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ADAPTIVENESS OF PROMISING LAVENDER AND LAVANDIN CULTIVARS UNDER in vitro CULTURE AND ex situ

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Abstract

Lavandula angustifolia Mill. and lavandin (Lavandula × intermedia Emeric ex Loisel) are promising fragrant plants with medicinal, aromatic and ornamental properties. To obtain high quality healthy planting material, in vitro cultures of valuable cultivars Belyanka, Record (lavender) and Rabat, Snezhnyi Bars (lavandin) were derived. Obtained regenerants were cultured for 4-5 months on Murashige and Skoog medium with 0.3 mg/l kinetin, 0.025 mg/l NAA и 0.25 mg/l GA₃ in growth chamber at 25 ± 1 °C under 16-h photoperiod and light intensity of 37.5 μ M · m⁻² · s⁻¹. Intact plants were studied during the growing season. In order to reveal plant morphogenetic capacity, biochemical stress indicators, indexes of photosynthetic activity, maximum fluorescence (F_m), stationary level of fluorescence (F_{st}) and water regime were determined. The proline content of lavender and lavandin plants grown ex situ was rather high (6.67-21.59 μ g/g). In in vitro micro-plants, although there was considerable hydration of the plant tissues, the proline concentration was higher than that in the intact plants (8.24-35.72 µg/g). Intact lavender and lavendin plants accumulated high amounts of phenolic compounds (1033-1492 mg/100 g) and ascorbic acid (14.96-20.06 mg/100 g). In plants under controlled conditions, the concentration of phenolic compounds and ascorbic acid was lower (490-777 and 4.95-5.98 mg/100 g, respectively), which is caused by significant waterlogging of tissues and lack of stress. Regardless of the growing condition, the level of phenolic compounds was higher in the lavandin cultivars compared to lavandula plants. Open field cultivated plants were distinguished by high activity of catalase (18.13-36.97 g $O_2 \cdot g^{-1} \cdot min^{-1}$) and superoxide dismutase (12.55-14.82 a.u./g). Under the hydrothermal stress effect ex situ, relative photosynthetic activity and viability index indicated minor decrease in assimilation processes in lavender cultivars but was within vital limits. In in vitro culture, the catalase activity of lavender cultivars was higher than that of lavandin. At the same time, SOD and PPO activity of lavender micro-plants in vitro was lower than that of lavandin micro-plants. In open field cultivation, leaf tissue hydration of tested plants was 56-62 %, with greater part of bound water. In plants cultured in vitro, the rate of hydration was high (70-77 %). with the same trend of water fractional composition. Under the controlled conditions and nominal heterotrophic nutrition type, photosynthetic activity was 0.28-0.55 a.u. with the maximum in the Rabat cultivar. Values of chlorophyll fluorescence induction and vitality index indicated no photoinhibition. It was found out the lavandin cultivars had better capacity for a wide use under various conditions.

Keywords: Lavandula sp., biochemical indicators, photosynthetic activity, water regime, in vitro, ex situ

Lavandula (*Lavandula* L.) is a valuable essential-oil-bearing, aromatic, decorative and medicinal culture. The main cultivated essential-oil-bearing plants include true lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.). They contain essential oils used in medicine, perfume, cosmetics and food industry [1]. Phenolic compounds,

which have a wide range of physiological effects, were also found in plant raw materials [2]. Traditional vegetative reproduction of lavender and lavandin is a complex, long-lasting and not always effective process. A high-quality planting material of essential-oil-bearing plants can be obtained in vitro. Biotechnological methods allow obtaining in the shortest possible time a significant amount of healthy plants, which are genetically identical to the original species, variety or form, in case of a lack of starting material [3. 4]. To study the adaptive capabilities of the micro shoots grown in vitro, it is necessary to take into account the functioning of the antioxidant system, which includes low-molecular protecting compounds and specific antioxidant enzymes [5, 6].

