Biochemistry (Moscow), Vol. 70, No. 3, 2005, pp. 316-321. Translated from Biokhimiya, Vol. 70, No. 3, 2005, pp. 390-396.

Effect of Salicylic Acid on Protein Composition of Tatar Buckwheat *Fagopyrum tataricum* Calluses with Different Ability for Morphogenesis

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Received March 26, 2004
Revision received June 22, 2004

Abstract—The effect of salicylic acid on the content of soluble proteins and individual polypeptides in Tatar buckwheat *Fagopyrum tataricum* calluses differing in ability for morphogenesis was studied. Changes in the protein composition of the calluses cultivated in the dark and in the light indicated the higher sensitivity of the non-morphogenic callus. Different response of callus cultures to salicylic acid and conditions of cultivation (light, darkness) is suggested to be associated with the antioxidant defense system, which is, in particular, characterized by the hydrogen peroxide content in the calluses. Salicylic acid increased the H_2O_2 content in non-morphogenic calluses more strongly than in morphogenic calluses, and the difference was more significant for the calluses cultivated in the light.

Key words: *Fagopyrum tataricum*, buckwheat callus, salicylic acid, hydrogen peroxide

Salicylic acid (SA) is involved in various physiological processes [1-4], and its content is known to markedly increase in the presence of elicitors, pathogens, and exogenous H_2O_2 [5-7]. Treatment of plants with SA increased the H_2O_2 content and strengthened the resistance against infection [1, 8, 9]. H_2O_2 is a source for generation of the strongest known oxidant in biosystems, the radical $^{\cdot}OH$ [10]. Reactive oxygen species (ROS) destroy DNA, proteins, and lipids, and, thus, damage cells [11, 12].

H_2O_2 and SA (which induces accumulation of H_2O_2) can act as signals for expression in uninfected cells of defense genes and generation of some pathogen-related proteins (PR-proteins), which determine the local and systemic resistance [1, 13, 14].

The morphogenic callus of Tatar buckwheat *Fagopyrum tataricum* (L.) Gaertn is interesting because it retains for a long time (up to 10 years) specific morphology, ability for regeneration, and chromosome stability, and these features rather seldom occur in *in vitro* cultures. A non-morphogenic callus appears on the surface of the morphogenic callus as separate foci, on average, with incidence of one case per 30-40 passages [15]. The morphogenic and non-morphogenic calluses are different in proliferative activity: the non-morphogenic callus displays more rapid accumulation of biomass and more active cell division.

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The purpose of this work was to compare the effect of SA on contents of soluble proteins and individual polypeptides in Tatar buckwheat calluses differing in morphogenicity.

MATERIALS AND METHODS

The study was performed on morphogenic and non-morphogenic calluses prepared from immature germs of Tatar buckwheat *F. tataricum* (L.) Gaertn. Buckwheat seeds were sterilized with 40% NaOCl. The germs were isolated aseptically and placed onto RX medium [15] containing salts by B5 [16] and supplemented with thiamine (2 mg/liter), pyridoxine (2 mg/liter), nicotinic acid (1 mg/liter), mesoinositol (100 mg/liter), casein hydrolyzate (2000 mg/liter), 2,4-dichlorophenoxyacetic acid (2 mg/liter), α-naphthaleneacetic acid (0.5 mg/liter), indole-3-acetic acid (0.5 mg/liter), kinetin (0.2 mg/liter), sucrose (25 g/liter), and agar (8 g/liter). The cultures were incubated in the dark at 25 ± 2°C. The generated calluses were separated from the germ tissues and cultivated separately on the RX medium at the same temperature in the dark or in the light. The passage duration was 21 and 28 days for the non-morphogenic and morphogenic calluses, respectively. The morphogenic callus consisted of proembryogenic cell complexes (PECC) and soft callus cells, which were derivatives of the PECC cells produced dur-

0006-2979/05/7003-0316

ing their loosening. The non-morphogenic callus was a loose actively proliferating culture, which consisted only of parenchyma cells. The ability of morphogenic callus for various kinds of morphogenesis (embryogenesis, organogenesis, histogenesis) has been shown [17].

