis is the idea that antioxidant levels are controlled by changes in their reduced/oxidized ratios.[1]

In this thesis this antioxidant hypothesis is tested by investigating the role of antioxidants in proliferating and developing wild carrot suspension cultures. This culture system served as a good test system because proliferative growth could be separated from development. GSH was the primary focus; however, AA and other antioxidants were used to test the GSH results and verify its antioxidant function. Glutathione disulfide (GSSG) and dehydroascorbic acid (DHA) were also included in the investigation to test the portion of the hypothesis concerned with changes in the reduced/oxidized ratios.

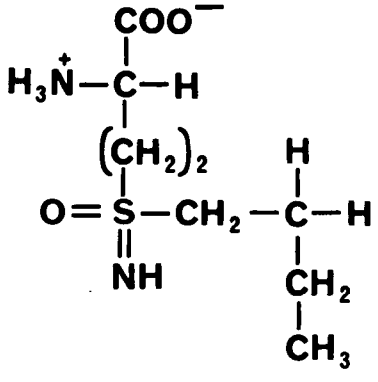
In plant studies, higher concentrations of auxin have also been associated with the reduced state.[10-13] An auxin-antioxidant interaction has been proposed to act synergistically to protect the reduced state.[10,11] A mechanism involving GSH, GSSG, AA and DHA in a respiratory pathway has been put forward previously as an explanation for the proposed auxin-antioxidant synergism. The association between higher auxin concentrations and higher antioxidant concentrations was further investigated in this thesis. In addition, the presence of the respiratory pathway, discussed above, also was investigated in wild carrot suspension cultures.

LITERATURE REVIEW

GLUTATHIONE METABOLISM

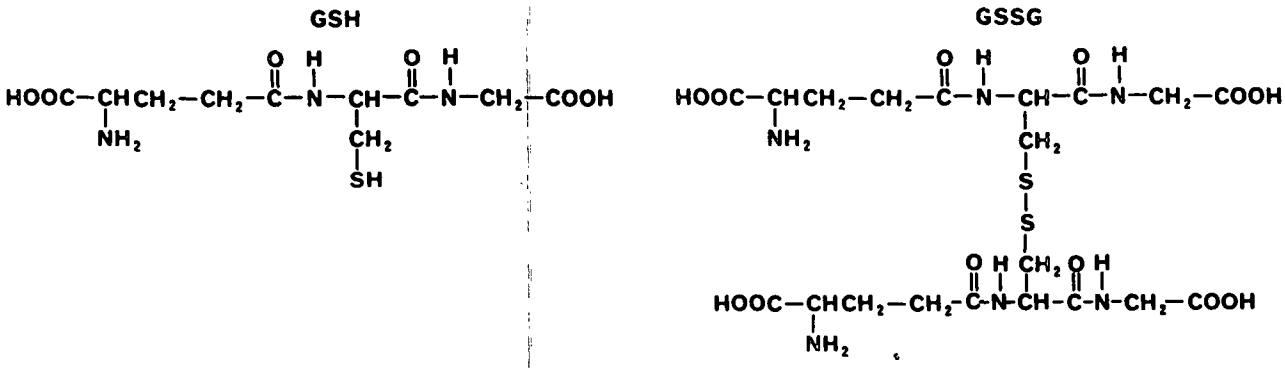
The tripeptide, glutathione, is thought to be a constituent of all living cells in the plant and animal kingdoms.[15] The majority of the research on glutathione metabolism has been done in animal systems; however, the limited research in plants indicates that at least GSH biosynthesis occurs in a similar manner.[14]

Glutathione exists in the reduced state as the thiol (GSH) or the oxidized state as the disulfide (GSSG). It is composed of the amino acids, glycine, cysteine and glutamic acid. It is synthesized as GSH by a two step process in animals and bacteria.[17] In the first step, γ-L-glutamyl-L-cysteine is synthesized by γ-glutamylcysteine synthetase. In the second step, glycine is added to yield glutathione and this reaction is catalyzed by glutathione synthetase. In plants, the dipeptide γ-L-glutamyl-L-cysteine or the corresponding disulfide has been isolated in wheat germ and garlic.[18] The existence of γ-glutamylcysteine synthetase has been indicated in bean seeds (Phaseolus vulgaris).[19] Little is known about the enzyme glutathione synthetase in plants, although the enzyme has been demonstrated in homogenates of tobacco cells.[20]



Chemical structures of glutathione and glutathione disulfide

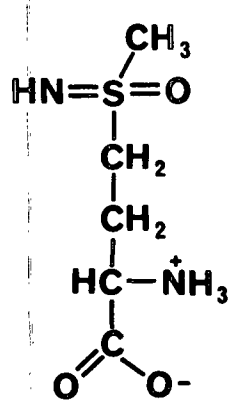
L-methionine-S-sulfoximine (MSO) inhibits glutathione biosynthesis in plants[21] and animals.[22] Other studies in plant and animal systems have shown that the effects of MSO are not specific and it also inhibits glutamine synthetase.[16,23]



Chemical structure of methionine sulfoximine

Studies with MSO in tobacco suspension cells revealed that exogenous GSH is taken up by the cells as a whole molecule.[21] In animals, GSH is taken up only after degradation to its corresponding amino acids.[22]

Buthionine sulfoximine (BSO) is another inhibitor of γ-glutamylcysteine synthetase in animals,[24] and a brief report suggests a similar function in plants.[25] BSO is believed to be a more specific inhibitor since it has no effect on glutamine synthetase.[26] The structure of BSO is shown below and the S-alkyl moiety of the sulfoximine is believed to bind to the active site of the enzyme.[24]



Chemical structure of buthionine sulfoximine

The mechanism of glutathione degradation has been investigated in tobacco suspension cultures supplied with glutathione as the sole sulfur source.[26] When tobacco suspensions, precultured under sulfur starvation conditions, were exposed to glutathione specifically labeled in the γ-glutamyl moiety, a large amount of the radioactivity inside the cells was found in 5-oxo-proline as well as in glutamate.[27] When glutamic acid labeled glutathione was added to tobacco cell homogenates, 5-oxo-proline was detected again. These observations indicate that the γ-glutamyl moiety of glutathione is cyclized to 5-oxo-proline, which is then converted to glutamate. Figure 1 shows the degradation mechanism established for animals.[20] The participation of γ-glutamyl transpeptidase in the degradation mechanism in plants has not been established and, as a result, a second degradation mechanism for plants has been proposed (Fig. 2).[20]

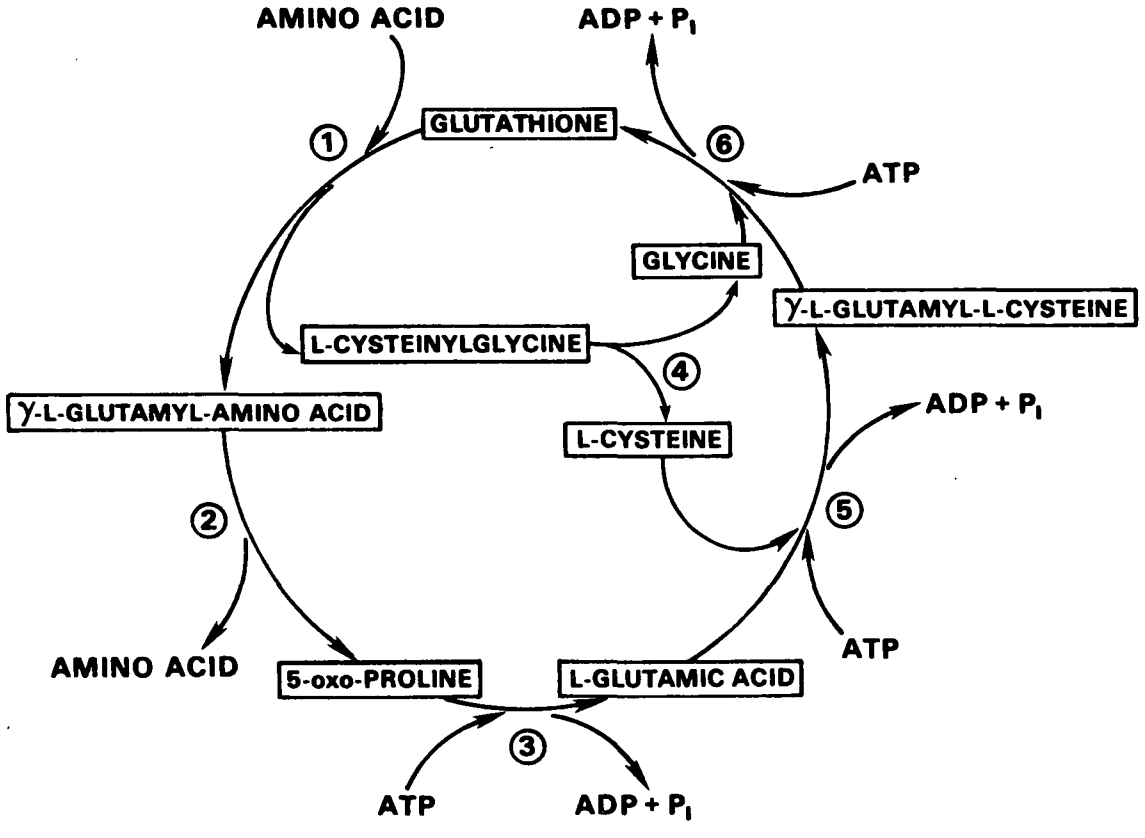


Figure 1. Synthesis and degradation of glutathione in the γ-glutamyl-cycle established for animals. 1. γ-glutamyl transpeptidase; 2. glutamyl cyclotransferase; 3. 5-oxo-prolinase; 4. dipeptidase; 5. γ-glutamyl-cysteine synthetase; 6. glutathione synthetase.

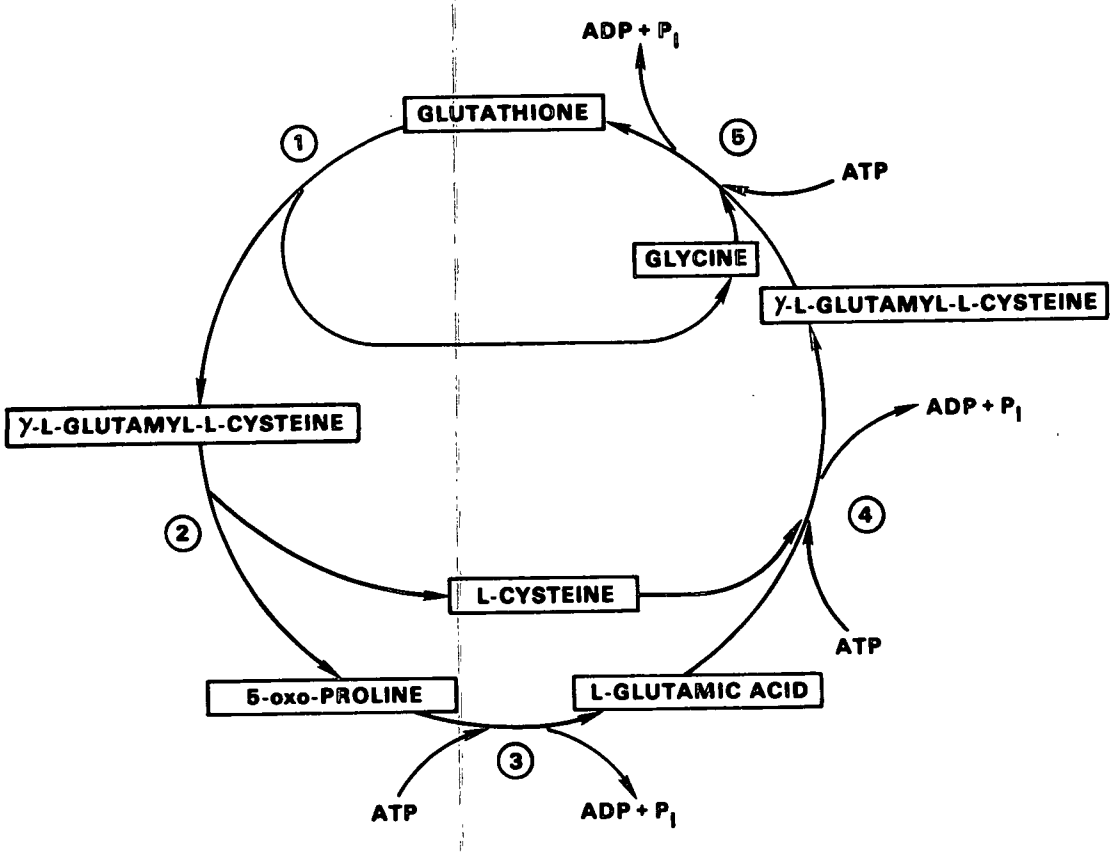
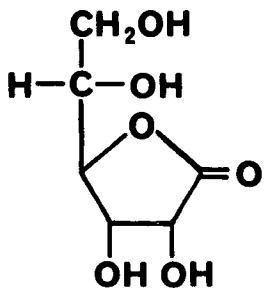


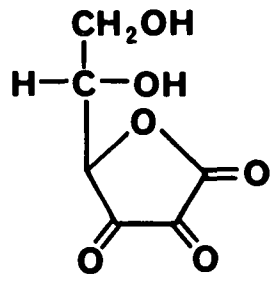
Figure 2. Synthesis and degradation of glutathione proposed for plants. 1. Carboxypeptidase; 2. γ-glutamyl cyclotransferase; 3. 5-oxo-prolinase; 4. γ-glutamylcysteine synthetase; 5. glutathione synthetase.

ASCORBIC ACID METABOLISM

Ascorbic acid (AA) is regarded as a normal constituent of higher plants and animals.[28] L-ascorbic acid is naturally occurring and is readily oxidized to dehydroascorbic acid (DHA); however, the reduced form is predominant under conditions of plant growth.[28] Elements of AA structure include a hexose, a L-1,4-lactone, and a hydroxymethyl group terminal to the side chain. The precise details of AA biosynthesis are not well understood,[29] and several pathways have been discussed in seedlings, fully grown leaves and wounded plant tissue from various plants.[30]



ascorbic acid (AA)



dehydroascorbic acid (DHA)

Chemical structures of ascorbic acid and dehydroascorbic acid

The degradation of AA is believed to occur through oxidation and ring cleavage.[28] AA oxidation occurs enzymatically and through reactions with oxidizing species. When AA is oxidized to DHA, the latter rapidly hydrolyzes to form threo-2,3-hexodiulosonic acid. Upon oxidation AA can be recycled by DHA reductase which catalyzes the following reaction:[31]

$$2GSH + DHA \xrightleftharpoons{\text{DHA Reductase}} AA + GSSG$$

The present knowledge of the processes which break down the carbon chain is meager, and most of the information is derived from studies with radioactive tracers.[32-33]

ANTIOXIDANTS

A cellular antioxidant can be defined as any electron donor which inhibits the oxidation of suitable labile substrates.[1] Antioxidants protect cellular components against attack by oxidants, and one type of nonspecific protection entails a general shift of the oxidation-reduction equilibria to a more reduced state.[1]

The shift of the oxidation-reduction equilibria toward a more reduced state has been associated with proliferatively growing plant tissue, and a more oxidized state has been associated with plant cell differentiation.[1] An extensive

discussion of the potential interrelationships among antioxidants, oxidants, and differentiation has been presented, and it has been proposed that the levels of antioxidants determine whether a cell differentiates or divides.[1,2]

The antioxidant functions of GSH and AA have received much attention in the literature. In plants, there are enzymes whose functions are to reduce the oxidized forms, GSSG and DHA, to GSH and AA, respectively.[31,34] These enzymatic mechanisms provide GSH and AA with a high antioxidant efficiency. DHA reductase has been discussed previously in this review; GSSG reductase is believed to be ubiquitous with the presence of GSH[34] and the reaction proceeds as follows:

$$GSSG + NADPH + H^+ \underset{}{\overset{\text{GSSG Reductase}}{\rightleftharpoons}} 2GSH + NADP^+$$

Evidence for the antioxidant function of GSH can be found in both plant and animal studies. When human embryonic cells were placed in a cysteine-free medium, cellular GSH levels decreased rapidly and cell death occurred after 18 hours.[4] Addition of the antioxidant, vitamin E, and other synthetic antioxidants extended cell life from 18 hours to 6 days even though the GSH levels were less than 1% of the normal levels.

Research on plants has localized some of GSH's antioxidant function in chloroplasts.[5] In one case, cotton leaves grown in a high oxygen environment were found to have elevated GSSG reductase levels as compared to a control grown in air. The authors proposed that the elevated enzyme level was a result of a greater antioxidant action of GSH, necessitated by the high oxygen environment.

A role for GSH in spindle formation during cell division in sea urchin eggs was proposed.[35] Another plant study supported that proposal by showing that the

addition of maleic hydrazide inhibited cell division.[36] The proposed reason for this effect was the ability of maleic hydrazide to react with protein SH groups, thus inhibiting the reduction of protein disulfide groups by GSH during mitosis.

