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Antioxidant Responses to Oleic Acid in Two-Liquid-Phase Suspension Cultures of *Taxus cuspidata*

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Abstract

Two-liquid-phase plant cell cultures employ the use of a partitioning system to redirect extracellular product into a second phase. After the addition of organic solvent, in order to understand the defense system of *Taxus cuspidata* cells to organic solvent in two-liquid-phase suspension cultures, we investigated cells' antioxidant metabolism. The results showed that *T. cuspidata* cells responded to oleic acid with oxidative bursts in both intracellular H_2O_2 and extracellular $O_2^{-\bullet}$ production. Inhibition studies with diphenylene iodonium suggested that the key enzyme responsible for oxidative bursts was primarily NADPH oxidase. Investigation of the relationship between reactive oxygen species (ROS) and defense responses induced by oleic acid indicated that 4% (v/v) oleic acid increased the levels of antioxidant enzymes of superoxide dismutase, ascorbate peroxidase, and catalase and the antioxidant capacity of reduced ascorbate and glutathione. However, when oleic acid content reached a critical value (6% [v/v]), no further increase in antioxidant enzymes and antioxidant capacity was observed, indicating that the defense responses played a role in a certain range of oleic acid content, beyond which the overall ROS scavenging machinery was not induced and the peroxidation of membrane lipids emerged.

Index Entries: Oleic acid; reactive oxidative species; defense response; two-liquid-phase suspension cultures; *Taxus cuspidata*.

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Introduction

Plant cell cultures have been regarded as a promising alternative to traditional cultivation methods or chemical synthesis routes for the production of many pharmaceuticals, fragrances, flavors, and dyes *(1,2)*, of which taxol is an effective chemotherapeutic agent against a wide variety of tumors, especially of the ovaries and breast *(2)*. However, the yield of taxol is very low owing to the inherent characteristics of plant cell cultures, including slow cell growth, and taxol decomposes easily. Therefore, many strategies have been proposed to improve taxol production from suspension cultures, and the two-liquid-phase system has been recognized as one of the most effective methods *(3)*. Because taxol exists mainly in intracellular *Taxus cuspidata* and partitions preferentially in the organic phase of two-liquid-phase cultures, a decrease in product feedback inhibition leads to an increase in taxol yield. Previous studies mainly focused on enhancing secondary metabolites in two-liquid-phase cultures *(4,5)*. Less attention was paid to the mechanism of organic solvents acting as a stimulating signal of defense responses and the relationship between reactive oxygen species (ROS) and ROS scavenging.

In plant metabolism, the increased production of toxic oxygen derivatives is provoked by both natural and stressful situations. The presence of a high concentration of ROS including superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) causes oxidative damage to the biomolecules such as lipids, proteins, and nucleic acids *(6)*, resulting in disruption of the cellular metabolism *(7)*. Recently, evidence has accumulated suggesting that oxidative stress is a major damaging factor in plants subjected to various environmental stresses such as laminar shear and rare earth metals *(8,9)*.

On the other hand, plant cell defense against the damaging factors of oxidative stress has been developed *(10)*. This means that a plant can scavenge ROS through various mechanisms. One of the protective mechanisms is the enzymatic antioxidant system that involves the sequential and simultaneous action of a number of enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APOX), and catalase (CAT). Actually, the activities of antioxidant enzymes are inducible by oxidative stress owing to exposure to abiotic or biotic stresses, thus representing a general plant response to adverse conditions *(11)*. Within the cellular mechanism protecting against the deleterious effects of ROS, ascorbate and glutathione also play a fundamental role *(12)*.

In two-liquid-phase cultures, oleic acid was selected as the organic solvent because of its good biocompatibility with cells *(13,14)* and the high partition coefficient of taxol in the organic solvent/liquid medium two-phase system *(4)*. Oleic acid probably contributes to the alternation of oxidant level in plants *(15)*. Correspondingly, plants will be induced to develop a defense system that is mainly composed of metabolites and scavenging enzymes of active oxygen. However, no information is available about the

effect of oleic acid on antioxidative responses in two-liquid-phase cultures of *T. cuspidata*, which is the focus of the present work.

Materials and Methods

Culture Conditions

The cell suspension line was derived from young stems of *T. cuspidata (3)* and subcultured on modified B_5 solid medium containing sucrose (25 g/L), α-naphthylacetic acid (2 mg/L), and 6-benzyl aminopurine (6-BA) (0.15 mg/L). The pH of the medium was adjusted to 5.8 prior to autoclaving. Cells were cultured for more than five generations in liquid medium at 25°C with shaking at 110 rpm in the dark. Fresh cells (3.0 g) of the fifth generation were inoculated in 50 mL of fresh modified B_5 medium in a 250-mL Erlenmeyer flask.

Two-Liquid-Phase Cultures

Oleic acid was selected as the organic solvent because of its good biocompatibility with cells *(13,14)* and the high partition coefficient of taxol in the organic solvent/liquid medium two-phase system *(4)*.

