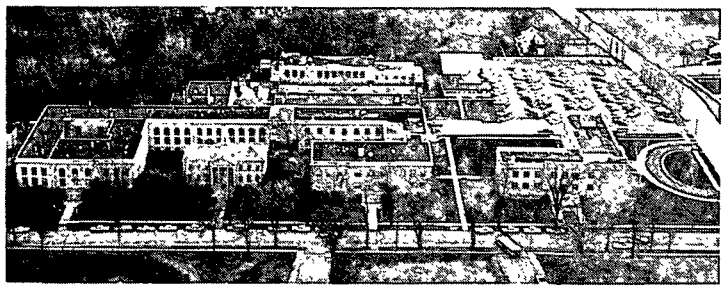

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CONTROL OF WILD CARROT SOMATIC EMBRYO DEVELOPMENT BY ANTIOXIDANTS: A PROBABLE MODE OF ACTION OF 2,4-D

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Control of Wild Carrot Somatic Embryo Development by Antioxidants: A Probable Mode of Action of 2,4-D

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ABSTRACT

As we previously reported for glutathione (GSH), both ascorbic acid (AA) and vitamin E were observed to suppress wild carrot (Daucus carota L.) somatic embryogenesis with little concomitant effect on biomass. Endogenous concentrations of AA were lower during embryo development than during cell proliferation, exhibiting a temporal pattern nearly identical to that of GSH. GSSG (oxidized GSH) reductase was found to be considerably more active in proliferating than in developing cultures, whereas no difference was evident in the case of dehydroascorbate (DHA) reductase. Both GSH and AA concentrations in these cells are governed by 2,4-D. These results show that redox status is a strong determinant of proliferative vs. developmental growth and indicate that the mode of action of 2,4-D in this system may be explained at least in part by its influence on endogenous antioxidant levels.

Cellular antioxidant levels have been shown to affect development in a number of living systems. We have shown previously by manipulation of glutathione (GSH) levels that decreasing concentrations are necessary to complete somatic embryo development in wild carrot suspension cultures (1). Those results upheld the concept that organization occurs in a more oxidizing environment than is needed for cell proliferation only.

In this investigation, the concept that antioxidants are important in determining whether cells proliferate or organize was further tested by investigating the effects of the antioxidants, ascorbic acid (AA) and vitamin E, on wild carrot somatic embryogenesis. The antioxidant efficiencies of GSH and AA were also investigated by determining the activities of glutathione disulfide (GSSG) reductase and dehydroascorbic acid (DHA) reductase in proliferating and developing wild carrot cultures.

In plants, an association exists between auxin and antioxidant levels, and, as a result, auxin-induced growth has been associated with a more reducing cellular environment (2,3,4). Although somatic embryogenesis may begin in the presence of 2,4-D, wild carrot suspension cultures are considered to grow proliferatively in the presence of auxin (2,4-D) and embryogenically when 2,4-D is absent from the medium (5). This characteristic provided an opportunity to further investigate the proposed auxin-antioxidant association.

MATERIALS AND METHODS

Cell Culture. Wild carrot (Daucus carota L.) cell suspensions were grown proliferatively in the presence of 2,4-D and embryogenically in its absence as described previously (1).

Determination of AA and DHA. Following extraction of cells with metaphosphoric/acetic acid and centrifugation as described for GSH/GSSG analyses (1), the resulting extracts were analyzed for both AA and DHA by the fluorimetric procedure of Deutsch and Weeks (6). In this assay AA is oxidized to DHA before derivatization with o-phenylenediamine. Excitation is at 350 nm and emission at 430 nm. Employment of these wavelengths plus the use of charcoal and boric acid complexes in the procedure proved to be quite suitable for these plant extracts.

Known amounts of AA added to wild carrot extracts as internal standards were recovered completely.

Antioxidant Addition Experiments. These experiments were conducted as described previously for GSH additions (1). The concentrations of GSH, AA, and water soluble vitamin E (DL-alpha-tocopherol phosphoric acid ester, ICN) used are given in Fig. 4 and 5. Antioxidant treatments were started on the seventh day after transfer of the cells to 2,4-D-free medium.

Enzyme Analyses. Crude extracts for enzyme analyses were prepared by extracting cells with 5 mM potassium phosphate buffer, pH 6.8, to which Polyclar AT (GAF) had been added (1.5 mg per mg fresh weight of cells). After homogenization in a ground glass grinder for 1 min, the homogenate was centrifuged at 17,000 g for 15 min. The supernatant was clarified further by passage through a Millipore filter before use.