The main protecting compounds of plants include proline, phenolic substances, and ascorbic acid. Proline is the source of energy, carbon and nitrogen in case of resource shortages caused by stress, and a decrease in the activity of synthesis enzymes [7]. Phenolic compounds and ascorbic acid are involved in the basic processes of plant cells activity, i.e. photosynthesis, respiration, protection from stress factors [8, 9]. Enzymes of the antioxidant system, i.e. superoxide dismutase (SOD), catalase (CAT), as well as polyphenol oxidase (PPO), bind excess amounts of reactive oxygen species (ROS), stop free radical chain reactions and thereby regulate the oxidative processes occurring in plants [10-12]. The activity of oxidation-reduction enzymes depends on the susceptibility of the organism to the stress factors and on the stage of plant development [13].

The photosynthetic activity and the vitality index are also sensitive parameters of the change in the functional state of plants. The amplitude and phase characteristics of the induction signal correlate with the physiological state of the tissue. The higher the speed and the amount of the change in optical parameters, the higher the functional activity of the plant [14]. The resistance of chlorophyll-containing tissues to excessive illumination is widely used in experimental biology as an integral criterion of the functional state of plants and adaptability to unfavorable environmental factors [15, 16]. The light-dark kinetics is discussed in connection with the response of photosystems I and II to changing conditions of cultivation [17, 18] and the effect of factors of abiogenic nature [19, 20].

Adaptation to new conditions of cultivation, namely the transfer of planting material from the open ground to the conditions of aseptic culture (in vitro), has a complex nature and is based on the lability and tolerance of biochemical and physiological parameters, the limits of which are determined by the genetic nature of the organism. Despite the high economic value of lavender and lavandin, data about the adaptive potential of this culture in vitro is insufficient. This report, for the first time, shows a comparative physiological and biochemical characteristics of promising lavender and lavandin varieties under various cultivation conditions.

The purpose of this paper is to identify the adaptive ability of valuable lavender and lavandin varieties in vitro and ex situ by determining their physiological and biochemical parameters.

Techniques. The study was conducted using valuable varieties of true lavender (Belyanka, Rekord) and lavandin (Rabat, Snezhnyy Bars) of the Nikitsky Botanical Garden (NBS-NSC, Crimea) from the gene pool collection. Intact plants in the phenological stage of technical maturity, grown ex situ on the NBS-NNC collection plots, as well as micro shoots cultured in vitro, were involved in physiological and biochemical studies. Samples were taken in the decades II and III of July 2016.

Apical meristems of auxiliary buds were used for in vitro tissue culture. Micro shoots were cultured for 4-5 months on a modified Murashige and Skoog medium with 0.3 mg/l kinetin, 0.025 mg/l α -naphthylacetic acid and 0.25 mg/l

gibberellic acid (Sigma, USA), 30 g/l sucrose and 8 g/l agar (Panreac, Spain). Explants in culture vessels were placed in an artificial climate chamber MLR-352-PE (Panasonic, Japan) at a temperature of 25 ± 1 °C, a 16-hour photoperiod and a light intensity of $37.5 \ \mu M \cdot m^{-2} \cdot s^{-1}$.

Biochemical indicators were determined by conventional methods: proline content was determined by the method of Chinard with ninhydrin reagent [21], the amount of phenolic substances was determined spectrophotometrically with Folin-Ciocalteu reagent (AppliChem GmbH, Germany) [22]. Calibration curves for proline content evaluation were constructed with L-proline (Appli-Chem GmbH, Germany), phenolic substances with gallic acid (Sigma, USA), flavonols with rutin (Sigma, USA). The amount of ascorbic acid was determined by iodometric titration [23], the catalase (CE 1.11.1.6) activity by the titrimetric method [24], polyphenol oxidase (CE 1.14.18.1) activity was measured in the presence of pyrocatechin (Sigma, USA) and p-phenylenediamine [25], superoxide dismutase (EC 1.15.1.1) by oxidation of quercetin (Sigma, USA) [26]. A spectrophotometer Evolution 220 UV/VIS (Thermo Fisher Scientific, USA) was used.

