

Effect of silicon chelates from plant raw materials on some physiological parameters in strawberry under *in vitro* conditions

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Abstract. For the first time, the effect mechanocomposite based on biogenic silica and green tea flavonoids (MC) on reduction of oxidative stress in microshoots of strawberry plantlets (cv. “Solnechnaya polyanka”) during *in vitro* multiplication, shoot elongation and rooting stages were established. Compared with the plantlets cultured in MC-free media, the ones cultured in media supplemented with MC showed increased key antioxidant enzyme activities (superoxide dismutase – SOD, peroxidase – POD, catalase – CAT). SOD activity increased from 1.1 to 1.4 times at 2.5 and 5.0 mg L⁻¹ MC, POD activity – from 1.8 to 3.8 times at 5.0 and 10.0 mg L⁻¹ MC, and CAT activity – from 1.1 to 1.4 times at 5.0 mg L⁻¹ MC depending on the stage of micropropagation. The high antioxidant enzyme activities correlated with the decrease of H₂O₂ content (up to 1.7 times compared with the control). The revealed changes in the physiological status of strawberry plantlets in the presence of MC contribute to the further successful acclimatization of regenerants to *ex vitro* conditions. Thus, these results have demonstrated the potential of “green chemistry” for the production of healthy plant material of strawberry cultivars.

1 Introduction

The world production and consumption of cultivated strawberry (*Fragaria × ananassa* Duch.) is increasing very fast. According to Faostat, about 9 million tons of fresh strawberry berries were grown and sold globally in 2017. In this regard, *in vitro* techniques are important tools for obtaining virus, bacteria and fungus-free strawberry basic planting material [1]. However growth and development of plants in *in vitro* conditions are stress-related processes which are accompanied by changing of metabolic reactions. Unfavourable *in vitro* factors (high relative humidity, low gas exchange, presence of exogenous carbohydrates, plant growth regulators and low rate of CO₂ absorption) shift the balance between oxidants and antioxidants toward oxidants, which stimulate development of intracellular oxidative stress [2]. Symptoms of the stress in *in vitro* conditions include a

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reduction of culture growth, inhibition of shoots, roots proliferation, culture ageing and finally cell necrosis. To achieve physiological stability of cultivated plant, the culture medium is optimized by the addition of exogenous compounds with antioxidant properties that activate the antioxidant (non- and enzymatic) defense systems in plant tissues to scavenge reactive oxygen species. In this regard, the selection of antioxidant preparations that minimize the oxidative processes in cells is a necessary step in micropropagation protocols.

A promising preparation is the mechanocomposite obtained from renewable plant raw materials (rice husk and green tea waste) [3]. Rice husk is a renewable source of amorphous silicon dioxide (silica) with a content of up to 29% by weight [4-5]. Green tea is rich in the flavanol group of polyphenols as catechins which may constitute up to 30% of the dry weight [6]. In previous studies, we have shown the growth-regulating and adaptogenic activities of the mechanocomposite based on biogenic silica and green tea flavonoids at the stages of *in vitro* rooting and *ex vitro* acclimatization of *F. × ananassa* plantlets [7-8]. Since mechanocomposite contains silicon compounds and catechins with antioxidant properties [9-10], in this work, for the first time, the potential antioxidant protective effect, including superoxide dismutase, catalase, and peroxidase enzymes, of this environmentally friendly preparation will be analyzed under micropropagation of strawberry. The use of "green chemistry" (mechanocomposite) as an inhibitor of *in vitro* stresses may become a technology recognized and in demand at the world.

Thus, in the present study we aimed to analyze the effect of different concentrations of mechanocomposite on the physiological parameters in strawberry plantlets under *in vitro* conditions, including the content of hydrogen peroxide (H₂O₂) and activity of key antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), peroxidase (POD)).

