Arch Biol Sci. 2017;69(1):119-127 DOI:10.2298/ABS160202085S

Response of Virginia (flue-cured) tobacco genotypes to water-deficit stress

Lydia Shtereva[1], Elisaveta Stoimenova[1], Marina Drumeva-Yoncheva[2], Bistra Michailova[1], Tanja Kartzeva[1] and Roumiana Vassilevska-Ivanova[1,*]

[1] *Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences*, 1113 Sofia, Bulgaria

[2] *Tobacco and Tobacco Products Institute*, Plovdiv, Bulgaria

*__Corresponding author__: ru_vas_bg@yahoo.com

Received: February 2, 2016; **Revised**: May 27, 2016; **Accepted**: June 24, 2016; **Published online**: September 23, 2016

Abstract: The effect of prolonged water deficit on four Virginia (flue-cured) tobacco genotypes, Line 842, Oxford 207, RG11 and Virgin D, was analyzed in whole plants. Drought stress was induced by withholding irrigation and subjecting plants to low, moderate and severe regimes. Some growth indices such as fresh weight, plant growth rate, number, color and area of new developed leaves, as well as proline, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content as a measure of oxidative stress were investigated to examine the role of genotype in water-deficit tolerance. Under stress, the weight of the aboveground parts of plants, plant growth height, number of new developed leaves and leaf area index decreased with the severity of treatment. The stressed plants accumulated more proline, malonildialdehide and hydrogen peroxide than control non-stressed plants under water-deficit conditions. The results showed that among the genotypes, Virgin D (VD) was the most sensitive to drought, while L 842 and Oxford 207 were moderately tolerant; RG11 was drought-tolerant. This suggests that the correlation between the physiological traits and level of antioxidative response exists and therefore it could be used as a rapid screening test to evaluate the drought tolerance of tobacco.

Key words: tobacco plant; drought stress; proline; hydrogen peroxide; lipid peroxidation

INTRODUCTION

Water-deficit, permanent or temporary, is one of the major abiotic factors that limits productivity of cultivated crops, and their ability to withstand such stress is of immense economic importance [1,2]. The numerous responses of plants to water deficits generally vary with the severity as well as with the duration of the water stress [3,4], which triggers a wide variety of plant reactions ranging from altered gene expression and cellular metabolism to changes in growth rate and plant productivity [5,6]. Like other abiotic stresses, water deficit leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) [7-9]. These ROS are highly reactive and can alter normal cellular metabolism through oxidative damage to nucleic acids, proteins, lipids, chlorophyll and any other organic constituent of a living cell [7,10]. Under optimal growth conditions, ROS are mainly produced at a controlled level in chloroplasts, peroxisomes and mitochondria, while under stress, their rate of production is dramatically elevated [11].

During water deficit, plants adapt to oxidative stress by accumulating certain protective compounds such as proline, glycine, betaine, polyols, trehalose, etc. [12]. Proline plays a predominant role in protecting plants from osmotic stress. The malondialdehyde (MDA) content, a product of lipid peroxidation, has been considered as indicator of oxidative damage and has been widely utilized to differentiate drought-tolerant and drought-sensitive cultivars [13-16]. Similarly, differential H_2O_2 accumulation has been reported in many plants in response to drought stress [17].

Tobacco (*Nicotiana tabacum* L.) Solanaceae is one of the most important industrial crops cultivated in many countries with different climatic conditions. The advantages of adequate soil-water content and appropriate irrigation in flue-cured tobacco production have been reported [18-22]. It was found that tobacco was the most sensitive to soil-water deficit during the

How to cite this article: Shtereva L, Stoimenova E, Drumeva-Yoncheva M, Michailova B, Kartzeva T, Vassilevska-Ivanova R. Response of Virginia (flue-cured) tobacco genotypes to water-deficit stress. Arch Biol Sci. 2017;69(1):119-27.

early developmental stages and period of leaf expansion [23,24]. Moreover, water deficit of different severity at the flowering and ripening stages of tobacco delayed harvesting time and resulted in a reduction of plant height, leaf number and leaf area [20-22,25].

