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Rosmarinic Acid and Antioxidant Enzyme Activities in *Lavandula vera* MM Cell Suspension Culture: A Comparative Study

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Abstract The growth and intracellular protein content of lavender (*Lavandula vera* MM) cell suspension culture was followed along with some antioxidant defense system members—non-enzymatic (rosmarinic acid) and enzymatic [superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6)]. It was found that the media content and the cultivation mode strongly influenced the production of plant defense compounds as well as the ratio between non-enzymatic and enzymatic ones. The bioreactor culture contains about two times more rosmarinic acid, superoxide dismutase, and catalase compared to the shake-flask cultivation. These findings are discussed with respect to the relative stress levels and plant antioxidant orchestra system. It was concluded that investigated defense system components (enzymatic and non-enzymatic) were closely associated in a complex balance. The three isoenzyme forms of SOD (Cu/ZnSOD, FeSOD, and MnSOD) in the cells of *Lavandula vera* were revealed by polyacrylamide gel electrophoresis analysis, and the FeSOD isoform exhibited highest activity.

Keywords Bioreactor · Catalase · Cell suspension culture · *Lavandula vera* · Rosmarinic acid · Superoxide dismutase

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Introduction

Metabolism of oxygen is connected with the formation of reactive oxygen species (ROS) such as superoxide anion radical ($^{\bullet}O^{-}_{2}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), which are an inevitable consequence of an aerobic lifestyle [1]. These molecules play an important role in the cytotoxic and mutagenic effects of oxygen and caused oxidization of membrane fatty acids, resulting in lipid peroxidation, oxidization of proteins, and DNA damage [2]. Aerobic organisms have developed strong mechanisms to prevent and repair oxidative injuries as they possess both enzymatic and non-enzymatic defense systems. The enzymatic ones form an important part of the cell's defensive repertoire against ROS [1]. The best well-known antioxidant enzymes are the intracellular ones [among others, superoxide dismutase (SOD) and catalase (CAT) are the most important], which protect against the toxic effects of oxidants generated within cells [3].

SODs (EC 1.15.1.1) are ubiquitous enzymes that catalyze dismutation of $^{\bullet}O^{-}_{2}$ to H_2O_2 and play a key role in the cellular defense against $^{\bullet}O^{-}_{2}$ stress. This enzyme is a metalloprotein with a redox metal in its active site. Four classes of SODs are distinguished, based on their metal cofactor: copper/zinc (Cu/ZnSOD), manganese (MnSOD), iron (FeSOD) [1, 4–9], and nickel (NiSOD). Barondeau et al. [10] noted that the nickel-containing SOD has been found only in *Streptomyces* and cyanobacteria so far. No information is available in scientific databases for the detection of NiSOD isoform in plant cells (source—www.scopus.com, accessed November 2008). In tobacco cells, the SOD isoforms are all nuclear encoded; however, the gene products are localized in different cell compartments [7]. MnSOD is present in the mitochondria, FeSOD is found in the chloroplasts, while Cu/ZnSOD is found both in the cytosol and the chloroplasts [7].

Catalase (EC 1.11.1.6) is a heme-containing enzyme that further catalyzes the conversion of the harmful H_2O_2 to H_2O and O_2. The enzyme is a tetramer with molecular mass more than 220 kDa and has a high K_m for H_2O_2, which makes it most efficient at scavenging high concentrations of H_2O_2 [9].

The non-enzymatic antioxidant mechanisms include a network of low molecular mass antioxidants [11]. Rosmarinic acid is a naturally occurring phenolic compound (an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid), being found to have a multitude of biological activities such as anti-inflammatory, anti-mutagenic, and antioxidant [12, 13]. This non-enzymatic low molecular antioxidant possesses also anti-viral, anti-bacterial, and anti-allergic properties [14, 15] and prevents the proliferation of human cancer cells [16]. Our previous studies demonstrated that the cell suspension culture *Lavandula vera* MM is a prominent producer of rosmarinic acid (RA) and the achieved volumetric yields were significantly higher compared to the nature-grown plant species [17–19]. Recently, we have reported on the *in vitro* antioxidant and radical-scavenging potential of extracts and preparations from a *L. vera* MM plant cell culture [20]. Thus, it is of particular interest to study the anti-oxidative defense system of *L. vera* MM cell suspension culture.