2 Materials and methods

2.1 Plant material and growth conditions

The physiological parameters of micropropagated plantlets of strawberry cultivar, 'Solnechnaya polyanka', under the influence of mechanocomposite during *in vitro* multiplication, shoot elongation and rooting stages were studied. The explants were cultivated on Gamborg-Eveleg's basal salt medium - B₅ [11] containing 20 g L⁻¹ sucrose, 6 g L⁻¹ Bactoagar (PanReac®, Spain) at all *in vitro* stages. To induce shoot multiplication, the microshoots (1.0 cm) with apical bud and two leaves were inoculated on medium supplemented with 0.75 mg L⁻¹ 6-benzylaminopurine (BA; ICN Biomedicals, Aurora, OH), and different concentrations of MC (0.0, 2.5, 5.0 or 10.0 mg L⁻¹). After 60 days, conglomerates with microshoots were placed on shoot elongation medium supplemented with 0.0, 2.5, 5.0 or 10.0 mg L⁻¹ of MC and were cultured for 45 days. After that plantlets were transferred to rooting medium supplemented with the same MC concentrations for 30 days. The MC-free medium was used as the control. All media were adjusted to pH 5.5 before autoclaving (121°C, 2.1 atm for 20 min), and MC was added to the media before autoclaving. The cultures were maintained in culture jars (15 mL medium per vessel) at 23 ± 2 °C with a 16-h photoperiod with 40 μmol m⁻² s⁻¹ of light intensity provided by cool white fluorescent lamps (Philips, Pila, Poland). Experiments were repeated thrice, and 30 microshoots were used for each MC concentration. At the end of the each cultivation period the endogenous H₂O₂ content and antioxidant enzyme activities were analyzed.

2.2 Biochemical analysis

The endogenous H₂O₂ level was analyzed using the standard H₂O₂ calibration curve at 560 nm according to Bellincampi et al. [12]. Data were expressed as μmol H₂O₂ per gram of fresh weight (FW) (μmol g⁻¹(FW)). To determine the antioxidant enzyme activities, about 0.1 g fresh shoot tissue was extracted in 50 mM Na-K-phosphate (pH 7.8) containing 100 mM phenylmethylsulphonyl fluoride. Then the homogenate was centrifuged at 12,000 rpm for 5 min at 4 °C and the supernatant was used for determination of the superoxide dismutase (SOD) and catalase (CAT) activities. Absorbance of samples was recorded using a SF-56 spectrophotometer (OKB-Spectr, Russia). Determination of SOD activity was performed by the enzyme's ability to inhibit the photochemical reduction of nitro blue tetrazolium as previously described by C. N. Giannopolitis and S. K. Ries with minor modifications [13]. The absorbance was measured at 560 nm and expressed in units (U) per gram of FW (U g⁻¹(FW)). CAT activity was determined by the decrease in optical density as a result of the decomposition of H₂O₂ at 240 nm [14] and expressed in units of H₂O₂ decomposed per min per g of fresh weight (U H₂O₂ g⁻¹ (FW) min⁻¹). For the analysis of peroxidase activity (POD) about 50 mg shoot tissues were macerated in 0.2 M acetate buffer (pH 4.5). After centrifugation (4°C, 12,000 rpm, 5 min), the supernatant was collected and immediately POD activity was measured by the decrease in the content of quercetin at 365 nm [15]. POD activity was expressed as U of quercetin oxidized per min per g of FW (U g⁻¹(FW) min⁻¹). Three biological and three analytical replications were performed for each analysis.

2.3 Statistical analysis

Data were expressed as means and standard errors (M ± SE). The data were subjected to a one-way analysis of variance (ANOVA) and the Duncan multiple range test was used to calculate significant differences at $p < 0.05$. Statistical evaluations were performed using Statistica 10.0 software (Statsoft Inc., Tulsa, OK, USA).

