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Role of Pyruvate and Ascorbate Production in Regulation of Antioxidant Enzymes and Membrane LPO Levels in *Fusarium Acuminatum*

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Abstract

The role of pyruvate and ascorbate in the regulation of superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase enzymes; and, therefore, membrane lipid peroxidation (LPO) levels in *Fusarium acuminatum* was investigated in media containing either glycerin or glucose as a carbon source, depending on the incubation period, in the range of 5–25 g/L. Increasing SOD activity between d 9 and 16 of the incubation period showed a positive correlation with a significant increase in pyruvate production up to 15 g/L of glycerin and glucose. In addition, maximum ascorbate production was observed at 15 g/L of glycerin as 82.5 ± 2.1 and 20 g/L of glucose as 54 ± 1.51, whereas CAT activity decreased with an increased concentration of both carbon sources. When compared with the LPO levels determined in media supplemented with glycerin and glucose, the minimum LPO level was 1.88 ± 0.028 nmol of malondialdehyde/g wet wt at 15 g/L of glycerin on d 16, at which it was also observed to have a maximum pyruvate and ascorbate production and SOD, CAT, and GSH-Px activities of 75 ± 1.42 μg/mL, 82.5 ± 2.1 μg/mL, 32.5 ± 0.634 μg/mL, 86.8 ± 2.58 IU/mg, and 1.867 IU/mg, respectively. These results indicate that the biosynthesis of pyruvate and ascorbate may be involved in the regulation of antioxidant enzymes, depending on the glycerin and glucose concentrations, and also this defense network was effective in preventing membrane damage from oxidative stress.

Index Entries: Antioxidant enzymes; ascorbate; carbon sources; *Fusarium* sp.; pyruvate.

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Introduction

All aerobically growing microorganisms encounter toxic reactive oxygen species (ROS) including the superoxide radical (O_2^-), H_2O_2, and the hydroxyl radical (OH·). ROS are mainly produced by leakage of electrons from the cellular electron-transport chain onto molecular oxygen, and they have been implicated in damage to virtually all types of major biologic macromolecules *(1–3)*. The rate of ROS production also plays an important role in regulating normal cellular function *(4)*. ROS metabolites modulate cellular redox state, signal transduction, and activation of transcription factors. It is possible that an age-related increase in electron leak and ROS production would be associated with an alteration in cell redox sate and impaired regulation of cell function *(5–7)*. Consequently, aerobic cells have evolved strategies in order to convert ROS into less toxic or nontoxic species. Although under normal physical conditions, the toxic effects of ROS are scavenged by antioxidants, oxygen radicals are scavenged by these antioxidants and oxygen radicals are implicated in damage to membrane lipids, proteins, and DNA, and their toxicity results when the degree of oxidative stress exceeds the capacity of the cell defense system *(8)*. The scavenging mechanisms are achieved by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, as well as nonenzymatic antioxidants, such as ascorbate, tocopherols, and glutathione *(9,10)*. Among these antioxidants, ascorbate is synthesized from glucose by means of a multistep pathway in which guanosine 5 diphosphate–mannose and comparatively rare sugar galactose are intermediates and galactano-γ-lactone is the last precursor. Some known functions of ascorbate, besides scavenging of free radicals, include an oxidation-reduction system in electron transport, a cofactor for a number of enzymes *(11)*, and a controlling factor in some eukaryotic organisms.

In aerobic cells, different metabolic routes, such as the glycolytic, the pentose phosphate, and the Enter-Doudoroff pathways, or carbon storage via glycogen or trehalose, are responsible for the net production or mobilization of carbon sources *(12)*. The effect of nutrient sources on pyruvate can also lead to changes in the rate of electron transport, which is the process in which most of the cellular ROS are generated.

In this article, we suggest what is the relationship between the production of pyruvate and ascorbate and the activities of antioxidant enzymes and lipid peroxidation levels in *F. acuminatum* depending on whether glycerin or glucose is the carbon source. The findings of our study may be helpful in learning more about the development and aging behavior of filamentous fungus as a eukaryotic model.