Proteins were extracted with 50 mM Tris-HCl buffer containing 5% mercaptoethanol (pH 7.4) at 4°C. Content of soluble proteins was determined by the Bradford method [18]. One-dimensional electrophoresis of proteins was performed in 12% polyacrylamide gel in the presence of 2% SDS by the Laemmli method [19] in vertical gel plates. The following proteins (Serva, Germany) were used as markers: bovine serum albumin (66 kD), ovalbumin (45 kD), trypsinogen (24 kD), and β-lactoglobulin (18.4 kD). The same amounts of the proteins were applied onto the gel start areas. After fixation, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Serva). The molecular weights of polypeptides were determined as described in [20].

Salicylic acid (Reakhim, Russia) was added into the media at final concentrations from 1 μM to 1 mM after sterilization through membrane filters with pore diameter of 0.22 μm. On acidification of the medium at the SA concentration of 0.1-1 mM, it was alkalified with 0.1 M NaOH before autoclaving.

The content of H_2O_2 was determined by our modification of the method described in [21]. The callus tissue was ground in cold acetone at the ratio of 1 : 2 and centrifuged for 30 min at 4000*g* in the cold. The supernatant fluid and the reagent were mixed at the ratio of 1 : 1, and after 1 h the absorption was determined with a KFK-3 photometer (Russia) at 560 nm. The H_2O_2 content was determined using a calibration curve constructed with known concentrations of H_2O_2. The reagent included 0.5 mM ammonium iron (II) sulfate, 50 mM H_2SO_4, 0.2 mM Xylenol Orange (Sigma, USA), and 200 mM sorbitol.

Experiments were performed in three biological replicates. Data were processed using the Statgraph program, and plots were prepared with Microsoft Graph program. On the plots, the data are presented as the means ± standard errors.

RESULTS AND DISCUSSION

Addition of SA (1 μM-1 mM) into the culture media of buckwheat grown in the dark decreased the content of soluble proteins in both morphogenic and non-morphogenic calluses by the passage termination, and the decrease was more pronounced in the non-morphogenic calluses (Fig. 1). The inhibitory effect was the greatest on cultivation of the non-morphogenic calluses in the presence of 1 mM SA. The decrease in the protein content in