GSH's strongly negative reduction potential allows it to reduce disulfide groups present in proteins and maintain them in a reduced state.[37] Barron first suggested that some enzymes require sulfhydryl groups for their activity and might be protected from inactivation by GSH.[8] Data obtained from rat liver homogenates supported Barron's view by showing that GSH prevented oxidation of protein SH groups by oxygen.[38]

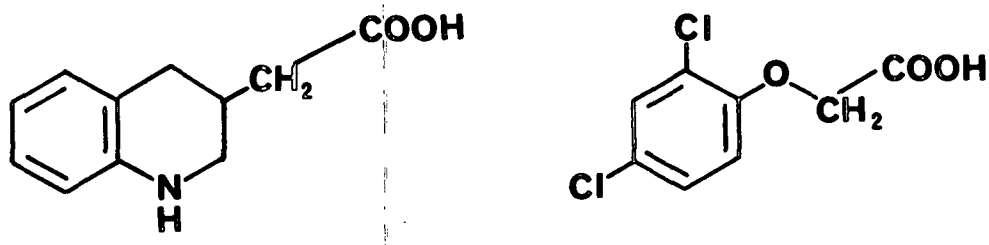
The antioxidant functions of both GSH and AA were indicated in experiments with excised leaves from rice plants.[6] Addition of hydrogen peroxide to the excised leaves accelerated senescence. When the leaves were presoaked in either an AA or a GSH solution, senescence was significantly retarded when the leaves were treated with hydrogen peroxide.

Other examples demonstrating the antioxidant function of AA can be found in animal and plant studies.[7,39] It is believed to play an important role in protecting plant tissue from singlet oxygen and peroxides.[39] Although AA has a more positive reduction potential than GSH, it is also capable of protecting protein SH groups.[40]

ANTIOXIDANTS: RELATIONSHIPS WITH PLANT AND CELL GROWTH

Glutathione's and ascorbic acid's roles in plant growth were thought to be mediated through their effects on auxin metabolism.[12] In experiments with pea segments, addition of indole 3-acetic acid (IAA) or 2,4-D induced proliferative growth and increased the GSH concentration, while measurement of GSSG showed a

corresponding decrease.[12] From these results it was proposed that auxins exert their growth effect by increasing the GSH/GSSH ratio, thereby affecting the redox state of the plant tissue.



Chemical structures of indole acetic acid (left), and 2,4-dichlorophenoxyacetic acid (right).

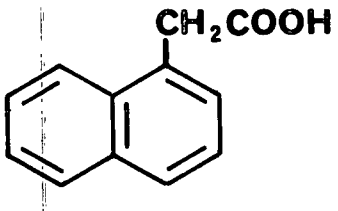
Also, in this article the relationships among GSH, GSSG, AA, DHA, and their effects on growth were discussed. Addition of AA to the pea segments inhibited growth and decreased the GSH/GSSG ratio. Upon further investigation, it was found that the exogenous AA was being oxidized to DHA, which was inhibiting growth. Other experiments showed that auxin inhibited AA oxidation, and as a result, auxin inhibition of AA oxidase was proposed to account for the effects of the antioxidants on growth.[12] This proposal received support from the finding that IAA also inhibited AA oxidase in lettuce seeds; however, the author argued that this effect could not be the total basis for IAA's effect on growth.[41]

A similar study was conducted with soybean tissue.[10] Treatment of mature soybean tissue with 2,4-D caused proliferative growth and elevated the levels of AA and total sulfhydryls. The authors proposed that the effects of auxin on growth are caused by its effects on the oxidation-reduction state in which

growth promotion is characterized by a more reduced state and growth inhibition is characterized by a more oxidized state. More recent work by this author led to the withdrawal of the above proposal.[42] In that study the enhancement of elongation in four different excised plant tissues by auxin was found not to be associated with a shift of AA to a more reduced state. These results indicated that no close association existed between the effect of auxins on growth and the reduction state of AA and sulfhydryl systems.[42] This conclusion was reached even in view of the fact that no data concerning the sulfhydryl systems was ever presented.

More evidence exists which supports an association between a more reduced state and proliferative growth. Pilet found that carrot callus had higher GSH levels than the differentiated tissue which served as the callus source.[9] In other work with Lens root, Pilet showed that GSH levels were observed to be highest in the meristematic tissue and lowest in the more mature tissue.[11] This concentration gradient of GSH from young to mature tissue was found to be paralleled by the IAA concentration in the plant.[11]

Support for Pilet was given by experiments with bean (Phaseolus radius).[13,14] Additions of IAA and a synthetic auxin, naphthaleneacetic acid (NAA), were found to significantly enhance root growth. This was accompanied by an increase in the total sulfhydryl levels and a decrease in peroxidase activity.[14] These results support the proposal that auxin induced growth is characterized by a more reduced state by showing the rise in sulfhydryls and the decline in the oxidizing activity of peroxidase. A more recent paper has indicated that AA blocks IAA peroxidase oxidation in vitro, strongly supporting its role as an auxin protector.[43]



Chemical structure of naphthaleneacetic acid

In experiments using NADH as an antioxidant it was observed that NADH inhibited peroxidase-catalyzed oxidation of IAA until most of the NADH was oxidized.[2] This result, combined with similar results using phenolics as antioxidants[44,45] led to the hypothesis that large quantities of antioxidants in a living system inhibit peroxidase catalyzed oxidation and serve as cellular poisers which may prove important in determining whether a cell differentiates or divides.[2]

ANTIOXIDANTS: DEVELOPMENT AND DIFFERENTIATION

An association between cellular antioxidants and differentiation was first proposed by Van Fleet.[46] In this classic paper three stages of differentiation were marked by changes in phenol/quinone ratios. The first stage he defined as active development, characterized by active cell division, when quinones were maintained in a reduced state by antioxidants. The second stage which showed differentiation was detected by the appearance of quinones. The third stage was characterized by quinone oxidation and polymerization, and all growth processes were observed to stop.

A more recent study evaluated the changes in concentration of nucleic acids, proteins, SH-groups and AA during embryogenesis of Limnophyton.[47] Using histochemical methods, it was found that all of these metabolites studied peaked at the globular embryo stage, and further development was marked by decreases in all of these metabolites. Another study, in which the GSH levels in proliferating

and developing wild carrot cultures were determined, showed that lower GSH levels were associated with development.[3] In this system proliferative growth occurred when 2,4-D was present, and development occurred when 2,4-D was absent from the medium.

In other studies the effects of antioxidants on cell differentiation have been investigated. The ability of N-methylformamide and N,N-dimethylformamide to induce differentiation in malignant animal cell lines was directly related to a decrease in endogenous GSH concentration.[48] Addition to GSH blocked differentiation in the N-methylformamide and N,N-dimethylformamide treated cells. In another animal study addition of phenolic antioxidants and water soluble vitamin E inhibited differentiation of mouse myeloid leukemia cells.[49]

Antioxidants (i.e., phenolics and AA) were analyzed before and during cotton fiber differentiation in young ovules.[50,51] The highest levels of these antioxidants were observed on the day of anthesis and showed a steady decline thereafter. This decrease in the antioxidants' levels correlated with the time of fiber differentiation. These results suggest that a shift in redox balance from reducing to oxidizing (which was accompanied by auxin destruction) may result in the initiation of fiber differentiation.

Patel *et al.* investigated the cytochemical events associated with formation of multiple shoots on cultured embryonic explants of *Pinus coulteri*.[57] In this system it was observed that peroxidase activity was most intense in the differentiating vascular strands. This result is in agreement with another study in which an increase in peroxidase activity was observed during the process of shoot differentiation in tobacco.[53] Patel also reported a localization of reducing substances, chiefly AA, in the surface meristemoids of the callus.

ASCORBIC ACID AND GLUTATHIONE INTERACTIONS

An interaction between glutathione and ascorbic acid was first postulated by Szent-Gyorgyi[54] whereby these two compounds were proposed to act as respiratory carriers. The reactions of this proposed respiratory pathway as proposed later by Mapson are shown in Fig. 3.[55]

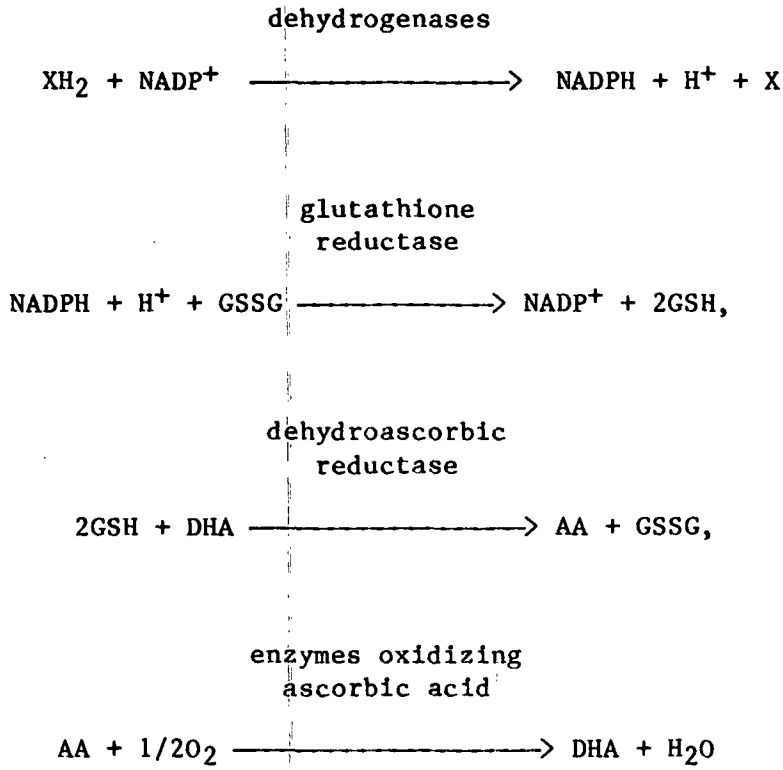


Figure 3. Respiration pathway proposed by Mapson et al.

Mapson et al. found that 25% of the total respiration in pea cotyledons passed over this GSH-AA system.[55] This conclusion was based upon the assumption that the oxygen consumption of cotyledon extracts was a reliable index of the respiration in vivo. The same procedures were also used with detached embryos; however, a lack of correspondence between the respiration of the embryo extracts and the intact tissue did not allow for a conclusion.[55]

Another study of the same nature as that described above was carried out on pea internode segments.[56] This study also indicated the existence of the GSH-AA system, but the system was estimated to be responsible for 20% of the total respiration, although some doubt was cast, even for this amount. The cellular location of the enzyme, AA oxidase, has also been disputed[57,58] although it is now believed to be present in many different areas of the cell.[59] AA oxidase has been shown not to be present in all plant systems investigated[60] and for this reason, the importance of this respiratory pathway to cellular processes is questionable.

MATERIALS AND METHODS

WILD CARROT CULTURE CONDITIONS

Wild carrot cultures were grown in the dark at 23°C. For the experiments involving chemical treatments, the cells were grown in roller tubes which were placed on roller drums and spun at 30 rpm. For all other experiments the cultures were grown in Erlenmeyer flasks of various sizes on oscillating or gyratory shakers. The basal medium was wild carrot medium[61] modified to contain 1.25 mM KH_2PO_4 and this medium will be designated as WCM-1. Cells were maintained in WCM-1 + 0.5 mg/L 2,4-D and were subcultured into fresh medium every two weeks at a 1:20 ratio. For somatic embryogenesis experiments, the standard launch protocol was to screen the cultures to yield cell aggregates of specific sizes (63 to 279 µm). These cells were inoculated into fresh medium lacking 2,4-D to yield a density of 0.5 µL of packed cells/mL of WCM-1. Proliferative growth was initiated by adding 0.5 mg/L 2,4-D to the medium.

CELL SAMPLE PREPARATION

In experiments which required analysis of a cellular compound, the cell samples were prepared in the following manner. Flasks or roller tubes containing the cell suspensions were removed from their respective mixing devices. The cells were collected by vacuum filtration and weighed to obtain a fresh weight. One hundred to 200 mg of cells were extracted in a ground glass homogenizer with 0.75 mL of 1.67M HPO_3 and 2.41M glacial acetic acid (HPO_3/HAOC) for 60 seconds. The resulting cell extract was centrifuged for 15 minutes at 17000 x g and filtered through an absorbent filter pad (Millipore, cat. No. AP10 024 00) to yield a clear supernatant which was used for the GSH, GSSG, AA, and DHA assays.

GLUTATHIONE AND GLUTATHIONE DISULFIDE ASSAYS

GSH and GSSG were assayed by determining the change in absorbance at 412 nm in an enzymatic assay.[62,63] The concentrations were determined by comparison against a standard curve. This assay yields the total glutathione (GSH + GSSH); GSSG was determined in the same manner, except that the GSH in the sample was first derivatized with 2-vinylpyridine (Sigma) to prevent its reaction in the assay.[63] The GSH concentration was obtained then by subtraction of GSSG from GSH + GSSG.

For GSH + GSSG determinations 20 µL of the cell extract was diluted into 1.0 mL 0.01N HCl; 0.4 mL of this solution was mixed with 0.4 mL reagent 1 plus 0.32 mL reagent 2. The components of reagent 1 and reagent 2 are listed in Table 1. The resulting mixture was incubated at room temperature for 5 min. Eighty microliters of 2.5 mg/mL NADPH (Sigma) was added to the mixture to initiate the reaction. The reaction was monitored for 2 min.

Table 1. Components of reagents used for glutathione assay.

Reagent 1	Reagent 2
2610 mg $NaH_2PO_4 \cdot 2H_2O$	9.25 mg EDTA-diNA salt
1116 mg EDTA-diNa salt	5.0 mg Bovine Serum Albumin (BSA, Sigma)
80 mg BSA	1.0 mL Imidazole buffer (Sigma) pH 7.2
24 mg 5,5-Dithio BIS (2-Nitrobenzoic acid) (Sigma)	30.0 I.U. GSH reductase (Sigma)
All in 200 mL H_2O	All in 20 mL H_2O

For the GSSG determinations 1.0 µL of 2-vinylpyridine per 100 µL of 0.1N HCl solution was found to be sufficient for full derivatization of the GSH in the

sample. The 2-vinylpyridine was added to the 0.01N HCl solution and mixed for 1 min; full derivatization occurred after 40 min at room temperature.

Internal standards run for these assays indicated that recovery was greater than 90%. A standard curve used for the glutathione determination is shown in Fig. 4. The standards used were GSH (Chemalog), GSSG (Chemalog) and were dissolved in the HPO_3/HOAC extracting solution; both standards were run with and without 2-vinylpyridine.