Materials and Methods

Medium and Growth Conditions

Cultures of *F. acuminatum* were maintained on supplemented potato dextrose agar. Spore production in the slants usually requires 5–7 d of

growth at 28°C. Spore concentration was determined by using a hemocytometer (3×10^8 conidia/mL). Cultures were grown in a synthetic medium called Armstrong Fusarium Medium (AFM) containing 1.1 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 1.6 g of KCl, 7.27 g of KNO_3, 2 ppm each for $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 2H_2O$, 1.0 mg of thiamine-HCl, and 0.05 mL of Tween-80 in 1 L of distilled water *(13)*. The culture medium was adjusted to pH 4.5. Cultures were incubated with agitation at 100 rpm at 28°C in 250-mL shaking flasks containing 100 mL of culture for 18 d. After the cultivation process, the cells were collected by centrifugation followed by washing twice with distilled water and kept at –20°C.

Preparation of Cell-Free Extracts

Wet *F. acuminatum* cells were frozen at –20°C and thawed overnight at +4°C and then suspended in a 20 m*M* phosphate buffer, pH 7.4, containing polypropylene glycol-1200 in a volume equal to 1.5 times its weight. A 600-µL cell suspension was ground in 1.5-mL plastic vials with 0.8 g of glass beads (0.5 mm ϕ) for 10 min. Cell debris was removed by centrifuging at 21,380*g* for 15 min.

Assay Methods in Cell-Free Extracts

SOD activity was measured in the crude extract by the method of Crosti based on the inhibitory effect of SOD on the spontaneous autooxidation of 6-hydroxydopamine (6-OHDA) at 490 nm *(14)*. One international unit is defined as the amount of SOD required to inhibit the initial rate of 6-OHDA autooxidation by 50%. In cell-free *F. acuminatum* cyanide-insensitive MnSOD activity was assayed in the presence of 5 m*M* KCN.

Catalase activity was determined in the crude extract by the method of Aebi *(15)*. According to this method, the decomposition of H_2O_2 is followed directly by the decrease in extinction at 240 nm and 37°C *(12)*.

GSH-Px activity was determined with Ransel kits by using a consecutive glutathione reductase reaction. The reaction was monitored by oxidation of NADPH followed at 340 nm and 37°C. This assay, based on the method of Paglia and Valentine *(16)*, requires cumene hydroperoxide as a substrate.

Pyruvate concentrations were determined by 2,4-dinitrophenylhydrazine. Colored complex formed that has a maximum absorbance at 520 nm *(17)*.

Vitamin C concentrations were immediately determined by 2,4-dinitrophenylhydrazine *(18)*.

Lipid peroxidation was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBA. The extinction coefficient at 532 nm of 153,000 M^{-1} cm^{-1} for the chromophore was used to calculate the MDA-like TBA produced *(19)*.

The protein content was determined by the method of Lowry et al. *(20)* using bovine serum albumin as the standard.

Statistical Analysis

Tukey test, one of the multiple comparisons, was used for analysis of statistical significance. The values are the mean of three separate experiments. The Pearson correlation was used to compare carbon sources at each given incubation time for each substrate and/or enzyme.

Results

The relationship among pyruvate and ascorbate production, antioxidant enzyme activities, and variations in membrane LPO level in *F. acuminatum* was investigated with respect to incubation period in the AFM medium when either glucose or glycerin was used as a carbon source in the range of 5–25 g/L.

Figure 1A shows that pyruvate production increased until d 16 for all glycerin concentrations ($r = 0.764$; $p < 0.01$), after which it decreased toward the end of the incubation period ($r = –0.425$; $p < 0.01$). In addition, pyruvate production increased significantly from 46.8 ± 1.49 to 75.0 ± 1.42 µg/mL when the glycerin concentration was increased from 5 to 15 g/L on d 16 ($p < 0.01$).

On supplementation with another carbon source, the production of pyruvate in *F. acuminatum* with respect to raised glucose concentration increased markedly at d 12 ($r = –0.582$; $p < 0.01$), and the maximum pyruvate production was determined as 48.2 ± 0.95 µg/mL at 15 g/L of glucose (Fig. 1B).