3 Results

3.1 Effect of MC on H₂O₂ content

A time dependent variation in the H₂O₂ content was found in the plantlets during *in vitro* shoot multiplication, shoot elongation and rooting stages (Table 1). An increased H₂O₂ content was observed in the plantlets at shoot multiplication and rooting stages. However, at shoot elongation stage, a decreasing trend was observed in H₂O₂ content up to *in vitro* rooting stage. At multiplication stage, the accumulation of H₂O₂ in the shoots was reduced by the addition of 10.0 mg L⁻¹ MC by 1.5 times compared with the control ($p \leq 0.05$). During the shoot elongation, H₂O₂ content significantly decreased under the influence of all MC concentrations. At *in vitro* rooting, the H₂O₂ content in shoots growing in the presence of 5.0 and 10.0 mg L⁻¹ MC was 1.7-fold lower than on control medium.

Table 1. Changes in H₂O₂ content (μmol g⁻¹ (FW) in microshoots of strawberry plantlets (cv. “Solnechnaya polaynka”) cultivated *in vitro* with different mechanocomposite concentrations during the shoot multiplication (SMS, after 60 d), shoot elongation (SES, after 105 d) and rooting stages (RS, after 135 d).

MC*, mg L ⁻¹	SMS	SES	RS
0.0	4.23± 0.01a	1.69± 0.06a	8.17± 0.76a
2.5	4.17± 0.14a	0.91± 0.02c	6.22± 0.85b
5.0	4.09± 0.08a	1.50± 0.01b	4.87± 0.04c
10.0	2.86± 0.12b	1.56± 0.01b	4.74± 0.54c

*MC, mechanocomposite based on amorphous silica and flavonoids of green tea. Data are mean ± SE (n=3). For each cultivar and variable, a single-factor analysis of variance was performed between means. Within a column, mean separation are indicated by different lowercase letters and determined by Duncan’s multiple range test (p ≤ 0.05).

3.2 Effect of MC on antioxidant enzyme activities

According to the data, SOD and CAT activities in microshoots increased significantly during the multiplication and elongation stages (Table 2). Moreover, SOD activity in shoots was extremely high at the elongation stage. At the rooting stage, their activities decreased. The application of MC had a significant influence on antioxidant enzyme activities in the strawberry shoots. The greatest SOD and CAT activities occurred in microshoots on media supplemented with 2.5 and 5.0 mg L⁻¹ MC. In particular, SOD activity increased up to 1.4 times, CAT activity – up to 1.8 times compared with the variants without MC. There was no noticeable change observed in POD activity in the microshoots during studied stages of micropropagation. Increasing POD activity in the microshoots was observed at 5.0 and 10.0 mg L⁻¹ MC (p ≤ 0.05) by 2.9 and 3.4 times during the shoot multiplication, by 2.2 and 1.8 times, respectively, during elongation stages. The data indicated that MC could markedly increase the antioxidant ability of plantlets.

Table 2. Changes in SOD (U g⁻¹ (FW)), CAT (U H₂O₂ g⁻¹ (FW) min⁻¹), and POD (U g⁻¹ (FW) min⁻¹) activities in microshoots of strawberry plantlets (cv. ‘Solnechnaya polaynka’) cultivated *in vitro* with different mechanocomposite concentrations during the shoot multiplication (SMS, after 60 d), shoot elongation (SES, after 105 d) and rooting stages (RS, after 135 d).

Parameter	MC*, mg L ⁻¹	SMS	SES	RS
SOD	0.0	14.62± 2.06bc	27.67± 0.93b	13.49± 0.18a
	2.5	19.67± 0.97ab	30.81± 0.12a	14.05± 0.26a
	5.0	20.42± 4.61a	30.65± 0.05a	13.92± 0.23a
	10.0	11.49± 3.23c	26.33± 0.22b	10.43± 0.58b
POD	0.0	0.37± 0.04b	0.12± 0.01d	0.91± 0.55a
	2.5	0.42± 0.09b	0.08± 0.01c	1.04± 0.38a
	5.0	1.07± 0.21ab	0.26± 0.01a	0.28± 0.05a
	10.0	1.42± 0.26a	0.21± 0.01b	0.15± 0.06a
CAT	0.0	10.8± 1.3b	3.14± 0.03b	1.61± 0.70a
	2.5	10.4± 2.7b	3.21± 0.01b	2.96± 0.92a
	5.0	15.1± 0.4a	3.43± 0.05a	1.82± 0.06a
	10.0	4.5± 0.4c	3.10± 0.09b	2.16± 0.99a

*MC, mechanocomposite based on amorphous silica and flavonoids of green tea. Data are mean ± SE (n=3). For each cultivar and variable, a single-factor analysis of variance was performed between means. Within a column, mean separation are indicated by different lowercase letters and determined by Duncan’s multiple range test (p ≤ 0.05).