The total water content in the leaves, the fractional composition of water, and the water deficiency were evaluated as physiological criteria characterizing the water regime when cultivating plants in the open ground [27]. The parameters of photosynthetic activity were measured using a portable fluorometer (Institute of Cybernetics named after V.M. Glushkov of the National Academy of Sciences of Ukraine) [28]. The maximum (F_m) and stationary (F_{st}) fluorescence values after the darkness adaptation were recorded as components of Kautsky's fluorescence induction kinetics. The viability index and photosynthetic activity were calculated [15].

The experiments were arranged in 3-fold biological and 3-fold analytical replications. The data obtained was processed using Statistica 6.0 software (StatSoft, Inc., USA). The tables show the average values of the indicators (M) and their standard deviations (\pm SD). The significance of differences between the variants was evaluated by the arithmetic mean and variation coefficient at p < 0.05.

Results. The Rekord variety of true lavender was obtained by the method of inbreeding. The plants are large, 55-60 cm high, semi-spreading. The variety is mead-season, winter hardy, high-yielding and high oleic. The content of essential oil is 1.8-2.0% of the raw mass of the inflorescences. The main components are linalool (34.6%), linally acetate (31.2%) and 1.8-cineol (3.7%). The Belyanka variety is a recessive form of the Rekord variety, isolated by individual selection. The plants are compact, 50-55 cm high. The variety is early-season and lowyielding. The content of essential oil in the inflorescences is up to 1% of the raw weight. The main components are linally acetate (14.3%), linalool (63.7%), cineole (2.7%), camphor (2.1%). The Snezhnyy Bars variety is a recessive form of lavandin of the Pervenets variety of clonal selection. The bush is compact, 80-90 cm high. The variety is winter hardy, high-yielding. The yield is 75-85 c/ha, the yield of essential oil is 225-240 kg/ha, the content of essential oil is 3% of the raw biomass. The main components: linalool (41.8%), linalyl acetate (19.4%), terpineol (7.4%), camphor (4.9%). The Rabat variety is an allotriploid hybrid, obtained by the method of distant hybridization of true lavender with spike lavender. The plants are compact. The yield is 110-120 c/ha, the yield of essential oil is 341 kg/ha, the content of essential oil is 3.1% of the raw mass. The main components are linalool (36.7%), linally acetate (32.1%), camphor (5.6%), 1.8-cineol (3.7%).

In the decades II and III of July 2016, the average daily air temperature was 27.0 °C (maximum air temperature was 31.0 °C). Relative air humidity was 51%, the minimum was 47%. The temperature on the soil at the time of sample

collection reached 57.5 °C, at a depth of 10 cm was 30.0 °C. As per the instrumental determination of the soil moisture, the reserves of productive moisture in a one-meter layer of soil were up to 22 mm (14% of the lowest moisture capacity). The rainless period that preceded the date of the analysis lasted 18 days. During this period, 6 days with conditions corresponding to dry hot wind were observed (relative air humidity dropped to 40%, the average daily air temperature was above 25 °C, wind gusts reached 15 m/s, average values were 5-8 m/s).

In open ground, the proline content in lavender and lavandin plants has a variety-specific nature (Table 1).

Phenolic compounds, Ascorbic Variety Conditions Proline, µg/g <u>acid, mg</u>/100 g mg/100 g 7.69±0.23 Belyanka Ex situ 20.06 ± 0.58 1033±26 Cv, % 8.5 7.1 82 In vitro 8.24 ± 0.24 5.61±0.16 645±17 Cv % 82 75 8.1 $12.95 {\pm} 0.37$ 18.92 ± 0.54 Rekord Ex situ 1181±31 Cv. % 8.1 8.0 7.4 33.75±0.99 In vitro 5.94±0.17 490±14 Cv, % 8.3 8.1 8.1 $6.67{\pm}0.20$ Rabat Ex situ 19.14±0.55 1305±34 Cv, % 8.5 8.1 7.4 35.72±1.04 4.95±0.13 668±19 In vitro Cv, % 8.2 7.4 8.0 21.59±0.63 14.96 ± 0.44 1492 ± 40 Snezhnyy Bars Ex situ Cv, % 8.3 8.3 7.6 35.32 ± 1.05 5.98±0.17 In vitro 777±22 8.4 8.0 Cv. % 8.0 N o t e. Cv — coefficient of variation at p < 0.05.