As shown in Fig. 2A, the biosynthesis of ascorbate in *F. acuminatum* increased markedly related to the increasing glycerin concentration from 5 to 20 g/L on d 9–12 and from 5 to 10 and 15 g/L at similar values on d 16. In addition, prolongation of incubation led to a decrease in ascorbate production ($p < 0.001$). The observed maximum ascorbate production for 20 and 25 g/L of glycerin was less when compared with 15 g/L. The highest ascorbate production was determined as 82.5 ± 2.1 µg/mL on d 16 of incubation in the medium containing 10 g/L of glycerin as a carbon source.

The increase in ascorbate production correlates well with the rise in glucose concentration used ($r = 0.565$; $p < 0.01$), and maximum ascorbate production was observed as similar values in media containing 20 and 25 g/L of glucose as 54.0 ± 1.51 and 52.1 ± 0.98 µg/mL, respectively, on d 16 (Fig. 2B).

As can be seen in Fig. 3A, the increase in SOD activity in *F. acuminatum* correlates well with the rise in glycerin concentration up to 15 g/L ($r = 0.697$; $p < 0.01$), and the highest activity was observed as 32.5 ± 0.634 IU/mg on d 16 of incubation. However, the maximum SOD activity values observed for 20 and 25 g/L of glycerin shifted to 16 d and were less when compared with 15 g/L of glycerine ($p < 0.001$). In addition, the alterations in SOD activity depending on the glycerin concentration during d 9–19 of the incubation period showed a positive correlation with the significant rise in pyruvate production ($r = 0.764$; $p < 0.01$), which may indicate that ROS were produced by an increase in pyruvate concentration.

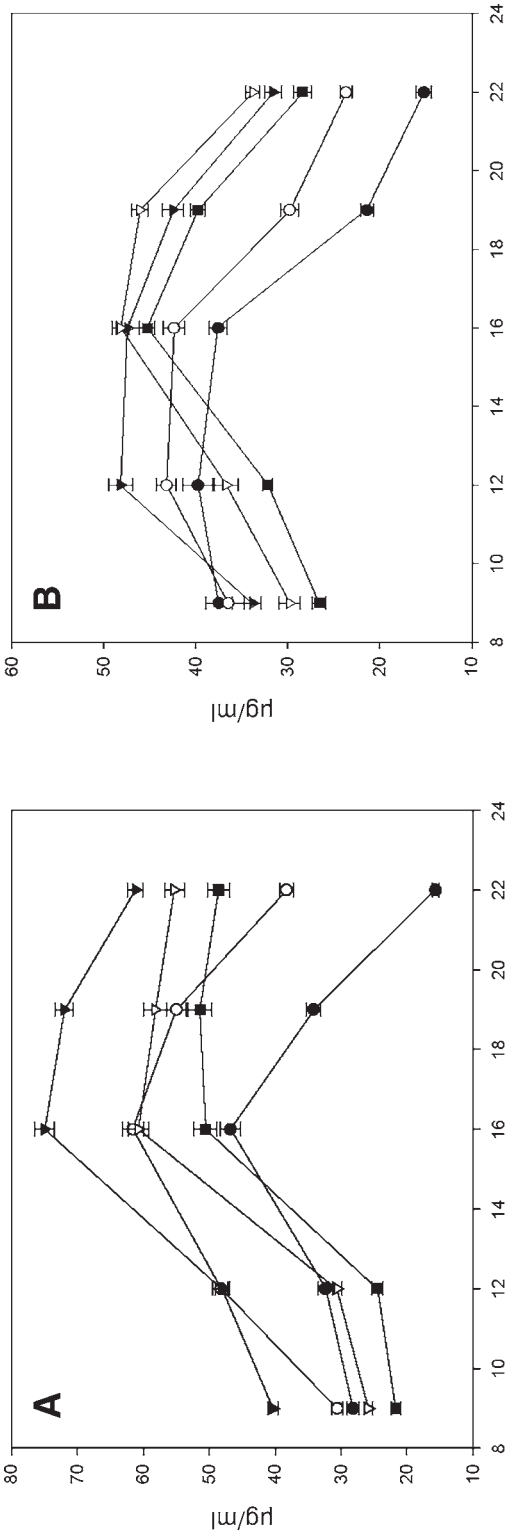

Fig. 1. Variations in pyruvate production in *F. acuminatum* depending on incubation period in media containing 5 (—●—), 10 (—○—), 15 (—▼—), 20 (—▽—), or 25 g/L (—■—) of **(A)** glycerin and **(B)** glucose. Values are the mean ± SEM for three separate experiments.