In Bulgaria, flue-cured Virginia tobacco is cultivated mainly in areas under irrigation. However, the amount of rainfall is unpredictable and generally insufficient during the critical growing stage of the plants [26]. Although tobacco is relatively drought-stress tolerant plant *per se,* reports demonstrated the loss of quality leaf yield in response to water-deficit stress [22,24,27-29]. Thus, improving the drought tolerance and water-use efficiency of flue-cured tobacco is one of the important subjects of studies that include both conventional breeding approaches and effective drought-tolerant screening systems.

Recently there has been an increasing interest in identifying attributes that contribute to water-deficit resistance and which can be used as selection criteria in tobacco-breeding programs [20,22,24,25]. However, the water-deficit tolerance experiments were performed mainly in fields where the growing conditions were difficult to control. This study was designed to develop a rapid laboratory screening method for testing tobacco genotypes to water deficit in the early ontogenetic stages by investigating: (i) the effects of water-deficit stress in four Virginia (flue-cured) tobacco genotypes on some morphological and physiological changes; (ii) the levels of some nonenzymatic stress-related markers – proline, malonildialdehide (MDA) and hydrogen peroxide (H_2O_2), and their relationship to the degree of water-deficit tolerance.

MATERIALS AND METHODS

Plant material

Four flue-cured Virginia tobacco (*Nicotiana tabacum* L.) genotypes of economic importance were chosen for this study: Line 842 (L 842) was developed in the Tobacco and Tobacco Products Institute (TTPI), Plovdiv by Prof. Chinchev [26]. It is an early-maturing line with moderately high yield potential and good quality. The cultivars Oxford 207 (Ox 207) and RG11 were introduced by the USA, while Virgin D (VD) was developed and released by Germany. L 842 and VD are resistant to potato virus Y (PVY), which is widespread in Bulgaria [30,31]. All experiments were carried out using seeds produced in the same year and under the same climatic conditions in the field of the TTPI.

Experimental site and experimental procedure

Tobacco (*Nicotiana tabacum* L.) seeds were germinated in a growth chamber (Float System for Producing Tobacco Seedlings). Seedlings at the fourth leaf stage were transplanted to individual pots (65/65/60 mm). Growth conditions were 25°/18°C (day/night) under a 16/8-h photoperiod with light intensity of 120 μmol m^{-2} s^{-1} and relative humidity (RH) of 75%. Only plants at the 6th true leaf stage and similar growth development were used in the water stress experiments.

Irrigation regime

Drought stress was imposed by cessation of watering for 4 weeks. Four irrigation regimes were applied: normal water supply (control plants that were watered to full capacity) and limited water supply provoking drought stress, which included irrigation with 20 mL distilled water (low stress), 10 mL distilled water (moderate stress) and 5 mL distilled water (severe stress); water was supplied once a week. All experiments were repeated at least twice, and each experimental treatment was performed with ten plants in four replicates.

Sampling

The length and width of the growing 6th leaf was measured at the beginning of water-deficit treatment. At the end of experiment (29th day after the start of the irrigation regime), the following traits were recorded: fresh weight (FW), plant absolute growth rate (AGR) of the whole aboveground parts (measured using the formula: $(H_2 - H_1)/(t_1 - t_2) \times 100 = \%$, where H_1 and H_2 are the initial and final plant height (cm) at the beginning (t_1) and end (t_2) of the measurement period, respectively) [32]. The green leaves of plants were used to determine the non-enzymatic stress-related compounds. Samples of the leaves were collected, cut into pieces and immediately frozen in liquid nitrogen. Leaf area index (LAI) was determined [33] using the following formula: $LAI = k\ (LW)$, where

k is an empirical constant 0.653; L and W represent the leaf length and width (cm). Leaf relative water content (RWC) was estimated as described in [34]. The calculation was made according to the equation: RWC (%) = (FW-DW)/(TW-DW) x 100, where FW is sample fresh weight, DW sample dry weight and TW sample turgid weight.

Proline content measurement

Free proline content was extracted from 0.5 g of leaf and samples of stems in 3% (w/v) aqueous sulphosalicylic acid and estimated by using ninhydrin reagent [35]. The absorbance of the fraction with toluene aspired from the liquid phase was read at 520 nm. Proline concentration was determined using calibration curve and expressed as μmol proline/g FW.

Hydrogen peroxide assay

The H_2O_2 content was colorimetrically measured as described [36]. About 500 mg of leaf and stem tissues were homogenized in an ice bath with 5 mL of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12000×g for 15 min at 4°C. The enzymatic reaction was started with 0.5 mL of supernatant and 0.5 mL of peroxidase reagent consisting of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1M KJ. The absorbancy of the supernatant was measured at 390 nm. H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2.