1. The content of protective substances in the plants of lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) of different varieties ($M\pm$ SD, 2016)

It is known that proline performs osmoregulatory functions and takes part in gene expression [29]. Proline content in plants grown in vitro, despite high water content tissues (56-62%), was significantly higher than in intact plants. This suggests that free proline has an effect on the growth and differentiation of lavender and lavandin cells. In vitro conditions are not stressful for plants because they are selected for optimal development of micro shoots and are characterized by constant temperature, humidity, and illumination.

Intact lavender and lavandin plants accumulated high amounts of phenolic compounds and ascorbic acid. An increase in the phenolic compounds, as a rule, is a response to the stress factors [30]. The content of phenolic compounds and ascorbic acid in the analyzed samples of the plants grown under controlled conditions was significantly lower, which is due to considerable water content in the tissues (56-62%) and the absence of stress. The content of phenolic compounds in lavandin varieties cultivated in the open ground was significantly greater than in lavender varieties.

Ex situ plants are characterized by high activity of catalase and superoxide dismutase (Table 2). In in vitro culture catalase activity in lavender varieties was 2 times higher than in lavandin. At the same time, the activity of SOD and PPO in lavender was lower than in lavandin. Comparative analysis showed that the minimum values of the catalase and polyphenol oxidase activity are typical for lavender and lavandin varieties grown in vitro. The decrease in enzyme activity is due to a high water content in tissues, a low content of ascorbic acid and phenolic compounds, and the absence of stress factors. The activity of SOD in lavandin varieties grown in the in vitro culture is similar to that in plants grown ex situ, and in lavender varieties in the in vitro culture, such activity was 50% lower than in intact plants.

2.	Activity of oxidation-reduction enzymes in the plants of lavender (Lavandula an-
	gustifolia Mill.) and lavandin (Lavandula × intermedia Emeric ex Loisel.) of dif-
	ferent varieties ($M\pm$ SD, 2016)

Variety	Conditions	Catalase, g $O_2 \cdot g^{-1} \cdot min^{-1}$	SOD, c. u./g	PPO, c. u. $\cdot g^{-1} \cdot s^{-1}$
Belyanka	Ex situ	30.68±0.87	12.98±0.32	0.524±0.013
	Cv, %	8.0	6.9	7.1
	In vitro	7.65±0.19	5.62 ± 0.14	0.103 ± 0.002
	Cv, %	7.0	7.1	5.5
Rekord	Ex situ	18.13±0.45	13.60 ± 0.33	0.628 ± 0.016
	Cv, %	7.0	6.9	7.2
	In vitro	6.80±0.16	6.12 ± 0.20	0.101 ± 0.003
	Cv, %	6.7	9.2	8.4
Rabat	Ex situ	31.45±0.77	12.55 ± 0.32	0.600 ± 0.015
	Cv, %	6.9	7.2	7.1
	In vitro	3.68±0.09	12.43 ± 0.31	0.112 ± 0.003
	Cv, %	6.9	7.1	7.6
Snezhnyy Bars	Ex situ	36.97±0.92	14.82 ± 0.38	0.377 ± 0.008
	Cv, %	7.0	7.3	6.0
	In vitro	2.98 ± 0.08	10.48 ± 0.28	0.124 ± 0.004
	Cv, %	7.6	7.6	9.1
N o t e. SOD $-$ variation at p <	superoxide dismuta 0.05.	ase, PPO – polyphenol oxidase; o	e. u. — conditional u	nits. Cv – coefficient of

When grown in the open ground, the water content in the leaves was 56-62% (Table 3), the proportion of bound water was 78-93% of its total content.