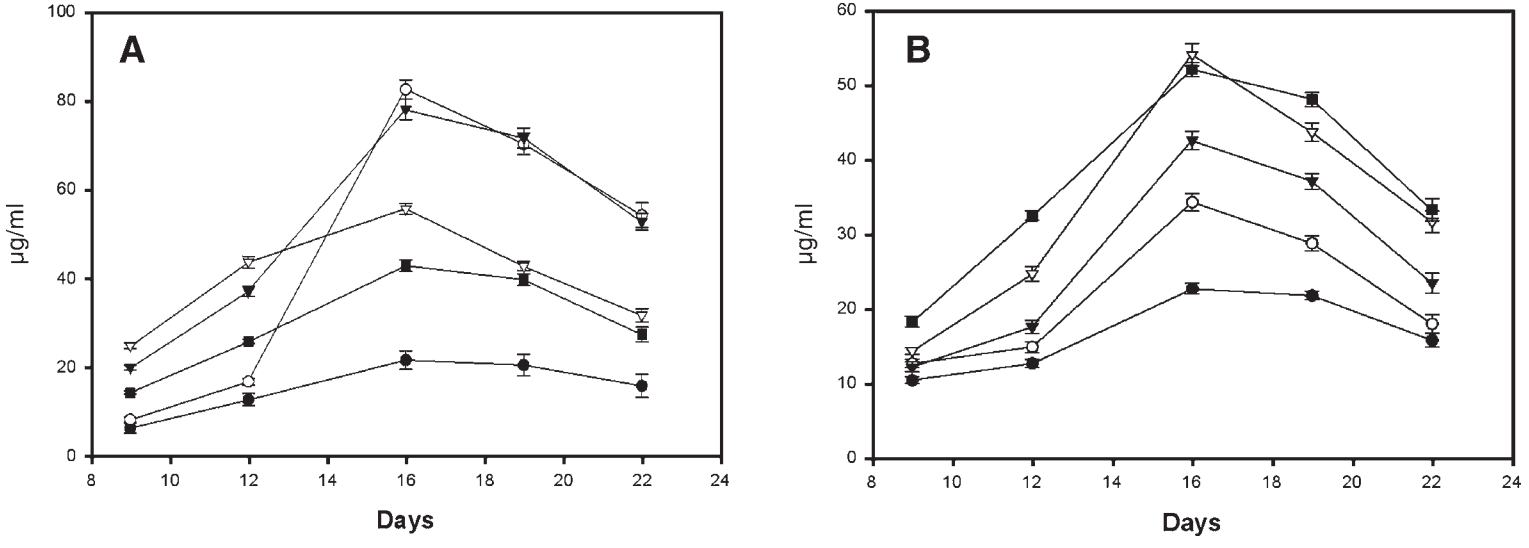

Fig. 2. Variations in ascorbate production in *F. acuminatum* depending on incubation period in media containing 5 (—●—), 10 (—○—), 15 (—▼—), 20 (—▽—), or 25 g/L (—■—) of **(A)** glycerin and **(B)** glucose. Values are the mean ± SEM for three separate experiments.