Oleic acid was preconditioned by mixing with the medium (10 mL of oleic acid/90 mL of medium) and then separated. The treated oleic acid was autoclaved at 120°C for 30 min before being added to the cultures. In two-phase culture experiments, a given volume of the sterilized solvent was added to each flask containing 50 mL of medium on d 10 of the cell culture. Afterward, the flasks were shaken continuously until completion of the culture period. Cell samples were collected periodically for analyses.

Measurement of $O_2^{-\bullet}$

Superoxide anions were assayed following the method of Wang and Lou *(16)* with a slight modification. Cell-free extracellular medium (0.5 mL) or suspension cultures (with cells) (0.5 mL) were mixed with 1 mL of 1 m*M* hydroxylammonium chloride and 0.5 mL of 50 m*M* phosphate buffer (pH 7.8), and the mixture was incubated at 25°C for 60 min. Then, 1 mL of sulfanilamide (17 m*M*) dissolved in 30% (w/w) acetic acid and 1 mL of naphthalene diamine dihydrochloride (7 m*M*) were added, and the mixture was incubated at 25°C for 20 min. Absorbance was detected at 530 nm using a spectrophotometer. A calibration curve of OD_{530} against NO_2^- concentration was constructed, and then NO_2^- concentration was calculated from the calibration curve. The concentration of $O_2^{-\bullet}$ was calculated as twice that of NO_2^- based on the following reaction: $2\ O_2^{-\bullet} + H^+ + NH_2OH \rightarrow H_2O_2 + H_2O + NO_2^-$.

Measurement of H_2O_2

H_2O_2 content was measured using the Ti-NH_3 method *(17)*. Briefly, fresh cells were collected and centrifuged at 25*g* for 5 min and homogenized in cold acetone. The homogenate was centrifuged at 10,000*g* for 20 min at

4°C. One milliliter of supernatant was transferred into another tube, and 0.1 mL of 20% $TiCl_4$ in 36% HCl and 0.2 mL of 28% ammonia were added in turn. After reaction and centrifugation at 570g for 10 min, the precipitate was resuspended in 4°C acetone and the procedure was repeated twice. Finally, the precipitate was dissolved in 3 mL of 1 *M* H_2SO_4, and the absorbance of the solution was detected at 410 nm against a water blank. H_2O_2 content was calculated from a standard curve prepared in a similar way.

Extraction of Relevant Enzymes and Activity Assay

Cells (1 g, fresh weight) were ground with 2 mL of 4°C phosphate buffer (0.1 *M*, pH 6.0) containing 0.1 m*M* EDTA, 0.3% (w/v) Triton X-100, and 4% (w/v) polyvinylpyrrolidone (PVP) for SOD assay or containing 2 m*M* EDTA, 4 m*M* dithiothreitol, and 2% (w/v) PVP for assays of other enzymes. The mixture was homogenized at 4°C and centrifuged at 10,000*g* for 25 min. The supernatant was collected for enzyme assay. SOD activity was assayed by using the photochemical nitroblue tetrazolium method. Peroxidase was assayed at 470 nm using guaiacol as a substrate and CAT was assayed at 240 nm using H_2O_2 as a substrate *(18)*. Proteins were determined by the Lowry method using bovine serum albumin as the standard.

Determination of Ascorbate and Glutathione

Extracts of ascorbate and glutathione were performed the same as for enzyme assays. The contents of ascorbate and glutathione were determined as described by Knörzer et al. *(19)* and Brehe and Burch *(20)*, respectively.

Lipid Peroxidation

Malonyl dialdehyde (MDA), a final product of lipid peroxidation, was measured to evaluate the extent of lipid peroxidation in two-liquid-phase suspension cultures of *T. cuspidata*. The MDA in the cell suspensions was measured by thiobarbituric acid (TBA) reaction *(21)*. Briefly, fresh cells (200 mg) were homogenized in 4 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 13,000*g* for 10 min. To a 2-mL aliquot of the supernatant was added 4 mL of 20% TCA containing 0.5% TBA. The mixture was heated at 95°C for 30 min and quickly cooled down in an ice bath. After centrifugation at 10,000*g* for 10 min, the absorbance of the supernatant was measured at 532 nm, and the nonspecific turbidity was exempted by subtracting the absorbance of the same sample at 600 nm. MDA content was calculated using a molar extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical Analysis

The results presented are the means of six independent experiments. Each sample was assayed twice. Sample variability was given as the standard deviation of the mean, which meant that the means given were based