The present study was conducted to evaluate the relationship between enzymatic (SOD and CAT) and non-enzymatic (RA) factors with impact in antioxidant machinery of cell suspension culture *L. vera* MM grown in different media (standard Linsmayer and Skoog media and a modified one) and under different cultivation conditions (shaken flasks and mechanically agitated laboratory bioreactor).

Materials and Methods

Plant Cell Culture

The callus culture from *Lavandula vera* MM (*L. vera* MM) was obtained from sterile stem explants of oil-bearing *L. vera*, variety "Drujba" [21]. It was maintained on a solid Linsmayer Skoog (LS) media [22], supplemented with 30 g/l sucrose, 0.2 mg/l 2, 4-dichlorphenoxiacetic acid, and 5.5 g/l phytoagar (Duchefa, Haarlem, The Netherlands, cat. no. P1003). The *L. vera* MM cell suspension culture used as inoculum (20% *v*/*v*) was cultivated in liquid LS media of the same composition on a shaker (110 rpm, 26°C, in dark) for 7 days.

Experimental Design

L. vera MM cell suspension culture was cultivated in both shake flasks and bioreactor under the following conditions/modes:

- on a shaker, in standard LS media (500-ml conical flasks with 1/5 net volume) under the abovementioned conditions;
- on a shaker, in modified LS media (the changes to the standard LS media are expressed in the enhanced sucrose concentration to 6% and the nitrate/ammonia ratio of 40; [18]) under the abovementioned conditions;
- in 3-l (2.25 l working volume) bioreactor (BioFlo 110, New Brunswick, USA), equipped with propeller impeller, at 50% DO_2, 400 rpm, 29.9°C [23] in modified LS media [18].

Growth of *L. vera* MM Cells

L. vera MM was cultivated for 14 days with sampling every 2 days. The cells were separated from the culture media through filtration. After washing with water, the cells were harvested (fresh biomass) and frozen. The growth of *L. vera* MM was monitored through determination of dry biomass (gravimetrically at 60°C for ~24 h).

Rosmarinic Acid Extraction and Determination

Rosmarinic acid was extracted from the frozen biomass with 50% (*v*/*v*) ethanol–water mixture at 70°C for 1 h (3×20 min). The extract was evaporated to dryness, the dry residue was dissolved in 70% (*v*/*v*) ethanol, and then stored for 24 h at −10°C [24]. The precipitate was then filtered off and the filtrate was used for spectrophotometric (at 327 nm) quantitative determination of RA (spectrophotometer Shimadzu UV/vis 1240) [25].

Cell-free Extract Preparation

The cell-free extract was prepared following the method described by Krumova et al. [26]. Briefly, plant biomass was harvested by filtration, washed consecutively with distilled water and cold 50 mM potassium buffer (pH 7.8), and then resuspended in the same buffer. The cell suspension was disrupted by homogenizer model ULTRA Turax T25 IKA WERK and filtered through filter paper at 4–6°C during treatment (by chilling in an ice–salt bath).

Cell-free extracts were obtained after centrifugation at 13,000 ×*g* for 20 min at 4°C and the supernatant was used for further experiments.

Enzyme Determination

SOD activity was measured by the nitro blue tetrazolium reduction method of Beauchamp and Fridovich [27]. One unit of SOD activity was defined as the amount of SOD required to inhibit the reduction of NBT by 50% of maximum (absorbance at 560 nm was measured). Heating at 100°C for 30 min was used to distinguish between total SOD activity and enzyme SOD activity. The enzyme SOD activity was determined indirectly as a total SOD activity minus the remaining SOD activity after temperature treatment. CAT was assayed by the method of Beers and Sizer [28]. The decomposition of H_2O_2 was followed by measuring the decrease in absorbance at 240 nm. One unit of CAT is the amount that decomposes 1 μmol of H_2O_2 for 1 min at pH 7.0 and 25°C. Protein was estimated by the procedure of Lowry et al. [29], using crystalline bovine albumin as standard.