Lipid peroxidation assay

The level of lipid peroxidation was determined by estimating the MDA content in 500 mg (fresh weight) leaves and stems [37]. MDA is a product of lipid peroxidation by thiobarbituric acid reaction. The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis

Standard errors of means were calculated for all parameters studied. Data were analyzed using ANOVA and Duncan's multiple range test at $P<0.05$ with the statistical package STATISTICA 7.0 (Stat-Soft, Inc., USA).

RESULTS

An equal developmental stage of tobacco plants was one of the prerequisite conditions of the current study. In our case, Ox 207 and RG11 genotypes germinated and developed much slower than the other two. Therefore, the screening for water-deficit stress began 2 weeks later. At the onset of water-deficit treatment, the oldest (basal) leaves of all four tobacco genotypes showed evidence of senescence – the first two leaves were completely desiccated, while visible yellowing, but without the loss of leaf turgor, occurred in the 3rd and the 4th leaves; the 5th and 6th were green with incomplete leaf development. On the top of plant, an apical 7th leaf with a length of less than 5 mm appeared.

Among the four genotypes tested, cv. Ox 207 exhibited the lowest values of plant growth parameters under normal irrigation (control plants) (Table 1). These data varied to a lesser extent in the remaining three genotypes, as the superiority in one parameter was compensated by the lower values in the other parameters. The rate of leaf initiation differed among the tested tobacco genotypes (Table 2). Both cultivars Ox 207 and RG11 revealed a slower rate of leaf initiation in control and water-deficit stress variants, thus indicating a genotype-dependence of that trait. However, the total number of green leaves in all the genotypes was almost equal over the course of the experiment.

Water deficit caused a significant decrease in plant growth rate (5-6-fold) compared with normal irrigated plants (Table 1). At the point of measurement, it appeared that drought stress affected the area of green leaves in all genotypes, but to a lesser extent their number (Tables 1 and 2). The number of green leaves in the L 842 line was reduced under low stress conditions and remained at that level under moderate and severe stress. The water shortage in moderate and severe stress dramatically increased the number of yellow leaves and reduced the number of green ones; the leaf size also diminished (Table 1). In the VD genotype, there were no significant differences between the number of yellow leaves, but with the increasing degree of drought the number of green leaves and their

Table 1. Morphological and physiological changes of tobacco plants under drought stress

