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PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF TEA (*Camellia sinensis* L.) MICROSHOOTS in vitro: THE NORM, OSMOTIC STRESS, AND EFFECTS OF CALCIUM

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Abstract

Stress tolerance is an important trait, that determines the productivity of plants under drought, hypothermia, mineral deficiency, and salinity. Numerous studies of various agricultural crops (J.K. Zhu, 2016; E. Fleta-Soriano, S. Munné-Bosch, 2016), including tea crop (*Camellia sinensis* L.), were aimed at solving this problem due to the global aridization of the climate. (T.K. Maritim et al., 2015; L.S. Samarina et al., 2019). Along with the sufficiently detailed physiological, biochemical and molecular studies of tea drought tolerance, the exogenous regulation of tolerance by using of chemical and biological substances is still not investigated. In addition, the important role of calcium ions (Ca^{2+}) in the cell recognition of an external stressor by the triggering signal transduction has been shown in many crops (M.C. Kim, 2009; E.G. Rikhvanov et al., 2014). In these studies, tissue culture media supplemented with the osmotically active substances (R.M. Pérez-Clemente et al., 2012; M.K. Rai et al., 2011) and artificial biosystems (microshoots and tissues in vitro), are often used as "drought models" to reveal cellular adaptation mechanisms. However, just a few studies were conducted aimed at deciphering the biochemical and molecular responses of tea plant to stress using tissue culture tool (L.S. Samarina et al., 2018; M.V. Gvasaliya et al., 2019). In this article, for the first time, we investigated the role of calcium in plant adaptation to long-term osmotic stress based on earlier published protocols of tea tissue culture (M.V. Gvasaliya, 2013) and osmotic stress induction protocols. We also demonstrated the prospect of studying the role of exogenous inducers in increasing plant tolerance using "drought models". This work aimed to identify the effect of different concentrations of calcium (Ca^{2+}) in the culture medium on the functional state of tea microshoots grown under mannitol-induced osmotic stress in vitro comparing with control. The changes in morphophysiological state of the leaves, leaves water content, cells membrane permeability, malondialdehyde, proline, and photosynthetic pigments were analyzed. It was found that increased Ca^{2+} content in the nutrient medium (from 440 to 880 mg/l) resulted the slower leaves development and significant decrease of malondialdehyde and cell membranes permeability of tea microshoots (by 50 %, $p \le 0.05$) during the long-term cultivation of tea microshoots in vitro (4 months), indicating inhibition of lipid peroxidation processes. The addition of mannitol (40 g/l) to the culture medium reduced the water content of the shoots (on average by 2 %, $p \le 0.05$), thereby forming light osmotic stress, which led to the accumulation of proline (an increase of 30-40 %, $p \le 0.05$), as well as to the structural and functional rearrangement of the photosynthetic apparatus (a decrease in the concentration of photosynthetic pigments by an average of 35-40 %). In addition, a significant decrease of malondialdehyde (by 50-70 %, $p \le 0.05$) and the intensity of electrolyte leakage from leaf tissues (on average by 50 %, $p \le 0.05$) were observed, indicating a less pronounced oxidative stress in comparison with control (without mannitol). An increase in the Ca^{2+} concentration in the nutrient medium (from 440 to 880 mg/l) (in the presence of mannitol) did not

significantly affect the water content in the leaves and the photosynthetic apparatus (content and ratio of chlorophylls/carotenoids). An insignificant effect of calcium (in the presence of mannitol) manifested itself in a significant decrease in malondialdehyde by 20 μmol/g dry weight. Consequently, the increased concentration of calcium (660-880 mg/l) in the nutrient medium provides an improvement in the functional state of long-term cultivated tea microshoots in vitro (4 months) by reducing the activity of lipid peroxidation in membranes and increasing their stability. The revealed patterns confirm the positive role of calcium ions in the reduction of combined oxidative stress caused by long-term cultivation of plants *in vitro* in combination with osmotic stress.

Keywords: tea plants, *Camellia sinensis* L., in vitro microshoots, calcium, mannitol, osmotic stress, pigments, proline, malondialdehyde

Stress resistance is essential to determine plant productivity [1, 2]. Osmotic stress caused by drought, hypothermia, and salinity leads to tissue dehydration and even plant death [3, 4]. The effect of stressors is traced by a complex of physiological, biochemical and molecular processes, including growth, water content and water potential of leaves, enzymatic activity, metabolomic profile, and gene expression [1, 5, 6].