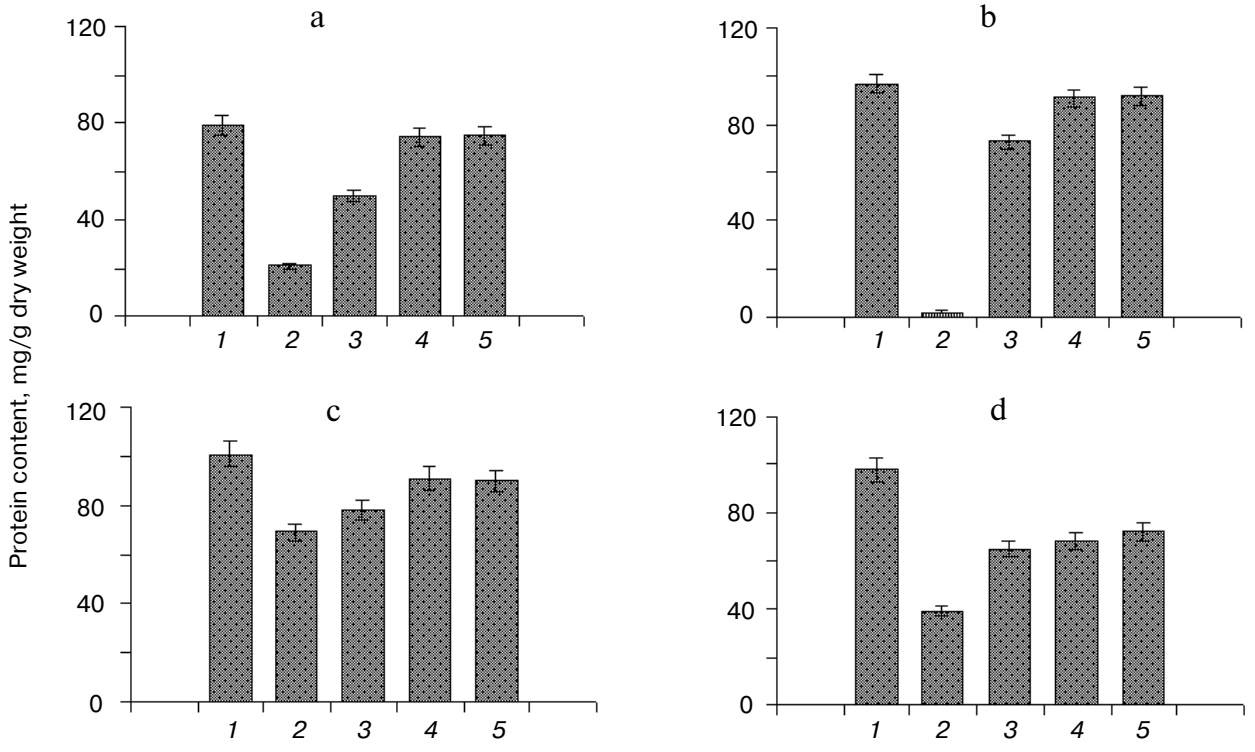

Fig. 1. Effect of salicylic acid on the content of soluble proteins in non-morphogenic buckwheat calluses in the dark (a) and in the light (b) and in morphogenic calluses in the dark (c) and in the light (d) in the presence of SA at the concentration of: 1 mM (*2*), 0.1 mM (*3*), 10 μM (*4*), 1 μM (*5*); *1*) control.

the presence of SA was also shown in other works, e.g., when pod and maize seeds were wetted in the presence of 10 μM-1 mM SA [22, 23].

The light differently influenced the contents of soluble proteins in calluses with different morphogenicity. In the morphogenic calluses, the content of soluble proteins in the light remained the same as in the calluses cultivated in the dark (Fig. 1). By the passage termination, in the non-morphogenic calluses the light caused an increase in the content of soluble proteins compared to the dark calluses. Light is known to activate synthesis of structural and enzymatic proteins [24-26], in particular, light increased activities of enzymes of the Benson–Calvin cycle via ATP or a reducer produced in chloroplasts [27-29].

In the non-morphogenic calluses 1 mM SA decreased the content of soluble proteins in the light more significantly than in the dark. Lower concentrations of SA (1 μM-0.1 mM) induced in the same calluses lesser changes in the protein content than in the control either in the light or in the dark. In the morphogenic calluses the same concentrations of SA (1 μM-0.1 mM) decreased the content of soluble proteins in the light more significantly than in the dark.

To study in more detail the SA effect on the contents of soluble proteins in morphogenic and non-morphogenic calluses, we determined their polypeptide composition. By termination of the passage changes in contents of some polypeptides were found in both types of calluses cultivated in the dark (Fig. 2). Figure 2 shows SA-caused changes in contents of some polypeptides in both morphogenic and non-morphogenic calluses. In the non-morphogenic calluses, the effect of SA was more pronounced. Salicylic acid (1 mM) significantly decreased the contents of 33-, 48-, 63-, 68-, and 76-kD polypeptides in the non-morphogenic calluses and of 40-, 41-, and 54-kD polypeptides in the morphogenic calluses cultivated in the dark. The content of 68-kD polypeptide was lowered in the non-morphogenic callus under the influence of all SA concentrations studied.

Salicylic acid changed the polypeptide spectrum in both types of calluses cultivated in the light (Fig. 2). In the non-morphogenic callus, SA (1 mM) decreased the levels of all polypeptides except for the 41-kD polypeptide, the content of which increased. Other concentrations of SA were less effective. In the morphogenic callus SA (1 mM) decreased the levels of 33-, 40-, 41-, 46-, and 54-kD polypeptides. The levels of 40-, 41-, and 54-kD polypeptides were also changed in the calluses cultivated in the dark. In the morphogenic callus cultivated in the light SA (1 μM-0.1 mM) increased the content of 41-kD polypeptide, the levels of other polypeptides being changed to a lesser degree. The SA-induced changes in the polypeptide levels by termination of the passage seem to be associated with appearance or disappearance of isoenzymes.

At present, reasons for the light-dependent effect of SA on proteins of calluses are not clear. This effect is suggested to be associated with the light-caused activation of enzymes of phenol metabolism (in particular, phenylalanine-ammonia lyase) and increase in their biosynthesis [30] that increases the content of endogenous SA.

It was suggested that the phytochrome system of plants could modulate the response to SA [31]. Thus, in response to infection with *Pseudomonas syringae* mutant *Arabidopsis* plants (phyA-phyB) deprived of phytochromes displayed a decreased production of damage foci and absence of induction of PR-proteins; by contrast, mutants with increased content of phytochromes (psi2) were characterized by an increased production of damage foci in the supersensitivity reaction and the increased expression of PR-protein mRNA.