GSSG reductase was assayed essentially by the procedure of Esterbauer and Grill (7). The sample and reference cuvettes each contained 0.2 µmoles NADPH (Sigma), 4.5 µmoles EDTA (Baker), 1.7 mg bovine serum albumin (Miles), plus enzyme extract in buffer (sample) or buffer only (reference). The reaction was initiated with 0.5 µmoles GSSG (Chemalog) added to the sample cuvette only after a baseline was established. The final volume in both cuvettes was 1.125 ml. DHA reductase was assayed by following the increase in absorbance at 265 nm due to AA formation. The DHA substrate was prepared by shaking AA solution with charcoal as in the Deutsch and Weeks procedure described above in which AA is converted to DHA. The sample and reference cuvettes each contained 115 µmoles DHA and enzyme extract in the phosphate buffer, pH 6.8. The reaction was

initiated by adding 14 µmoles GSH (Chemalog) to the sample cuvette only. The final volume in both cuvettes was 2.825 mL.

Relationship Between 2,4-D Concentration and Antioxidant Levels. Cells grown in the presence of 0.5 mg/L 2,4-D were subcultured normally but to media containing 0, 0.025, 0.25, and 0.5 mg/L 2,4-D. After 12 days of growth, the cells were harvested and analyzed for GSH, GSSG, AA, and DHA. Except for the zero level of 2,4-D, no signs of significant development were apparent from microscopic examination.

RESULTS AND DISCUSSION

Previous studies in which the concentrations of GSH and GSSG were determined in proliferating and developing wild carrot cultures showed higher levels of GSH in proliferating cultures, supporting the concept that the reduced state promotes unorganized growth (1). The levels of GSSG were found not to account for the differences observed in the GSH levels between proliferating and developing cultures. This result did not support that portion of a general oxidation/reduction hypothesis which states that changes in antioxidant levels are accompanied by inverse changes in the concentrations of the corresponding oxidized forms (8). AA and DHA levels for proliferating and developing wild carrot cultures (Fig. 1A and 1B) parallel the results previously obtained for GSH and GSSG (1), thus verifying that lower antioxidant levels are associated with development and that any changes in reduced/oxidized ratios reflect mainly changes in concentrations of reduced forms, since DHA levels like GSSG levels remained very low.

Since GSH and AA showed the same pattern with respect to their levels as a function of time in proliferating and developing wild carrot cultures, it was of

interest to study the activities of GSSG reductase and DHA reductase under the same conditions. These enzymes have been shown to regenerate GSH and AA upon their oxidations and may serve as indicators of the antioxidants' efficiencies (9,10).

GSSG reductase (Fig. 2) exhibited a higher activity in the proliferating cultures, indicating that the potential for GSSG reduction is higher under these conditions. This result also suggests that the antioxidant action of GSH may be utilized to a greater extent in proliferating cultures, thereby supporting a role of GSH as an antioxidant in this reductive mode of growth. Other studies also support this interpretation, e.g., a report showing that when cotton plants were grown at elevated oxygen levels, the GSSG reductase activity rose (11). This was interpreted to indicate a greater use of GSH as an antioxidant except that, in cotton, GSH was proposed specifically to protect the plant from the harmful effects of oxygen species. This may also obtain to some degree here in the wild carrot system.

The data for DHA reductase (Fig. 3) differed from that for GSSG reductase in that the activity was essentially the same for both proliferating and developing cultures, indicating that the potential for DHA reduction is similar under both growth situations. The Km of DHA reductase for DHA has been reported to be high in cauliflower florets, 2 mM (12). The assay conditions used here for DHA reductase from wild carrot cultures also indicated a high Km for DHA. Accurate activity readings could be obtained only when at least 14 mM DHA was used. The highest DHA concentration measured during a culture period was approximately 0.5 mM (Fig. 1B), so at this concentration and lower the enzyme's effectiveness would be limited.

Although these results do not suggest a high antioxidant efficiency for AA by this enzymatic mechanism, DHA can be reduced directly by GSH. In animals, DHA reductase is not present and DHA reduction occurs via direct chemical reduction with GSH (10).

Exogenous GSH was observed previously to inhibit wild carrot somatic embryogenesis, and its antioxidant activity was proposed to account for this effect (1). To further test this proposal, AA and a water soluble form of vitamin E were added to developing cultures on day 7; GSH was also added to a separate set of culture tubes as a control. On day 17 embryos were counted (Fig. 4) and fresh weights were determined (Fig. 5) for each treatment.

Figure 4 shows that the addition of AA and vitamin E at 0.5 mM inhibited embryogenesis as did GSH, although the latter was the more potent inhibitor. The concentration used (0.5 mM) was found to be near optimum for illustrating this effect for the compounds tested. The fresh weight results (Fig. 5) show that GSH and AA both inhibited growth to some extent. However, the water soluble form of vitamin E did not influence the fresh weight yield, suggesting that it may be more effective than the other antioxidants in this situation.

These results indicate that the effect of GSH on embryo development occurs as a result of its antioxidant function. Also, the data indicate that, in general, a more reduced state (higher antioxidant levels) is detrimental to completion of the embryogenesis process. In animal studies, reports exist which show a similar relationship between the addition of antioxidants and differentiation. For example, N-methylformamide's and N,N-dimethylformamide's abilities to induce differentiation in malignant cell lines were inhibited by the addition

of GSH to the system (13). Another study showed that differentiation of mouse myeloid leukemia cells was inhibited by the addition of phenolic antioxidants and vitamin E (14). To our knowledge, this and our earlier report (1) comprise the first evidence from a tissue culture system that endogenous or exogenous antioxidants can affect plant development.