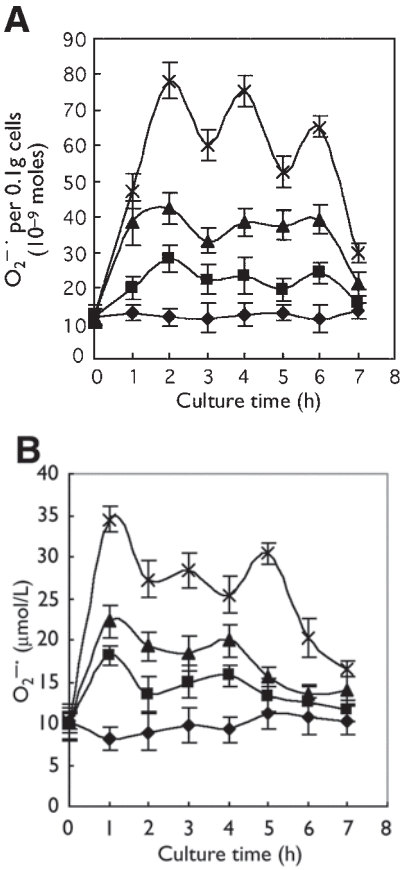

Fig. 1. Time course of $O_2^{-\bullet}$ burst in suspension cultures of **(A)** *T. cuspidata* and **(B)** extracellular medium induced by oleic acid. The preconditioned oleic acid was added to flasks on d 10 of cell culture. Oleic acid content (% [v/v]): (◆) 0 (control); (■) 2; (▲) 4; (×) 6. The data presented are the average of six independent experiments (means ± SD).

on 12 data points. The significance of differences between mean values was determined by a nonparametric Mann-Whitney rank sum test. Differences at $p < 0.05$ were considered significant.

Results and Discussion

Effects of Oleic Acid on Oxidative Stress

After the introduction of oleic acid, a significant oxidative stress was recorded in two-liquid-phase cultures of *T. cuspidata* cells. Figure 1A shows that at an oleic acid content of 6% (v/v), the amount of $O_2^{-\bullet}$ increased with time and reached a maximum after 2 h, which was 6.6-fold higher than that of the control culture. Figure 1B indicates that the accumulation of $O_2^{-\bullet}$ in the extracellular medium sharply increased after the addition of 6% (v/v) oleic acid and reached a maximum at about 1 h, which was 4.2-fold higher

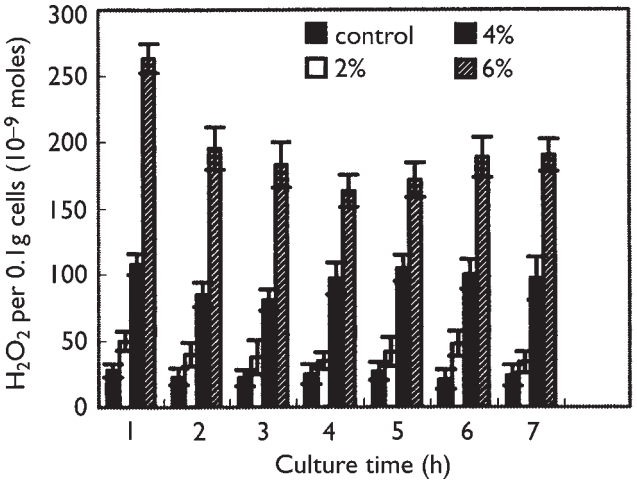

Fig. 2. Time course of intracellular H_2O_2 production induced by oleic acid in suspension cultures of *T. cuspidata*. The preconditioned oleic acid was added to flasks on d 10 of cell culture. The data presented are the average of six independent experiments (means ± SD).

than that of the control culture. In the case of 4% (v/v) oleic acid, the accumulation of $O_2^{-\bullet}$ increased compared with that of the control culture, but its intensity was much lower than that in the case of 6% (v/v) oleic acid. In the case of 2% (v/v) oleic acid, the accumulation of $O_2^{-\bullet}$ slightly varied compared with that of the control culture. Figure 2 shows that the maximal release of H_2O_2 was recorded at 1 h for oleic acid contents of 6, 4, and 2% (v/v), but the maximal amount of H_2O_2 released was, respectively, 9.4-, 3.9-, and 1.8-fold higher than that of the control sample. The amounts of $O_2^{-\bullet}$ and H_2O_2 released hardly changed with time for the control cultures and the cultures treated with 2% (v/v) oleic acid.

These results showed that *T. cuspidata* cells responded to organic solvents in two-liquid-phase cultures with oxidative stress. A number of studies indicated that H_2O_2 is a main component of oxidative bursts, with the possible involvement of $O_2^{-\bullet}$ *(22)*. Generally, there is a biphasic oxidative burst in response to an avirulent microbial pathogen *(23)*. The massive burst of $O_2^{-\bullet}$ in phase II, usually appearing at 2–6 h after challenge, only occurs as a response to avirulent pathogen, whereas the weak burst of $O_2^{-\bullet}$ in phase I, occurring within 1 h, is a nonspecific response to both avirulent and virulent pathogen. In our case, both the H_2O_2 and $O_2^{-\bullet}$ bursts were observed. The burst of $O_2^{-\bullet}$ began rapidly after the addition of oleic acid and lasted 7 h (Fig. 1A). It is likely that oleic acid induces the $O_2^{-\bullet}$ burst in a manner similar to an avirulent pathogen.