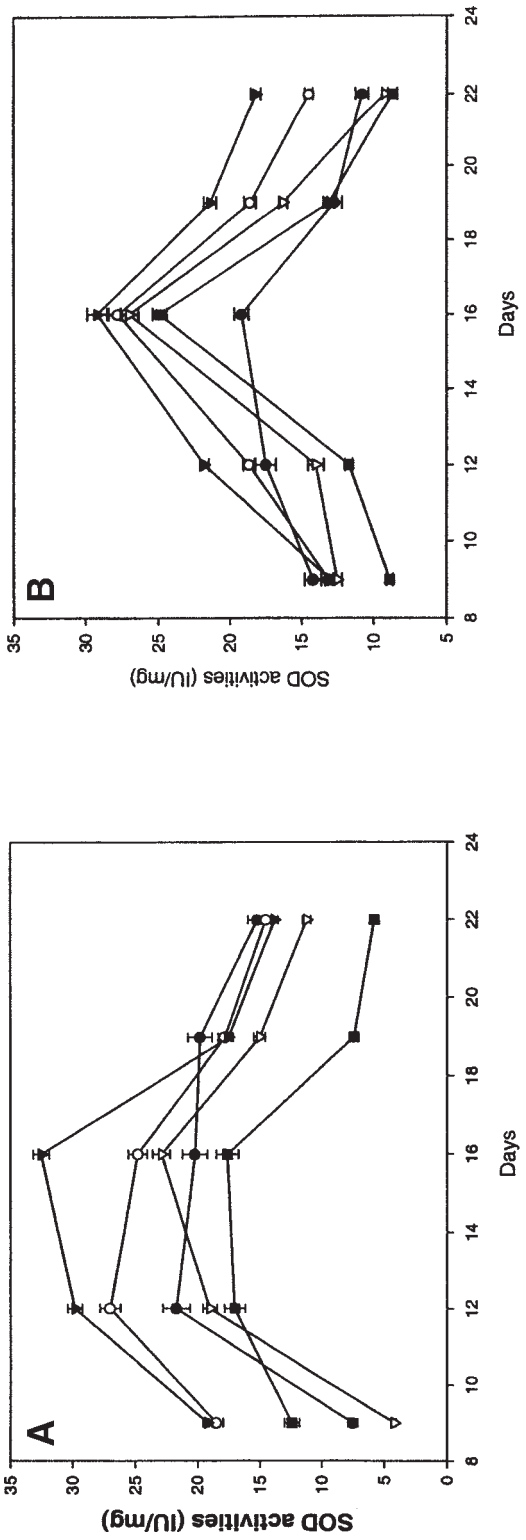

Fig. 3. Variations in SOD activity in *F. acuminatum* depending on incubation period in media containing 5 (—●—), 10 (—○—), 15 (—▼—), 20 (—▽—), or 25 g/L (—■—) of **(A)** glycerin and **(B)** glucose. Values are the mean ± SEM for three separate experiments.

As shown in Fig. 3B, SOD activity increased for all glucose concentrations used, reaching a maximum at 16 d ($r = 0.867$; $p < 0.01$), after which they began to decline ($r = –0.826$; $p < 0.01$). Variations in SOD activity depending on the glucose concentration were markedly increased from 19.2 ± 0.49 to 29.2 ± 69 IU/mg by raising the glucose concentration from 5 to 15 g/L, respectively, on d 16 ($p < 0.001$). A close quantitative relationship between pyruvate production and SOD activity was also observed during d 9–16 of incubation in medium supplemented with glucose ($r = 0.870$; $p < 0.01$) as well as glycerin.

Glutathione peroxidase activities for the media containing 15 g/L of glycerin and glucose, which were observed to have the highest SOD and CAT activities, were determined to be 1.867 and 1.172 IU/mg, respectively.

Figure 4A shows that maximum CAT activities were observed at d 12 for 5 and 10 g/L of glycerin, whereas they shifted to d 16 of incubation for 15–25 g/L of glycerin ($p < 0.001$). CAT activities tended to decrease as the glycerin concentration increased for d 9–12 ($r = –0.746$; $p < 0.01$), whereas they increased at 15 g/L during the 16- to 22-d incubation period ($r = 0.718$; $p < 0.01$). The highest increase in CAT activity was determined to be 105.8 ± 1.26 IU/mg in medium containing 5 g/L of glycerin on d 12 ($p < 0.01$).