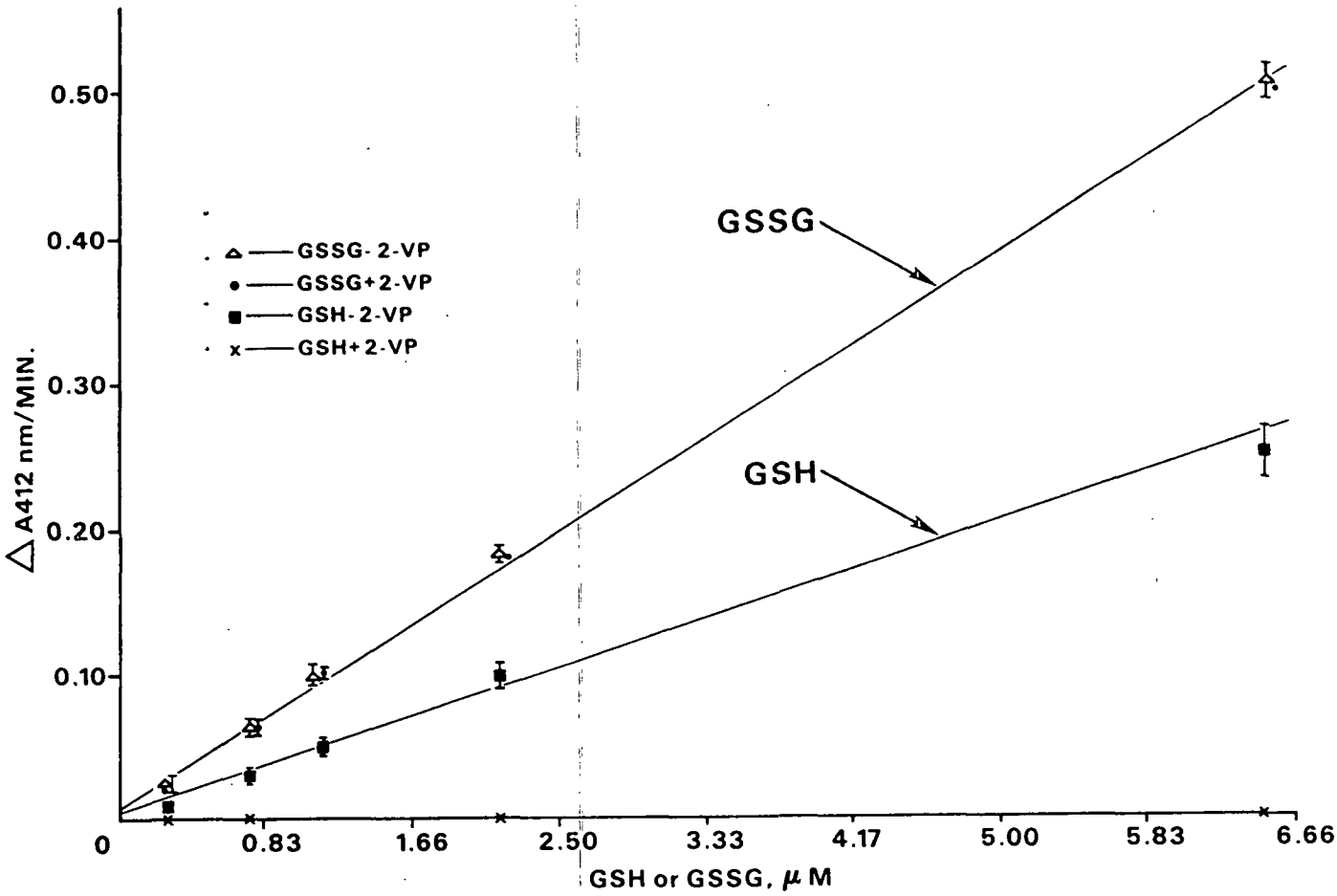
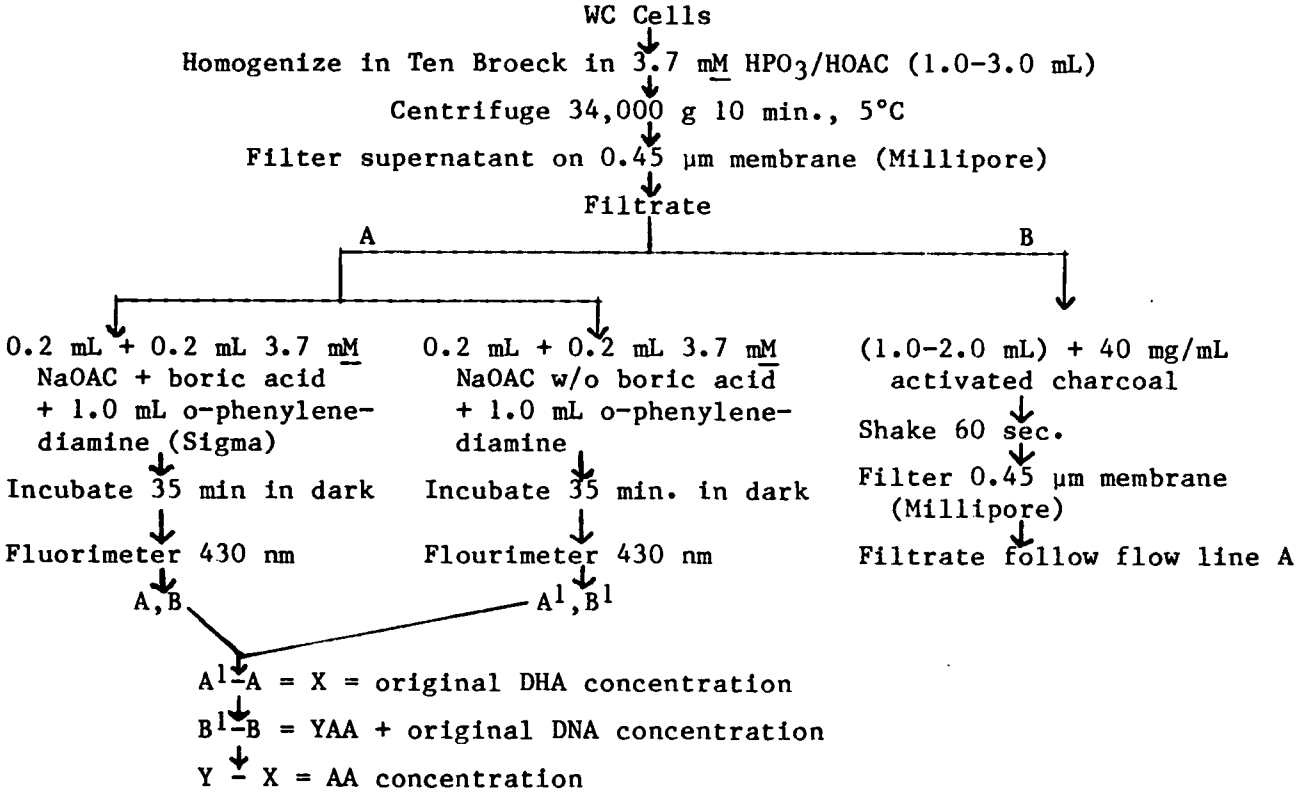


Figure 4. GSH and GSSG standard curves, with and without 2-vinylpyridine.

ASCORBIC ACID AND DEHYDROASCORBIC ACID ASSAYS

AA and DHA were assayed by a fluorimetric procedure,[64] and a flow chart describing the procedure is shown in Scheme I. The AA concentration was determined via route B of the scheme as DHA by first oxidizing the AA to DHA by shaking the solution with activated charcoal (40 mg activated charcoal/mL of AA solution). The AA concentration was obtained by subtracting the original concentration of DHA in the sample which was determined separately (route A), from Y (AA + original DHA concentration).



Scheme I. Flow chart of ascorbic acid assay.

Internal standards run for this assay using AA as the standard indicated complete recovery of the standard with wild carrot cell extracts. A standard curve for DHA is shown in Fig. 5.

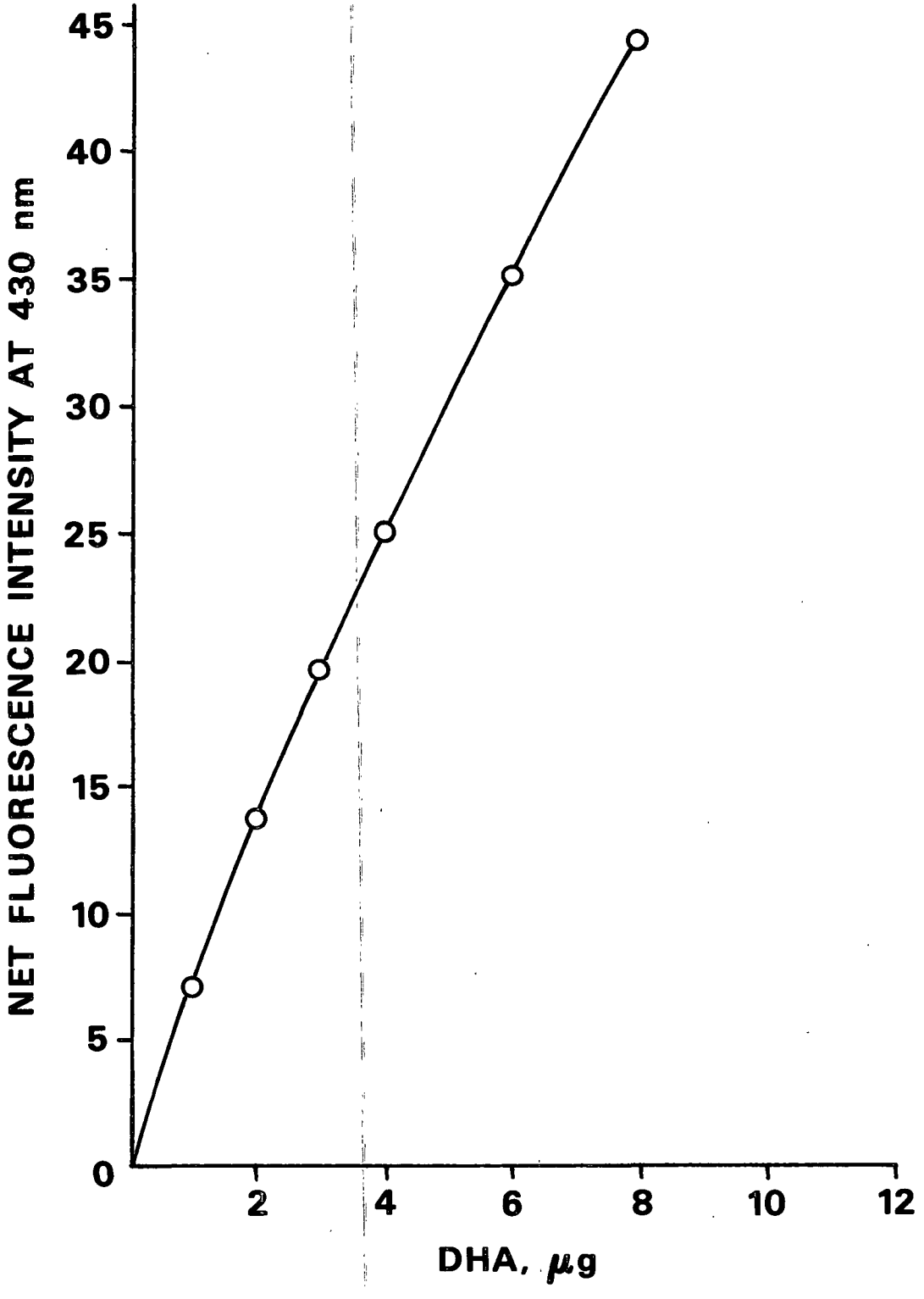


Figure 5. DHA standard curve.

GLUTATHIONE, BUTHIONINE SULFOXIMINE AND OTHER ANTIOXIDANT ADDITION EXPERIMENTS

All chemical additions were made on day 7 of a culture period for the results presented in this thesis. Before the chemicals were added to the medium they were sterilized by filtration through a 0.22 μm Millipore filter. Sterilized water was added to the untreated controls to maintain constant volumes in all tubes.

After complete embryo formation was visually observed, the embryos were harvested by vacuum filtration and fresh weights were determined. The cell populations were plated on 0.2% Noble agar (DIFCO) with 2.0% merthiolate (Lilly Co.) for embryo counting. The embryos were counted on a Quebec Colony counter and were counted if they showed both root and cotyledon development.

^{14}C-2,4-DICHLOROPHENOXYACETIC ACID STUDY

^{14}C-2,4-D (Pathfinder Laboratories), uniformly ring labeled, with a specific activity of 10.36 mCi/mmole, was added to wild carrot cultures at a level of 0.5 mg/L. The carrot cells were subcultured into ^{14}C-2,4-D at a ratio of 1 part cells/20 parts fresh medium. After a subculture period (two weeks), the carrot cells containing the ^{14}C tag were launched as was previously described under Wild Carrot Culture Conditions into medium without 2,4-D. After 15 days of growth, the embryos were collected on an 864 μm screen. Those cells passing through the screen were labeled "less developed."

Fresh weights were determined on each cell sample from the two fractions, after which the cells were placed in scintillation vials with 10 mL of Isolab Solvent Free liquid scintillation fluid. The samples were counted to ± 2% error and each fraction was run in triplicate and cpms were converted to dpms via a computer program.

ENZYME EXTRACTION

Five millimolar potassium phosphate buffer, pH 6.6, was used as the extraction solution for all enzyme assays. Polyclar AT (GAF Corp.), 1.5 mg/mg fresh weight of cells was also added to the solution to minimize enzyme inactivation by phenolic compounds. The cells were homogenized in a ground glass homogenizer for 60 seconds, centrifuged for 15 minutes at 17000 x g and then filtered through an absorbent pad (Millipore, Cat. No. AP10 024 00) to yield a clear supernatant, which was used for the enzyme assays.

Glutathione Disulfide Reductase Assay

The procedure used to assay the activity of GSSG reductase was that of Esterbauer and Grill.[65] The activity was measured by following the decrease in absorbance of NADPH at 340 nm. The sample and reference cells each contained 1.6 µmoles NADPH (Sigma), 10.5 µmoles EDTA (Baker Chemicals), 1.0% bovine serum albumin (Miles Laboratories) and 0.5 mL of the enzyme extract. The reaction was initiated by adding 10 µmoles of GSSG (Chemalog) to the sample cell. The final volumes in the sample and reference cells were 1.13 mL.

Dehydroascorbic Acid Reductase Assay

The assay conditions used were a modification of a procedure by Smits.[66] The activity was measured by following the increase in absorbance of ascorbic acid at 265 nm. The sample and reference cells each contained 25 mM DHA and 0.075 mL of enzyme extract. The reaction was initiated by adding 5 mM GSH to the sample cell. The final volumes in the sample and reference cells were 2.83 mL.

Ascorbic Acid Oxidase Assay

The assay conditions were taken from Smits[66] and the activity was measured by following the decrease in absorbance of the sample at 265 nm. The sample and

reference cells each contained 2.0 mM AA and the reaction was initiated by adding 0.2 mL of enzyme extract to the sample cell. The total volumes in the sample and reference cells were 2.85 mL.

AA oxidase from pumpkin (Boehringer Mannheim GmbH.) was used in place of the enzyme extract to test the assay conditions. It was also added to the enzyme extract to test the extract's effect on this enzyme's activity.

CELL COUNTING

Experiments comparing the two cell counting methods detailed in this section are presented in Appendix I.

Cell Dispersion

Wild carrot suspension cell clumps or developed structures were dispersed by digesting the cells in a pectinase solution for 1 hour at 35°C. The pectinase solution, which was developed at IPC[67] consisted of 2.0 mL of cells in 1% NaCl, 1.0 mL water, 1.0 mL 0.25M acetate buffer, pH 4.5, and 0.3 mL 50% pectinase (Sigma). After digestion, the cells were drawn in and out of a syringe with 18 gage (840 μm), 21 gage (515 μm), and 25 gage (260 μm) needles in this order, five times in and out for each needle size. Following this treatment the cells were observed microscopically to be separated and ready for counting.

Coulter Counting

The cell counts were determined on a model TA11 Coulter counter. The operation manual supplied the necessary details for operation.[68] A 100 μm orifice tube (Coulter Electronics Corp.) was used for all counting and 0.5 mL of sample was used per count. Between 25 μL and 100 μL of carrot cells suspension was added to 10 mL of 1% NaCl which had been previously filtered 2X through a 0.22

μm filter (Millipore) to eliminate background counts. The diluted cell solution was shaken in a Coulter cell counting vial (Thomas Scientific) and a cell count was determined. Each cell sample was run in triplicate and the counts were derived from channels 7-12 on the Coulter counter. These channels were chosen because they represent the size range of wild carrot cells determined by microscopic sizing (i.e., 7.0-20.0 μm).[67]

Manual Counting

Dispersed cells were added to a Levy hemocytometer counting chamber via capillary action. All grids except the center grid were counted and each sample was counted in triplicate. The cells were counted under a microscope using 20X magnification.

PLANT STUDIES

GSH, GSSG, AA, and DHA were determined in the following mature carrot plant sections: root tips, meristem, young leaves, young plant stems, root pieces, mature plant stems and mature leaves. The assay procedures as well as the extraction method (i.e., same as suspension cultures) have been described above, and the results are tabulated on a gram fresh weight basis, for reference purposes only, in Appendix II.

THESIS OBJECTIVES

Several studies indicate that an association exists between unorganized growth and elevated levels of GSH and/or AA and between comparatively lower levels of these compounds and organization. These associations support the hypothesis that unorganized growth occurs in a more reduced state and organization is characterized by a more oxidized state with respect to antioxidant levels.[20]

The purpose of this thesis was to further test this hypothesis by using wild carrot suspension cultures as the test system. This system is an excellent choice for this purpose because, for practical purposes, unorganized growth (proliferation) can be separated from organized growth (somatic embryogenesis). Proliferative growth occurs when the cell suspensions are grown in the presence of 2,4-D, and embryogenesis occurs when the cell suspensions are grown in the absence of 2,4-D.

To test the hypothesis the following specific objectives were defined:

1. Determine if higher GSH and AA levels are associated with proliferating cultures when compared to developing cultures.
2. Determine if any difference in the antioxidants' levels between proliferating and developing cultures results from a change in their respective reduced/oxidized ratios by determining the concentrations of the oxidized forms of GSH and AA (i.e., GSSG and DHA, respectively).
3. Manipulate the antioxidant levels in developing cultures to test if the levels are important for development.

4. Investigate a proposed association between auxin and the antioxidant levels.[10,12-14]

5. Investigate the enzymes in the proposed redox chain involving GSH, GSSG, AA, and DHA[55] and determine if these enzymes exist in wild carrots and what their activities are in proliferating and developing cultures. If the redox chain exists, does it operate to control the antioxidant levels in proliferating and developing cultures?

RESULTS AND DISCUSSION

DETERMINATION OF THE LEVELS OF GLUTATHIONE, GLUTATHIONE DISULFIDE, ASCORBIC ACID, AND DEHYDROASCORBIC ACID IN PROLIFERATING AND DEVELOPING WILD CARROT CULTURES

Endogenous GSH and GSSG levels were determined in proliferating and developing carrot cells on various days during a culture period. In all experiments the GSH concentration rose to and remained at higher levels in proliferating cultures than in developing cultures after 6 or 7 days of growth. The data in Fig. 6A and 6B are representative results from proliferating and developing cell cultures for the GSH and GSSG levels, respectively. The association of lower GSH levels with the developing cells after day 6 is consistent with the concept that development occurs in a more oxidized environment than does proliferation.

Also, it was proposed that the decrease in levels of antioxidants during differentiation is associated with a rise in the corresponding levels of oxidants.[20] Figure 6B does not support this expectation, since proliferating and developing cultures showed similar GSSG levels. Occasionally, in six repeat experiments, and on day 9 in Fig. 6B, differences in the GSSG levels between proliferating and developing cultures were observed. These differences, however, paralleled the analogous differences in the GSH levels between proliferating and developing cultures.