Treatments	Genotypes	Leaf area index of the 6th leaf	Plant growth rate (%)	Fresh weight/ plant (g)	Growth area of green leaves / plant (cm^2)	RWC Mean±SD (%)
	L842	1.56±0.16 $_{a}^{a}$	64.5±3.0 $_{a}^{a}$	2.33±0.05 $_{a}^{b}$	53.0±8.4 $_{a}^{a}$	87.2±2.6 $_{a}^{ab}$
	VD	1.03±0.12 $_{a}^{b}$	65.9±4.9 $_{a}^{a}$	2.55±0.07 $_{a}^{a}$	48.2±5.3 $_{a}^{a}$	84.9±1.2 $_{a}^{b}$
Control (C)	RG11	0.68±0.04 $_{a}^{c}$	61.7±3.3 $_{a}^{a}$	2.41±0.07 $_{a}^{ab}$	41.2±3.9 $_{a}^{ab}$	91.1±1.3 $_{a}^{a}$
	OX207	0.41±0.05 $_{a}^{c}$	57.5±3.0 $_{a}^{a}$	2.12±0.08 $_{a}^{c}$	31.6±2.6 $_{a}^{b}$	89.6±0.9 $_{a}^{a}$
	L842	1.37±0.11 $_{ab}^{a}$	52.4±5.3 $_{a}^{a}$	1.99±0.09 $_{b}^{ab}$	42.7±2.9 $_{a}^{a}$	84.4±2.1 $_{a}^{ab}$
Low	VD	0.72±0.12 $_{b}^{b}$	55.1±4.2 $_{b}^{a}$	2.17±0.09 $_{b}^{a}$	26.7±1.1 $_{b}^{b}$	82.7±1.9 $_{a}^{c}$
stress (LS)	RG11	0.42±0.04 $_{b}^{c}$	39.5±4.6 $_{b}^{b}$	1.82±0.07 $_{b}^{b}$	22.6±2.4 $_{b}^{bc}$	88.4±2.5 $_{a}^{a}$
	OX207	0.28±0.05 $_{ab}^{c}$	49.9±5.3 $_{b}^{ab}$	1.47±0.07 $_{b}^{c}$	17.2±2.0 $_{b}^{c}$	86.6±1.0 $_{b}^{a}$
	L842	1.13±0.13 $_{b}^{a}$	14.6±3.7 $_{b}^{c}$	1.32±0.02 $_{c}^{a}$	15.8±3.2 $_{bc}^{a}$	78.9±2.2 $_{bc}^{ab}$
Moderate	VD	0.46±0.05 $_{bc}^{b}$	35.0±1.8 $_{c}^{ab}$	1.11±0.06 $_{c}^{b}$	15.6±1.2 $_{c}^{a}$	71.6±2.6 $_{c}^{b}$
stress (MS)	RG11	0.30±0.07 $_{b}^{b}$	35.4±3.6 $_{b}^{a}$	1.17±0.04 $_{c}^{b}$	14.6±0.6 $_{c}^{a}$	80.4±2.6 $_{c}^{a}$
	OX207	0.24±0.04 $_{b}^{b}$	23.6±3.7 $_{b}^{bc}$	1.07±0.06 $_{c}^{b}$	8.7±1.1 $_{c}^{b}$	82.9±2.8 $_{c}^{a}$
	L842	0.64±0.06 $_{c}^{a}$	10.4±2.7 $_{b}^{a}$	0.64±0.06 $_{d}^{a}$	4.6±0.8 $_{c}^{a}$	47.6±2.9 $_{d}^{c}$
Severe	VD	0.34±0.05 $_{c}^{b}$	12.5±1.6 $_{d}^{a}$	0.68±0.05 $_{d}^{a}$	4.6±1.5 $_{d}^{a}$	49.3±1.5 $_{d}^{bc}$
stress (SS)	RG11	0.08±0.02 $_{c}^{c}$	12.7±2.1 $_{c}^{a}$	0.57±0.03 $_{d}^{a}$	3.4±0.8 $_{d}^{a}$	54.3±2.8 $_{d}^{ab}$
	OX207	0.16±0.03 $_{b}^{c}$	11.2±2.7 $_{c}^{a}$	0.67±0.03 $_{d}^{a}$	3.4±0.6 $_{c}^{a}$	59.2±2.6 $_{d}^{a}$

Numbers indicate mean±standard error (n=10); Values are significantly different at 5% significance level when compared between variants. Mean values followed by the same letter are not significantly different; subscript letters indicate the differences between variants of treatments and superscript letters indicate the differences between genotypes. Plant growth rate was measured at the beginning and at the end of the experiment.

Table 2. Number of yellow and green leaves after water-deficit stress in four tobacco genotypes

Treatment	Genotypes	Number of leaves	
		Yellow leaves	Green leaves
Control (C)	L842	2.6±0.51 a	4.6±0.40 a
	DV	3.0±0.45 a	4.0±0.32 a
	RG11	0.0±0.00 b	4.2±0.20 a
	OX207	0.0±0.00 b	3.8±0.20 a
Low stress (LS)	L842	1.8±0.37 a	4.0±0.00 a
	DV	2.2±0.20 a	3.2±0.20 b
	RG11	0.0±0.00 b	3.2±0.20 b
	OX207	0.2±0.20 b	3.2±0.20 b
Moderate stress (MS)	L842	3.8±0.20 a	2.0±0.32 b
	DV	2.4±0.24 b	2.4±0.25 ab
	RG11	0.0±0.00 d	2.8±0.20 a
	OX207	1.0±0.00 c	1.8±0.20 bc
Severe stress (SS)	L842	4.0±0.32 a	1.4±0.24 a
	DV	2.2±0.20 a	0.8±0.20 a
	RG11	1.2±0.37 b	1.2±0.20 a
	OX207	1.4±0.32 b	1.4±0.25 a

Mean values followed by the same letter are not significantly different ($P < 0.05$)

size decreased (Table 2). It was found that in RG11 and Ox 207 genotypes drought led to the appearance of single yellow leaves; in RG11 this occurred under severe stress only, but for Ox 207 their number was statistically significantly lower under moderate and severe stress (Table 2). It was observed that stress affected the area of the green leaves mainly (Table 1) and to a lesser extent their number. Also, water-deficit treatment dramatically affected the fresh weight of treated plants in all four genotypes examined (Table 1).