It has been reported that GSH, sulfhydryl groups and AA increase in the presence of auxins (2,3,4), and this has been substantiated in this report in the case of AA (Fig. 1). This relationship was further investigated in wild carrot cultures by growing the cultures at various levels of 2,4-D and determining the GSH and AA levels on day 12 when development would normally be well along if it were occurring and maximal differences in antioxidant content relative to proliferative cultures would be expected. Figure 6 shows the GSH and AA levels determined for cultures grown in the presence of four concentrations of 2,4-D. These results are in agreement with those of previous investigators and suggest an even more direct relationship, since these data indicate a dependence of the antioxidant levels on the 2,4-D concentration.

The AA levels, although higher in the proliferative cultures than in developing cultures, were not as clearly dependent as the GSH levels for the 2,4-D concentrations tested. Both AA and GSH are known to have biological functions beyond their roles as antioxidants; however, these results indicate that the antioxidant function may be more significant for GSH than for AA in the morphogenic phenomena under study here.

The corresponding results (not shown) for the oxidant levels showed no correlation between the oxidant levels and the 2,4-D concentrations. However,

this finding might have been expected considering the substantial GSSG reductase activity in these cultures.

This study adds to the body of evidence supporting a hypothesis that a lowering of antioxidant concentrations is necessary for complete embryo development. A recent report on the role of GSH in slime mold development (15) reveals strong parallels with our recent findings. In the carrot system the concentrations of antioxidants may be directly controlled by the 2,4-D added to the cultures as indicated by the data presented here. Reports from Sung's laboratory indicate that complete carrot somatic embryogenesis is suppressed by 2,4-D plus an unidentified factor produced by cells grown at high population densities (5). Although additional corroboration should be sought, the relationship of 2,4-D to antioxidants presented here is feasible and considered tentatively established at this juncture.

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LITERATURE CITED

1. Earnshaw BA, MA Johnson 1985 The effect of glutathione on development in wild carrot suspension cultures. Biochem Biophys Res Commun 133:988-993

2. Key JL, F Wold 1961 Some effects of 2,4-dichlorophenoxyacetic acid on the oxidation-reduction state of soybean seedlings. J Biol Chem 236:549-553

3. Marre E, O Arrigoni 1957 Metabolic reactions to auxin I. The effects of auxin on glutathione and the effects of glutathione on growth of isolated plant parts. Physiol Plant 10:289-301

4. Ram C, D Balasimha, MN Tewari 1976 Effect of gibberellic acid and auxins on growth, sulfhydryl content and peroxidase activity in Phaseolus radiatus L. seedlings. Plant Biochem J 3:128-133

5. Borkird C, JH Choi, ZR Sung 1986 Effect of 2,4-dichlorophenoxyacetic acid on the expression of embryogenic program in carrot. Plant Physiol 81:1143-1146

6. Udenfriend S 1969 Fluorescence Assay in Biology and Medicine, Vol II, Academic Press, New York, pp 321-322

7. Esterbauer H, D Grill 1978 Seasonal variation of glutathione and glutathione reductase in needles of Picea abies. Plant Physiol 61:119-121

8. Siegel SM, F Porto 1961 Oxidants, antioxidants and growth regulation. In RM Klein, ed, Plant Growth Regulation. Iowa State University Press, Ames, pp 341-353

9. Ziegler DM 1985 Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. Ann Rev Biochem 54:305-329

10. Fahey RC 1977 Biologically important thiol-disulfide reactions and the role of cyst(e)ine in proteins: an evolutionary perspective. J Exp Med Biol 86A:1-30

11. Foster JG, JL Hess 1980 Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen. Plant Physiol 66:482-487

12. Crook EM 1941 The system dehydroascorbic acid-glutathione. Biochem J 35:226-236

13. Cordeiro RF, TM Savarese 1984 Reversal by L-cysteine of the growth inhibitory and glutathione-depleting effects of N-methylformamide and N,N-dimethylformamide. Biochem Biophys Res Commun 122:798-803

14. Takenaga K, Y Honma, M Hozumi 1981 Inhibition of differentiation of mouse myeloid leukemia cells by phenolic antioxidants and alpha-tocopherol. Gann 72:104-112

15. Allen RG, KJ Farmer, PL Toy, RK Newton, RS Sohal, C Nations 1985 Involvement of glutathione in the differentiation of the slime mold Physarum polycephalum. Develop Growth & Differ 27:615-620