Calcium (Ca^{2+}) plays a special role in maintaining plant resistance to adverse environmental factors [7, 8]. A change in its concentration in the cytosol is the first stage in the cell recognition of an external stimulus and the triggering of the signal transduction system for a response [7, 9, 10]. An important group of sensors involved in the cascade of calcium ion signals in higher plant cells are Ca-dependent protein kinases [11, 12]. Under the action of calcium, changes are noted in the growth, photosynthesis and water-air regime of plants, the functions of stomata, and accumulation of stress proteins [7, 13]. The induction of the antioxidant system reduces the oxidative damage [14, 15].

Tea plants (*Camellia sinensis* L.) are cultivated in drought-prone regions of the world [16-18]. According to a number of researches [9, 16, 18], including our own [17], hydrothermal stress leads to the loss of more than 50% of the tea yield under dryland conditions. Progressive climate aridization imposes urgency on the problem of plant drought resistance. Much attention is paid to the mechanisms of tea plant resistance to drought, the search for the most informative markers of drought resistance, and creation of new resistant varieties [4, 9, 15]. However, in-depth understanding of the young tea shoot metabolism under the exogenous effects of biogenic elements and other substances are practically not covered, though being widely studied in other crops where agar nutrient media with the addition of osmotically active substances are used as “drought models” [19-21]. The “drought models” are suitable to control water potential, which is essential to ensure high accuracy, reproducibility and comparative assessment of various experiments [22].

In vitro plant tissues and microshoots are informative model systems for studying metabolic processes and cellular responses to stresses, including physiological and biochemical changes. This technique was used to select resistant genotypes of potato [23], lathyrus culture [24], cucumber [25], and beans [26] under osmotic stress (drought). However, little research has focused on tea plants [27, 28], and these studies have mainly elucidated biochemical and molecular responses to drought for better understanding the changes it causes in plant proteome and metabolome.

In this paper, we have established for the first time the role of calcium in the adaptation of tea microshoots to stress caused by prolonged in vitro growing combined with osmotic stress, and confirmed the prospect of agar culture media added with mannitol as a “drought models” for studying effects of exogenous inducers on plant resistance.

The aim of the work was to assess the reaction functional performance of

in vitro grown tea microshoots under optimal conditions and upon the mannitol-simulated weak osmotic stress as influenced by different concentrations of calcium (Ca^{2+}) in the nutrient medium.

Materials and methods. Microshoots of tea (*Camellia sinensis* L.) local population were grown in vitro on the Murashige-Skoog nutrient medium supplied with 6-benzylaminopurine (BAP, 6 mg/l), 1-naphthaleneacetic acid (NAA, 1 mg/l), gibberellic acid (HA, 2 mg/l) [29]. The treatments were 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. For each treatment, 10-15 microshoots were grown for 4 months in a factorostatic chamber (16/8 h photoperiod, 25±1.0 °C, humidity 70%, illumination 3000 lx, lamps L 36 W/765, OSRAM GmbH, Germany). Leaves of microshoots were collected for analysis.

The water content in leaves was measured by drying samples in a thermostat (BD-115, Binder GmbH, Germany) at 70 °C to constant weight [30].

To assess cell membrane stability, a portion of leaves (50 mg) was placed in deionized water (50 ml). Electrical conductivity was measured in 0, 60, 120 minutes, and 60 minutes after boiling the plant sample (a portable ST300C conductometer, STCON3 sensor with verification, OHAUS Corporation, USA). The relative electrical conductivity of the solution was calculated as $L_1/L_2 \times 100$ %, where L_1 is the electrical conductivity in 0 min, L_2 is the electrical conductivity in the cooled solution after boiling in a water bath for 1 h at 100 °C [31].

Lipid peroxidation (LPO) was assessed the malondialdehyde (MDA) concentration [32]. Leaves of tea microshoots were homogenized in 0.1 M Tris-HCl buffer (pH 7.5) with 0.35 M NaCl followed by adding a 0.5% solution of thiobarbituric acid in a 20% aqueous solution of tri-chloroacetic acid. The reaction mixture was incubated for 30 min in a boiling water bath (WB-4MS, BioSan, Latvia), cooled, and the optical density of the supernatant liquid was measured (SF-46 spectrophotometer, LOMO, Russia) at $\lambda = 532$ nm.