AA and DHA were also determined in these experiments and the data are shown in Fig. 7A and 7B, respectively. The data for AA yield a similar result as was observed for GSH (Fig. 6A), supporting the concept that development occurs in a more oxidized environment. The DHA data parallel the AA result and support the analysis done above for the GSSG data.

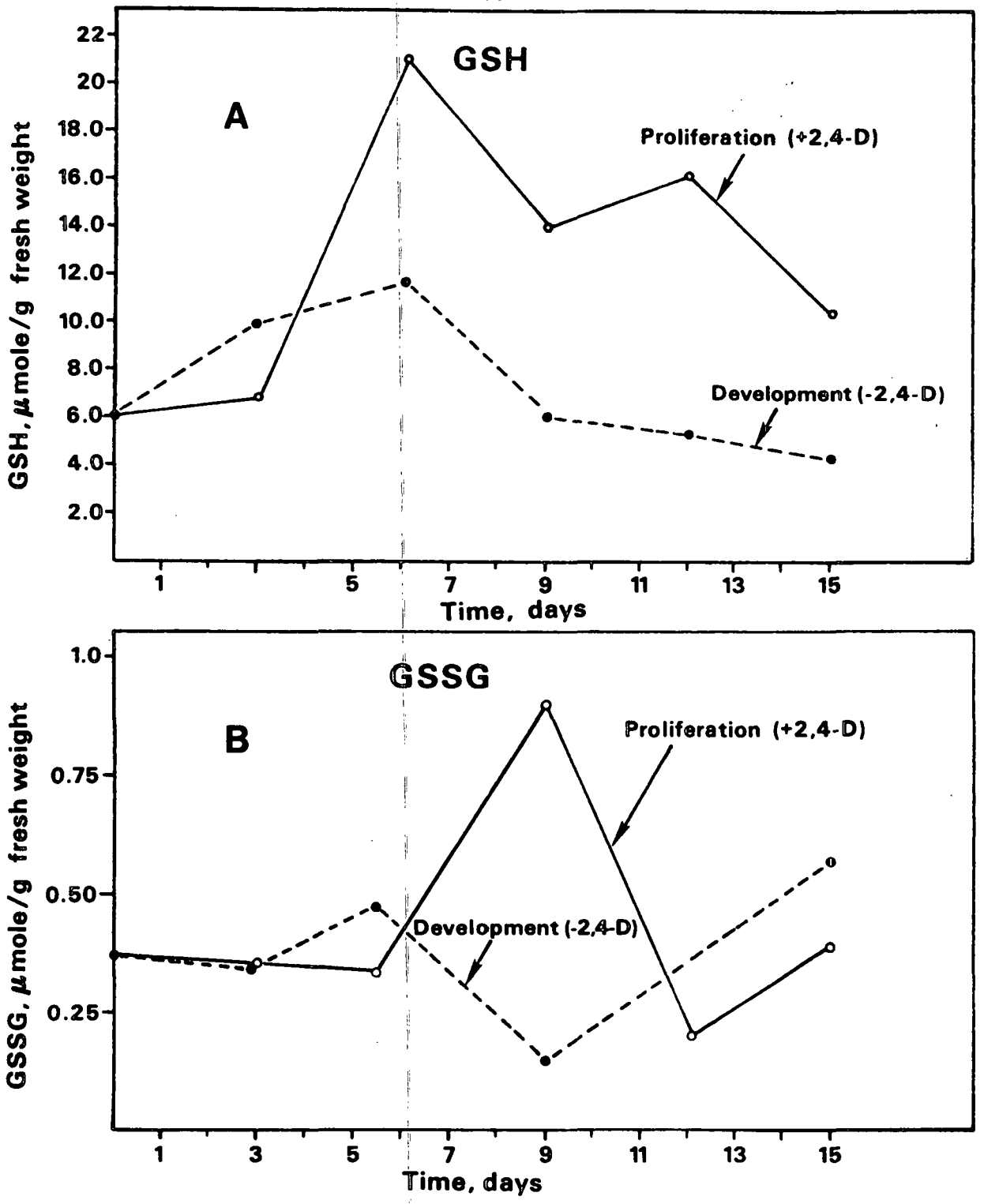


Figure 6. GSH(A) and GSSG(B) levels for proliferating and developing wild carrot cultures. The GSH values of proliferating and developing cultures are significantly different ($P < 0.05$, Duncan's New Multiple Range Test) on and after day 6. The GSSG values of proliferating and developing cultures are significantly different ($P < 0.05$) only on day 9. Each data point is a result of triplicate determinations and the experiment has been repeated six times.

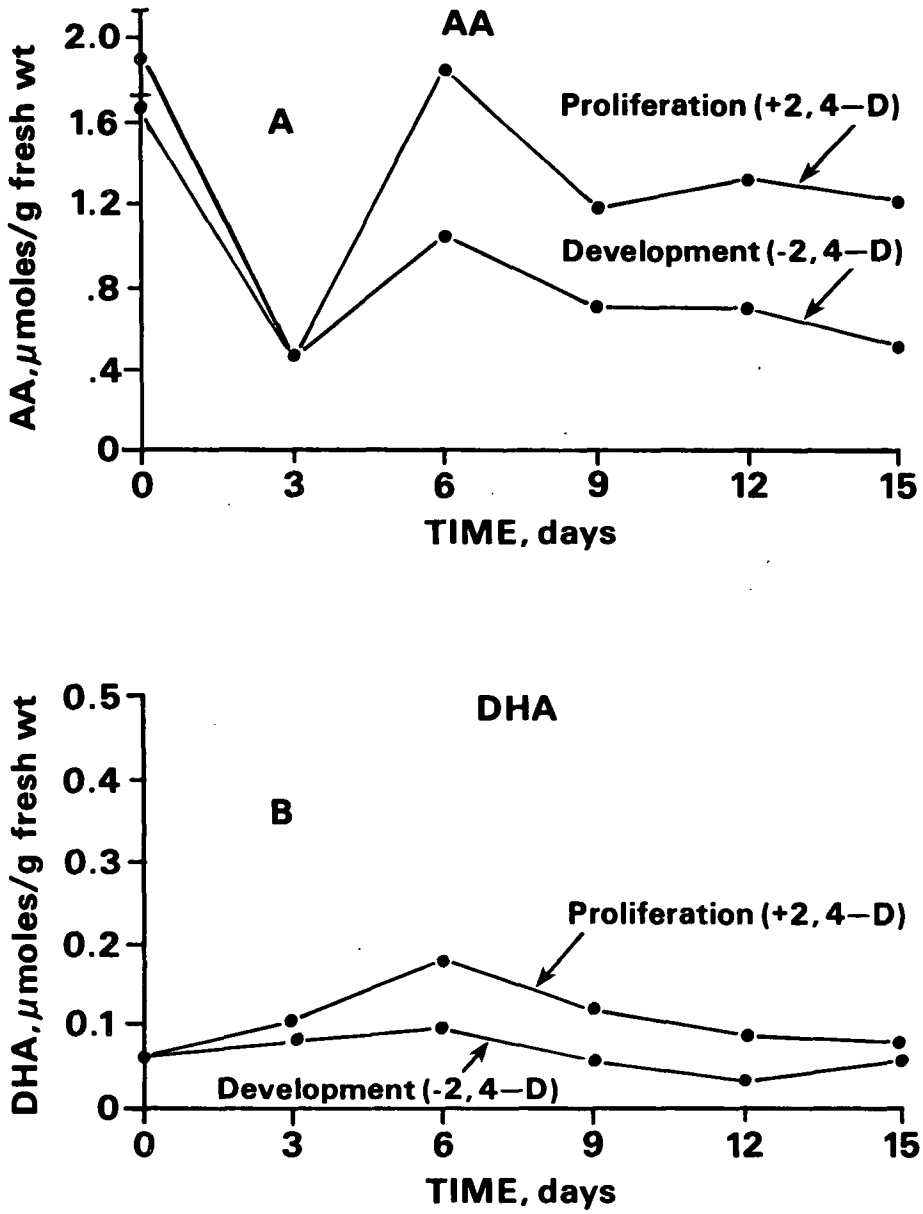


Figure 7. AA(A) and DHA(B) levels for proliferating and developing wild carrot cultures. The AA values of proliferating and developing cultures are significantly different ($P < 0.05$, Duncan's New Multiple Range Test) on and after day 6. The DHA values of proliferating and developing cultures are significantly different ($P < 0.05$) on days 6 and 12. Each data point is a result of triplicate determinations and the experiment has been repeated six times.

In developing cultures only a portion of the cell clusters form embryos. The remaining cells are either less developed or undeveloped at the end of a culture period. This observation yielded another method for investigating whether development occurs in association with lower antioxidant levels. Proliferating and developing cell suspensions were grown for 15 days or until complete embryo formation had occurred. Two fractions were obtained by screening the developed culture through an 874 μm screen. The fraction remaining on the screen was composed of embryos, while the fraction passing through the screen was composed of less developed structures plus undeveloped cell clusters. GSH, GSSG, AA, and DHA were determined in these two fractions as well as in the proliferatively grown cells.

The data for GSH and GSSG are shown in Fig. 8. Differences in the GSH levels existed among all three fractions, and the embryo fraction had the lowest level. No differences were observed in the GSSG levels among the three fractions.

The corresponding data for AA and DHA are shown in Fig. 9. The AA levels paralleled the GSH levels, and the DHA level was significantly lower in the undeveloped fraction; however, no difference in the DHA levels was observed between the embryo fraction and the proliferative cells. This result also indicated no correlation between the oxidants' levels and the degree of development.

Another experiment was done to test the antioxidant hypothesis; however, it was not replicated and the data are shown in Appendix III. In this experiment, a nonembryogenic cell line was used in which embryogenesis could be induced after restoration of competence. This cell line was labeled incompetent because development would occur only to a torpedo stage after 17 days of growth. Competency (complete embryogenesis) was restored after 3 successive subcultures in medium lacking 2,4-D. For each subculture, the cells were screened 63-279 μm and

inoculated at 0.5 µL of cells/mL of medium lacking 2,4-D. The competent culture yielded lower levels of GSH and AA throughout the culture period. These results are in line with the other results presented in this section (Fig. 6A and 7A) because the restored competent culture was composed of globular and heart shaped embryos at day 0 and normally, in a developing culture, these structures occur between days 4 and 8.

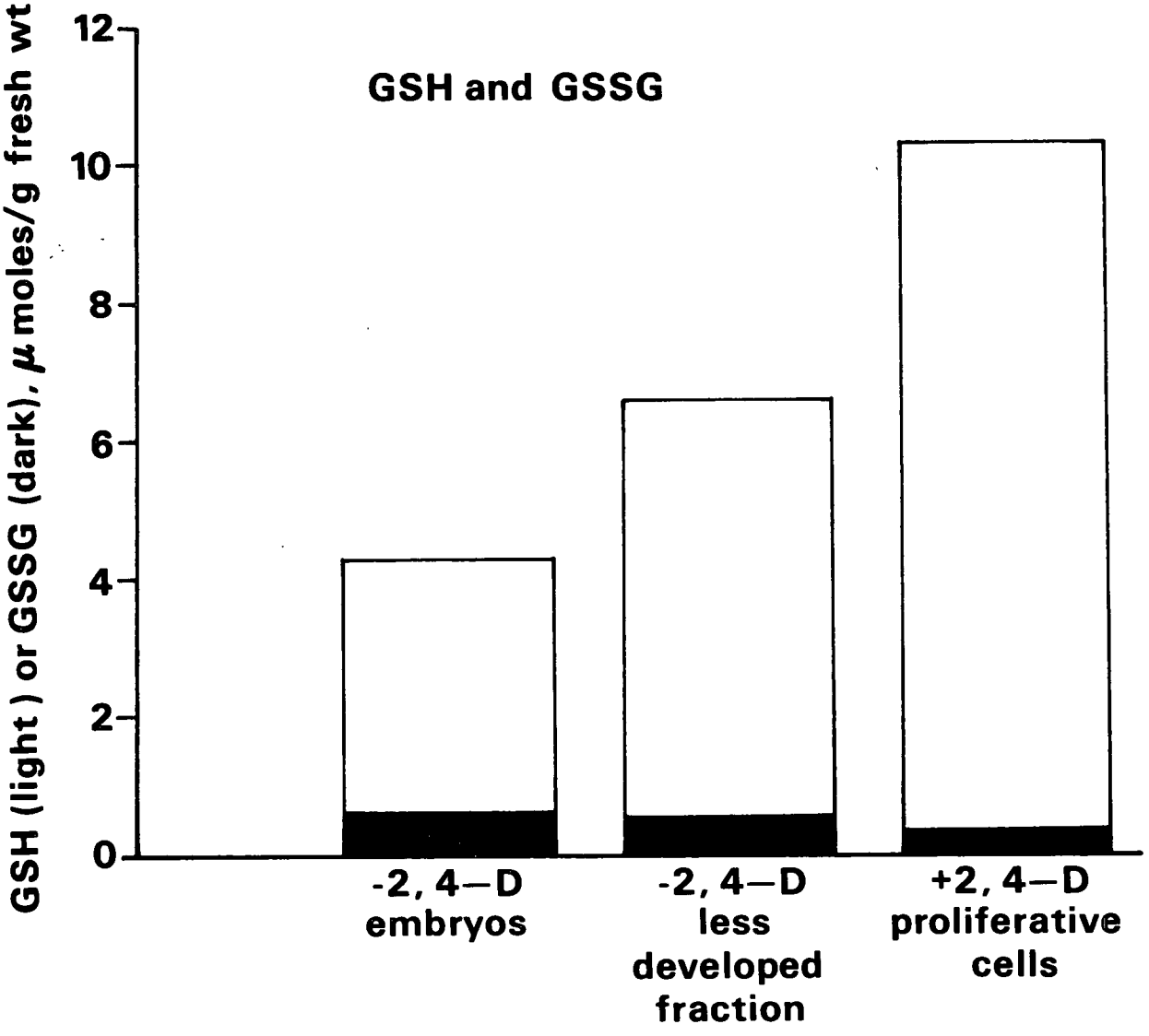


Figure 8. GSH (light) and GSSG (dark) levels of embryos, a less developed fraction and proliferative wild carrot cells determined on day 15 of a culture period. The GSH levels are significantly different ($P < 0.05$, Duncan's New Multiple Range Test) in all instances and the GSSG levels show no significant differences ($P < 0.05$). Each data point is a result of triplicate determinations, and the experiment has been repeated three times.

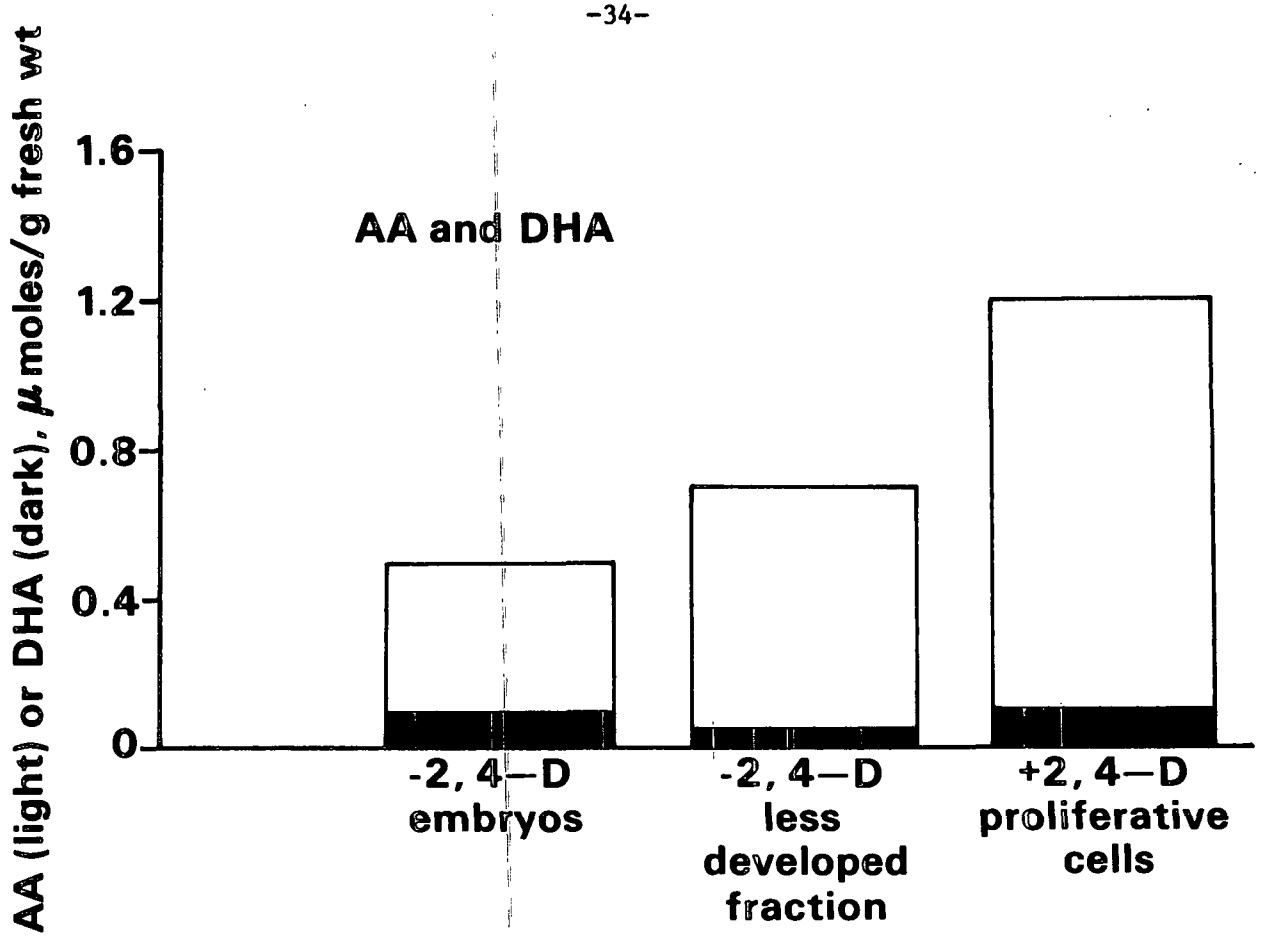


Figure 9. AA (light) and DHA (dark) levels of embryos, a less developed fraction and proliferative wild carrot cells determined on day 15 of a culture period. The AA levels are significantly different ($P < 0.05$, Duncan's New Multiple Range Test) in all instances, and the DHA level for the less developed cells is significantly lower than for the other fractions ($P < 0.05$). Each data point is a result of triplicate determinations, and the experiment has been repeated three times.