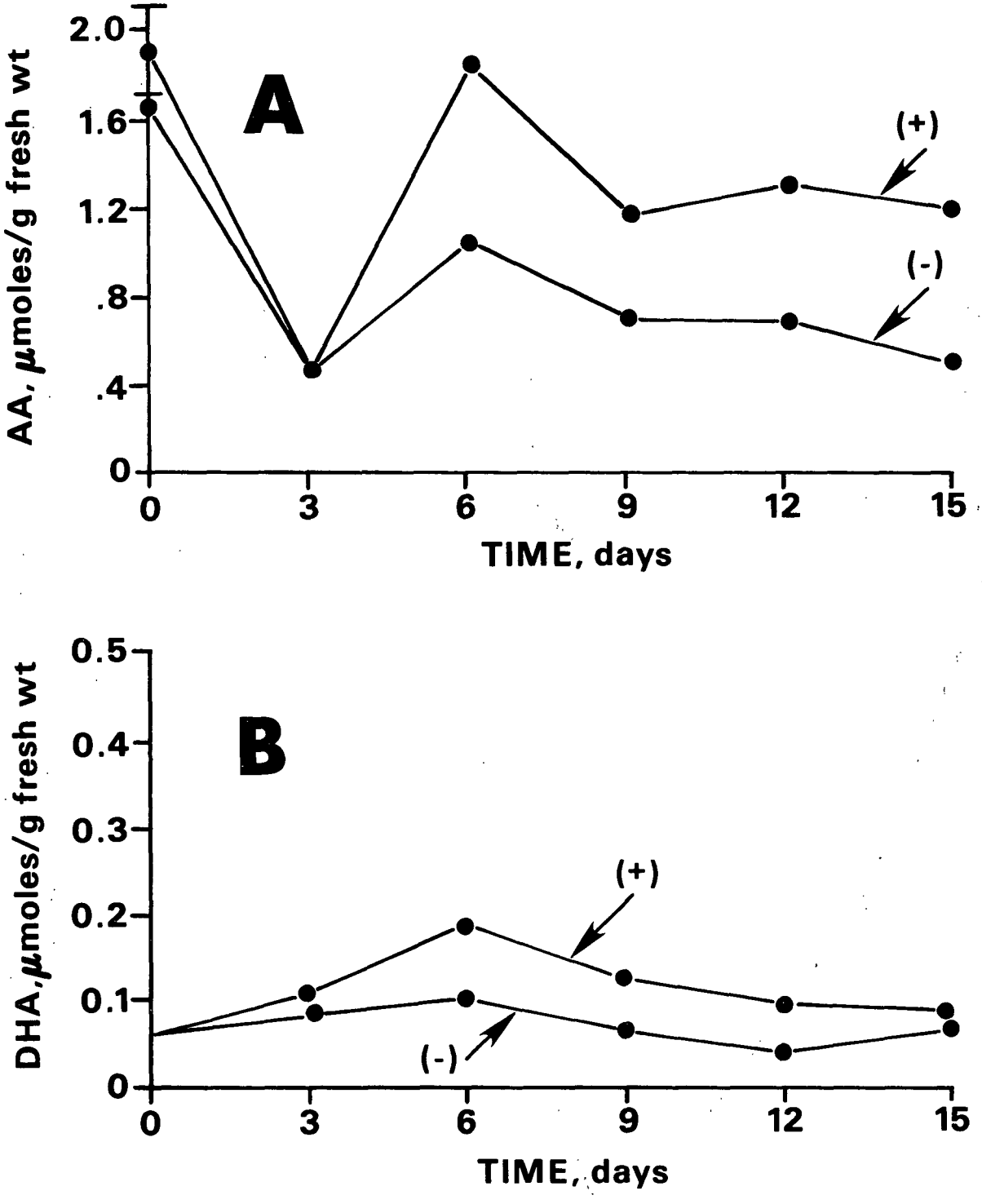

Fig. 1. AA(A) and DHA(B) levels for wild carrot cultures growing in the presence (+) and absence (-) of 2,4-D. The AA values of (+) and (-) cultures are significantly different on and after day 6 ($P < 0.05$, Duncan's New Multiple Range Test). The DHA values of (+) and (-) cultures are significantly different on days 6 and 12 ($P < 0.05$). Each data point is a result of triplicate determinations and the experiment was repeated six times.