It is known that SA affects activities of enzymes of oxidative metabolism [32, 33]. In the presence of SA, the H_2O_2 content changes rather rapidly (e.g., accumulation of H_2O_2 in leaves of *Arabidopsis* was recorded even 2 h after incubation with SA). The H_2O_2 content in plant tissues directly depends on the SA concentration used [9]. Unlike other ROS, the H_2O_2 molecule is relatively stable [34]. Therefore, the increased content of H_2O_2 remains for a long time in the presence of an inducer (e.g., in the callus of *Astragalus adsurgens* until the 28th day of cultivation on medium with 0.15 mM SA) [35].

The significant decrease in the contents of soluble proteins and individual polypeptides observed by us in the presence of 1 mM SA was probably mediated by production of H_2O_2, because treatment with H_2O_2 has been recorded to decrease the protein content in rice leaves [26].

To elucidate the possible mechanism of the unfavorable effect of high concentration of SA (1 mM) on the protein metabolism of calluses, we determined the content of H_2O_2 in both types of calluses cultivated in the presence of SA in the light and in the dark.

The content of H_2O_2 in plant tissues varies over a wide range depending on the plant species and organ. Thus, the H_2O_2 content in the embryogenic callus of *Astragalus adsurgens* was during the passage, on average, 0.175 [35], in tobacco leaves 5.7 [21], and in rice leaves 45 μmol/g wet weight [36].

The morphogenic and non-morphogenic buckwheat calluses were significantly different in the H_2O_2 content. The non-morphogenic and morphogenic calluses contained 9.1 and 2.8 μmol H_2O_2/g wet weight, respectively (3.2-fold difference), on average, for 7 days of cultivation in the dark.

Changes in the H_2O_2 content in the non-morphogenic buckwheat calluses cultivated in the dark and in the light were different (Fig. 3). The content of H_2O_2 sharply increased even after 1 day of cultivation in the light, as compared to the dark culture. After 3 days of cultivation in the light, the H_2O_2 content significantly lowered. In the morphogenic calluses, the H_2O_2 content