Involvement of NADPH Oxidase in Expression of Oxidative Bursts

It has been reported that NADPH oxidase is the main enzyme responsible for ROS generation in plants *(24)*. To assess the action of NADPH

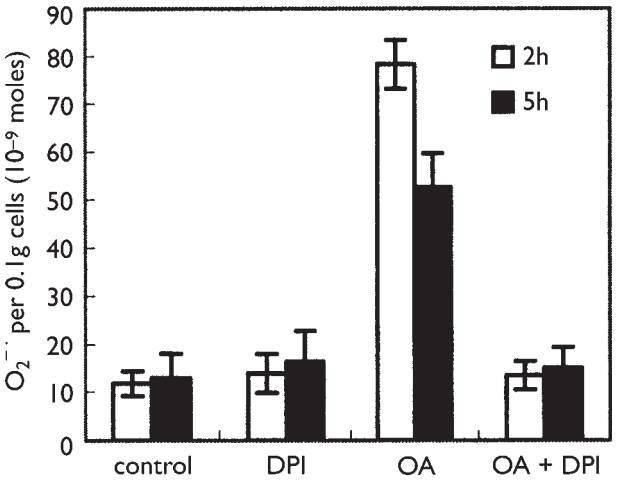

Fig. 3. Inhibition of oleic acid–induced $O_2^{-\bullet}$ burst by DPI (5 μ*M*). $O_2^{-\bullet}$ in suspension cultures was detected at (□) 2 and (■) 5 h after the addition of 6% (v/v) oleic acid (OA), respectively. DPI was added to the culture system 30 min before the addition of oleic acid. The data presented are the average of six independent experiments (means ± SD).

oxidase, diphenylene iodonium (DPI), a suicide inhibitor of NADPH oxidase, was introduced into the suspension cultures 30 min prior to the addition of 6% (v/v) oleic acid, and the amount of $O_2^{-\bullet}$ was detected at 2 and 5 h after the addition of oleic acid, respectively. Figure 3 shows that the $O_2^{-\bullet}$ burst induced by 6% (v/v) oleic acid was almost completely blocked by 5 μ*M* DPI, whereas DPI alone (without oleic acid) did not affect the $O_2^{-\bullet}$ level. These results suggest that oleic acid induced the $O_2^{-\bullet}$ burst through an NADPH oxidase-dependent pathway. The $O_2^{-\bullet}$ burst was also observed in the extracellular medium (Fig. 1B). This phenomenon might be explained as follows. The NADPH oxidase transferred electrons from intracellular NADPH to extracellular molecular oxygen and the generated $O_2^{-\bullet}$ was unable to penetrate through the biologic membrane owing to its negative charge, leading to the existence of the $O_2^{-\bullet}$ produced from the $O_2^{-\bullet}$ burst mainly in outside protoplasts and the release of only a small part of $O_2^{-\bullet}$ into the extracellular medium. It seems that activation of the cellular mechanisms of ROS generation was dependent on the $O_2^{-\bullet}$ originating from NADPH oxidase when it is considered that the pretreatment with DPI could completely inhibit the $O_2^{-\bullet}$ burst occurring at 2 and 5 h (Fig. 3).

Effects of Oleic Acid on Activities of Antioxidant Enzymes

The activities of the protective enzymes including SOD, APOX, and CAT changed on the addition of oleic acid (Figs. 4–6). Antioxidant enzyme activities were almost unchanged compared with those of the control cultures when the oleic acid content was 2% (v/v), which was consistent with less change in O_2^{-}. Intracellular SOD, APOX, and CAT were activated after the addition of 4% (v/v) oleic acid, and their activities reached maximum

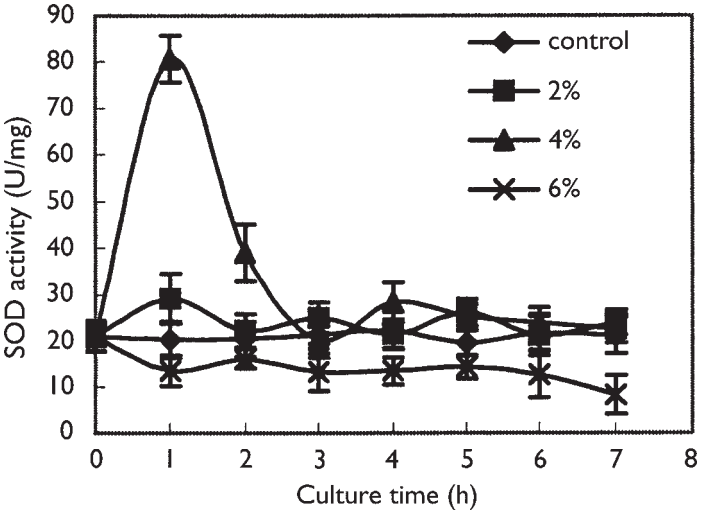

Fig. 4. Time course of intracellular SOD activity induced by oleic acid in suspension cultures of *T. cuspidata*. Preconditioned oleic acid was added to the flasks on d 10 of cell culture. One enzyme unit is defined as 50% inhibition of the colorimetric reaction. SOD activity is expressed as the enzyme units contained per milligram of intracellular proteins. Oleic acid content (% [v/v]): (◆) 0 (control); (■) 2; (▲) 4; (×) 6. The data presented are the average of six independent experiments (means ± SD).