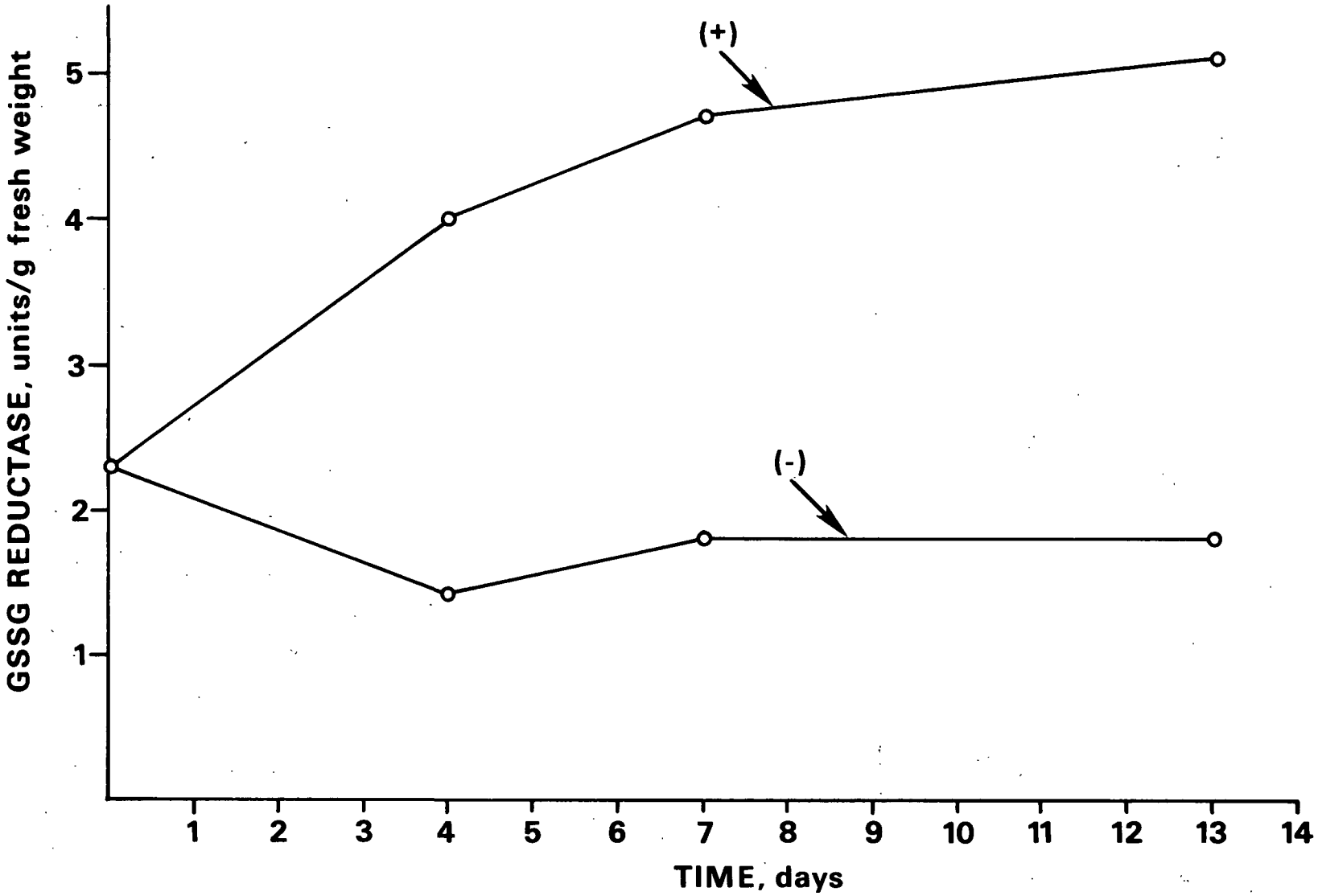

Fig. 2. GSSG reductase activity in units (μmole/min) for wild carrot cultures growing in the presence (+) and absence (-) of 2,4-D. The activities of (+) and (-) cultures are significantly different on and after day 4 ($P < 0.05$, Duncan's New Multiple Range Test). Each data point is a result of triplicate determinations and the experiment was repeated twice.