Pigments were extracted from leaves using 96% ethanol [32]. After centrifugation of the homogenate (13000 rpm, 5 min), the chlorophyll a (Chla) ($\lambda = 665$ nm), chlorophyll b (Chlb) ($\lambda = 649$ nm), and carotenoids (Car) ($\lambda = 440$ nm) concentrations were measured in the supernatant. The concentrations of pigments were calculated by the standard method [33].

Proline was extracted from leaves by a standard method and measured with a ninhydrin reagent [27] at $\lambda = 520$ nm (a USF-01 device, VNIIOFI, Russia).

All measurements were conducted in three biological and three analytical replicates. Statistical processing was performed using Microsoft Excel 2010 and SigmaPlot 12.2 (http://www.sigmaplot.co.uk) programs. The table and figures show the arithmetic mean values (*M*) and their standard errors (±SEM). The significance of the differences between the means was assessed by the Student's *t*-test; values marked with different Latin letters differ at $p \leq 0.05$.

Results. Mannitol is known to cause in vitro osmotic stress [27, 34]. In our tests, tea microshoots grown for 4 months in vitro were low, which was also noted earlier [27], and had bright green leaves (Fig. 1). High Ca^{2+} concentration in the basal nutrient medium (880 vs. 440 mg/l $CaCl_2$, that is, 2 times higher than the normal Ca^{2+} level) caused a pronounced decrease in the microshoot height (see Fig. 1, A). Therefore, an increase in Ca^{2+} concentration slowed down the formation and development of leaves in tea microshoots. This tendency continued under osmotic stress, i.e., the higher the Ca^{2+} concentration in the medium, the smaller the leaf size (see Fig. 1, B). Nevertheless, the leaves were well developed, that is, we did not observe apparent stress effects,

which may evidence the Ca^{2+} protectiveness found in other studies, for example, in potatoes [23]. Note that in the available literature, we did not find any reports on the drought modeling in tea plants in vitro, except for our work.

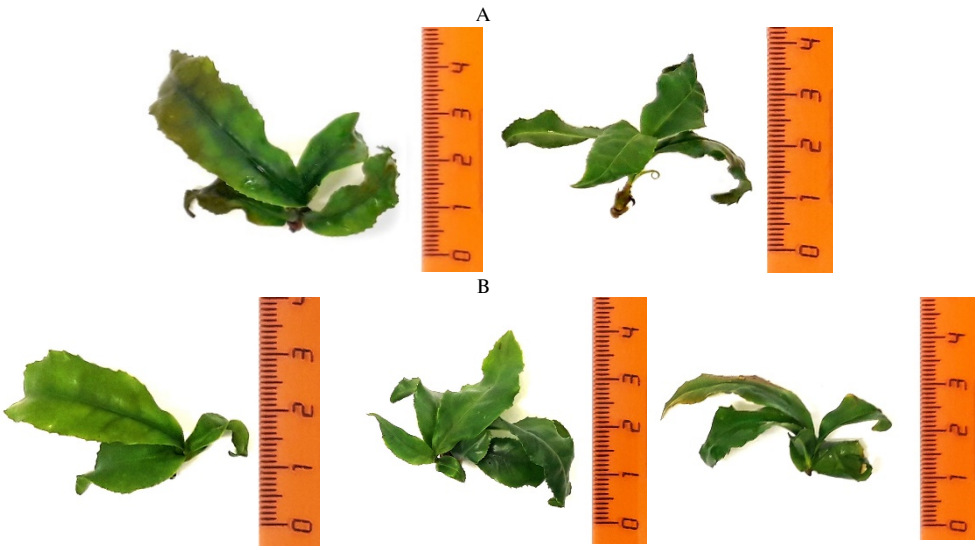

Fig. 1. Microshoots of tea plants (*Camellia sinensis* L.) grown in vitro on the nutrient media with (A) **and without** (B) **osmotic agent mannitol** (40 g/l) **and different concentrations of $CaCl_2$** (top row — 400 and 800 mg/l, bottom row from left to right 400, 600, and 800 mg/l).