4 Discussion

The main task of micropropagation remains the production of a maximum number of high quality regenerants. Nutrients of medium are a critical for the improved growth and development of plants in *in vitro* conditions. Under stressful conditions, the overproduction of reactive oxygen species (ROS) can cause oxidative damage to plant macromolecules and cellular structures. Among the ROS, H_2O_2 is one of the major markers of oxidative stress and key signaling molecule involved in the process of differentiation, growth, and development of plants [16]. Consequently, it is important to elaborate a successful micropropagation procedure to obtain plants phenotypes with optimal physiological parameters that can survive the environmental conditions when directly placed in a greenhouse or field.

In this study, we found a significant decrease in H_2O_2 content in strawberry microshoots in response to MC application under *in vitro* propagation (Table 1). The maximum of H_2O_2 content reduction (up to 1.7 times) was observed at the end of *in vitro* rooting stage. This indicates enhanced resistance of plantlets to oxidative stress. It is known, enzymatic antioxidants play an important role in protection of the cell against the damaging effects of ROS. Enzyme SOD constitutes the first line of plant defense, converting superoxide anion radical ($O_2^{\cdot-}$) to H_2O_2 , which is quickly metabolized by POD and CAT [17]. In current experiments, a high SOD activity and low content of H_2O_2 in microshoots at 2.5 and 5.0 mg L^{-1} MC, especially at elongation stage, was detected suggests its role in preventing oxidative stress (Table 2). Decrease of the SOD activity at the rooting stage evidence that the plants adapt to environmental *in vitro* conditions. CAT and POD are the components of the antioxidant defense system involved in H_2O_2 detoxification. In this study, their activities increased in microshoots of plantlets on media supplemented with 5.0 mg L^{-1} MC. An increase in the activity of these enzymes indicates the synthesis of plant defense compounds against special *in vitro* conditions.

A similar patterns correlated with enhancing the activity of antioxidant enzymes in plants by silicon (Si) treatment were reported [18-19]. The results of study of *Ajuga multiflora* Bunge indicated enhancing the activity of some antioxidant enzymes such as ascorbate peroxidase (APX), CAT, and SOD on the media supplemented with Si compounds [20]. In microshoots of *Ornithogalum dubium* Hoult., in the presence of Si, the activity of antioxidant enzymes, ascorbate oxidase, APX, and glutathione peroxidase, significantly increased [21]. In the medicinal plant *Lonicera japonica* Thunb., at the salt stress, the use of Si compounds stimulated the activity of CAT and SOD [22].

The revealed increase in the activity of key antioxidant enzymes (SOD, CAT, and POD) during the multiplication, shoot elongation and *in vitro* rooting stages indicates the correct functioning of the mechanism of the antioxidant defense system in strawberry plants in the presence of MC.

5 Conclusion

In the present study, the mechanocomposite based on silicon chelates from plant raw materials can be used as a potential component of nutrient media for *in vitro* propagation of strawberry (cv. "Solnechnaya polaynka"), since it positively influences physiological status of the plants. MC modified the production and detoxification of H_2O_2 and intensifies activity of the key antioxidant enzymes (SOD, CAT, and POD) in the shoot tissues of plantlets. Determined physiological changes in microplants grown with MC will further contribute to successful *ex vitro* acclimatization of regenerants more critical stage of micropropagation. Thus, regulation of physiological status through MC addition could be a promising way to obtain better growth and development of this popular berry crop.

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