PAGE Analysis

Isoenzymes of SOD were separated on 10% non-denaturing polyacrylamide gel electrophoresis (PAGE) at 4°C. Equal amounts of 50 μg protein per lane were loaded. After electrophoresis, SOD activities in gels were visualized according to González et al. [30]. SOD isoenzymes were determined by a pre-stain incubation for 30 min in 50 mM potassium phosphate buffer (pH 7.8), containing 5 mM KCN or H_2O_2. Cu/ZnSODs were inhibited by cyanide, FeSODs were resistant to cyanide but were inactivated by hydrogen peroxide, and MnSODs were resistant to both inhibitors.

Statistical Evaluation of the Results

The results obtained in this investigation were evaluated from at least three repeated experiments using three parallel runs. The statistical comparison between different evaluations was determined by the Student's *t* test for mean interval estimation and by one-way analysis of variance followed by *F* test, with a significance level of 95% (α=0.05).

Results

Growth and Antioxidant Capacity During Cultivation in Shake Flasks in Standard Medium

The experiments were performed in the shake flasks containing standard LS media. Cell suspension growth attained its double time and maximum (15.5 g dry weight/l) after 6 and 8 days of cultivation, respectively, and then the beginning of stationary phase was observed (Fig. 1). The time profile of the intracellular protein content of *L. vera* MM cultures was measured during the cultivation period as well (Fig. 1). It showed an early increase in protein content up to a maximum at days 6–8 of cultivation (~135 mg/g dry weight) followed by a decrease of more than 30%.

The antioxidant capacities (SOD, CAT, and RA) of the shake-flask culture were followed as well (Fig. 2). RA was accumulated until day 8 of the cultivation process when the maximum production (75 mg/l) was achieved. The increase in RA production correlates well with the rise in biomass concentration. During the preliminary experiments, we found

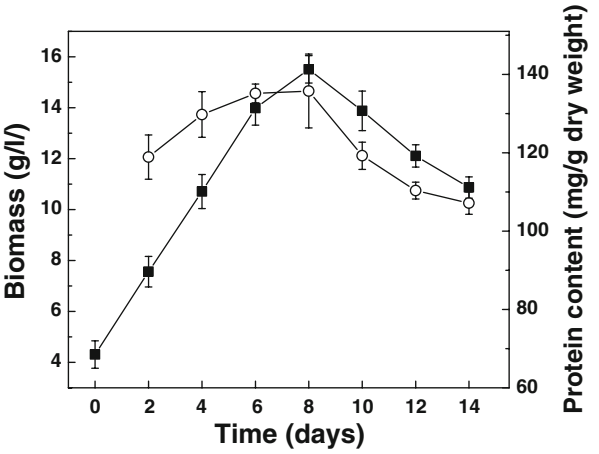

Fig. 1 Time courses of growth (■) and intracellular protein content (○) of *L. vera* MM cell suspension cultures during batch cultivation in shake flasks in standard medium

out that SOD activity consists of two components: one, which is a temperature-dependent (enzyme SOD) fraction and another, which is a temperature-independent fraction (containing the whole non-enzymatic defense machinery of lavender cells). In our previous experiments, we found that extract and preparation from *L. vera* MM with enriched content of rosmarinic acid exhibited strong superoxide radical-scavenging activities. Thus, to distinguish between SOD-like and SOD enzyme activity, high temperature was applied. It was found that the SOD biosynthesis in *L. vera* cultures demonstrated an opposite trend of rosmarinic acid one—it was highest from the beginning of the cultivation (1,665 U/g dry weight) and gradually diminished (1,082 U/g dry weight). CAT synthesis did not show significant changes during the cultivation exposing maximum between 10 and 12 days.

Kinetics of Cell Growth and Antioxidant Capacity During Cultivation in Shake Flasks in Optimized Medium

When the modified medium [18] was used for cultivation in shake flasks, *L. vera* MM cell suspension showed better growth and protein synthesis than the standard medium (Fig. 3). The growth curve was in the exponential phase up to day 10 of cultivation showing a

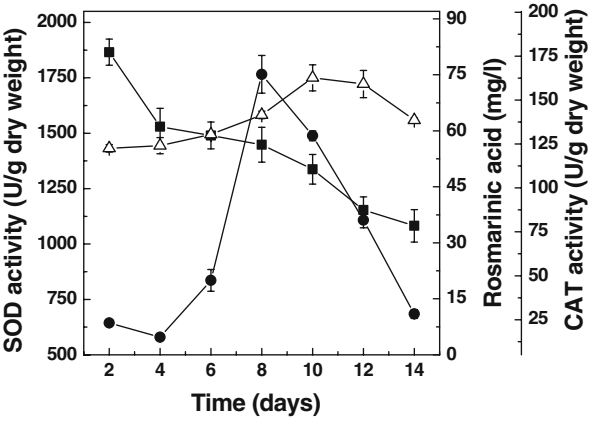

Fig. 2 Time courses of enzymatic and non-enzymatic antioxidant production in *L. vera* MM cells during batch cultivation in shake flasks in standard medium (△ SOD activity, ■ CAT activity, ● RA production)

