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Screening Smoke Tree (*Cotinus coggygria* Scop.) on Osmotic Stress using Polyethylene Glycol 6000 *in vitro*

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ABSTRACT

Background: Droughts during the growing season are becoming increasingly common due to climate change and global warming. The study's goal study was to assess the adaptation mechanisms of *C. coggygria* Scop. under simulated water deficit conditions using indicators characterized changes in the leaf stomatal apparatus and photosynthesizing system.

Methods: The influence of osmotic polyethylene glycol 6000 on the research object *C. coggygria* regenerants was studied *in vitro*. Such indicators as morphological changes, pigment content, stomatal density and size and stomatal slit size were quantified.

Result: Due to the influence of osmotic stress, there was a pigment content increase in the leaf plate on osmotic media. Stomatal density raised by 27.0-29.8% in explant cells cultivated on PEG 4.0-6.0%. Under the osmotic influence, there was a drop in the stomatal cells' area and shape and the ratio of stomatal length to width in the regenerant leaves decreased from 1.5 to 1.0-1.1. These results indicate the adaptation of *C. coggygria* regenerants to stress caused by an artificial drought *in vitro*. The changes' identification in the stomatal apparatus and the *C. coggygria* pigment ratio will accelerate the selective screening of drought-resistant plants for use in protective forestry.

Key words: Drought, *In vitro*, PEG 6000, Pigments, Stomata.

INTRODUCTION

Droughts during the growing season are becoming increasingly common due to climate change and global warming. The average weather data analysis of the Volgograd region's dry steppe zone over the past 60 years shows the average annual air temperature in this area increased by 1.9°C. Average annual precipitation decreased from 1993 to 2015 (Protopopov, 2019) Data analysis from 1969 to 2020 (Yuferev and Tkachenko, 2021) shows an increase in average daily temperatures during the growing season from April to October by 16°C.

The lack of precipitation affects almost all processes of plant growth and development and leads to cellular changes in plants: a decrease in the cell volume, disturbance of inter- and intracellular water potential gradients, cell turgor loss, impairment of the membrane's integrity, decreasing substance concentration and protein denaturation (Kruglova *et al*., 2018). In some years, during the summer, the development of tree species is delayed by drought, which may lead to a decrease in the drought resistance of plants and their winter hardness (Kolmukidi, 2015).

The study and evaluation of plants' drought tolerance with laboratory methods gives the ability to simulate specific environmental conditions and evaluate plant responses to them. Researchers use selective agents: polyethylene glycol (PEG) with different molecular masses (600, 4000, 6000, 8000), D-mannitol, sucrose and NaCl (Hanasz *et al*., 2022). For example, using PEG 6000, mannite and sorbit as osmotics showed the advantage of the first for *Vigna radiata* L (Gnanaraj *et al*., 2022).

Currently, systems for selection of drought-resistant forms are used for major crops such as wheat (Faisal *et al*. 2019), rice (Purbajanti *et al*., 2019; Veronica *et al*., 2022), barley (Hellal *et al*., 2018), chickpea (Hussain *et al*., 2022), potatoes (Hanasz *et al*., 2022; Sajid and Aftab, 2022), alfalfa (Elmaghrabi *et al*., 2018) and sorghum (Badigannavar *et al*., 2018). At the same time, the osmotic influence on tree species *in vitro* is mentioned less often (Ahmad *et al*., 2020; Gul *et al*., 2021; Pasaribu *et al*., 2021; Vuksanovic *et al*., 2022).

In vitro selection for drought stress resistance has comparative advantages: reduced screening times, no need for large spaces and easy control. The decrease in water available to plants causes a number of biochemical and physiological changes. It leads to the development of adaptive mechanisms to keep the organism functioning normally. The anatomical and morphological leaf

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characteristics used to determine the drought plant's tolerance are evaluated (Banks *et al*., 2019; Nen'ko *et al*., 2019). Informative indicators of the osmotic stress influence on plants are the regulatory compounds' content, the stomatal apparatus changes and the photosynthetic system state (Kalinina and Lyasheva, 2018; Din *et al*., 2020; Jiroutova *et al*., 2021).

Analysis of climate change shows how important it is to investigate the tree crops' response to drought stressors. It is necessary for the subsequent stage of selective selection of suitable genotypes for drought resistance.