The data presented in this section support the hypothesis that development occurs in a more oxidizing state or in association with lower antioxidant levels. These results also agree with results from other reports. For example, in plant studies it has been reported that the highest levels of antioxidants are observed in the meristematic regions, which are characterized by high rates of cell division.[9,53] In domestic carrot, higher levels of GSH were associated with proliferative callus tissue than with the differentiated tissue which served as the source of the callus growth.[9]

In the experiments used to test the antioxidant hypothesis, the oxidant levels yielded one of two trends: a) no difference between unorganized and organized growth or b) parallel differences with the corresponding antioxidant levels. In either case, the data yield little support for the idea that decreases in the levels of antioxidants during development occur in conjunction with increases in the oxidants' levels.[1]

Many previous investigators dealing with antioxidants, cell growth and development have not measured the oxidant levels. Exogenous GSSG has been observed to affect enzyme activities as well as the rate of protein synthesis in rabbit reticulocytes.[70] The levels needed to cause these effects were significantly higher than their physiological concentrations, and for this reason it is not believed that GSSG carries out these functions in vivo. The level of GSSG is small in relation to the total glutathione content in all systems studied, typically less than 5%, and the presence of GSSG reductase is thought to maintain these low levels of GSSG.[69]

In earlier studies the ratios of GSH/GSSG and AA/DHA were observed to change in response to auxin treatment.[10,12] Growth stimulating auxin treatments were found to increase the ratios, while growth inhibiting concentrations were found to lower these ratios. With respect to the AA/DHA ratio this observation was dismissed as not being causative of the growth response.[42] With respect to the GSH/GSSG ratios, Marre and Arrigoni[12] measured ratios are much lower or the percentage of GSSG determined is higher than levels subsequently determined in other systems, and for this reason their analytical method is questionable.

EFFECTS OF GLUTATHIONE, ITS CORRESPONDING AMINO ACIDS AND OTHER ANTIOXIDANTS ON WILD CARROT DEVELOPMENT

Results from the previous section indicate that lower levels of GSH are associated with development, thus creating a more oxidizing environment. Conversely, high GSH levels (or a more reduced state) are associated with proliferative growth. If the level of GSH is important for development, then causing changes in its level should affect development. If the antioxidant function of GSH affects development, then cysteine, the amino acid responsible for GSH's antioxidant function, may also have a similar effect. Also, addition of other antioxidants should yield similar results if sufficiently reducing and if the observed responses are not specific for GSH.

GSH was added to developing cultures to increase the GSH levels and simulate the proliferative growth situation. BSO was added to decrease the GSH levels which would produce a more oxidized state. The treatment's effects on development and growth were analyzed by counting embryos and determining fresh weights, respectively, at the end of an experiment. In these experiments the cells were treated on day 7 because the difference in the GSH level between proliferating and developing cultures begins to occur on or near this day (refer Fig. 6A). Trials were made in which treatment occurred on earlier days, but the results were not as significant.

The GSH and GSSG levels were determined on day 9, two days after the treatments with BSO and GSH, and again at the end of the experiment, day 17. The data for GSH measured on days 9 and 17 are shown in Fig. 10A and 10B, respectively.

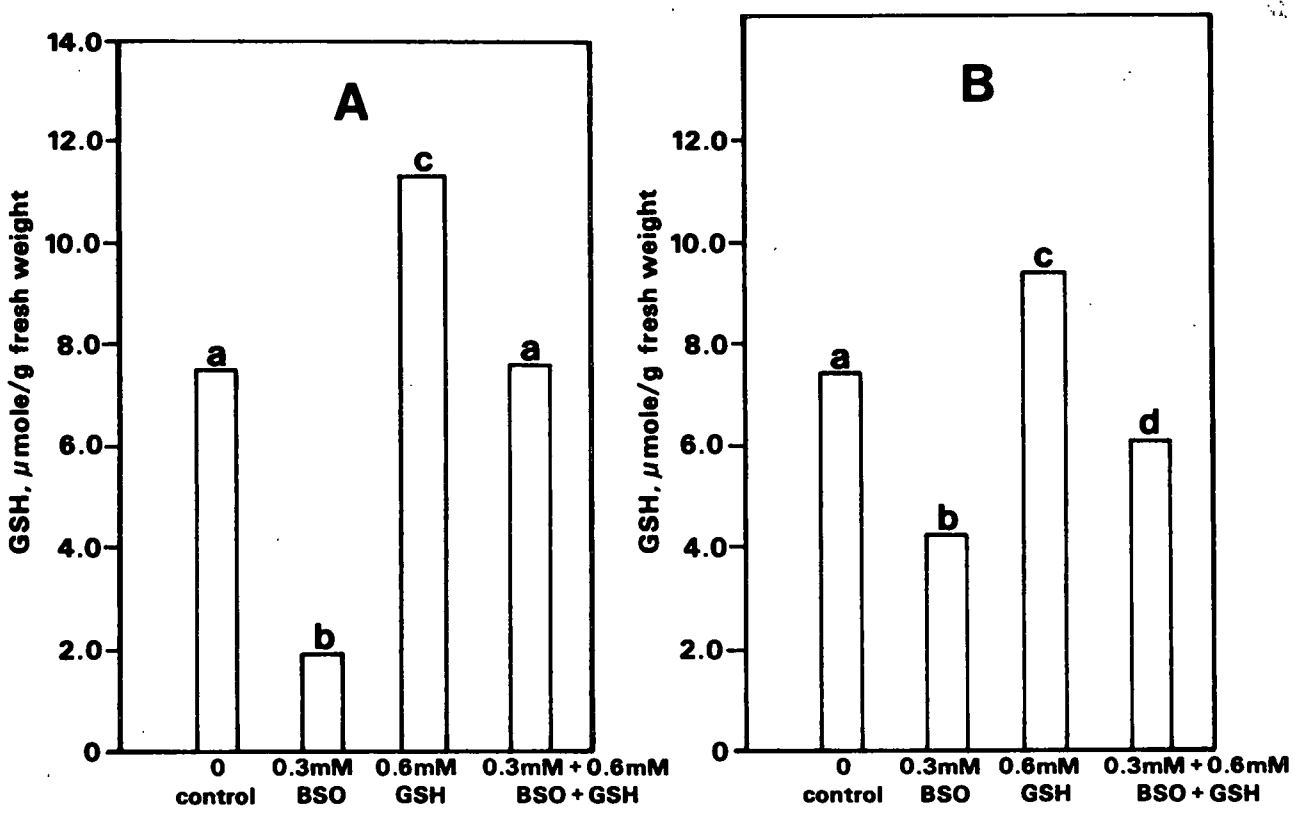


Figure 10. Effect of BSO, GSH, and BSO + GSH on the GSH levels in developing wild carrot cultures. The levels were determined on day 9(A) and day 17(B). Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. Each data point is a result of triplicate determinations and the experiment has been repeated three times.

Addition of BSO lowered the GSH levels while addition of GSH increased the levels on days 9 and 17. With the exception of a brief report,[25] to our knowledge this is the first evidence that BSO lowers plant cellular GSH levels. BSO has been shown to inhibit GSH synthesis in animals by specifically inhibiting glutamylcysteine synthetase.[24] The results shown here indicate that this mechanism may also operate in wild carrot cells. Treatment of the cells with BSO + GSH resulted in a maintenance of the GSH levels at control levels on day 9. On day 17, the GSH level in the GSO + GSH treated cells was between that of the control and the BSO treated cells. The GSSG levels are shown for day 9 (Fig. 11A) and

day 17 (Fig. 11B). On day 9, the levels paralleled the GSH levels, and on day 17 no trends were recognized.

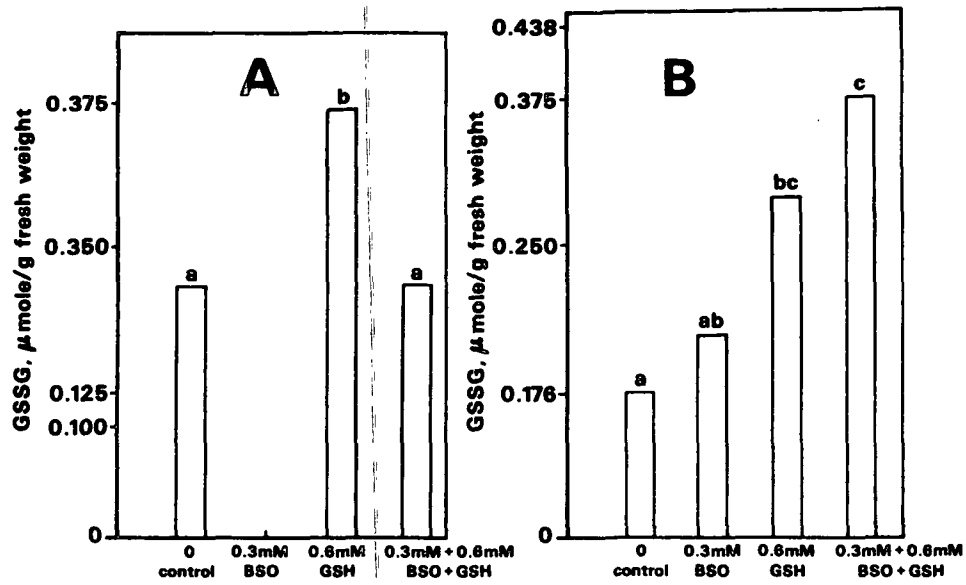


Figure 11. Effect of BSO, GSH, and BSO + GSH on the GSSG levels in developing wild carrot cultures. The levels were determined on day 9(A) and day 17(B). Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. Each data point is a result of triplicate determinations and the experiment has been repeated three times. N.D. = not detected.

On day 17, embryogenesis was stimulated by BSO treatment and inhibited by GSH treatment (Fig. 12).

The fresh weight data (Fig. 13) suggest that the treatments' effects were specifically on development. BSO had no effect on fresh weight, while GSH had only a minimal effect, probably due to its almost complete inhibition of development. BSO + GSH treatment yielded development and growth similar to control levels. These results, together with the results from the GSH determinations, indicate that BSO is acting specifically to reduce GSH levels and not through some nonspecific toxic effect.

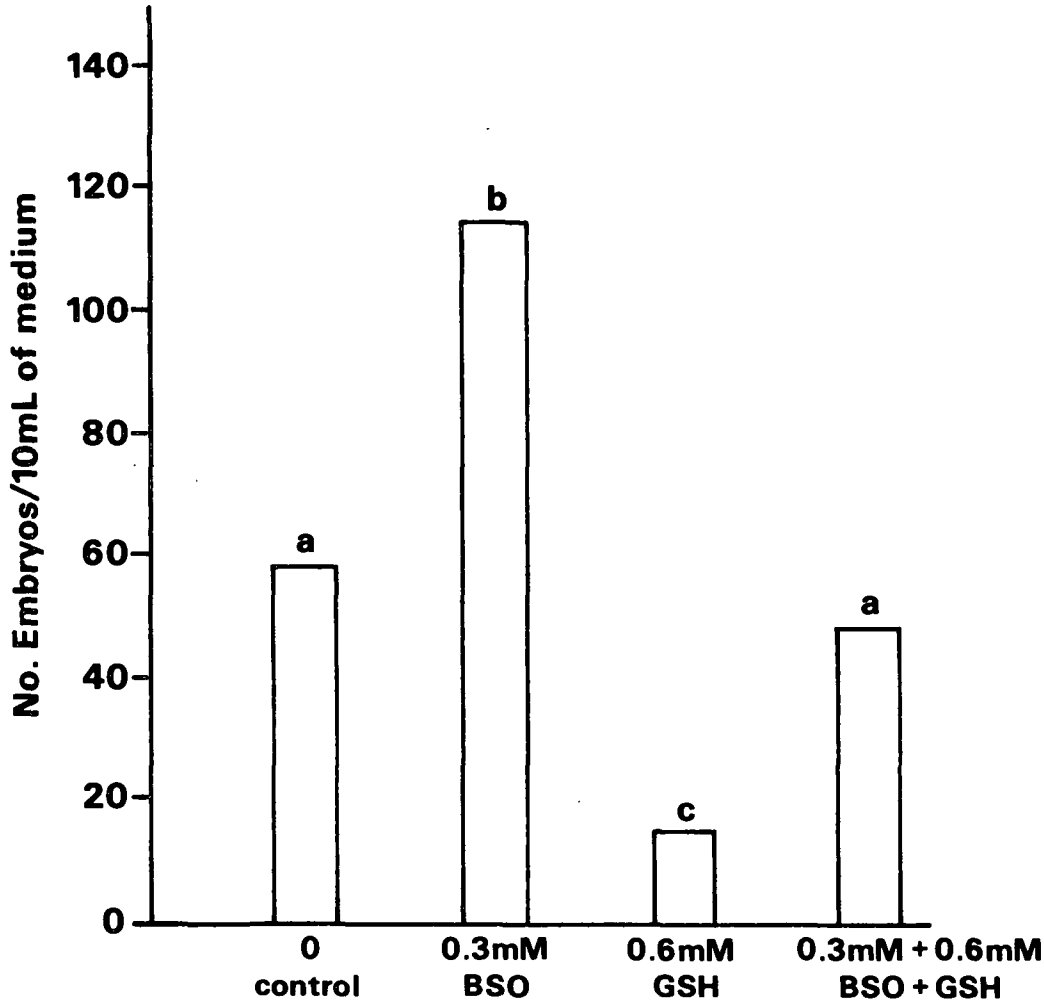


Figure 12. Effect of BSO, GSH, and BSO + GSH treatments on wild carrot embryogenesis. The treatments were made on day 7 and embryo counts were determined on day 17. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. All treatments were done in quadruplicate and the experiment has been repeated three times.

On day 17, the GSH levels (Fig. 10B) were inversely correlated with the degree of development (Fig. 12), consistent with the antioxidant concept. The GSSG levels paralleled the GSH levels on day 9 (Fig. 11A), while on day 17 (Fig. 11B) the GSSG levels showed no correlation with development. These results provide additional evidence that GSH, not GSSG, is responsible for the effects of glutathione on development.

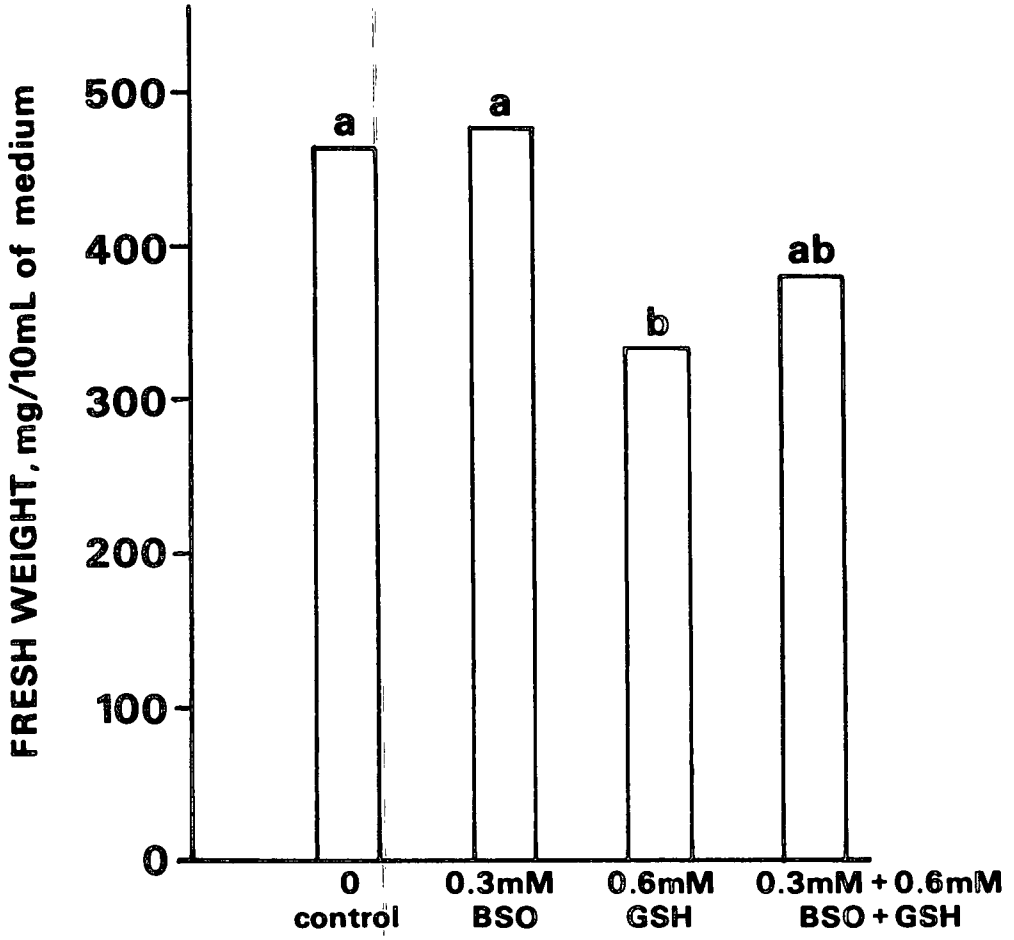


Figure 13. Effect of BSO, GSH, and BSO + GSH treatments on fresh weight determinations for developing wild carrot cultures. The treatments were made on day 7, and fresh weights were determined on day 17. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. All treatments were done in quadruplicate, and the experiment has been repeated three times.