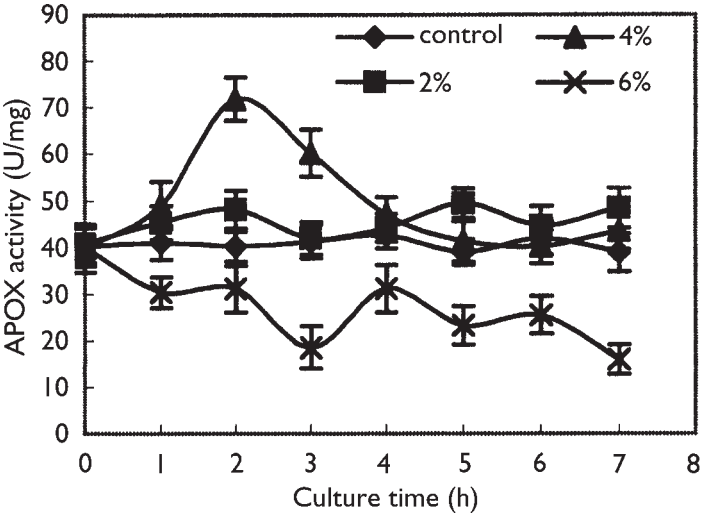

Fig. 5. Time course of intracellular ascorbate peroxidase (APOX) activity induced by oleic acid in suspension cultures of *T. cuspidata*. Preconditioned oleic acid was added to the flasks on d 10 of cell culture. One enzyme unit is defined as the variation of absorbance of 0.01 s^{-1}. APOX activity is expressed as the enzyme units contained per milligram of intracellular proteins. Oleic acid content (% [v/v]): (◆) 0 (control); (■) 2; (▲) 4; (×) 6. The data presented are the average of six independent experiments (means ± SD).

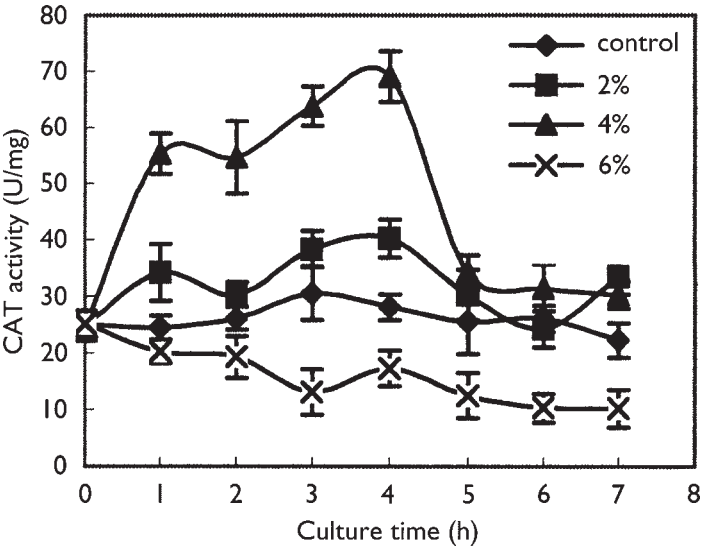

Fig. 6. Time course of intracellular CAT activity induced by oleic acid in suspension cultures of *T. cuspidata*. One enzyme unit is defined as the variation of absorbance of 0.01 min^{-1}. Preconditioned oleic acid was added to the flasks on d 10 of cell culture. CAT activity is expressed as the enzyme units contained per milligram of intracellular proteins. Oleic acid content (% [v/v]): (◆) 0 (control); (■) 2; (▲) 4; (×) 6. The data presented are the average of six independent experiments (means ± SD).

at about 1, 2, and 4 h, respectively. However, after the addition of 6% (v/v) oleic acid, the activities of the protective enzymes were much lower than that of the control. A transient decrease in APOX and CAT activities was observed at 3 h ($p < 0.01$).

A variety of abiotic stresses cause molecular damage to plant cells either directly or indirectly via the formation of ROS. The enzyme SOD dismutates $O_2^{-\bullet}$ to H_2O_2 and oxygen, CATs scavenge H_2O_2, and peroxidase utilizes H_2O_2 in the oxidation of various inorganic and organic substrates *(9)*. In the present study, oleic acid (4% [v/v]) elevated the levels of SOD, APOX, and CAT, the central components of the antioxidant defense system in the two-liquid-phase suspension cultures of *T. cuspidata*, and the effects varied with oleic acid content and treatment time of the cells. This pattern of antioxidative enzyme activity could imply that the tolerance mechanism involves a system that removes free radicals, preventing the accumulation of $O_2^{-\bullet}$ and increasing the requirement for the antioxidative enzymes. However, no increase in antioxidant enzymes was observed at an oleic acid content of 6% (v/v). This shows that *T. cuspidata* cells had a certain capacity to adapt to moderate organic solvent stress (4% [v/v] oleic acid) by developing an antioxidative defense system. However, at a high oleic acid content (6% [v/v] or more), the deleterious effect became more severe *(15)*, demonstrating that organic solvent toxicity irreversibly hindered the development and function of *T. cuspidata* cells. This might indicate that the defense responses played a role in a certain range of oleic acid content.

When the content of oleic acid reached a critical value (6% [v/v]), the overall ROS scavenging machinery was not induced.

