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Changes in the Growth of Tartary Buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) Calli with Different Ability for Morphogenesis Induced by Salicylic Acid

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ABSTRACT

The effect of salicylic acid (SA) on the growth processes of tartary buckwheat calli (*Fagopyrum tataricum* (L.) Gaertn) with different morphogenic potential have been studied. The changes of biomass and mitotic index of two callus types refer to a high sensitivity of non–morphogenic callus to the SA treatment. The different sensitivity of calli to SA may be connected with different activity of enzymes being parts of oxidative metabolism in these calli.

Keywords: mitotic index, oxidative stress

Abbreviations: MC, morphogenic callus; **MI**, mitotic index; **NC**, non–morphogenic callus; **PECC**, proembryogenic cell complexes; **SA**, salicylic acid

INTRODUCTION

Salicylic acid (SA) is an endogenous growth regulator of phenolic nature, which participates in various physiological processes in plants. SA, for example, is able to induce flowering in a range of plants (Cleland and Tanaka 1979), to control ion uptake by roots (Raskin 1992). Moreover, SA plays a crucial role in stress resistance in plants by modifying the expression of genes involved in cell protection and in signal transduction (Blanco *et al.* 2005). It might serve as a regulator of the hormonal status of plants (Shakirova *et al*. 2003), fruit ripening (Srivastava and Dwivedi 2000) and other processes. SA applied exogenously enhances somatic embryogenesis in plants (Luo *et al*. 2001; Hao *et al*. 2006; Kumar *et al.* 2007).

In modern biotechnology, cell and tissue cultures of plants are used as model systems to study morphogenetic mechanisms. Investigation of growth processes of callus cultures capable of plant regeneration is of great interest and can help in plant selection. However, the information about growth processes of buckwheat calli cultured on medium with SA is practically unknown.

The morphogenic callus (MC) of tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) is interesting because it retains its specific morphology, its regeneration ability, and its diploid chromosome number for a long time (up to 10 years), and these features occur in *in vitro* cultures rather seldom. Non-morphogenic callus (NC) appears on the surface of the MC as separate foci, on average, with an incidence of one case per 30-40 passages (Rumyantseva *et al.* 1998). MC and NC have different proliferative activity: NC shows more rapid accumulation of biomass and more active cell division than MC.

The objective of this work was to compare the effects of SA on biomass growth and mitotic index (MI) in tartary buckwheat calli differing in morphogenetic potential.

MATERIALS AND METHODS

The study was performed on MC and NC prepared from immature embryos of tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.). Buckwheat seeds were sterilized with 40% NaOCl. Embryos were isolated aseptically and placed onto RX medium (Rumyantseva *et al.* 1998) containing salts by B5 (Gamborg *et al*. 1968) and supplemented with thiamine (2 mg/l), pyridoxine (2 mg/l), nicotinic acid (1 mg/l), mesoinositol (100 mg/l), casein hydrolysate (2000 mg/l), 2,4-dichlorophenoxyacetic acid (2 mg/l), α-naphthaleneacetic acid (0.5 mg/l), indole-3-acetic acid (0.5 mg/l), kinetin (0.2 mg/l), sucrose (25 g/l), and agar (8 g/l). Cultures were incubated in the dark at 25 ± 2°C. The developed calli were separated from the embryo tissues and cultivated separately on RX medium at the same temperature in the dark. The duration between passages was 21 and 28 days for NC and MC, respectively. MC consisted of proembryogenic cell complexes (PECC) and soft callus cells, which were derivatives of the PECC cells produced during their loosening (**Fig. 1A**). NC was a loose, actively proliferating culture, which consisted only of parenchymal cells (**Fig. 1B**). The ability of MC for various types of morphogenesis (embryogenesis, organogenesis, histogenesis) has been shown (Rumyantseva *et al.* 1992).

SA (Reakhim™, Russia) was added into the growth media at final concentrations from 1 μM to 1 mM after sterilization through membrane filters with a pore diameter of 0.22 μm. The media containing SA were alkalified with 0.1 M NaOH to bring to pH to 5.8 before autoclaving.