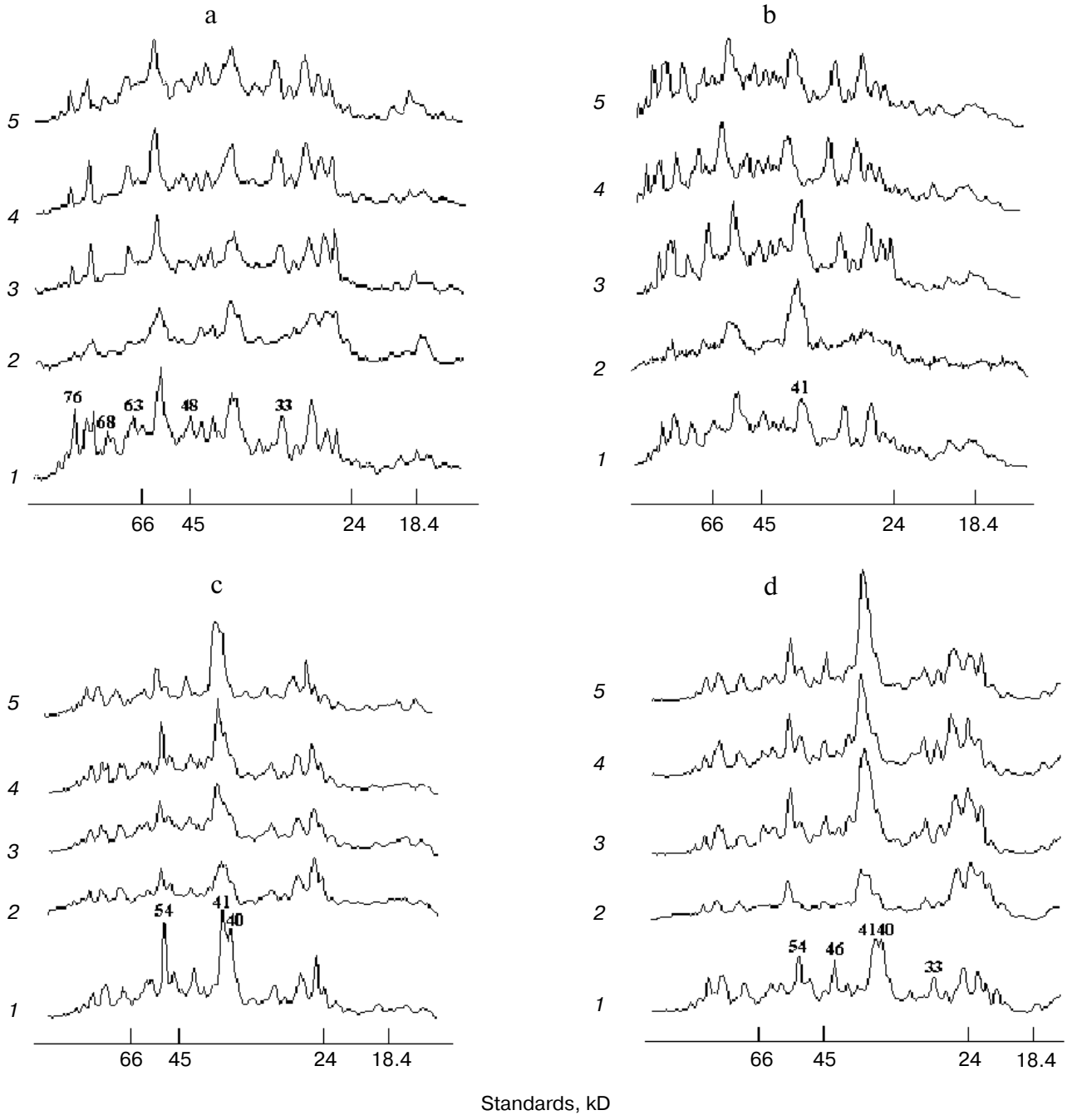

Fig. 2. Effect of salicylic acid on the polypeptide spectrum in non-morphogenic buckwheat calluses in the dark (a) and in the light (b) and in morphogenic calluses in the dark (c) and in the light (d) in the presence of SA at the concentration of: 1 mM (*2*), 0.1 mM (*3*), 10 μM (*4*), 1 μM (*5*); *1*) control.

changed insignificantly during the first 7 days of cultivation in the light and in the dark (Fig. 3). The amount of ROS usually increases in response to drought, salinification, extreme temperatures, nitrogen shortage, and contamination of the atmosphere [36]. The decrease in the H_2O_2 content in the cells of the non-morphogenic calluses after 3 days of cultivation was probably associated with activation of antioxidant enzymes in the light.

Cultivation of calluses in the presence of SA in the light and in the dark resulted in changes in the H_2O_2 content as compared to the control. In the non-morphogenic callus cultivated for 1 day in the dark, the content of H_2O_2 was slightly increased; on the 3rd day of the cultivation, the content of H_2O_2 differed from the control more significantly (Fig. 3). In the presence of SA, the content of H_2O_2 in the non-morphogenic callus