The water content in the cells is an important parameter to characterize physiological state of a plant, especially under drought conditions [19]. Our study showed that the water content in leaves of tea microshoots grown on the basal nutrient medium did not depend on the effective concentration of Ca^{2+} and was practically equal for both treatments (Table 1).

Mannitol slightly but reliably decreased water content in leaves (by 2 %, $p \leq 0.05$), and this effect did not depend on the $CaCl_2$ concentration in the medium. Based on this, we assumed that Ca^{2+} contributed to the maintenance of water homeostasis of leaves in vitro in tea microshoots under osmotic stress, and this effect was practically the same at all studied concentrations (from 440 to 880 mg/l). There are reports that Ca^{2+} is involved in the regulation of plant responses to the adverse effects of drought [35]. In seedlings of *Vernicia fordii* Hemsley and *Hordeum vulgare* L. treated with this macronutrient, the leaf water content increased, and this effect depended on the time of exposure, Ca^{2+} concentration, and plant genotype [36, 37].

1. Water content in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the osmotic nutrient media

Mannitol	Treatment	$CaCl_2$ concentration, mg/l	Water content, %
Basal medium (no mannitol)	1	400	66.22 ± 1.92^a
	2	800	66.73 ± 2.28^a
Basal medium added with mannitol (40 g/l)	3	400	64.79 ± 0.11^b
	4	600	64.59 ± 0.76^b
	5	800	64.40 ± 1.45^b

[a, b] Differences between values marked with different Latin letters are statistically significant at $p \leq 0.05$.

Stressful conditions, including drought, often promote generation of reactive oxygen species in plant cells and intensify oxidative processes [1, 4, 19, 35]. Thence, LPO parameters in cells are determined, e.g., by the measuring MDA concentration [19, 38]. We noted a higher MDA level in the leaves of microshoots grown on the basal medium with low Ca^{2+} concentration (440 mg/l $CaCl_2$), on

average, approximately 2 times as much as for other treatments (Fig. 2). Perhaps this effect is due to the lack of this compound in the nutrient medium during long-term cultivation of tea microshoots in vitro. With an increase in Ca^{2+} concentration in the medium (up to 880 mg/l $CaCl_2$), the MDA concentration significantly decreased and the values detected under osmotic stress in combination with different Ca^{2+} concentrations. So, in the presence of mannitol in the nutrient medium, the MDA concertation for treatment 3 (440 mg/l $CaCl_2$) was 51.8% lower than for treatment 1 (control), 60.7% lower than for treatment 4 (660 mg/l $CaCl_2$), and almost 70% lower than for treatment 5 (880 mg/l $CaCl_2$) ($p \leq 0.05$). These results draw us to the conclusion that not only mannitol, but also Ca^{2+} (especially in high concentrations) has a regulatory effect on the antioxidant system of tea microshoots, which is confirmed by the data on a decrease in lipid peroxidation level upon treatment 2-5 compared to control treatment 1. As noted, drought conditions contribute to the development of oxidative stress, disrupting the balance between production and detoxification of reactive oxygen species [39]. In this case, Ca^{2+} ions are important secondary messengers in the transduction of intracellular signals in plants and in the regulation of oxidative reactions [7, 8]. The exogenous application of Ca^{2+} was shown to reduce negative impact of drought on *Helianthus annuus* L. seedlings [40]. Our results also indicate a significant decrease in the intensity of oxidative processes in the cells of tea microshoots due to Ca^{2+} application under osmotic stress.

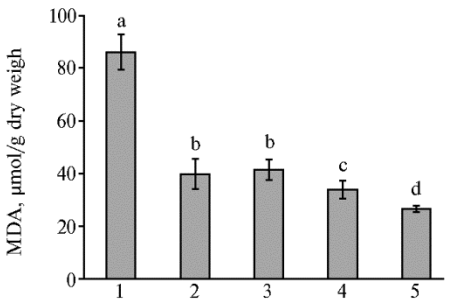

Fig. 2. Malonic dialdehyde (MDA) concentration in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the nutrient media without (1, 2) **or with** (3-5) **mannitol** (40 g/l): 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. Statistically significant differences between mean values ($p \leq 0.05$) are marked by different Latin letters.