Growth was determined as changes in dry weight of MC and NC during *in vitro* cultivation.

Chromosomes were stained with 2% acetoorcein. MI was calculated with the formula MI=M/N×100%, where M is the number of dividing cells and N is the total number of cells. For each treatment, at least three thousand cells were examined.

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 deviation. Means were compared by the *t*-test or Fisher's least significant difference test at $P<0.05$ in all cases.

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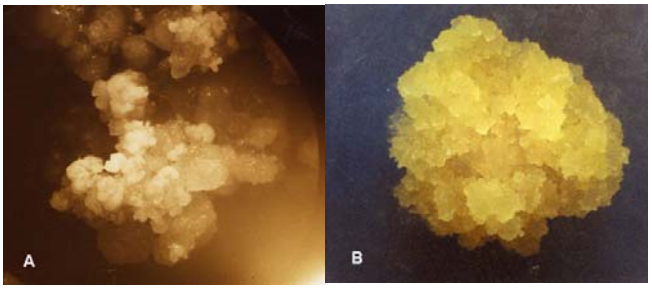

Fig. 1 Morphology of tartary buckwheat calli. (**A**) Morphogenic callus. (**B**) Non-morphogenic callus.

RESULTS AND DISCUSSION

Since SA is an endogenous growth regulator, it was important to study the influence of exogenous SA on growth processes in both types of calli during *in vitro* cultivation.

SA is involved in the response to abiotic stress. However, the effects of SA on plant resistance were found to be contradictory. Generally, deficiency of SA or a very high level of SA increases the plant's susceptibility to abiotic stress (Yuan and Lin 2008). Ali *et al.* (2007) showed a significant decrease in fresh and dry weights of *Panax ginseng* root suspension cultures grown after exposure to SA for 9 days (200 μM). In contrast, SA at a low level plays an important role in modulating the redox balance and in protecting rice plants from oxidative stress (Yang *et al*. 2004). At the termination of cultivation, the effects of SA (1 μM – 1 mM) on the growth of buckwheat cells in MC and NC were different (**Fig. 2**). The inhibitory effect was greatest during cultivation of NC in the presence of 1 mM SA. Lower concentrations of SA (10 μM – 0.1 mM) led to lesser inhibition of callus growth. Treatment of the NC with SA (1 μM) significantly ($P<0.05$) increased the callus biomass compared with the control. At the same time, the decrease in dry weight of MC treated with SA (1 mM) was not considerable

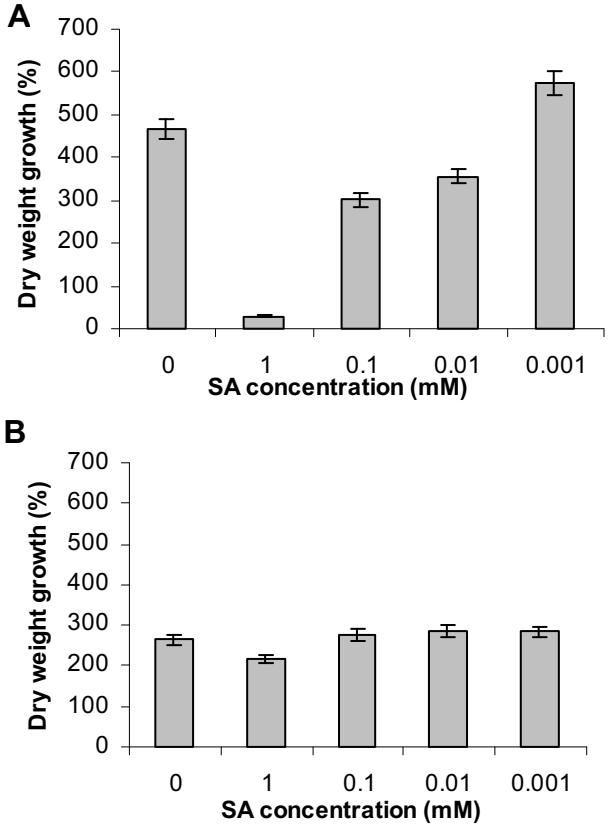

Fig. 2 Dry weight growth of buckwheat calli at the end of the cultivation period on media with salicylic acid (SA). (**A**) Non-morphogenic callus. (**B**) Morphogenic callus.