The research object was "smoke tree" (*Cotinus coggygria* Scop), one of the species constituting the family *Anacardiaceae*. It is a perennial deciduous shrub or a small tree with a height of up to 5 m and is widespread in areas of central China, southern Europe, Greece and southern Russia, in both arid and subarid zones. *C. coggygria* grows on dry and rocky soils, is resistant to drought and is used in steppe forestry (Matica *et al*., 2016; Zholobova *et al*., 2022). The plant is used in folk medicine all over the world and its medicinal properties have been studied. The value of *C. coggygria* is based on the presence of useful extracts and essential oils in its various plant organs (Teixeira da Silva *et al*., 2018; Thapa *et al*., 2020), which have antibacterial and antitumor effects (Gospodinova *et al*., 2021; Iliev *et al*., 2021).

The present study aimed to investigate the influence of PEG 6000 on *C. coggygria* when modeling water deficiency *in vitro*. The research problem was to estimate the adaptation mechanisms of *C. coggygria* under these conditions by introducing PEG 6000 into the nutrient medium.

Materials and Methods

The research area was the Laboratory of Biotechnology, FSC of Agroecology RAS, Volgograd, Russian Federation in 2022. *C. coggygria* explants (protopopov, 2019). Data (length= 1.5 - 2.0 cm) were used as plant materials. Ingredients for planting media included MS (Murashige and Skoog, 1962), PEG 6000 (Croda Europe Limited, the Great Britain); Dimethyl sulfoxyde (Scharlau, Spain) used to isolate pigments.

The hormone-free nutrient medium of MS used for the cultivation of *C. coggygria* explants. To create osmotic stress, MS added PEG with a molecular mass of 6000 in the required amount to obtain the studied concentrations of 2, 4 and 6% (m/v). On each osmotic variant and the control medium samples were cultivated on the rack for plant cultivation STELLAR-FITO LINE (ANO "AVTech", Russia). The conditions were sustained for 8 weeks: a photoperiod of 16 h, an intensity of lighting 80.5 - 87.5 umol s^{-1} m^{-2}, t = 24°C. Each treatment was repeated three times and each replication consisted of 10 plants.

For evaluation of the PEG effect on *C. coggygria* regenerants morphological changes, pigment content (chlorophyll, carotenoids), stomatal density and size and stomatal slit size were quantified. Pigments were extracted using dimethyl sulfoxide (DMSO), 99% (m/v). Each leaf (3-6 mg) was placed into a micropipette and 250 microliters of DMSO were added. The samples were kept in the thermoshaker TS-100 C (Biosan, Latvia) at a temperature of 65°C for 15 minutes and then cooled to 23-25°C. Then extracts were measured with the microplate spectrophotometer SPECTROstar NANO (BMG Labtech, Germany) at 480, 649 and 665 nm.

Pigments' content was calculated by the formulas (Staruhina *et al*., 2021; Gan *et al*., 2022). Chlorophyll or carotenoids were calculated by the formula (Wang *et al*., 2022). Total chlorophyll was defined as the sum of chlorophyll *a* and chlorophyll *b* concentrations. A stomatal count of 0.1 mm^2 and stomatal size were calculated by formulas (Gao *et al*., 2021).

The lower epidermis of *C. coggygria* leaves were separated from the mesophyll using tape for the study of the stomatal apparatus. The resulting samples were microcopied (×100, ×400) with the microscope LUM 1 LED (Altami, Russia) and the camera Levenhuk M500 BASE (Levenhuk, USA). The experimental data were processed using LevenhukLite ×64 (Levenhuk, USA) and ImageJ (USA) software.

The Mann-Whitney criterion ($p \leq 0.05$) was used to determine the validity of the differences. The results were processed using the Microsoft Excel package.

Results and Discussion

The research to create PEG-induced stress on *C. coggygria* regenerants showed no root formation in the mediums with different PEG 6000 contents. A 100% rootability was observed in the absence of PEG 6000 (Fig 1).

The PEG-affected explants experienced gradual leaf wilting for 7-14 days and the new ones' formation adapted to the available water scarcity. The leaves' number under osmotic stress decreased and the leaf plate was darker than the control. Structural changes were observed in the leaf apparatus: with the increase in osmotic concentration, the leaf plate density increased and the stomata were submerged in the epidermal layer, which testifies to the work of the keeping mechanism of a more negative water potential, water extraction, retention and conservation. The statistically reliable reduction of the total leaves' number and the leaf plate area between the experimental and control group samples was determined. At the end of the research, the absence of non-viable explants was recorded (Table 1).