The results described above indicate that the concentration of GSH is important in determining whether carrot cells develop or grow proliferatively. As a control, an experiment was designed to test if the individual amino acids which make up glutathione have a similar effect on development. On day 7, glycine, glutamic acid, and cysteine were added to developing cultures, and GSH was used as a control. The concentration used for the amino acids was 5×10^{-4}M since

GSH was added at this level. On day 14, embryos were counted and fresh weights were determined for each treatment.

The amino acids' effects on development and growth are illustrated in Fig. 14 and 15, respectively. Glycine and glutamic acid showed no effect on development or growth at the level tested. Cysteine was toxic at the level tested, and apparently the incorporation of cysteine into glutathione has a detoxifying effect on this amino acid. This is reasonable because in tobacco cultures glutathione is believed to function as the major storage form of reduced sulfur, rather than cysteine.[20] The toxic effect of cysteine has also been observed in other plant and animal systems and may result from its oxidation to the disulfide, cystine.[72,73]

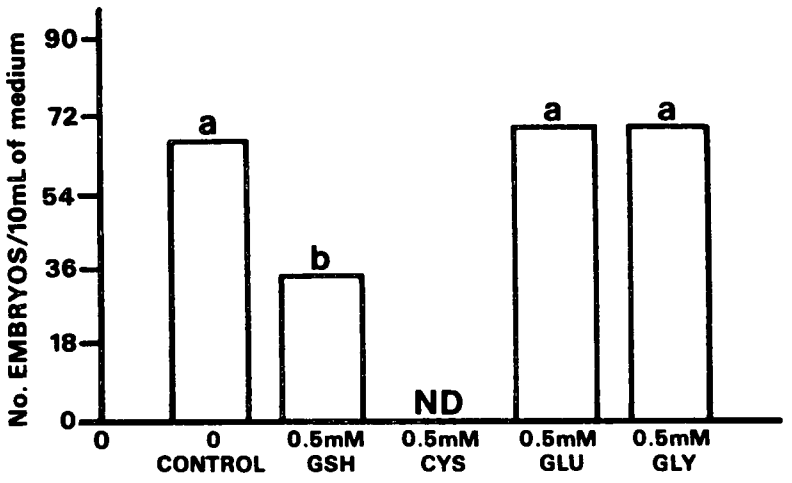


Figure 14. The effect of GSH and its corresponding amino acids, cysteine (cys), glutamic acid (glu) and glycine (gly) on wild carrot somatic embryogenesis. The treatments were made on day 7 and embryo counts were determined on day 17. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. All treatments were done in quadruplicate and the cysteine result has been repeated one time. N.D. = not detected.

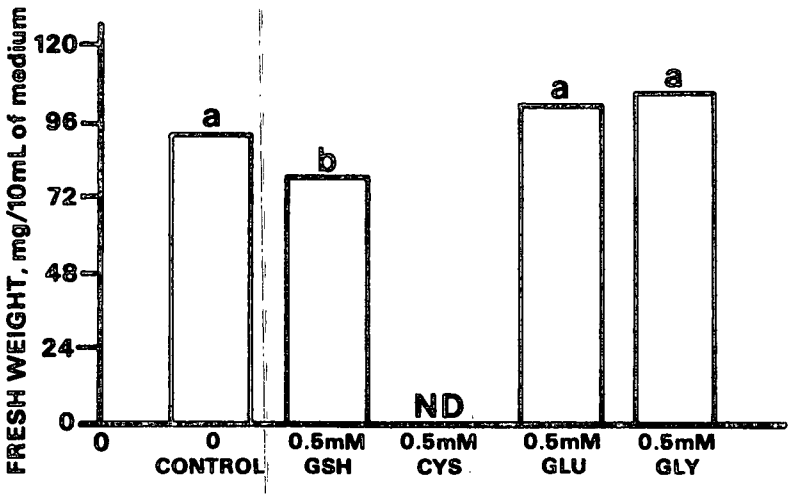


Figure 15. The effect of GSH and its corresponding amino acids, cysteine (cys), glutamic acid (glu) and glycine (gly) on fresh weight determinations for developing wild carrot cultures. The treatments were made on day 7 and fresh weights were determined on day 17. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. All treatments were done in quadruplicate and the cysteine result has been repeated one time. N.D. = not detected.

The experiment described above yielded no information concerning the mechanism of how GSH affects development. It was thought that cysteine might also inhibit development, thus indicating the involvement of the SH group and suggesting an antioxidant effect. As a result of the observed toxic effect of cysteine, another experiment was designed to determine if the effect of GSH on development was resulting from its antioxidant action. In this experiment, developing cells were treated with other antioxidants on day 7. AA, α-tocopherol-phosphoric acid [a water soluble form of vitamin E (vit E)], and a synthetic antioxidant, n-propyl gallate (NPG), were used as the test antioxidants and GSH was used as a control.

Chemical structures of vitamin E-phosphoric acid (left) and n-propyl gallate (right)

Embryo counts (Fig. 16) and fresh weights (Fig. 17) for each treatment were determined on day 17 of the culture period. The data for development show that GSH was the most potent inhibitor; however, the other antioxidants tested inhibited development to some extent. Only AA at 1 x 10^{-4}M was not inhibitory to development. The fresh weight results show that yields from treatments with GSH, AA at 5 x 10^{-4}M and NPG at 1 x 10^{-4}M were less ($P < 0.05$) than the control. It is of interest that vit E inhibited development, but did not influence fresh weight. This suggests that vit E may be more effective than the other antioxidants tested at producing proliferative growth in this situation.

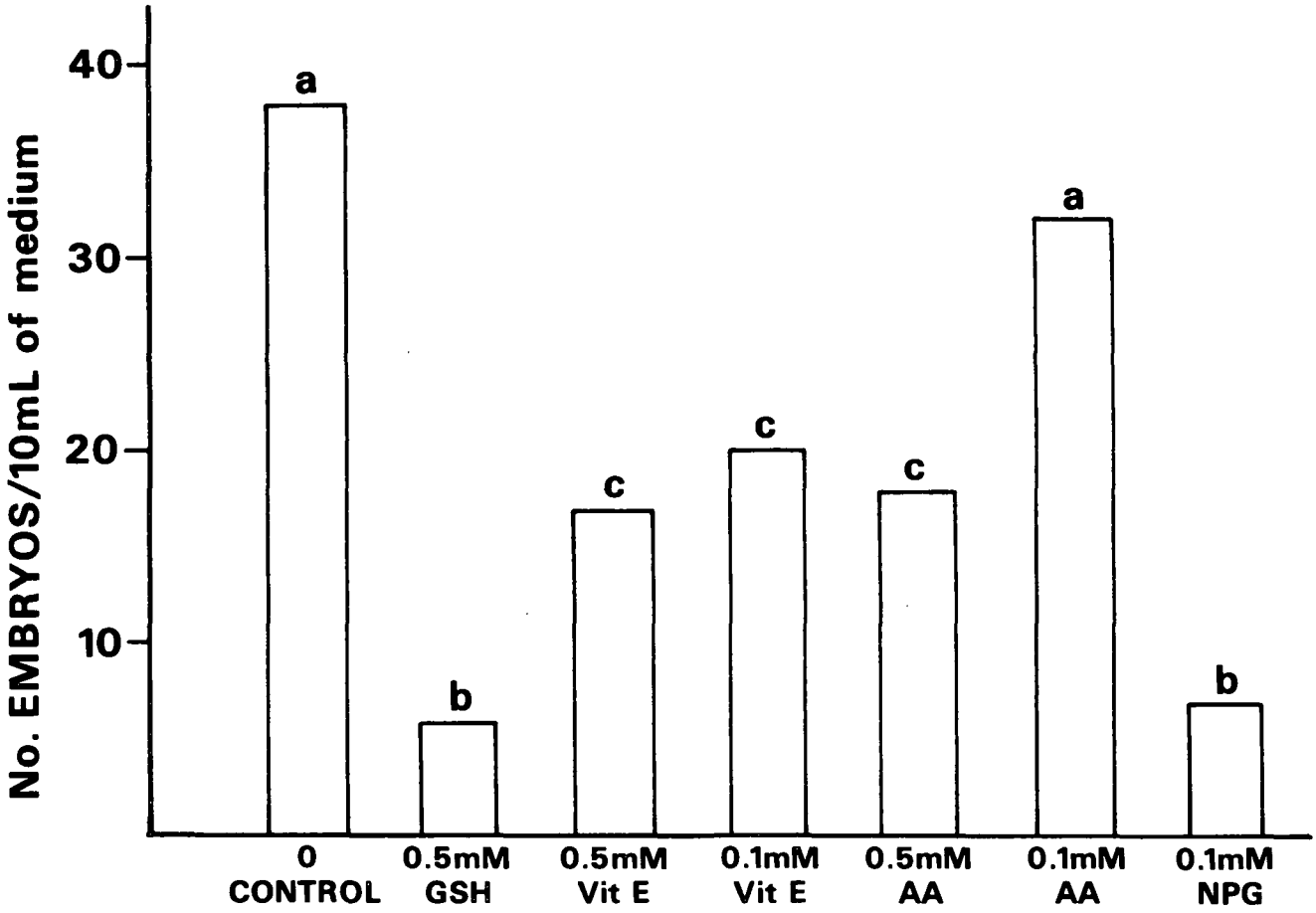


Figure 16. Effect of antioxidants on wild carrot somatic embryogenesis. The treatments were made on day 7, and the embryos were counted on day 17. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. All treatments were done in quadruplicate and the experiment has been repeated one time.

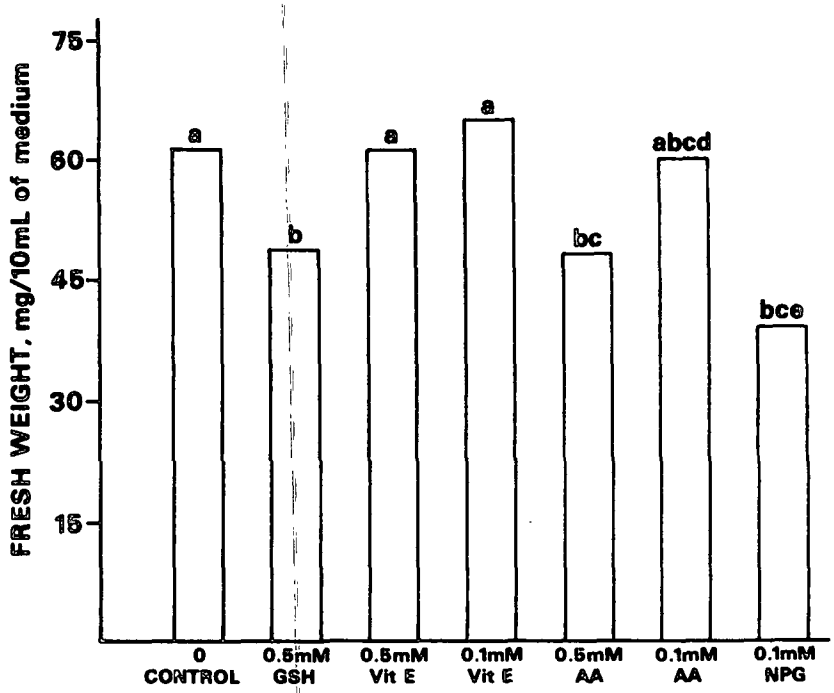


Figure 17. Effect of antioxidants on fresh weight determinations for developing wild carrot cultures. The treatments were made on day 7, and fresh weights were determined on day 17. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. All treatments were done in quadruplicate and the experiment has been repeated one time.

NPG at 5×10^{-4}M was tested but was found to be toxic and no growth occurred. Repeating this experiment with another cell line yielded similar results in terms of GSH, AA, and vit E. Lower concentrations of NPG were used in this experiment since it was believed that 1×10^{-4}M NPG used in the first experiment may have been bordering on toxicity as the cells were a brownish-green color indicating phenolic involvement. However, no effect on development was observed when NPG was run at lower concentrations, so the effects of this synthetic antioxidant on wild carrot development remain unknown.

In spite of the NPG results, the response to the addition of the other antioxidants indicates that GSH's effect on development occurs as a result of its antioxidant function. Also, the results indicate that, in general, a more reduced state (higher antioxidant levels) is detrimental to embryogenesis. To

my knowledge, this is the first time that the addition of antioxidants has been shown to affect plant development in a tissue culture system. In animal studies, reports exist which show a similar relationship between the addition of antioxidants and cell differentiation in tissue culture systems. For example, N-methylformamide's and N,N-dimethylformamide's ability to induce differentiation in certain malignant cell lines was inhibited by the addition of GSH.[48] Another study showed that differentiation of mouse myeloid leukemia cells was inhibited by the addition of phenolic antioxidants and vitamin E.[49]

One possible explanation for the effect of antioxidants on development in wild carrot cultures can be found in the effects antioxidants have on protein SH groups. It has been reported that GSH and AA can reduce protein disulfide bonds.[36,39] Other research has shown that, in general, enzyme catalyzed protein degradation occurs more readily when the disulfide bonds of the proteins are reduced to SH groups.[74] It is possible that increased antioxidant concentrations maintain proteins in a more reduced state, thus making them more susceptible to degradation. This hypothesis is supported by results from a recent publication which indicates that protein degradation is 5-10 X higher in proliferative wild carrot cultures than in developing wild carrot cultures.[75]

RELATIONSHIPS AMONG AUXIN, GLUTATHIONE, AND ASCORBIC ACID

It has been reported that GSH, sulfhydryl groups and AA increase in the presence of auxins.[10,12-14] Results presented in this thesis show that higher levels of antioxidants are associated with auxin induced proliferation. In this section the proposed association between 2,4-D and antioxidants was further tested.

Previously in this report it was shown that in a developing culture only a portion of the cell clusters develop into complete embryos, and the remaining

cells are either less developed or undeveloped. A screening technique was described which served to divide the culture into two fractions. One fraction was comprised of mostly whole embryos and the other was comprised of less developed structures or undeveloped cell clusters. In these two fractions differences in the GSH (refer to Fig. 8) and AA (refer to Fig. 9) concentrations were observed.

The procedure described above also revealed a method to test the proposed associations between auxin (2,4-D) and the antioxidants. It would be expected, if the proposed association between auxins and antioxidants exists, that a difference should also exist between the residual 2,4-D in these two fractions. The residual 2,4-D present in these cultures results from the carryover that occurs when cells are taken from the +2,4-D proliferative growth conditions and grown in -2,4-D medium.

2,4-D cannot be easily assayed so ^{14}C-2,4-D was used in this experiment. Cells were grown at 0.5 µL of cells/mL of medium containing ^{14}C-2,4-D for two weeks. At the end of this period the cells were placed in -2,4-D medium according to the standard launch protocol described in the Materials and Methods section. After 15 days, complete embryo development had occurred and the cells were screened, as described previously, to yield an embryo fraction and a less developed fraction. The two fractions of cells were then placed into a scintillation counter to determine the relative levels of the ^{14}C label.

Figure 18 illustrates the results from this experiment. A greater concentration of the ^{14}C was observed in the less developed fraction than in the embryo fraction, indicating that under -2,4-D growth conditions the 2,4-D concentration may play a role in determining the degree of development. To my knowledge, this is the first time that an apparent quantitative association has been shown

between the levels of 2,4-D and the degree of development. This result correlates with the results for GSH and AA previously shown in Fig. 8 and 9, respectively, showing the expected association between 2,4-D and the antioxidants.