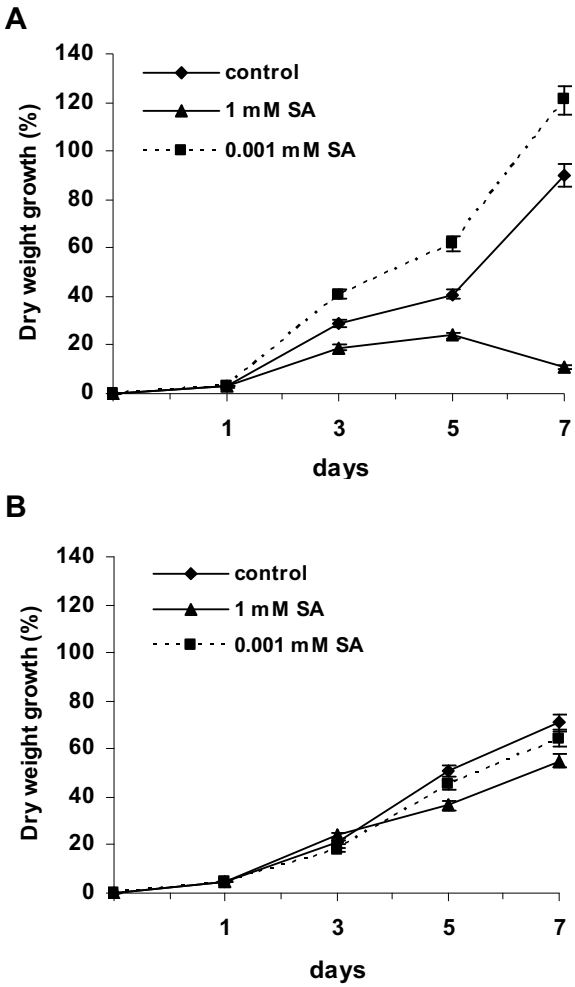

Fig. 3 Dynamic in dry weight growth of buckwheat calli on media with salicylic acid (SA). (**A**) Non-morphogenic callus. (**B**) Morphogenic callus.

(**Fig. 2**). SA at concentrations of 1 μM – 0.1 mM did not change the growth of MC biomass ($P>0.05$).

It is known that SA can act as signal and induce gene expression and synthesis of pathogenesis-related (PR) proteins (Raskin 1992; Delaney *et al.* 1994). Galls and Matsuoka (2007) showed that in tobacco BY-2 cells in total 376 genes were at least 2-fold upregulated by SA compared to their expression levels in control cells. Therefore, it was interesting to study the effects of SA on mass growth of both types of callus at the first 7 days of cultivation when cells are more sensitive to environmental conditions. The data on the effects of SA on the growth of the MC and NC are presented in **Fig. 3**. SA at 1 mM significantly ($P<0.05$) decreased the biomass growth of NC from the 3rd day of cultivation, and this effect was more pronounced on the 7th day of cultivation of the callus. In contrast, SA (1 μM) increased ($P<0.05$) the biomass of NC from day 3 to 7 in comparison with the control. Significant changes in the growth of MC treated with SA (1 μM and 1 mM) were not observed (**Fig. 3**).