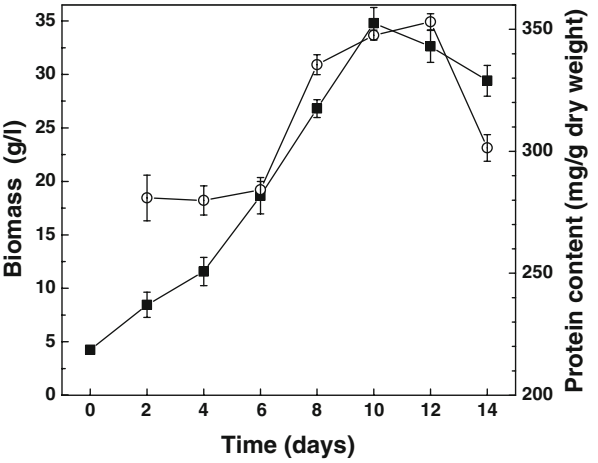

Fig. 3 Time courses of growth (■) and intracellular protein content (○) of *L. vera* MM cell suspension cultures during batch cultivation in shake flasks in optimized medium

growth profile similar to those in Fig. 1, but there was a significant difference in the rate of biomass formation. The cultivation in the optimized medium resulted in a 2.2-fold increase in the maximum dry weight of cells in comparison with the standard medium (34.8 vs. 15.5 g/l). A similar trend for protein synthesis was observed as well (353 vs. 135 mg/g dry weight).

The accelerated growth coincided with a significant improvement in RA biosynthesis compared to standard medium. The intracellular content of RA increased sharply until day 12 when ~19-fold higher maximum value was achieved followed by a sudden decrease of 35% (Fig. 4). SOD activity did not appear to be affected by the change of the medium components, but the time course of enzyme synthesis showed some differences. The cultivation started with high activity of SOD, which then decreased to 70–85% between days 8 and 10. Unlike the experiments on standard medium (Fig. 2), SOD biosynthesis in optimized medium demonstrated a sharp increase at the end of cultivation reaching the maximal value. On the contrary, CAT activity was increased about two times compared to the standard medium. It should also be mentioned that the time course of CAT synthesis exhibited an opposite trend to those of RA production.

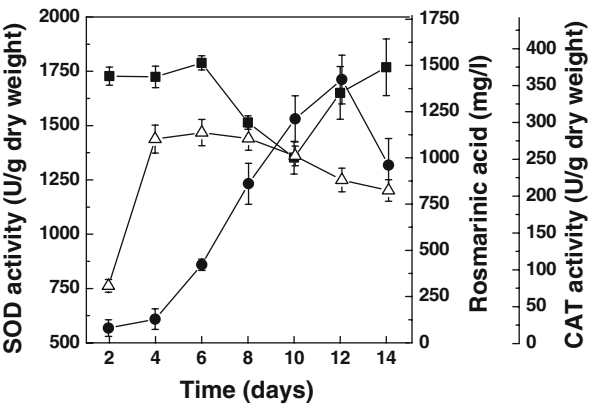

Fig. 4 Time courses of SOD activity (△), CAT activity (■), and RA production (●) changes in shake-flask cultures of *L. vera* MM, cultivated in optimized medium