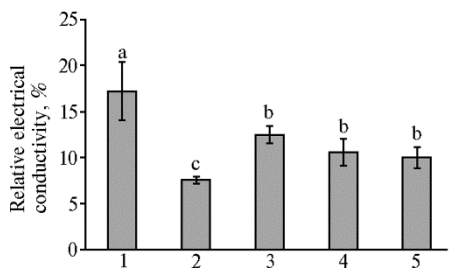

Fig. 3. Relative electrical conductivity which reflects the electrolytes leakage form leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the nutrient media without (1, 2) **or with** (3-5) **mannitol** (40 g/l): 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. Statistically significant differences between mean values ($p \leq 0.05$) are marked by different Latin letters.

An altered state of plant cell membranes resulted in a change in relative electrical conductivity is one of the initial stages of plant response to stressors [7, 10]. Cell membrane regulation, being a part of the entire regulation systems in the plant, is the most important adaptation mechanism that determines the preservation of viability, and the permeability of plant cell membranes (the rate of release of electrolytes from tissues) is an indicator of plant resistance to stresses, including the osmotic stress [41]. By analogy with the MDA level, the highest electrolyte efflux from leaf tissues occurred in tea microshoots grown on the basal medium with Ca^{2+} (Fig. 3). With an increase in the Ca^{2+} concentration (880 mg/l $CaCl_2$) in the presence of mannitol, the leakage of electrolytes decreased 1.5-2.0 times on average ($p \leq 0.05$), which indicates an increase in the stability of cell membranes.

Under osmotic stress, the effect was more apparent at higher concentrations of Ca^{2+} (660 and 880 mg/l $CaCl_2$) when we noted a decrease in relative electrical conductibility (see Fig. 3). Such a decrease indicated a less pronounced lipid peroxidation in cell membranes of these in vitro grown microshoots upon application of higher concentrations of Ca^{2+}, which is in line with literature data [14, 15]. The changes in the analyzed parameter directly correlate with the MDA levels in leaves, demonstrating a more pronounced development of lipid peroxidation processes in cells when Ca^{2+} concentrations in the medium during long-term culture is insufficient (up to 440 mg/l $CaCl_2$).

It is known that stress enhances hydrolytic processes, which leads to the accumulation of so-called stress metabolites, for example, proline, a low-molecular-weight osmotically active compound capable of forming hydrophilic colloids which protects proteins from denaturation under various stresses [9]. Our studies have shown an increase in the free proline in tea microshoots under osmotic stress (Fig. 4). An increase in the Ca^{2+} concentration to 880 mg/l significantly decreased proline concentration in the leaves of tea microshoots. The ratio of the absolute content of proline in microshoots for +mannitol/–mannitol treatments (after stress/before stress) which characterizes the rate of proline production was the highest for 400 mg/l $CaCl_2$ and amounted to 1.45 vs. 1.36 for 880 mg/l $CaCl_2$.

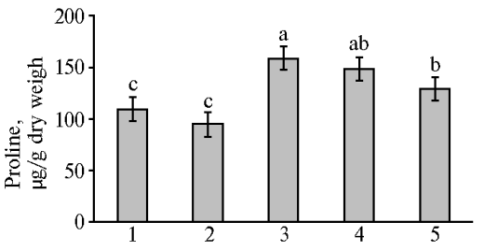

Fig. 4. Proline concentration in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the nutrient media without (1, 2) **or with** (3-5) **mannitol** (40 g/l): 1 — basial nutrient medium with $CaCl_2$ (440 mg/l, control); 2 — basal nutrient medium with $CaCl_2$ (880 mg/l); 3-5 — basal nutrient medium with 40 g/l mannitol and 440, 660, and 880 mg/l $CaCl_2$, respectively. Statistically significant differences between mean values ($p \leq 0.05$) are marked by different Latin letters.