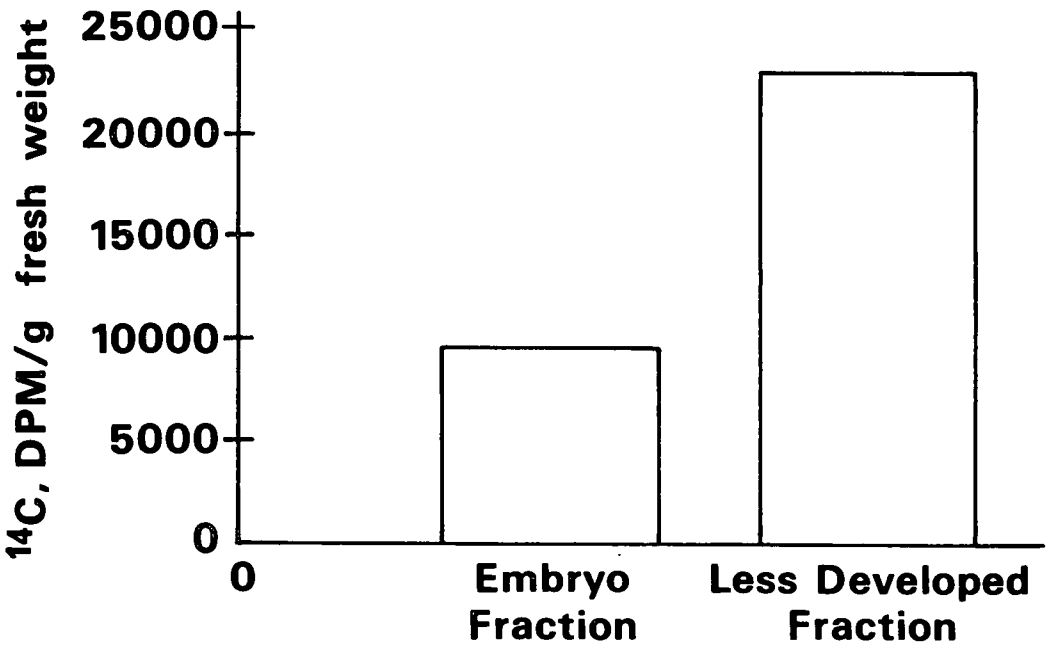


Figure 18. Concentration of ^{14}C in cell fractions containing embryos and less developed cells; the two fractions are significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. The values result from triplicate determinations.

To further test this association cells were grown in various concentrations of 2,4-D, and GSH, GSSG, AA, and DHA were determined for each 2,4-D concentration on day 12 of a culture period. Day 12 was chosen because previous data for this particular cell line indicated that the greatest difference in the GSH and AA concentrations between proliferating and developing cultures was on day 12 (refer to Fig. 6A and 7A, respectively).

The data for GSH and AA are shown in Fig. 19. The data for GSH show that as the 2,4-D concentration decreased, so did the GSH concentration, and with the

exception of the zero level 2,4-D treatment, no visual signs of development were observed in any of the cultures. The AA levels showed similar results; however, no difference was observed between the 0.5 mg/L and 0.25 mg/L 2,4-D treatments. The corresponding data for the GSSG and DHA levels are shown in Fig. 20. The GSSG levels show no significant differences for the treatments and the DHA levels are significantly different only in the case where 2,4-D was not present.

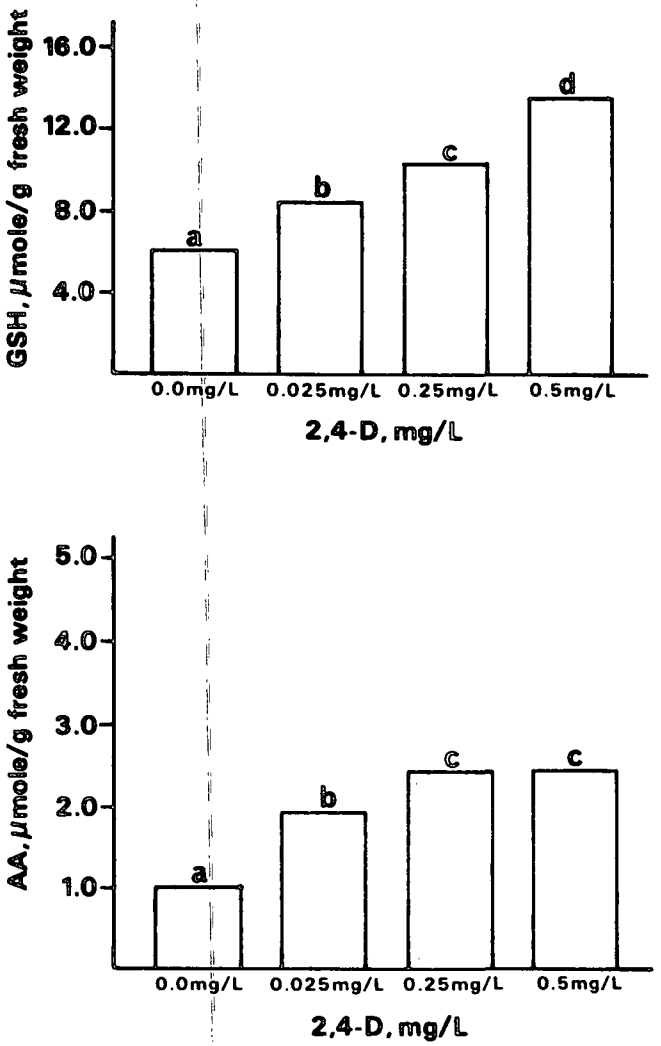


Figure 19. GSH (top) and AA (bottom) levels determined on day 12 of wild carrot cultures grown in various levels of 2,4-D. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Multiple Range Test) from each other. All values are a result of triplicate determinations and the experiment has been repeated three times.

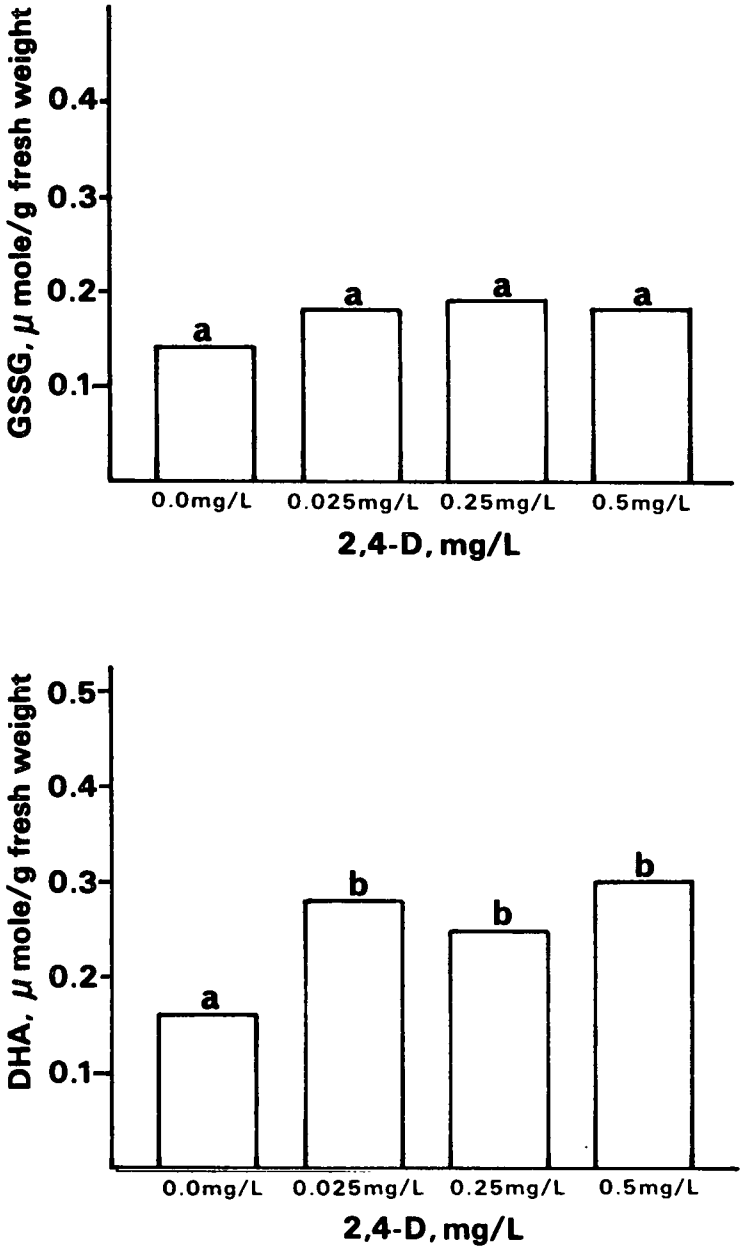


Figure 20. GSSG (top) and DHA (bottom) determined on day 12 of wild carrot cultures grown in various levels of 2,4-D. Values with common letters are not significantly different ($P < 0.05$, Duncan's New Mulitple Range Test) from each other. All values are a result of triplicate determinations and the experiment has been repeated three times.

These results provide additional evidence for an association between the reduced state (higher antioxidant levels) and auxin concentration. Two other repeat experiments of this type indicated that GSH was consistently related to

the 2,4-D concentration in the manner shown above. AA always showed higher levels in the proliferative cells than in developing cells; however, the relationship shown between GSH and 2,4-D was not as clear in the case of AA. This may indicate that the functions of GSH in the cells are more dependent upon its antioxidant action, while in the case of AA, it is known to have other functions independent of its antioxidant action.[76] The results from the experiment where GSH and AA were added to developing cultures (Fig. 16) also indicate that the antioxidant function of GSH plays a more important role in development than AA, since GSH was the more potent inhibitor of development.

The results shown here are in agreement with those of previous investigators, who showed that higher GSH and AA levels were associated with auxin-induced growth. The results also suggest a more direct relationship, since the associations indicate a dependence of the antioxidant levels on the 2,4-D concentration. In spite of the above evidence, it may also be possible that these associations result from indirect biochemical changes that are necessary for development and occur gradually as the 2,4-D concentration is decreased. With respect to the level of the oxidants; the results show that there is no correlation with the 2,4-D concentration and support the results presented in previous sections concerning the oxidants' levels (Fig. 6B, 7B, 8, 9, 10B, 11B).

The data presented thus far in this thesis indicate that lower antioxidant levels are associated with developing wild carrot cultures when compared to proliferating cultures. Other data indicate that the lower levels associated with development are a prerequisite for development. In this section evidence supporting a 2,4-D-antioxidant association has been presented. In the following section a proposed redox chain involving GSH, GSSG, AA, and DHA was investigated, which could have revealed a direct association between the antioxidant levels and 2,4-D.

INVESTIGATION INTO A POSSIBLE MECHANISM FOR CONTROL OVER THE ANTIOXIDANT LEVELS

The redox chain linking GSH, GSSG, AA, and DHA levels (refer to Fig. 3) has been shown to exist in some plant species.[55,56] It has also been observed that ascorbic acid oxidase can be inhibited by auxin in some plant species.[12,41] Auxin inhibition of ascorbic acid oxidase could lead to a build up of reducing power in the form of GSH and AA and explain the association between higher levels of these compounds and auxin induced growth. This information led to the following limited investigations of the enzymes involved in this redox chain.

GSSG reductase, DHA reductase and ascorbic acid oxidase activities were determined on various days over a culture period in proliferating and developing cultures. The data for GSSG reductase are shown in Fig. 21. The proliferating cultures show higher activities for GSSG reductase than the developing cultures. This result indicates that the potential for GSSG reduction is higher in proliferative cultures. This result also suggests that the antioxidant action of GSH is utilized to a greater extent in the proliferative cultures, thus supporting the antioxidant role of GSH under the reduced state conditions.

Additional backing for this suggestion is given by the report showing that when cotton plants were grown in elevated oxygen levels, the GSSG reductase activity rose.[5] This was interpreted to indicate a greater use of GSH as an antioxidant; however, in this case, GSH was proposed to protect the plant from the harmful effects of oxygen, which may also be possible in this case with wild carrot.

The data for DHA reductase in Fig. 22 show similar levels for the proliferating and developing cultures. Unlike in the case of GSSG reductase, this

result indicates that the potential for DHA reduction is similar for both growth situations. The Km for DHA reductase has been reported to be high in cauliflower florets (2 mM).[77] The assay conditions used for DHA reductase here also indicate a high Km for DHA in wild carrot cells. Accurate activity readings could only be obtained when at least 14 mM DHA was used. The highest level of DHA observed during the culture period was approximately 0.5 mM, so at this concentration and lower the enzyme's effectiveness is limited.

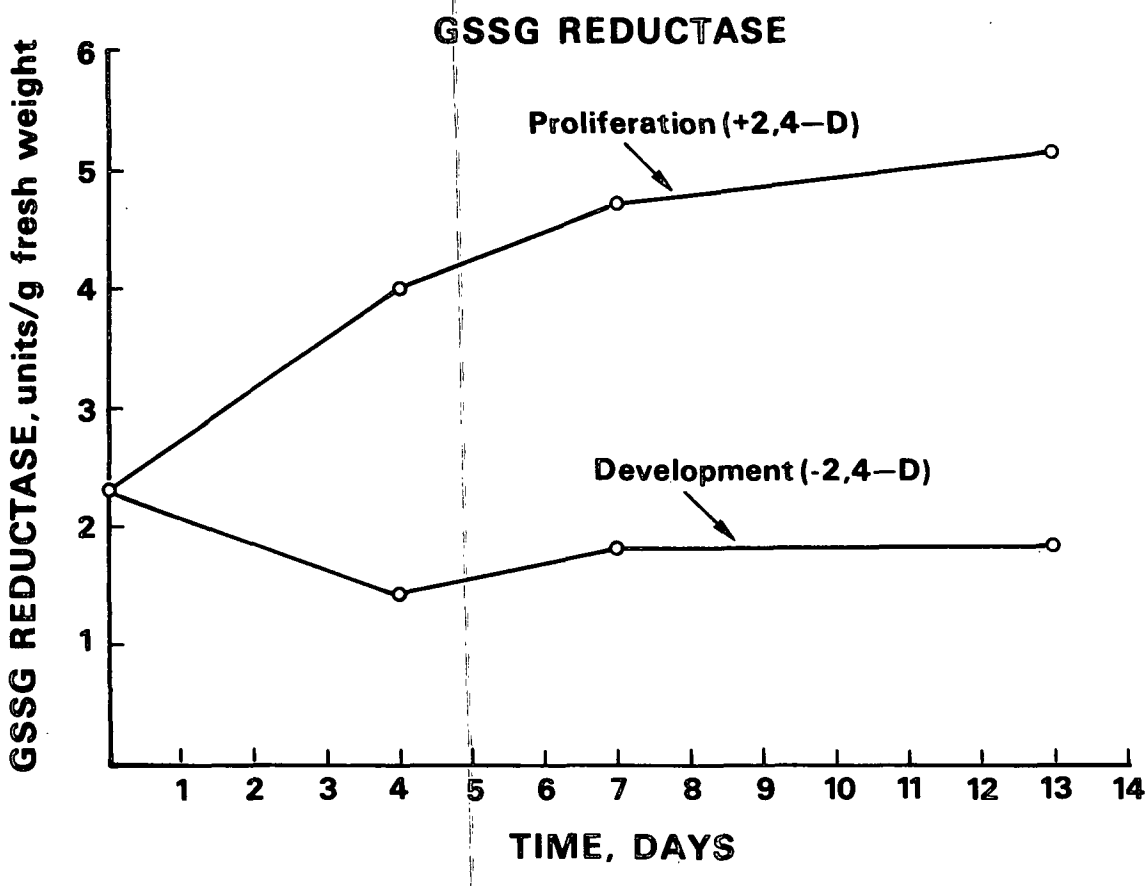


Figure 21. GSSG reductase activity in units (μmole/min) for developing and proliferating wild carrot cultures. The activities of proliferating and developing cultures are significantly different ($P < 0.05$, Duncan's New Multiple Range Test) on and after day 4. Each data point is a result of triplicate determinations, and the experiment has been repeated twice.