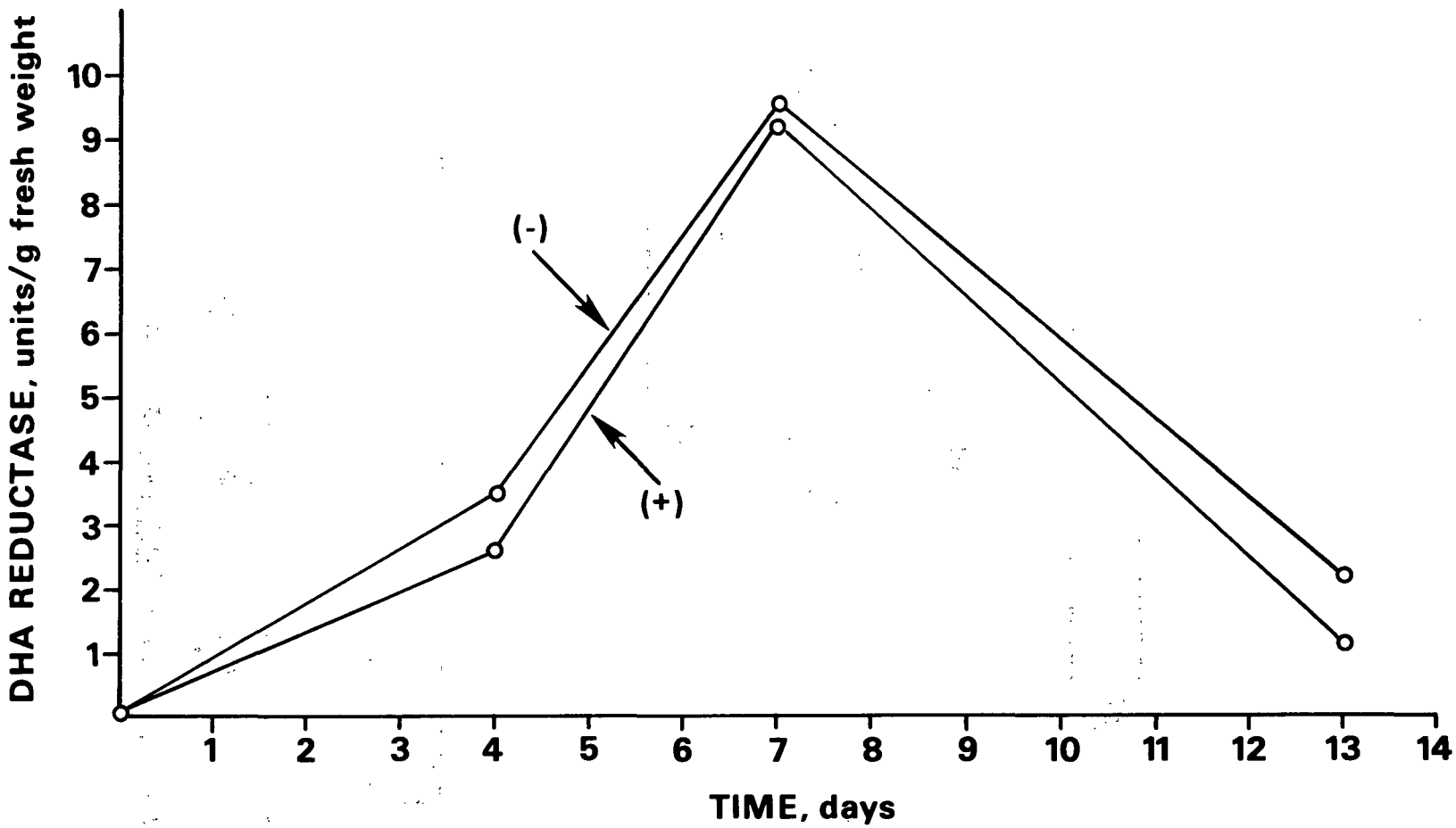

Fig. 3. DHA reductase activity in units (μmole/min) for wild carrot cultures growing in the presence (+) and absence (-) of 2,4-D. The activities of (+) and (-) cultures are significantly different only on day 13 ($P < 0.05$, Duncan's New Multiple Range Test). Each data point is a result of triplicate determinations and the experiment was repeated twice.