The control medium explant leaves had an area of 37.5 mm^2 and an average leaf count of 10 pieces per explant. On media containing PEG in the studied concentrations, the average leaf plate area varied from 9.8 mm^2 to 11.5 mm^2 and the average leaf number per explant was almost halved (4.0-5.4 pcs). The leaf shape itself was not changed. The plate area decreased threefold (PEG, 2%) and nearly fourfold (PEG, 6%). Data analysis showed that stomatal density increased statistically from 36.2 pcs in 0.1 mm^2 under the osmotic influence (on the control medium) to 46.0-47.0 pcs on media containing PEG. The data shows the regenerants adapted to the artificial water shortage by adding PEG 6000.

The drastic reduction in leaf size and leaf count in the explant led to a significant reduction in the stomatal cell number in the plant's leaves, which were experiencing water deficiency. The size changes in the closing cells and the closure of the stomatal slot were observed (Fig 2).

The mean polar axis length L (Fig 3A) and the equatorial diameter D (Fig 3B) changed when PEG 6000 was added to the growth media. The statistically correct difference is fixed for the ratio (L/D) × 10 between the PEG 2%, 4% and 6% media (Fig 3C). The stoma area of the leaf plate explants in the control group was 149.8 μm^2 and the PEG-added media decreased to 142.2-104.9 μm^2 (Fig 3D). There was also a statistically significant decrease in the stomatal gap area between the control and experimental groups. This was reduced in the control medium to 13.7 μm^2 in compartments with 2%, 4% and 6% concentrations to 6.3, 4.1 and 5.6 μm^2 (Fig 3E).

Fig 1: Osmotic stress effect on *C. coggygria* explants after 7 weeks of cultivation on MS medium with different PEG 6000 content, % (m/v).

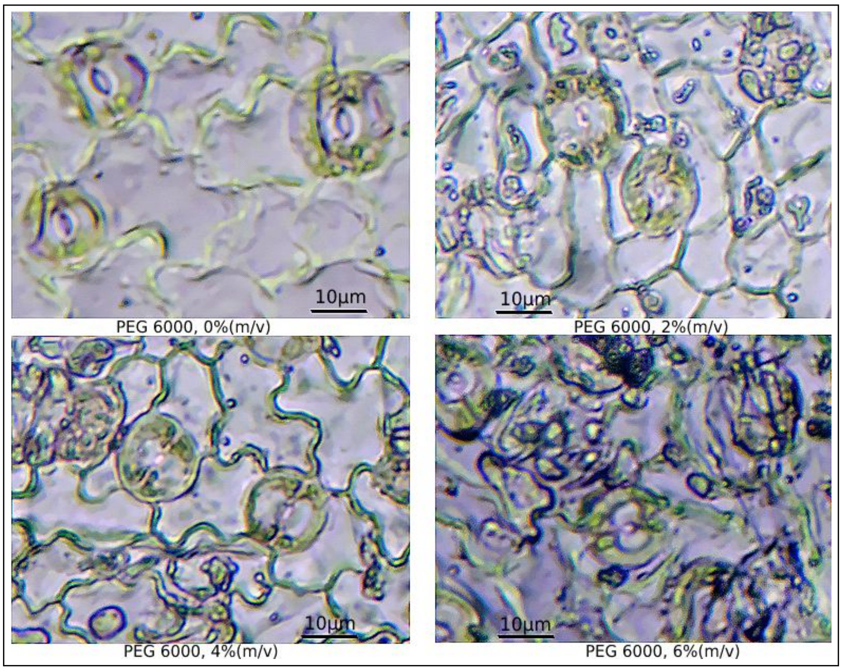

Fig 2: Microscopy of *C. coggygria* leaf plates after cultivation on MS medium with different PEG content.hy.

Under the PEG-stress influence, there was a statistically reliable increase in the pigment content in the leaf plate between the control and the osmotic media. In the control group, the total chlorophyll amount was 12 mg per leaf plate and in leaves formed by increased osmotic stress, the chlorophyll content was 20-24 μg per leaf. The carotenoid content of the leaf explants formed on the control medium was 1.5 g. When osmotic was added, it increased to 3.2-3.7 μg per leaf (Fig 4).

Under the influence of osmotic stress, changes in the ratio of pigment composition were noted (Table 2). While the osmotic potential of the medium increased, the ratio of chlorophyll a to chlorophyll b rose by 20.8-29.0%. Reliable differences were observed between the control medium (standard osmotic potential) and PEG media. There are no statistically valid differences between osmotic-containing media. The increase in the ratio of chlorophyll a to chlorophyll b indicates a rise in the photochemical activity of plants under osmotic stress. The indicator's increase shows a change in carbon dioxide assimilation from C_3 to C_4. The photorespiration pathways of the plant are twice as effective at capturing CO_2, thus increasing the intensity of dry biomass synthesis (Turmanidze and Dolidze, 2014).