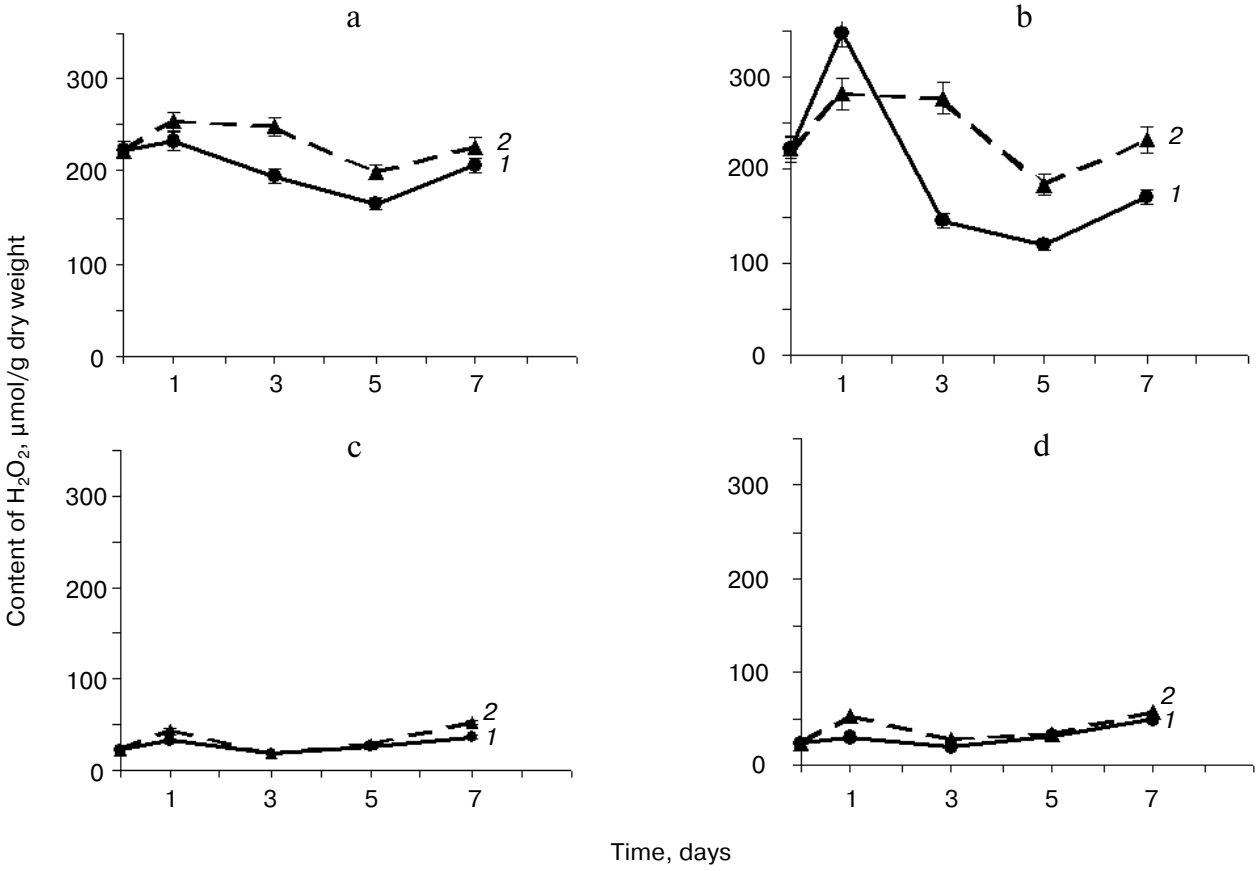

Fig. 3. Effect of salicylic acid on the H_2O_2 content in non-morphogenic buckwheat calluses in the dark (a) and in the light (b) and in morphogenic calluses in the dark (c) and in the light (d) in the presence of 1 mM SA (*2*); *1*) control.

increased after 1 day of cultivation in the light, but this increase was lower than in the control. The difference in the H_2O_2 content in the control and experimental non-morphogenic calluses was maximal after 3 days of cultivation in the light. On cultivation of morphogenic calluses in the presence of SA, the content of H_2O_2 was slightly increased after 1 day in the light as compared to the dark-cultivated calluses. During the following days of cultivation, the contents of H_2O_2 were virtually the same in the control and experimental morphogenic calluses, in the dark and in the light. Thus, SA increased the H_2O_2 content mainly in the non-morphogenic calluses, and the difference was more significant in the light cultures.

The content of H_2O_2 in plants depends on conditions of illumination. Light changes activities of enzymes of oxidative metabolism such as peroxidases [37, 38], superoxide dismutase and glutathione reductase [24], ascorbate peroxidase, and catalase [26, 39].

Oxidative stress is a result of disturbance in the balance between ROS and antioxidant defense because of active generation of ROS or decreased level of antioxidants [10]. In the non-morphogenic buckwheat calluses, the extent of oxidative stress seems to be higher than in the morphogenic calluses, and this is supported by data on the H_2O_2 (one of the reactive oxygen species) content in these types of calluses. The antioxidant defense in the morphogenic calluses seems to be higher than in the non-morphogenic ones, and this is in agreement with data on the higher activity of ion-bound peroxidase in the morphogenic callus [15].

The buckwheat calluses are different in their structure. The non-morphogenic callus is represented by cells of the same type, which are specialized for rapid growth under certain conditions of cultivation, whereas the morphogenic callus has cells of various types capable of differentiation with changes in conditions of cultivation. Thus, we conclude that the changes found in the contents of soluble proteins and individual polypeptides in buckwheat calluses with different ability for morphogenesis cultivated in the dark and in the light indicate the higher sensitivity of the non-morphogenic callus. The different response of callus cultures to SA and conditions of cultivation (light, darkness) can be associated with the antioxidant system, which is, in particular, characterized by the H_2O_2 content in the calluses.

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