Buckwheat MC and NC are characterized with different mitotic activity during *in vitro* growth (Rumyantseva *et al.* 1998). We have found that after 1 day of passage there were only few dividing cells (0.1-0.2%) in both NC and MC in the control and in the presence of SA (**Fig. 4**). Callus and suspension cultures have a lag phase at the first 1-3 days of cultivation (Bychkova and Butenko 1978). **Fig. 4** shows that mitotic activity of MC and NC increased after the lag phase. A maximum mitotic activity in both callus types was observed on the 3rd day of the cultivation. Maximum MI was 1.95% in the MC control and 3.4% in the NC control. It is a known fact that maximum proliferative activity of a cell culture varies from 0.01 to 10-15% (Danilina and Danilov

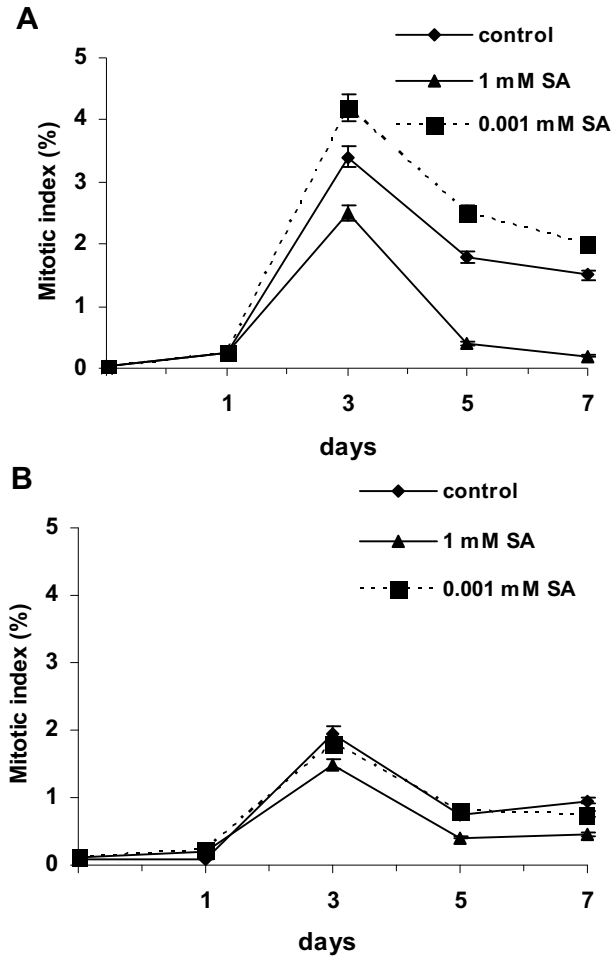

Fig. 4 Mitotic index dynamics of buckwheat calli on media with salicylic acid (SA). (**A**) Non-morphogenic. (**B**) Morphogenic callus.

1978). Differences in proliferative activity of MC and NC may be explained by the differences in the structure of these types of calli. In NC, one type of cells occurs, and these cells are able to divide, whereas in MC, only meristematic cells of PECC have the ability to divide.

SA affected the mitotic activity of buckwheat calli during the early stage of cultivation (**Fig. 4**). After 3 days of passage MI in NC cultivated on 1 mM SA decreased by 0.9% in comparison with the control, and after 7 days decreased to 0.2%. According to Chens *et al*. (2001), 0.2 mM SA inhibited cell proliferation in tobacco suspension culture on the 3rd day of cultivation. The effect of 20 μM SA on biomass growth of tobacco cells was comparable with the control level. In our experiments 1 μM SA increased MI by 0.8% on the 3rd day of cultivation. The stimulating effect of 1 μM SA on MI was maintained during the first 7 days of cultivation. The stimulation of NC growth by 1 μM SA may be caused by the induction of cell proliferation. 1 mM SA had a lower inhibitory effect on the MI of the MC than that of NC. In addition, SA at 1 μM had no effect on the mitotic activity of MC during the first 7 days of callus cultivation.