3. Parameters of the water regime and the relative quantum efficiency of the photosystem-2 in lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) of different varieties ($M\pm$ SD, 2016)

Tu dianta n	Conditions	Variety					
Indicator		Belyanka	Rekord	Rabat	Snezhnyy Bars		
Total water content,%	Ex situ	61.1±3.0	57.9±2.5	56.3±4.8	62.3±2.1		
	Cv, %	13.9	12.2	24.1	9.5		
	In vitro	76.1±3.3	72.3±2.9	77.0 ± 2.5	74.4±3.2		
	Cv, %	12.3	11.4	9.2	12.2		
Fraction of bound water, % of total water	Ex situ	78.3±4.9	90.6±3.5	82.1±4.3	93.2±1.3		
content	Cv, %	17.7	10.9	14.8	3.9		
	In vitro	69.5±4.1	58.1±2.2	68.3±4.8	49.4±6.1		
	Cv, %	16.7	10.7	19.9	34.9		
Water deficiency, %	Ex situ	26.9±1.4	24.8±2.9	23.1±2.9	29.1±1.2		
	Cv, %	14.7	33.1	35.5	11.6		
Relative photosynthetic activity, $(F_m-F_{st})/F_m$	In vitro	0.68 ± 0.09	0.70 ± 0.05	0.75 ± 0.10	0.71 ± 0.05		
	Ex situ	0.28 ± 0.10	0.45 ± 0.05	0.55 ± 0.08	0.45 ± 0.09		
Viability index, F _m /F _{st}	In vitro	2.61 ± 0.50	2.51±0.61	3.18 ± 0.52	2.94 ± 0.70		
	Cv, %	54.2	68.7	46.2	67.3		
	Ex situ	1.41 ± 0.03	1.71 ± 0.12	2.36 ± 0.37	2.00 ± 0.36		
	_Cv, %	6.0	19.8	44.3	50.9		
N ot e. F_m and F_{st} – the maximum and stationary values after darkness adaptation, respectively. Cv – coefficient of variation at $n \le 0.05$							

After a long (18 days) drought period, the total water content in the vegetative organs decreased, while the proportion of bound water increased. The maximum water-retaining capacity was typical for tissues of vegetative organs of the Snezhnyy Bars and Rekord varieties due to the bound water fraction. The degree of water deficiency in the leaves of lavender and lavandin varieties varied from 23% to 29%. The water content in the leaves of in vitro micro shoots was higher in lavandin varieties (74-77%); there were no significant differences between varieties for this parameter. However, the smallest variability of water content in micro shoots during cultivation and the maximum ratio of bound and free water fraction maks it possible to highlight the Rabat and Belyanka varieties.

Changes in the water regime to a greater extent affected the photosynthetic activity of lavender varieties. They were characterized by the decrease in the relative quantum activity of photosystem II, photochemical reactions and the efficiency of energy capture by open reaction centers. The viability index of all studied varieties is within the standard, but in lavandin of the Rabat variety, it is significantly higher.

The apical meristem in the in vitro conditions after 5-6 subculturings formed 2-5 micro shoots of 23-82 mm in height, each micro shoot had 10-26 leaves, the leaves were lanceolar, 9-15 mm in length. The micro shoots leaves showed high photosynthetic activity. When cultivating under controlled conditions in vitro and at a relatively heterotrophic nutrition, the viability index was also normal, its values were of a variety-specific nature. Parameters of the functional state of the studied in vitro plants indicate the absence of photoinhibition, the normal functioning of photosystems both during the operation of lightharvesting systems and at the moment of oxidation of electron donors in the reaction center of the photosystem II.

Thus, the content of phenolic compounds, ascorbic acid, and the activity of catalase, superoxide dismutase, and polyphenol oxidase are maximal in the plants of lavender and lavandin in the open ground. There are no significant differences between varieties of lavender and lavandin in terms of these parameters. The content of proline in the in vitro micro shoots is higher, and the content of phenolic compounds, ascorbic acid, and enzymatic activity is lower than in intact plants. Changes in the water regime of the studied varieties to a greater extent affect the photosynthetic activity of lavender varieties. The viability index is normal, there is no photoinhibition. The adaptive potential of lavandin varieties under different cultivation conditions is determined to be higher than that of lavender varieties.

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