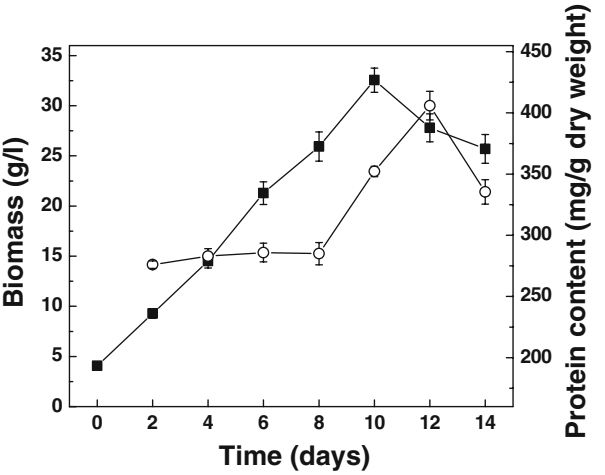

Fig. 5 Time courses of growth (■) and intracellular protein content (○) of *L. vera* MM cell suspension cultures during batch cultivation in bioreactor in optimized medium

Growth and Antioxidant Response of Cell Suspension Cultivated in Bioreactor in Optimized Medium

For comparing growth and antioxidant potential in different cultivation systems, *L. vera* MM cell suspensions were cultivated in a 3-l stirred tank reactor using the optimized medium. Experimental results in Fig. 5 showed that the biomass content and intracellular protein accumulation did not differ from those of the shake-flask cultures cultivated in the same medium (Fig. 3). In contrast to these findings, cultivation in bioreactor significantly improved the efficiency of RA biosynthesis (Fig. 6). The time course demonstrated a continuous increase up to the end of the cultivation, resulting in ~2-fold increase in intracellular RA content compared to the shake-flask cultures.

Comparison of both cultivation systems indicated that the up-scaling from shake flasks to bioreactors leads to approximately 20% enhancement of SOD production (Fig. 6). The time course of SOD synthesis showed a bell-curve-like fashion with maximum at days 6 and 8 (~2,095 U/g dry weight). The subsequent decrease in the SOD activity after day 8 coincided with strong enhancement of RA production. CAT activity showed a similar trend with a maximum a day 6 (~240 U/g dry weight).

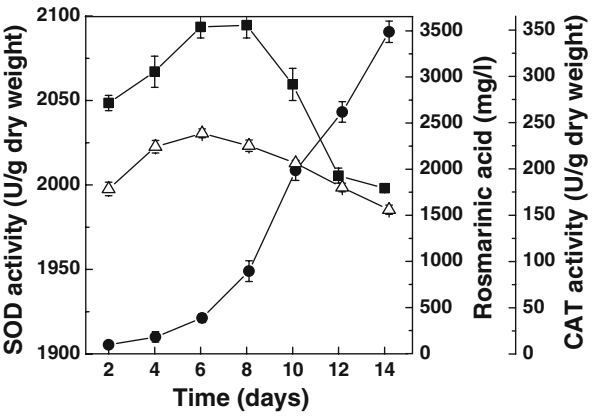

Fig. 6 Time courses of SOD activity (△), CAT activity (■), and RA production (●) changes in bioreactor cultures of *L. vera* MM in optimized medium

Isoenzyme Profiles of SOD in *L. vera* MM Cell Suspension Cultures

A characteristic SOD isoenzyme banding pattern for *L. vera* MM cells was found in native PAGE gels (Fig. 7, lanes 1, 2, and 3). Examination of SOD isoenzyme profiles in *L. vera* MM cells revealed three isoforms. According to the inhibitory analysis, two weak isoenzyme forms of SOD were found—Cu/ZnSOD, which disappeared completely upon KCN treatment but were revealed upon hydrogen peroxide, especially Cu/ZnSOD1, and FeSOD, which was sensitive to H_2O_2. The FeSOD isoenzyme is more specific for *L. vera* MM suspension cells. The activity of FeSOD is well pronounced at the early stage of cell culture cultivation (Fig. 7, lanes 1 and 2). One very weak MnSOD, resistant to KCN and H_2O_2, was revealed for a short time.

Discussion

Plant cells possess efficient systems for scavenging ROS and protecting themselves from harmful oxidative reactions [31, 32]. In the present study, the changes in the part of the enzymatic and non-enzymatic antioxidant defense system in *L. vera* MM grown in different cultivation modes were examined.