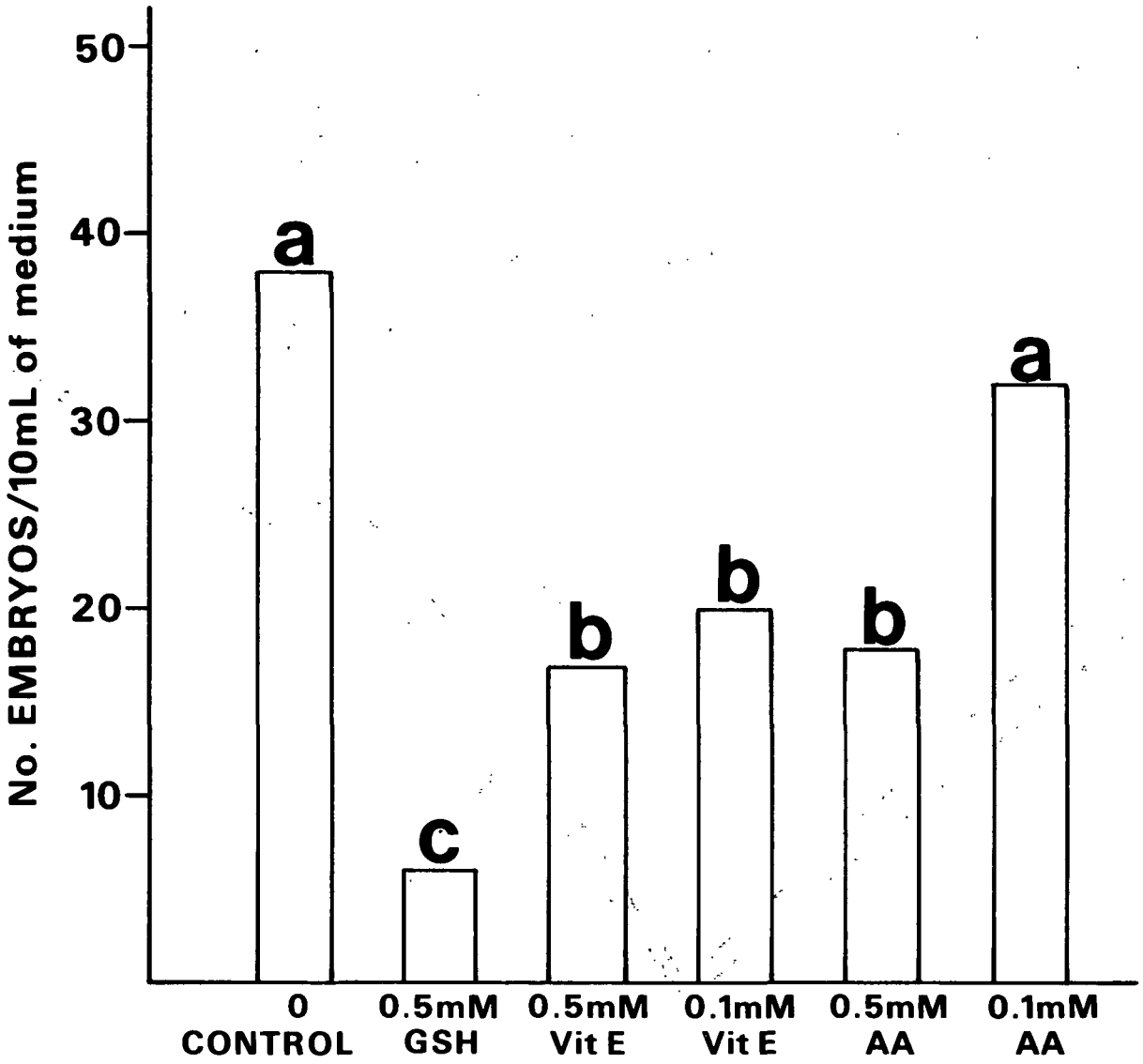

Fig. 4. Effect of antioxidants on wild carrot somatic embryogenesis. The treatments were made on day 7, and the embryos were counted on day 17. Values with common letters are not significantly different from each other ($P < 0.05$, Duncan's New Multiple Range Test). All treatments were done in quadruplicate and the experiment was repeated once.