Effects of Oleic Acid on Changes in Ascorbate and Glutathione

To investigate further the antioxidant mechanism, ascorbate and glutathione contents were detected. Within the cellular mechanism protecting against the deleterious effects of ROS, ascorbate and glutathione also play a fundamental role. The reduced ascorbate content did not change considerably in treated cells, except for a 35 ($p < 0.001$) and 26.7% ($p < 0.05$) decrease observed at 48 and 120 h after the addition of 6% (v/v) oleic acid, respectively (Table 1). However, the addition of 6% (v/v) oleic acid led to a substantial increase in cellular dehydroascorbate (DHA) content starting from 24 h, ranging from 227 ($p < 0.001$) to 52% ($p < 0.01$) at 24 and 120 h after the addition of 6% (v/v) oleic acid, respectively. A transient increase in total ascorbate content was observed at 24 (31.6%; $p < 0.01$) and 72 h (29.6%; $p < 0.01$) after the addition of 6% (v/v) oleic acid. Along with the increase in DHA content, AsA/total ascorbate ratios decreased markedly in the treated cells over the 120-h period. The range of decrease in redox state of the treated ascorbate pool was from 0.71 ($p < 0.01$) to 0.59 ($p < 0.001$), respectively. In the case of 4% (v/v) oleic acid, AsA content was much higher and DHA content was much lower than those in 6% (v/v) oleic acid-treated cells. Ascorbate contents were slightly changed compared with that of the control cultures when the oleic acid content was 2% (v/v).

By contrast, GSH and total glutathione contents declined significantly in treated cells after the addition of 6% (v/v) oleic acid (Table 2). GSH decreased about sevenfold, from 14.1 to 2.4 nmol at 24 and 120 h, respectively. GSH content was from 30 to 51% ($p < 0.05$) lower than that of the control cells at 48 and 120 h, respectively. The total glutathione content in treated cells changed in a similar way. The GSSG content when compared with that of the control at 24, 72, and 120 h after the addition of 6% (v/v) oleic acid was higher but declined to 18 and 40% of that of the control at 48 and 96 h. However, the control values varied markedly during the experimental period, so the changes were statistically nonsignificant. The GSH/total glutathione ratios in control and treated cells were comparable. Only at 120 h was the glutathione redox state in treated cells significantly ($p < 0.01$) lower than that of the control. A similar but less pronounced decrease in both GSH and total glutathione content, about threefold, was also observed in the control cells during the experimental period. Moreover, in both control and treated cells, the changes in GSH content were strongly time dependent (Table 2); similar trends were observed for the total glutathione content. Consistent with ascorbate content, in the case of 4% (v/v) oleic acid, GSH content was much higher and GSSG content was much lower than those of 6% (v/v) oleic acid-treated cells. Glutathione content was slightly changed compared with that of the control cultures when the oleic acid content was 2% (v/v), which was consistent with the lesser change in H_2O_2 and $O_2^{-\bullet}$.

Table 1
Changes in Ascorbate Content of Cells Cultured With Oleic Acid in Two-Liquid-Phase Cultures

	Time (h)					
Metabolite content[a]	0	24	48	72	96	120
Control						
AsA	35.2 ± 3.5	32.1 ± 4.8	37.3 ± 5.0	33.6 ± 7.7	27.1 ± 6.3	35.9 ± 7.0
DHA	5.3 ± 1.4	5.5 ± 1.3	5.3 ± 1.4	7.0 ± 1.6	5.1 ± 1.7	6.9 ± 1.9
Total ascorbate	40.5 ± 5.1	37.6 ± 5.7	42.6 ± 5.5	40.6 ± 8.8	32.2 ± 6.0	42.8 ± 9.1
AsA/total	0.87	0.85	0.88	0.83	0.84	0.84
2% Oleic acid						
AsA	35.2 ± 3.5	33.6 ± 3.1	35.1 ± 4.5	33.1 ± 5.1	26.9 ± 6.0	33.7 ± 6.7
DHA	5.3 ± 1.4	6.9 ± 2.2	7.3 ± 1.9	9.2 ± 2.4	6.9 ± 2.0	7.4 ± 2.7
Total ascorbate	40.5 ± 5.1	40.5 ± 4.6	42.4 ± 5.0	42.3 ± 5.9	33.8 ± 7.1	41.1 ± 6.2
AsA/total	0.87	0.83	0.83	0.78	0.80	0.82
4% Oleic acid						
AsA	35.2 ± 3.5	34.9 ± 3.9	28.2 ± 4.1*	33.8 ± 4.9	26.2 ± 4.2	32.7 ± 5.0
DHA	5.3 ± 1.4	12.1 ± 4.3*	9.2 ± 3.9*	14.6 ± 4.7**	8.3 ± 3.5	7.9 ± 3.8
Total ascorbate	40.5 ± 5.1	47.0 ± 5.2*	37.4 ± 3.4	48.4 ± 4.1	34.5 ± 4.9	40.6 ± 4.7
AsA/total	0.87	0.74*	0.75*	0.70**	0.76	0.81
6% Oleic acid						
AsA	35.2 ± 3.5	31.5 ± 4.2	24.1 ± 4.9***	30.8 ± 5.3	24.9 ± 6.1	26.3 ± 6.5**
DHA	5.3 ± 1.4	18.0 ± 5.3***	13.9 ± 4.2**	21.8 ± 5.9***	10.7 ± 3.7**	10.5 ± 3.1**
Total ascorbate	40.5 ± 5.1	49.5 ± 6.9**	38.0 ± 4.1	52.6 ± 6.5	35.6 ± 5.7	36.8 ± 5.1
AsA/total	0.87	0.64**	0.64***	0.59***	0.70**	0.71**