It was found that the replacement of standard medium by a modified one and the transfer from shake flasks to stirred bioreactor caused a significant increase in both the enzyme and non-enzyme antioxidant activities, especially those of RA. The presented results showed that, in shake flask cultures, RA content was 18 times higher on optimized medium than that obtained from the standard medium. Furthermore, when the cells were transferred from shake flasks to stirred bioreactor, the RA accumulation raised again two times. It is known that the formation of secondary metabolites in plant cells under stress is part of the defense mechanism [33]. RA, which belongs to the family of polyphenols, is a member of non-enzyme defense machinery of the plant cells. The antioxidant activity of phenolic compounds is mainly due to their redox properties [34]. As has been observed in our previous study, preparations from *L. vera* MM with enriched RA content possess strong radical scavenger activity [20]. Thus, to reduce the oxidative stress, *L. vera* MM accelerated the biosynthesis of RA, which traps free radicals.

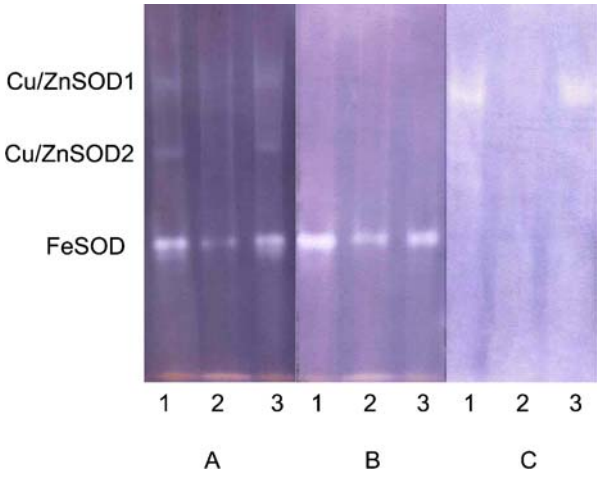

Fig. 7 SOD isoenzyme pattern of cell-free extracts from *L. vera* MM cultures after first (*1*), fourth (*2*), and seventh (*3*) days of cultivation in bioreactor. *A* revealing of SOD isoforms; *B* revealing of SOD isoforms after pre-treatment of gels with KCN; *C* revealing of SOD isoforms after pre-treatment of gels with H_2O_2. Similar results were obtained in two independent experiments with two gels

The increase in non-enzymatic antioxidant levels coincided with an increase in antioxidant enzyme activities, SOD, and CAT (Figs. 4 and 6). The increase in enzyme defense was more clearly expressed in bioreactor cultures than in shake-flask ones. Now, the obtained results showed that SOD activity remained unchanged with the use of optimal medium in shake-flask cultures. This could be partially explained by the fact that suspension cultures may be submitted to a lower oxidative stress in shake flasks than in bioreactor from one side. From the other side, RA, in which levels increased drastically in optimized medium in the flasks, could scavenge effectively $^{\bullet}O_2$ radicals instead of SOD [20]. A similar scavenging activity of RA from different plants has been experimentally estimated by several authors [35–37].

On the contrary, the transfer from flasks to bioreactor caused equal increase in enzymatic and non-enzymatic antioxidant defense (about two times for RA, SOD, and CAT). Most probably, bioreactor environments amplify the generation of reactive oxygen species in higher degree than in flasks. Previously reported data by a number of authors demonstrated that mechanical and shear stress in bioreactor induced oxidative bursts in plant cell suspension cultures [38–40] associated with a change in antioxidant enzyme activities [41]. In addition, the relatively higher dissolved oxygen levels (50% of air saturation) contributed to the enhanced oxidative burst.

It was found that the *L. vera* MM suspension cells possess an abundant FeSOD, indicating that this isoenzyme performs important antioxidant function together with the Cu/ZnSOD and/or MnSOD. According to Bowler et al. [42], the FeSOD isoenzymes are often not detected in plants. Nevertheless, Rubio et al. [43] proposed the possibility of other functions of FeSOD in nodules and leaves of alfalfa not connected with the scavenging of superoxide radicals generated during photosynthesis; it could be assumed supposedly that the abundant FeSOD isoenzyme in *L. vera* MM suspension cells has an additional antioxidant protection role essential for cell functioning.

It is worthwhile mentioning that the regulation of the components of both enzymatic and non-enzymatic defense is closely associated in a complex balance (Figs. 2, 4, and 6). At the beginning of the cultivation, when RA concentration was very low, SOD and CAT as primary cell defense compounds maintained high levels of activity. The increase in amount of RA led to a decrease in antioxidant enzyme activities. Probably, both antioxidant defense elements may compensate each other during cultivation, displaying the orchestra-like work of the plant cells.

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