Figure 4B shows that CAT activity in *F. acuminatum* increased markedly in the whole range of glucose concentrations for the first 16-d-period ($r = 0.485$; $p < 0.01$) and began to decrease in the following days of incubation ($r = –0.334$; $p < 0.01$). CAT activity, in contrast to SOD, tended to decrease as the glucose concentration increased for all incubation periods ($r = –0.808$; $p < 0.001$). The CAT activity decreased approx 6.68-fold for medium containing 25 g/L of glucose compared with medium containing 5 g/L of glucose on d 16.

Variations in LPO level as an indicative marker of oxidative cell damage in *F. acuminatum* reached a minimum on the incubation day when SOD and CAT activities reached maximum values (Fig. 5A).

As can be seen in Fig. 5B, the minimum LPO levels in *F. acuminatum* were determined on d 16 of the incubation period, when maximum SOD and CAT activities were also observed. The LPO level had a lower value in 15 g/L of glycerin than in 15 g/L of glucose whereas SOD, pyruvate, and CAT activities were at their highest.

Discussion

In aerobic metabolism, energy expenditure is directly related to oxygen consumption. Approximately 90% of the oxygen consumed by aerobic organisms is reduced at the mitochondrial inner membrane's electron transport system (ETS) during oxidative phosphorylation *(21,22)*. The respiratory chain, which plays a central role in the control of metabolic activity, is also a powerful source of ROS. Besides oxygen, alternative carbon sources such as glucose and glycerin can influence the rate of reactions in the glycolysis and citric acid cycles, as well as glyoxylate shunt *(23)*, and change

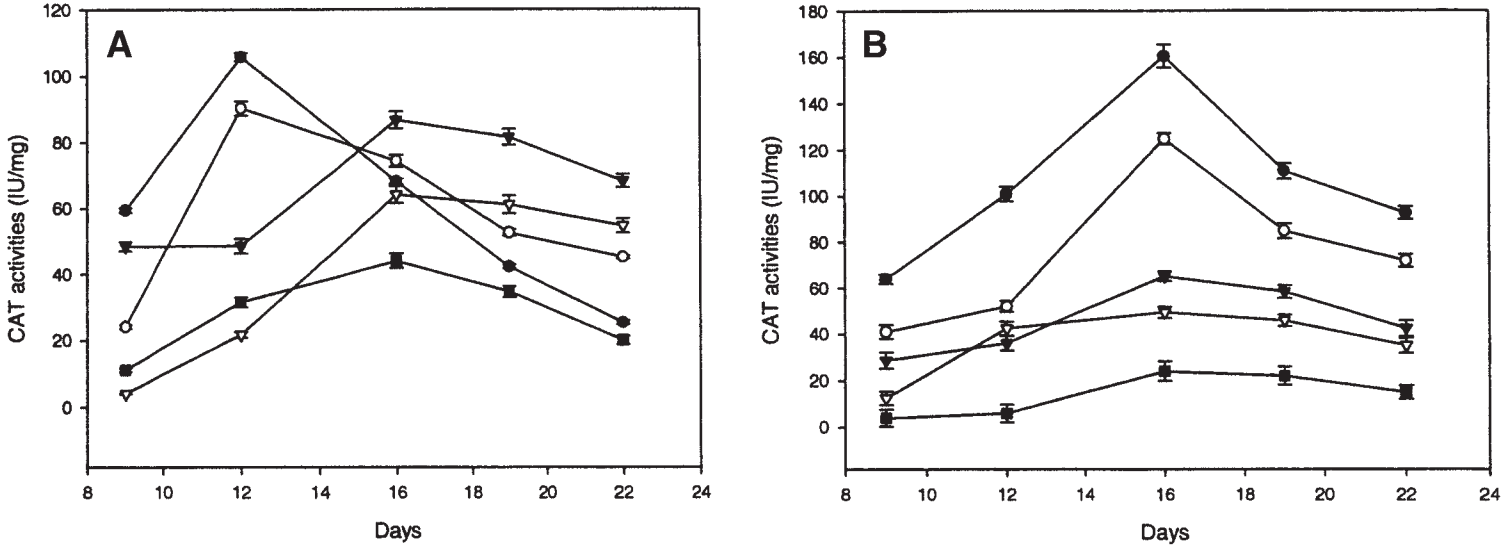

Fig. 4. Variations in CAT activity in *F. acuminatum* depending on incubation period in media containing 5 (—●—), 10 (—○—), 15 (—▼—), 20 (—▽—), or 25 g/L (—■—) of **(A)** glycerin and **(B)** glucose. Values are the mean ± SEM for three separate experiments.