The non-toxicity of PEG 6000 in relation to living objects and its lack of participation make it promising for use in laboratory evaluation of the tree crops' sustainability. PEG 6000 reduces the water potential of the nutrient medium but does not penetrate the cell itself (Kovalikova *et al*., 2020, Mohanlal *et al*., 2021). The PEG effect includes the structural and functional restructuring of the photosynthetic apparatus and inhibition of photosynthesis. Reducing the leaves' size and number of stomatal cells and increasing their number per leaf area are characteristic features of woody plants' xerophytization (Belova and Kravchenko, 2018) to adapt to water scarcity. Under stress conditions, photosynthetic activity is increased and maintained at a high level for the

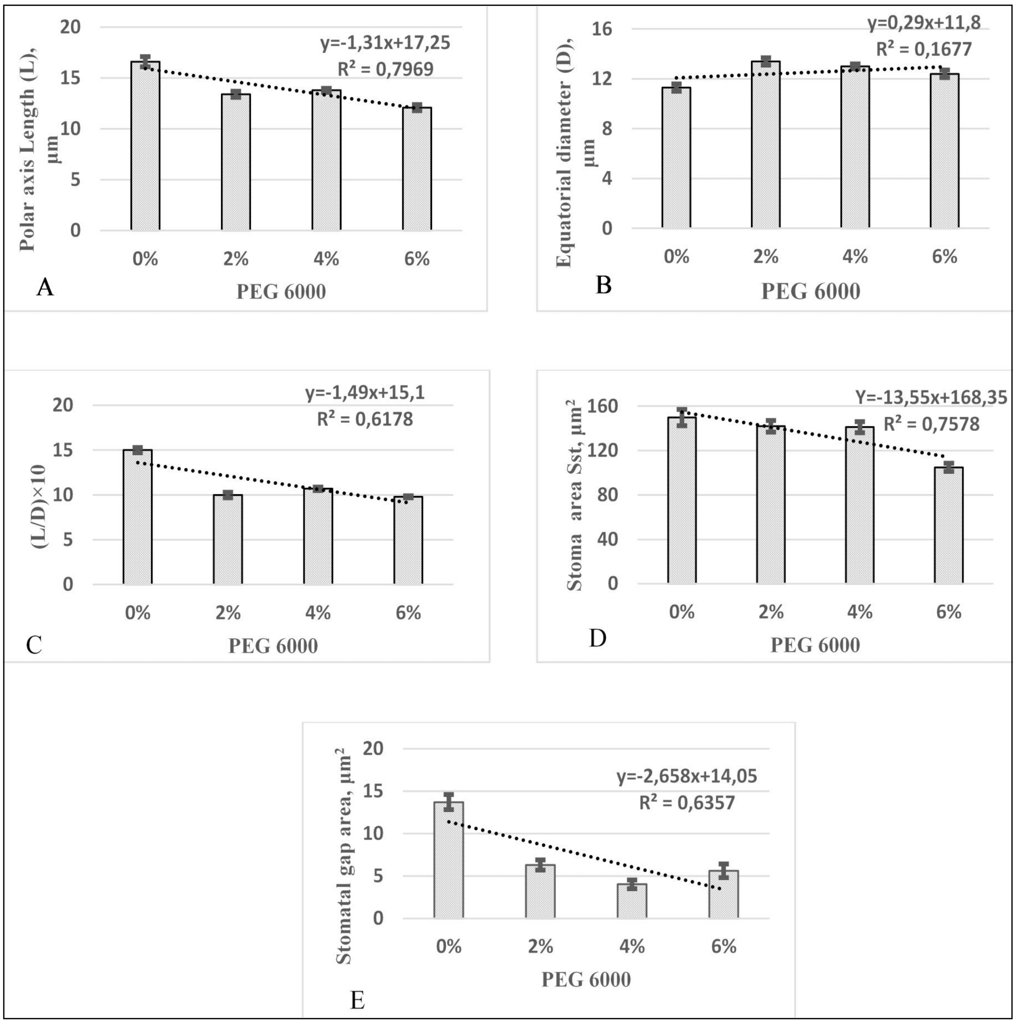

Fig 3: Stomatal cells' quantitative traits of *C. coggygria* under osmotic stress induced by PEG 6000 added to the medium at levels of 2%, 4% and 6%: A- polar axis length, B- equatorial diameter, ratio (L/D), D- stoma srea, E- stomatal gap area.