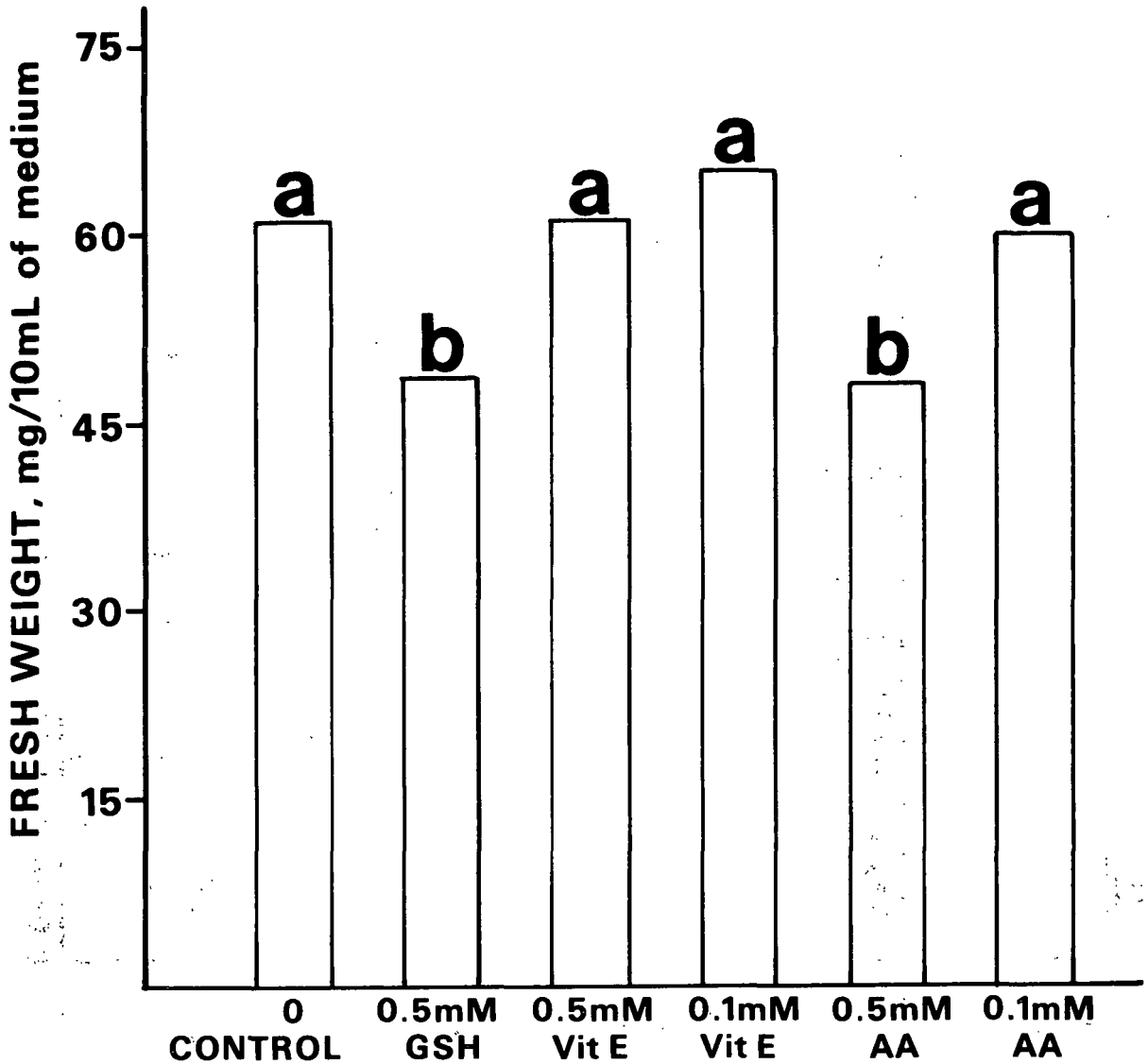

Fig. 5. Effect of antioxidants on fresh weight determinations for developing wild carrot cultures. The treatments were made on day 7, and fresh weights were determined on day 17. Values with common letters are not significantly different from each other ($P < 0.05$, Duncan's New Multiple Range Test). All treatments were done in quadruplicate and the experiment was repeated once.

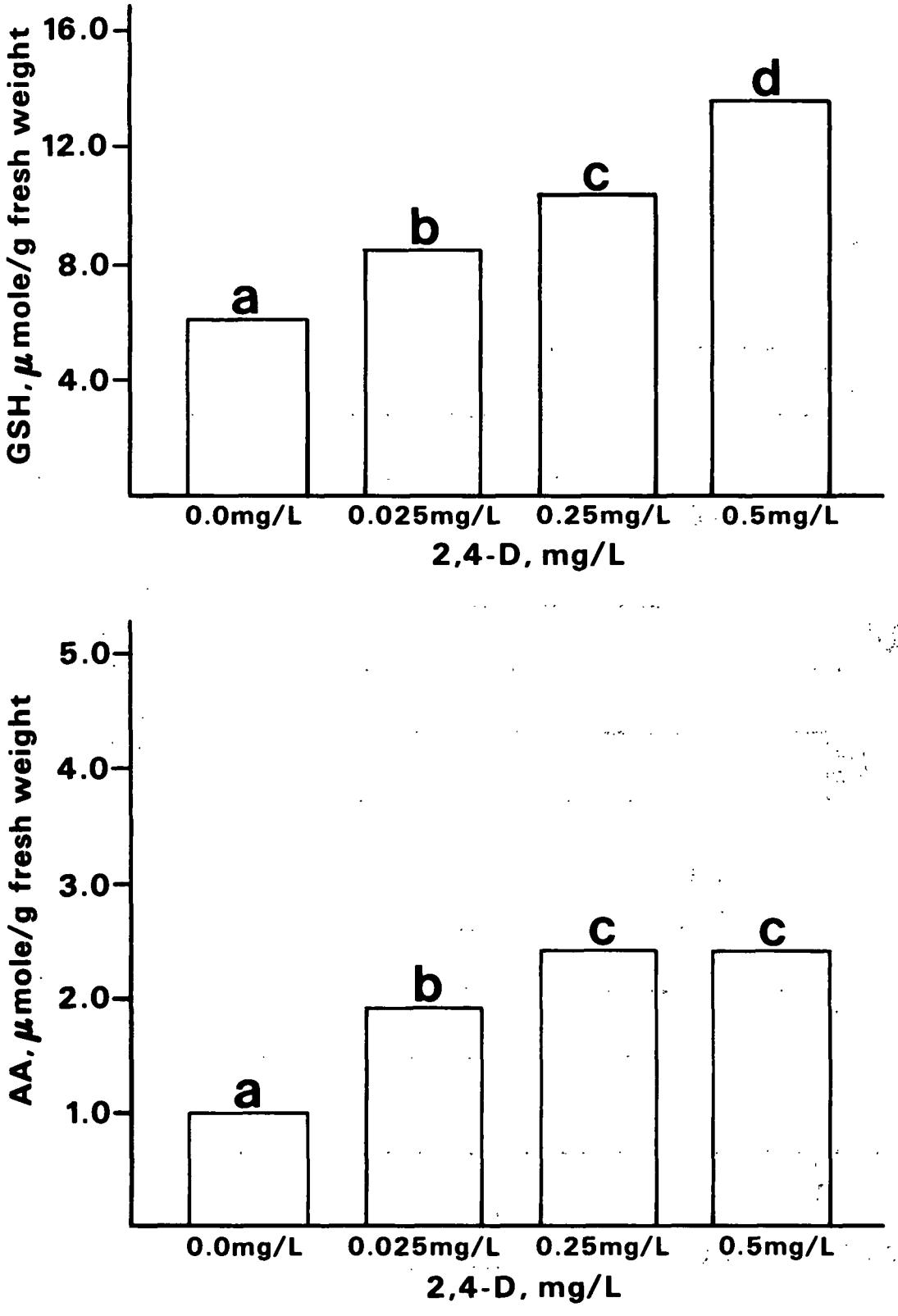

Fig. 6. GSH (top) and AA (bottom) levels determined on day 12 for wild carrot cultures grown in various levels of 2,4-D. Values with common letters are not significantly different from each other ($P < 0.05$, Duncan's New Multiple Range Test). All values are a result of triplicate determinations and the experiment was repeated three times.