According to the literature, the content of endogenous IAA in MC is higher in comparison to NC. Thus, it may be an indicator of embryogenic competence in cell cultures. Moreover, when the embryogenic calli loose their competence to regenerate because of prolonged cultivation, the endogenous concentrations of free IAA reduced to those of the non-embryonic culture (Jiménez and Bangerth 2001). Total levels of endogenous cytokinins from callus culture of *Medicago arborea* L. were higher in the non-embryonic calli than in embryonic calli (Pintos *et al*. 2002). It is well known that SA treatment may result in the perturbation of endogenous concentration of phytohormones. For example, following SA treatment, the total level of cytokinins increased in the tissues of phlox (*Phlox paniculata* and *Phlox setacea*) leaves (Talieva and Kondrat'eva 2002). SA in low concentrations also stimulated ethylene production in carrot (*Daucus carota*) cell suspension cultures (Nissen 1994) and in the culture tubes of peach rootstock regenerants of three genotypes: Cadaman (*Prunus persica* × *P. davidiana*), GF-677 (*Prunus amygdalus* × *P. persica*), and Myrobolan 29C (*Prunus cerasifera*) (Molassiotis *et al*. 2005). The treatment of wheat (*Triticum aestivum*) plants with 50 μM SA increased the level of cell division within the apical meristem of seedlings roots causing an increase in plant growth and elevated productivity (seeds weight in ear, weight of 1000-seeds and yield) (Shakirova *et al*. 2003). These authors suggested that this stimulation was caused by change in the concentration of endogenous phytohormones in cells.

The stimulatory effect of 1 μM SA on growth processes (mitotic index and biomass growth) of NC and its absence in MC may be explained by both different level of endogenous hormones and change of their ratio under SA action.

It is known that SA induces a rise in the concentration of reactive oxygen species (ROS) in cells (Rao *et al.* 1997; Tarchevsky 2002; Fawavardeh and Rabbani 2006; Wang and Li 2006; Bóka *et al*. 2007), which results in both oxidative stress and cell cycle damage (Van Breusegem *et al*. 2001; Ali *et al* 2006). Thus, the inhibitory effect of SA applied in higher concentration on the growth of buckwheat calli may be caused by the toxic action of H_2O_2 on cells. In the NC buckwheat calli, the extent of oxidative stress seems to be higher than in the MC, and this is supported by data on the H_2O_2 content in these types of calli. Early it was shown that MC and NC buckwheat calli were significantly different in the H_2O_2 content. The NC and MC contained 9.1 and 2.8 μM H_2O_2/g wet weight, respectively (a 3.2-fold difference), on average, for 7 days of cultivation (Maksyutova *et al.* 2005). Moreover on cultivation of calli in the presence of SA the H_2O_2 content increased mainly in NC callus (Maksyutova *et al.* 2005). All these facts may explain why high SA concentration inhibited the biomass growth of NC more than MC.

It has been shown that 1 mM SA greatly inhibited the rate of cell proliferation in wheat seedlings after 2 h treatment (Gordon *et al*. 2002). Among others, the SA-induced change in the proton permeability of membranes (especially the plasma membrane) was suggested to be the main reason for inhibited proliferation. As a result, SA caused a decrease in cytoplasmic pH and an increase in energy expenditure for restoration of ion homeostasis (Gordon *et al*. 2002).

Our results on the growth patterns (dry weight growth and MI) of tartary buckwheat calli with different morphogenic ability demonstrated that NC is more sensitive to SA than MC. As mentioned above, NC consists of one cell type adapted to specific cultivation conditions, while MC is represented by several cell types able to differentiate in changing conditions.

It may not be excluded that different reactions of NC and MC to SA treatment may be related to differences in the activity of redox enzymes and this is in agreement with our data on the higher activity of ion-bound peroxidase in the MC than NC (Rumyantseva *et al.* 1998). Oxidative stress is a result of the imbalance between ROS and the antioxidant defense system (Van Breusegem and Dat 2006). It was proposed that SA may bind to catalase and directly inhibit its activity (Kawano *et al*. 1998). SA can also rapidly enhance superoxide dismutase activity (Wang and Li 2006), thus increasing the intracellular concentration of $O_2^{\cdot-}$. Since superoxide dismutase catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2, which may enhance the level of H_2O_2 in SA-treated cells. Thus, if the activities of peroxidase and other antioxidant enzymes in SA-treated cells are not sufficient to prevent H_2O_2 accumulation, cells suffer oxidative stress. However, this problem needs investigation and will involve a study of the activity of antioxidant enzymes in calli with different morphogenic abilities.

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