[a]Given in nanomoles of ascorbate extracted by the standard procedure from 1 g of fresh cells. Values are the means of six batches of experiments ± SD. Differences from the relevant control values are taken as significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Table 2
Changes in Glutathione Content of Cells Cultured With Oleic Acid in Two-Liquid-Phase Cultures

	Time (h)					
Metabolite content[a]	0	24	48	72	96	120
Control						
GSH	16.9 ± 4.7	14.5 ± 5.7	16.7 ± 6.0	9.1 ± 2.8	8.7 ± 4.3	4.9 ± 3.5
GSSG	0.5 ± 0.1	0.9 ± 0.3	1.0 ± 0.2	0.6 ± 0.2	0.9 ± 0.4	0.6 ± 0.3
Total glutathione	17.4 ± 3.2	15.4 ± 5.1	17.7 ± 6.2	9.7 ± 3.8	9.6 ± 4.3	5.5 ± 2.9
GSH/total	0.97	0.94	0.94	0.94	0.91	0.89
2% Oleic acid						
GSH	16.9 ± 4.7	14.8 ± 4.3	14.9 ± 3.7	8.1 ± 2.5	7.6 ± 3.9	4.0 ± 2.6
GSSG	0.5 ± 0.1	1.0 ± 0.4	0.7 ± 0.2	0.4 ± 0.2	0.5 ± 0.3	0.6 ± 0.2
Total glutathione	17.4 ± 3.2	15.8 ± 5.2	15.6 ± 4.6	8.5 ± 3.4	8.1 ± 3.0	4.6 ± 2.7
GSH/total	0.97	0.94	0.96	0.95	0.94	0.87
4% Oleic acid						
GSH	16.9 ± 4.7	14.7 ± 5.9	13.1 ± 6.1	7.0 ± 2.3*	6.8 ± 2.7*	3.5 ± 1.8
GSSG	0.5 ± 0.1	1.1 ± 0.5	0.8 ± 0.3	0.5 ± 0.2	0.6 ± 0.3	0.7 ± 0.4
Total glutathione	17.4 ± 3.2	15.8 ± 6.1	13.9 ± 6.5	7.5 ± 2.9*	7.4 ± 2.3*	4.2 ± 1.9
GSH/total	0.97	0.93	0.94	0.93	0.92	0.83
6% Oleic acid						
GSH	16.9 ± 4.7	14.1 ± 6.1	11.7 ± 5.3	5.1 ± 2.2**	4.9 ± 2.8**	2.4 ± 1.9*
GSSG	0.5 ± 0.1	1.2 ± 0.4	0.9 ± 0.3	0.7 ± 0.2	0.8 ± 0.3	0.9 ± 0.4
Total glutathione	17.4 ± 3.2	15.3 ± 7.7	12.6 ± 6.1**	5.8 ± 1.9**	5.7 ± 2.6**	3.3 ± 1.8*
GSH/total	0.97	0.94	0.93	0.88	0.86	0.73**

[a]Given in nanomoles of glutathione extracted by the standard procedure from 1 g of fresh cells. Values are the means of six batches of experiments ± SD. Differences from the relevant control values are taken as significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

The reduced forms of ascorbate and glutathione together with antioxidant enzymes are involved in ROS scavenging. Ascorbate and glutathione also act as primary antioxidants reacting nonenzymatically with singlet oxygen, superoxide, and hydroxyl radicals. As a secondary antioxidant, ascorbate participates in the regeneration of α-tocopherol operating in trapping alkylperoxyl radicals resulting from membrane lipid peroxidation and protects enzymes containing prosthetic transition ions *(25)*. Moreover, ascorbate has recently been shown to be involved in the signal transduction pathway leading to the salicylic acid-induced resistance to pathogens *(26)*. Glutathione, in addition to participating in ROS detoxification through the ascorbate–glutathione cycle, is a key component of the cellular redox balance, protecting SH-containing enzymes against oxidation and being involved in defense gene induction and used by glutathione-*S*-transferase in the detoxification of xenobiotics *(27)*. It was obvious that the reduced forms of ascorbate and glutathione were much higher at 4% (v/v) oleic acid than at 6% (v/v) in treated cells. Therefore, the ROS scavenging mechanism played a role in a certain range of oleic acid concentration (below 6% [v/v]). Beyond this, the overall ROS scavenging machinery was not induced.

Significant decreases in GSH and total glutathione pools were found. It has been reported that aging watermelon seeds undergo a significant decrease in glutathione content *(28)*. In our case, we speculated that the conjugation with glutathione should be a major route for the cells' detoxification, and that the resulting glutathione depletion might make the cells more susceptible to oxidation; for example, the addition of 6% (v/v) oleic acid generated more H_2O_2 and O_2^-.