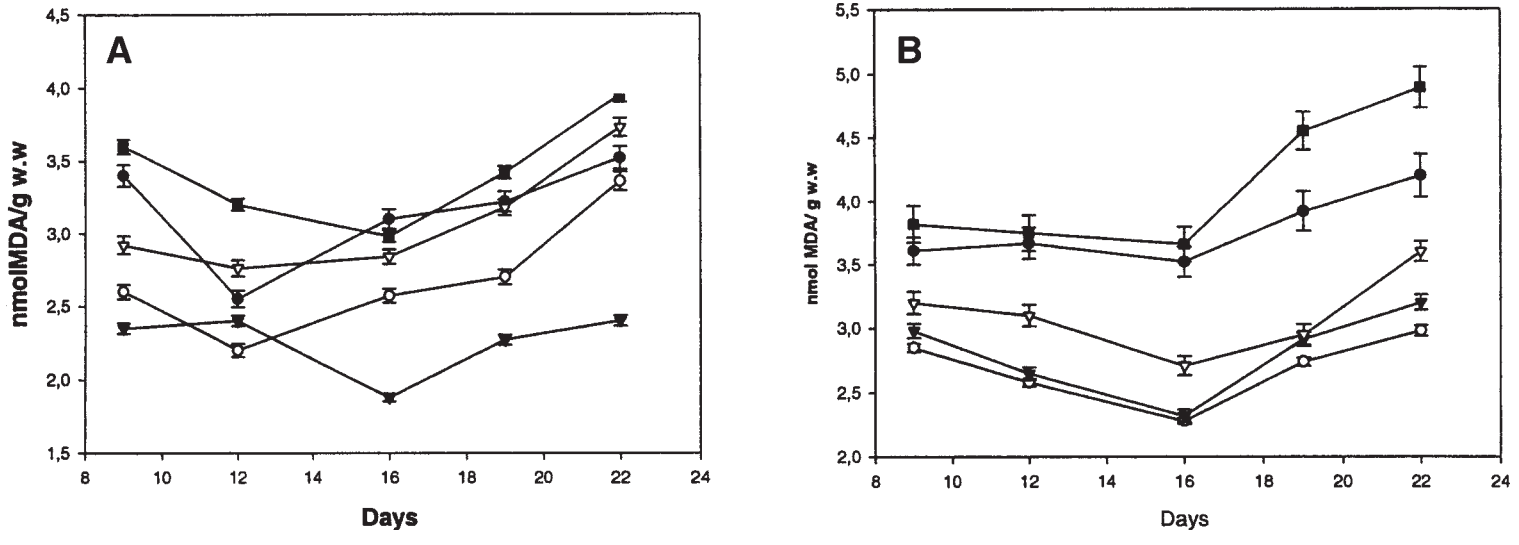

Fig. 5. Variations in LPO levels in *F. acuminatum* depending on incubation period in media containing 5 (—●—), 10 (—○—), 15 (—▼—), 20 (—▽—), or 25 g/L (—■—) of **(A)** glycerin and **(B)** glucose. Values are the mean ± SEM for three separate experiments.

the rate of production of ROS via ETS; therefore, variations in antioxidant enzymes and LPO level may change.