2. Concentration (mg/g dry weigh) **of chlorophyl a** (Chla)**, chlorophyl b** (Chlb), **and carotenoids** (Car) **in leaves of tea (*Camellia sinensis* L.) microshoots grown in vitro, as influenced by $CaCl_2$ concentration in the osmotic nutrient media**

Treatment	Chla	Chlb	Car	Chla + Chlb	(Chla + Chlb)/Car
Basal medium without mannitol:					
440 mg/l $CaCl_2$	3.68±0.84[a]	0.90 ±0.30[a]	1.74±0.34[a]	4.58	2.63
880 mg/l $CaCl_2$	3.91±0.46[a]	0.95 ±0.17[a]	1.82±0.20[a]	4.86	2.67
Basal medium with mannitol (40 g/l):					
440 mg/l $CaCl_2$	2.39±0.32[b]	0.73±0.05[a]	1.26±0.12[a]	3.12	2.48
660 mg/l $CaCl_2$	1.95±0.11[b]	0.54±0.04[b]	0.98±0.04[b]	2.49	2.54
880 mg/l $CaCl_2$	2.07±0.16[b]	0.55±0.04[b]	1.07±0.05[b]	2.62	2.45

[a, b] Differences between values marked with different Latin letters are statistically significant at $p \leq 0.05$.

Osmotic compounds can cause a stress response in plants, including that leading to structural and functional rearrangement of the photosynthetic apparatus and inhibition of photosynthesis [42, 43]. The leaves of photosynthetic pigments in tea microshoots grown on the basal nutrient medium with Ca^{2+} were the highest and almost equal at both Ca^{2+} concentrations (Table 2). This may be a consequence of the structural and functional rearrangement of the photosynthetic apparatus, as well as a change in the concertation of reactive oxygen species, which explains the high level of LPO and the release of electrolytes (see Fig. 2 and 3) in long-term culture of microshoots on a nutrient medium with low concentrations of Ca^{2+} (440 mg/l $CaCl_2$). This aspect seems to us interesting and will be further studied.

When microshoots were grown on the basal medium with various Ca^{2+}

concentrations, the Chla levels in their leaves were the highest, while when mannitol was added, the Chla decreased upon all treatments by 35-40% ($p \leq 0.05$), that is, almost equally (see Table 2). A similar trend is characteristic of the Chlb levels. The level of chlorophyll b was high in the control (440 mg/l $CaCl_2$), mannitol added to this medium, decreased Chlb concentration by 20% ($p \leq 0.05$). For combination of mannitol and higher concentrations of Ca^{2+} (660 and 880 mg/l $CaCl_2$), the decrease was 40% compared to the control ($p \leq 0.05$). A decrease in the concentrations of chlorophylls in plant tissues is considered a manifestation of oxidative stress and can be the result of both degradation of pigments and structural reorganization of chloroplasts [43, 44].

The pigment system of plants is not only chlorophylls but also carotenoids that absorb the blue spectrum light, protect photosynthetic apparatus from photodegradation, and perform other protective functions [45]. As follows from our data, the trends in Car concentration changes were largely similar to those for chlorophylls, especially for Chlb (see Table 2). A higher content of carotenoids was characteristic of the leaves of tea microshoots grown on the basal medium with Ca^{2+}; the mannitol added to the medium, decreased carotenoids, but only for 660 and 880 mg/l $CaCl_2$ (treatments 4 and 5).

The revealed changes in the levels of pigments reflect the structural and functional reorganization of the photosynthetic apparatus of tea microshoots under low osmotic stress caused by mannitol. It is also possible that the observed changes occur under in vitro conditions and are largely due to in vitro differentiation of plant tissue structures, which was repeatedly reported [21, 23, 29].

Thus, a Murashige-Skoog nutrient medium added with mannitol (40 g/l) and 440-880 mg/l $CaCl_2$ causes changes in physiological and biochemical properties of tea microshoots in in vitro culture. This is manifested in a decrease in leaf water content (by 2%, $p \leq 0.05$), which is indicative of weak osmotic stress, in the accumulation of free proline in leaves, and in a decrease in the levels of photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids). When mannitol is added and the concentration of Ca^{2+} increases, the relative electrical conductivity and the concentration of malondialdehyde decrease, which indicates a decrease in cell membrane lipid peroxidation. Our study discloses some cellular mechanisms of action and the role of exogenous Ca^{2+} in tea microshoots during long-term culture and under osmotic stress. These data are of great importance with regard to depositing tea plants in biotechnological collections. In addition, they testify to the significant role of osmolytes in the preservation and maintenance of plant viability, especially in vitro.

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