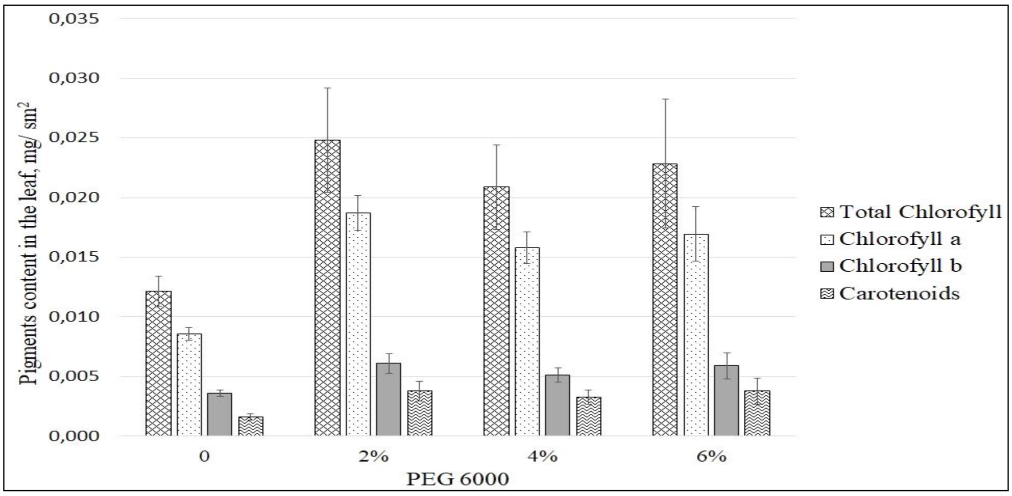

Fig 4: Pigment composition of *C. coggygria* leaves formed under the influence of PEG 6000. Data are represented as mean ± SD. Means with the same letter are not significantly different for each other (P<0.05).

Table 1: *C. coggygria* leaf plate parameters.

Quantative trait	PEG added to MS medium, % (m/v)			
	0 (control)	2%	4%	6%
Leaf plate area, mm^2	37.5±3.3	12.6±1.1	10.9±1.2	9.8±1.1
Polar axis length (L) of leaf plate, (L) mm	9.1±0.5	5.0±0.2	4.7±0.3	4.7±0.3
Equatorial diameter (D), mm	5.6±0.2	3.1±0.2	2.8±0.2	2.8±0.2
(L/D) ×10	16.3±0.6	16.3±0.4	16.9±0.5	16.8±0.7
Stomatal count of 0.1 mm^2	36.2±2.6	47.0±0.7	46.3±2.6	46.0±2.6
Number of leaves per explant, pcs.	10.0±1.1	5.4±0.6	4.71±0.52	4.00±0.63

Table 2: The main groups' ratio of photosynthetic pigments under the influence of osmotic stress.

Pigment	PEG, % (m/v)			
	0	2%	4%	6%
Chlorophyll *a* + *b*/Carotenoids	7.7±0.6	6.5±0.1	6.5±0.2	6.0±0.2
Chlorophyll *a*/Chlorophyll *b*	2.4±0.1	3.1±0.1	3.1±0.1	2.9±0.1

synthesis and accumulation of di- and oligosaccharides. Under these conditions, chlorophyll is correlated with the level of membrane-bound protein components of the photosynthetic apparatus (Yakovets, 2010).

The study noted a 15.5-22.0% decrease in the ratio of total chlorophyll to carotenoids. In the control medium, this indicator was 7.7 and under the action of osmotic stress it is reduced to 6.0-6.5. A statistically valid difference is recorded between the control and PEG media. The decrease in this indicator shows faster leaf aging due to osmotic stress (Lichtenthaler and Babani, 2004).

According to the research results, all *C. coggygria* explants have successfully adapted to the soil drought's effect, modeled *in vitro* on media with PEG concentrations of 2%, 4% and 6%. PEG-induced osmotic stress describes the physiological and biochemical mechanisms of *C. coggygria's* response to water stress. The results showed a decrease in the open stomata number.

CONCLUSION

The addition of PEG to the nutrient medium caused a reduction of the stomatal opening in the cells, indicating the water-saving ability of the leaves grown *in vitro*. In the samples subjected to osmotic stress, the content of chlorophyll and carotenoids increased, which is due to the leaf plate's change. The osmotic pressure of the external solution leads to a significant restructuring of pigment systems in drought-resistant plants. The research uncovered some cellular mechanisms that occur when the photosynthetic apparatus of *C. coggygria* is subjected to osmotic stress. The results may be useful in developing a strategy for initial screening and selection of drought-resistant C. coggygria genotypes.

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Conflict of interest: None.

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