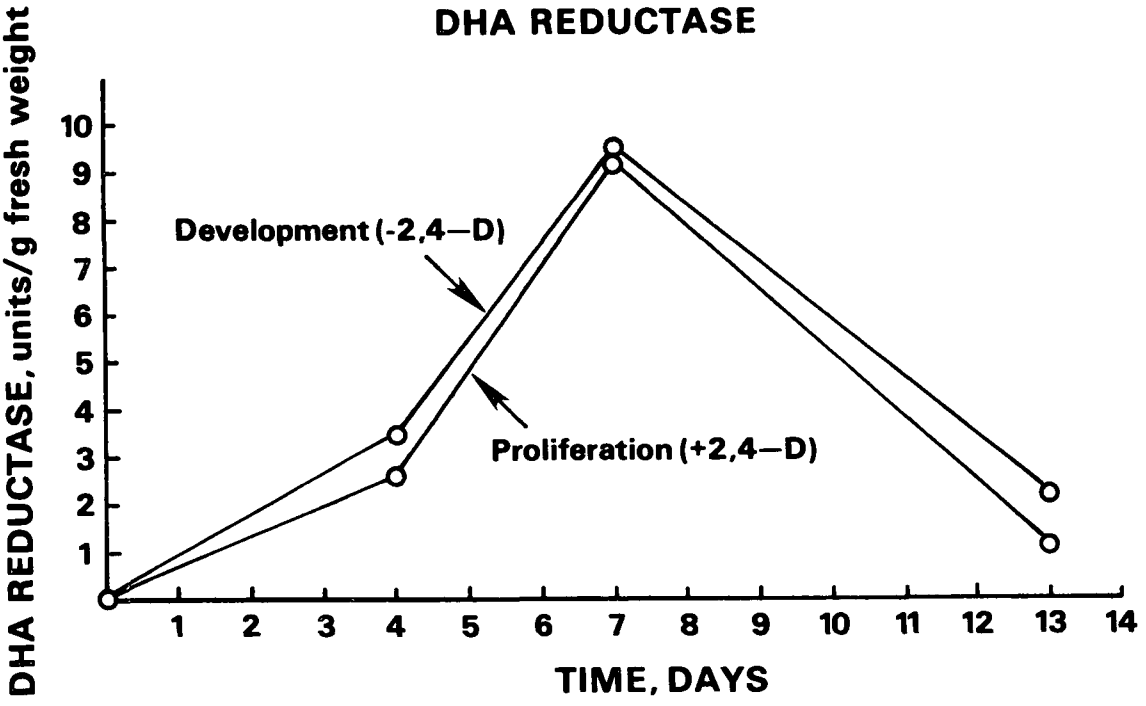


Figure 22. DHA reductase activity in units (mole/min) for developing and proliferating wild carrot cultures. The activities of proliferating and developing cultures are significantly different ($P < 0.05$, Duncan's New Multiple Range Test) only on day 13. Each data point is a result of triplicate determinations and the experiment has been repeated twice.

Activity for ascorbic acid oxidase could not be detected under the assay conditions used. The assay conditions were tested by using a commercial ascorbic acid oxidase, and this enzyme revealed activity under the assay conditions imposed. The commercial enzyme was also added to the cell extracts to determine if the enzyme was being inhibited; no loss of activity was observed. These experiments indicate that ascorbic acid oxidase is not present or is extremely weak in wild carrot cultures. Other investigations have shown that ascorbic acid oxidase is not present in all other plant species studied.[60]

With the apparent absence of ascorbic acid oxidase in this system and the indication of the high Km for DHA in DHA reductase, these results indicate that the proposed redox chain is not operating in wild carrot cells. It would also appear that this proposed mechanism of control over the antioxidant levels is not possible in wild carrot cultures.

CONCLUSIONS

The results from this thesis investigation show that more oxidizing conditions with respect to the antioxidant levels are necessary for wild carrot somatic embryogenesis, and this is consistent with the antioxidant hypothesis. Lower levels of GSH and AA are present during wild carrot development when compared to proliferative growth; however, the differences in the GSH and AA levels between proliferating and developing cultures could not be accounted for by changes in the GSSG and DHA levels. These results do not support the portion of the hypothesis that antioxidant levels are controlled by changes in the antioxidant/oxidant ratios.

BSO and GSH were added to developing cultures to test whether changes in the GSH concentration affect wild carrot development. The addition of BSO lowered the GSH concentration and promoted development. With the exception of a brief report, to our knowledge this is the first time in a plant system that BSO has been shown to lower GSH levels. This result suggests that the same mechanism by which BSO inhibits GSH synthesis in animals may also occur in wild carrot. These results are in line with the antioxidant hypothesis because lower levels of GSH or more oxidizing conditions enhanced development.

Addition of GSH to developing cultures raised the GSH levels and inhibited development. The antioxidant function of GSH in mediating this effect on development was demonstrated in experiments with other antioxidants. The addition of AA and vit E to developing cultures also inhibited development. To this author's knowledge, these experiments mark the first time that additions of antioxidants have been shown to affect plant development in a tissue culture system, although

similar results exist in animal studies dealing with cell differentiation. These experiments are also in line with the antioxidant hypothesis, since more reducing conditions with respect to the antioxidant levels inhibited development.

The levels of GSH and in most cases AA were found to correlate with the amount of 2,4-D added to wild carrot cultures. Higher levels of 2,4-D were associated with higher levels of GSH and AA. In some cases the AA level did not change with the level of 2,4-D and this may result from the fact that AA has other functions in the plant which are independent of its antioxidant function. These results agree with those of other investigators[10,12-14] and indicate that an auxin-antioxidant association exists.

One plausible mechanism which could explain this association was investigated. This mechanism is a redox chain involving GSH, GSSG, AA, and DHA and the enzymes GSH reductase, DHA reductase and AA oxidase. AA oxidase could not be detected in wild carrot cultures and, as a result, this proposed mechanism of control over the antioxidant levels was rejected. Other results showed that the Km of DHA reductase for DHA was too high in relation to the cellular concentration of DHA and would limit the effectiveness of DHA reductase. GSSG reductase activity was observed to be higher in proliferating cultures than in developing cultures. This result reinforces the antioxidant hypothesis by suggesting that the antioxidant function of GSH is utilized to a greater extent in the proliferative cultures. This is consistent with the other results from this thesis which indicate that proliferative growth is in a more reduced state.

RECOMMENDATIONS FOR FUTURE RESEARCH

This research has established that antioxidant concentrations affect wild carrot development and as a result support the hypothesis that organization occurs in a more oxidizing environment then does unorganized growth. The most obvious direction for future research is to investigate specifically how antioxidants mediate their effects on development.

In this thesis one possible mechanism for how antioxidants function was discussed and this was concerned with their possible influence on protein degradation; however, other possibilities exist. A function for antioxidants as IAA protectors has been established previously and elevated levels of IAA have been associated with undifferentiated growth. Further elucidation of this relationship may serve to explain antioxidants' effects on plant development. Van Fleet has shown that the presence of quinones characterizes the differentiated state. Antioxidants may act to maintain quinones in a reduced state, thereby restricting their biochemical action, which may affect differentiation.

The recommendations above only consider antioxidants' effects on plants; however, the literature also shows parallel effects of antioxidants on cell differentiation in animal systems. From this it may be suspected that the effects of antioxidants are of a more general nature which cannot be explained only by their effects on auxin metabolism or plant phenolics. One explanation of this type would be their proposed effects on protein SH groups. It would be of interest to determine the concentration of protein disulfide groups in proliferating and developing cultures. If antioxidants are acting to reduce protein disulfide groups, then a lower protein disulfide concentration should be associated with the proliferative cultures, since these cultures have higher

antioxidant levels. Another explanation may exist in antioxidants' proposed effects on the protein, tubulin, which is present in both plants and animals.[78] Tubulin is cross-linked with disulfide groups and would be susceptible to the same perturbations by antioxidants as are other proteins.[79]

Other related work on a smaller scope would be to understand how the GSH levels are regulated in proliferating and developing wild carrot cultures. The observed change in the GSH levels between proliferating and developing cultures apparently does not occur through a change in the antioxidant/oxidant ratios, so some other mechanism must be functioning. Initially, the activities and properties of the enzymes involved in GSH synthesis should be examined in proliferating and developing cultures, as should the enzymes involved in degradation and conjugation.

Some experiments described in this thesis should be repeated in conifer tissue culture systems. Preliminary results not reported in this thesis indicate that addition of BSO to excised tissue from slash pine and loblolly pine inhibited callus growth, while the addition of GSH enhanced callus growth in these systems. The use of these compounds may be beneficial in controlling the degree of dedifferentiation of excised tissues in tissue culture.

An AA synthesis inhibitor (lycorine) has been reported in the literature.[80,81] It would be of interest to investigate the effects of this compound on wild carrot somatic embryogenesis. The results should be similar to the BSO results to the extent that AA has the same effect on embryogenesis as GSH.

THESIS ABBREVIATIONS

GSH - Glutathione

GSSG - Glutathione disulfide

AA - Ascorbic acid

BSO - Buthionine sulfoximine

Vit E - Vitamin E-phosphoric acid

2,4-D - 2,4-Dichlorophenoxyacetic acid

MSO - Methionine sulfoximine

NADPH - Nicotinamide adenine dinucleotide phosphate, reduced form

IAA - Indole 3-acetic acid

NAA - Naphthaleneacetic acid

NADH - Nicotinamide adenine dinucleotide, reduced form

EDTA - Ethylenediaminetetraacetic acid

2-VP - 2-Vinylpyridine

NPG - n-propyl gallate

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This thesis is dedicated to my grandfather, Ervin John Earnshaw, whose memory served as inspiration throughout this educational experience.

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APPENDIX I

INVESTIGATION INTO THE APPROPRIATE BASIS FOR EXPRESSING THE RESULTS

A problem encountered in this thesis was how to base the results for the GSH, GSSG, AA, and DHA concentrations. Two bases were tested in this study, fresh weight and per cell. It was initially thought that a per cell basis would be more reliable since the number of cells per mg of fresh weight may change during a culture period and may not be reflected in the fresh weight determinations.

Cells were counted via a Coulter counter after the cells were digested in the pectinase solution and mechanically treated to yield single cells. The Coulter counter results were checked by manually counting the cells in a hemocytometer chamber under a microscope after the pectinase digestion.

The data for the number of cells per milligram fresh weight grown in the presence and absence of 2,4-D as determined by the Levy count and Coulter counter methods are shown in Fig. 23 and 24, respectively. The number of cells determined for each time point are similar for the two methods, showing that the Coulter counter method was reliable.

A greater number of cells/mg fresh weight is shown for the developing cultures between days 3 and 8. There is also a peak at day 8 which has also been observed in another similar study using wild carrot suspension cells.[66] In other repeat experiments similar results or results showing no significant differences between the cell number/mg fresh weight for proliferating and developing cultures were obtained.

The proliferating cultures were never observed to have a greater number of cells/mg fresh weight than the developing cultures and for this reason cell

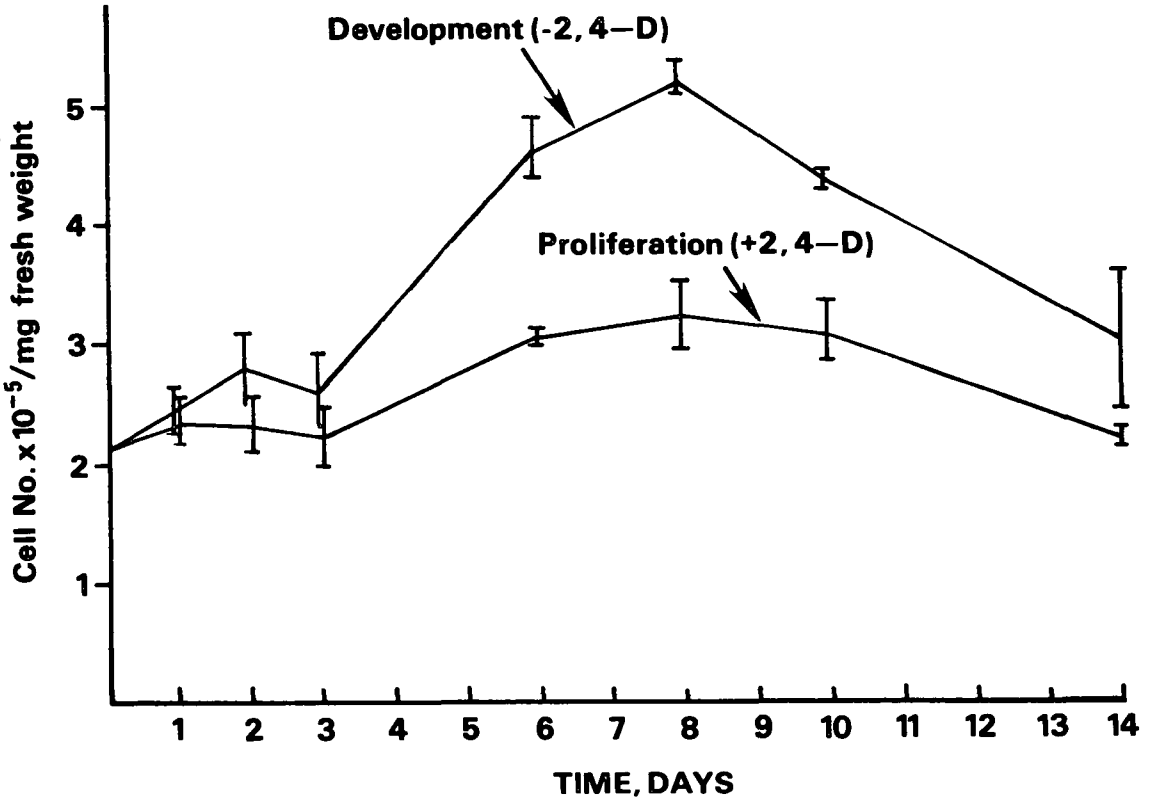

Figure 23. Levy counts, each data point is a result of triplicate determinations and is expressed as $\bar{X} \pm$ S.D.

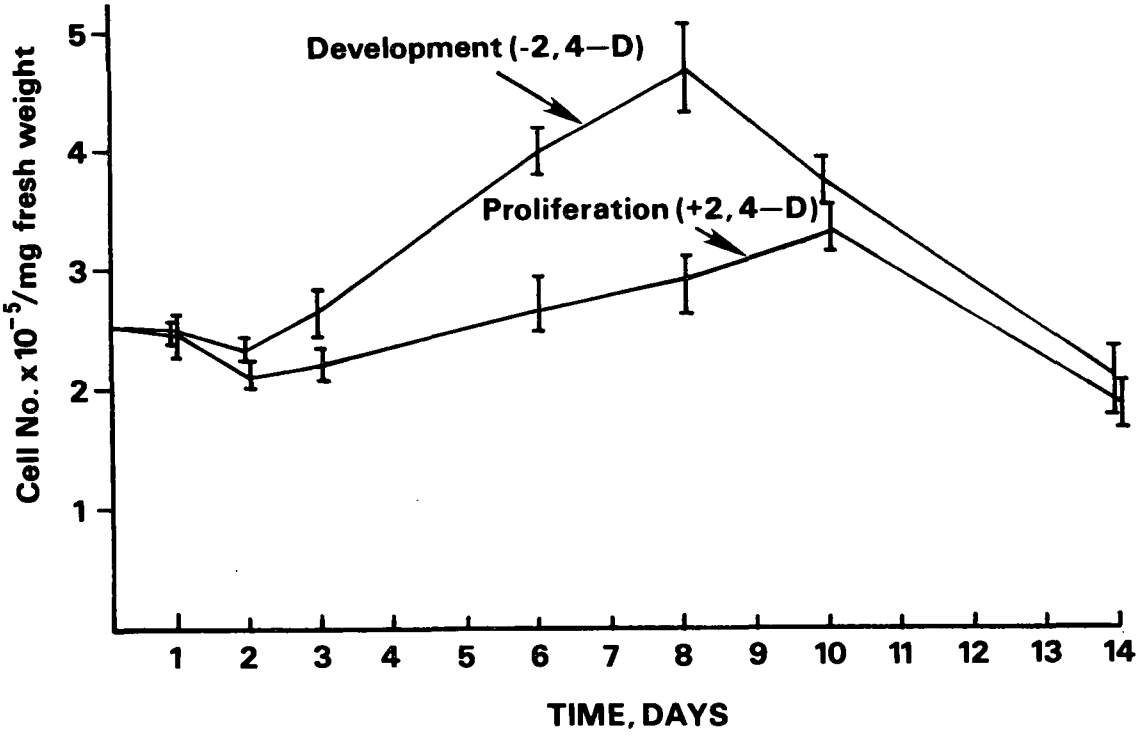

Figure 24. Coulter counts, each data point is a result of triplicate determinations and is expressed as $\bar{X} \pm$ S.D.

number was not used as the basis for expressing the results in this thesis. The reason for this is because this basis would only serve to exaggerate the already statistically significant differences between proliferating and developing cultures obtained by using the fresh weight basis. Since fresh weight was easier to obtain, it was chosen as the basis for all results presented in this thesis.

APPENDIX II

GLUTATHIONE AND ASCORBIC ACID VALUES FOR PLANT STUDIES

Glutathione Values

Carrot Plant Parts	GSH ± SD µmole/g fresh weight	GSSG ± SD µmole/g fresh weight
Root tips	8.02 ± 2.58	0.200 ± 0.076
Meristem	9.10 ± 3.46	0.420 ± 0.085
Young leaves	7.10 ± 0.63	0.264 ± 0.021
Young plant stems	6.38 ± 1.08	0.331 ± 0.053
Root pieces	6.04 ± 0.48	0.317 ± 0.042
Mature plant stems	4.12 ± 1.08	0.198 ± 0.022
Mature leaves	3.70 ± 0.32	0.189 ± 0.042

Ascorbic Acid Values

Carrot Plant Parts	AA ± SD µmole/g fresh weight	DHA ± SD µmole/g fresh weight
Root tips	0.661 ± 0.161	0.179 ± 0.028
Meristem	0.836 ± 0.261	0.168 ± 0.023
Young leaves	2.79 ± 0.670	0.283 ± 0.068
Young plant stems	0.364 ± 0.093	0.174 ± 0.042
Root pieces	0.379 ± 0.051	0.146 ± 0.017
Mature plant stems	0.470 ± 0.102	0.064 ± 0.019
Mature leaves	2.28 ± 0.670	0.261 ± 0.010

APPENDIX III

GSH AND AA DETERMINATIONS IN INCOMPETENT, COMPETENT AND PROLIFERATIVE WILD CARROT CULTURES