Ascorbate and glutathione were induced as a response to oleic acid. This finding was consistent with that of a report by Kuźniak and Sklodowska *(29)* that in necrotrophic *Botrytis cinerea*-infected tomato leaves, glutathione content decreased. There was also a report that glutathione content increased and ascorbic acid content decreased in susceptible barley after biotrophic *Erysiphe graminis f. sp. hordei* infection *(30)*. It seems that the pathogen-activated antioxidative processes, including changes in ascorbate and glutathione turnover, could also be modified depending on the type of plant–pathogen interaction.

In contrast to the GSH results, slight modulations in AsA content and a concomitant burst of DHA formation were observed in the treated cells, suggesting that an appropriate concentration of oleic acid induced changes in AsA turnover. Heber et al. *(31)* reported that the light-dependent accumulation of univalent AsA oxidation product, which reflected the oxidative stress being suffered by plant cells, was strongly enhanced in old leaves.

Therefore, a suitable concentration of oleic acid affected cellular AsA and GSH. The decrease in antioxidant capacity of AsA and GSH was manifested by the accumulation of DHA in parallel with the decrease in ascorbate redox status in the glutathione pool, and these decreases should be at least partially the result of oleic acid-induced promotion of senescence.

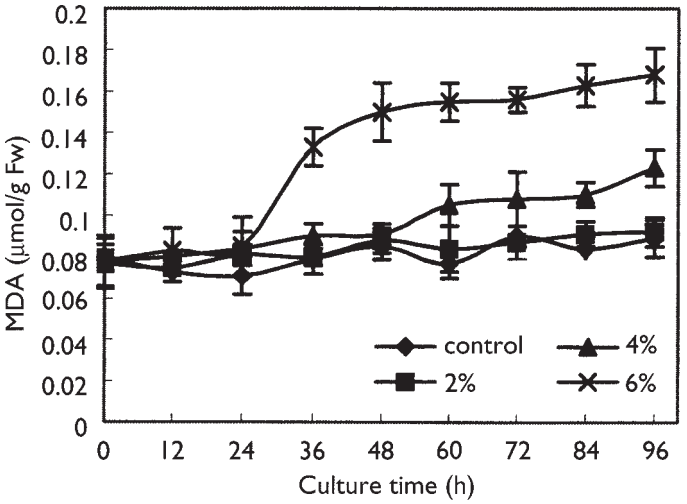

Fig. 7. Effect of oleic acid on membrane lipid peroxidation as revealed by MDA accumulation in two-liquid-phase cultures of *T. cuspidata*. Preconditioned oleic acid was added to the flasks on d 10 of cell culture. Oleic acid content (% [v/v]): (◆) 0 (control); (■) 2; (▲) 4; (×) 6. The data presented are the average of six independent experiments (means ± SD). FW, fresh weight.

This was consistent with a report by Ge et al. *(32)* that 6% (v/v) oleic acid induced apoptosis of *T. cuspidata* cells.

Membrane Lipid Peroxidation Induced by Oleic Acid

The transient generation of ROS is a hallmark of plant defense responses to a broad range of biologic and physical stimuli *(7)*. Protonation of $O_2^{-\bullet}$ can produce hydroperoxyl radicals ($^{\bullet}OH$, H_2O_2), which can convert fatty acids into toxic lipid peroxides, destroying biologic membranes. Measurement of MDA levels is routinely used as an index of lipid peroxidation under stress conditions. In the present study, MDA content increased significantly when *T. cuspidata* was subjected to 6% (v/v) oleic acid compared to controls (Fig. 7). This finding was consistent with the previous results that 6% (v/v) oleic acid caused irreversible damage to *T. cuspidata* cells and could not induce an antioxidant response. This suggested that increasing levels of endogenous *T. cuspidata* indirectly produced superoxide radicals, resulting in increased lipid peroxidation (MDA) and oxidative stress. Lipid peroxidation is a free-radical-mediated process *(16)*. The striking increase in lipid peroxidation when oleic acid was 6% (v/v), might be a reflection of the decline of antioxidative enzymes and the decrease in antioxidant capacity of AsA and GSH.

Conclusion

T. cuspidata cells sensed oleic acid and responded with an oxidative stress of triphasic characteristics. The roles of $O_2^{-\bullet}$ and H_2O_2 bursts had a

close relationship with defense responses induced by oleic acid. Our results indicated that the defense responses played a role in a certain range of oleic acid concentration. However, when oleic acid content reached a critical value (6% [v/v]), the overall ROS scavenging machinery was not induced. This was evidenced by no further increase in antioxidant enzymes and a decrease in antioxidant capacity of AsA and GSH that were manifested by the accumulation of DHA in parallel with the decrease in ascorbate redox status and the decline in the glutathione pool, leading to the emergence of peroxidation of membrane lipids of *T. cuspidata*. The reduced antioxidant machinery and altered redox homeostasis affected the protoplast fate, resulting in a programmed cell death.

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