In addition, pyruvate, which is an important central metabolite in carbohydrate metabolism, not only functions in a metabolite reaction but is also involved in the regulation of metabolic pathways at several levels. In the present study, pyruvate production in *F. acuminatum* markedly increased when the concentration of glycerin and glucose increased from 5 to 15 or 20 g/L after d 16 or 12 of incubation, respectively. However, pyruvate production determined in glycerin-supplemented medium was higher than in glucose-supplemented medium. Glucose enters the carbon mechanism from the beginning of the glycolysis pathway; nevertheless, glycerol enters via glyceraldehyde-3-phosphate *(24)*. These systems may also depend on the posttranscriptional event *(25)*. In addition, according to some reports, glucose is transported by a proton symport when the organism is grown at low glucose concentration, but facilitated diffusion operates when the cells are grown at higher glucose concentration with respect to energy expenditure *(26,27)*. On the other hand, because glycerin is lipid soluble, uptake of this carbon source may occur by passive diffusion *(28,29)*. This change in pyruvate production in glycerol- and glucose-supplemented media depending on the concentrations can be explained by the altering of the carbon source's entering the carbon mechanisms and by the transport rate, which can also affect energy metabolism, specifically the electron transport in the inner mitochondrial membrane. This transport is one process in which most of the cellular ROS are generated, which may have caused the variation in antioxidant enzyme activities.

During the incubation period, ascorbate production gradually increased in parallel with the glucose concentration ($r = 0.565$; $p < 0.01$). This correlation was also determined in medium containing glycerin as an alternative carbon source up to 15 g/L ($r = 0.420$; $p < 0.01$), but this situation was inversely related to concentrations for 20 and 25 g/L ($r = –0.422$; $p < 0.01$). This difference could be owing to the fact that ascorbate is synthesized by different metabolic pathways with respect to carbon sources and concentrations in the growth media *(29)*.

The activities of SOD in *F. acuminatum* for 15 g/L of glycerin and glucose increased significantly as did pyruvate production. This situation shows that the induction of SOD activity in glycerin and glucose media is accompanied by a corresponding increase in pyruvate production.

In contrast to pyruvate production, ascorbate production, and SOD activity, CAT activity had a negative correlation in the investigated range of glycerin and glucose concentrations during all incubation periods ($r = –0.444$; $r = –0.808$; $p < 0.01$). This negative correlation between CAT and ascorbate is thought to be the result of two factors: (1) ascorbate through ascorbate peroxidase scavenges H_2O_2, a toxic byproduct arising from mitochondrial electron transport *(30)*; and (2) CAT was inactivated by ascorbate because semidehydroascorbate is an irreversible inhibitor acting probably on a histidine residue that is placed in the active center of CAT *(31)*.

According to our results, in spite of the decreases in CAT activity in *F. acuminatum*, LPO levels also declined with elevating SOD activity and pyruvate-ascorbate production up to a concentration of 15 g/L of glycerin during the first 16 d of incubation. This result indicates that SOD as well as ascorbate plays a key role in protecting the *F. acuminatum* cells against the potentially deleterious effects of ROS *(32)* that may have been produced by the increase in pyruvate production. In addition, the highest SOD activity and ascorbate production and minimum LPO level were determined in medium containing 10 g/L of glucose. This result also showed the protective effect of this antioxidant against lipid peroxidation.

The decline in pyruvate production and SOD and CAT activities in both carbon sources at 25 g/L compared with 20 g/L caused a sharp increase in membrane LPO levels during the entire incubation period. This may suggest that an osmotic upshift occurred for higher carbon concentrations than the optimum level because of the decreasing carbon concentration uptake of activity *(33)*.

Our results showed that pyruvate and ascorbate production and SOD and CAT activities were significantly decreased in glycerin (r = –0.425, –0.388, –0.720, and –0.524; $p < 0.01$) and glucose (r = –0.725, –0.574, –0.826, and –0.334; $p < 0.01$) whereas LPO levels increased after d 16 (r = 0.437, 0.454; $p < 0.01$). This result may indicate that as mitochondria including the proteins of the respiratory chain become more and more damaged, higher amounts of ROS are generated depending on the aging. As a result of an increase in the ROS production, it is possible that the impact of antioxidant enzyme systems decreases with aging. The link between mitochondrial ROS and antioxidant defense mechanisms supports the free-radical theory of aging *(34,35)*.

In summary, the data presented suggest that variations in antioxidant enzymes and ascorbate in *F. acuminatum* are closely related to pyruvate production. In addition, this scavenging network plays an important role in protecting biomembranes against activated ROS, which may change in the cell environment, by using different carbon sources and concentrations.

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