In this study, leaf relative water content (RWC) was also followed (Table 1). The control plants had 84.9-91.1% of RWC. Cessation of water supply resulted in a slow decline of RWC. A decline between 2.5-3.5% was determined in the leaf RWC under low stress conditions, while under moderate stress the decline ranged from 9 to 16%; under severe water stress, a decline of approximately 41-52.5% was observed.

In situations of water deprivation, the free proline level was found to increase in all four genotypes in parallel with the severity of the stress (Fig. 1). At low stress, the Ox 207, L 842, RG11 and VD genotypes showed higher proline amounts in comparison to the control. Under moderate stress conditions, free proline content also increased from 3.5- (L 842) to 9-fold (RG11 line). Also, the changes in proline levels in tobacco plants caused by severe stress (compared with the control) were similar to those observed under low and moderate stress. The level of proline was, as expected, much higher in the RG11 line (30-fold) compared with the control. It appears that the levels of free proline in the leaves of tobacco plants were offset by the significantly

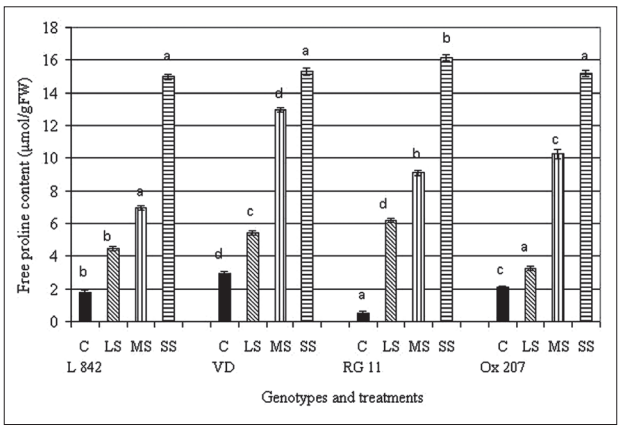

Fig. 1. Effect of water-deficit stress on the free proline content in leaves of four Virginia (flue-cured) tobacco genotypes. Each column indicates the mean±SE obtained from four replicates of at least two independent experiments; different letters indicate significant differences between the genotypes under various treatment options assessed by Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

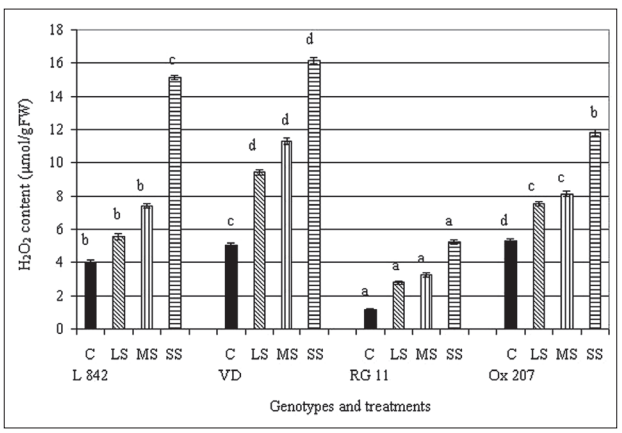

Fig. 2. Effect of water-deficit stress on H_2O_2 content in leaves of four Virginia (flue-cured) tobacco genotypes flue-cured. Each column indicates the mean±SE obtained from four replicates of at least two independent experiments; different letters indicate significant differences between the genotypes under various treatment options assessed by Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

lower amount of proline in the RG11 line in comparison with the three other tobacco genotypes.

Fig. 2 shows the effect of water stress on H_2O_2 content in the examined tobacco genotypes. The level of H_2O_2 increased in the leaves of all genotypes, and this increase was a function of water-deficit stress. The pattern of H_2O_2 increase was different in the genotypes examined. Among unstressed plants, the lowest level of H_2O_2 was that of RG11. Water deficit gradually increased the H_2O_2 concentration, as the highest concentration was measured in VD under severe water stress. The result indicated that among the four tobacco genotypes, the most sensitive were L 842 and VD (Fig. 2). A significant number of yellow and withered leaves was established in both L 842 and VD, pointing to hydrogen peroxide-induced accelerated aging of leaves [38].

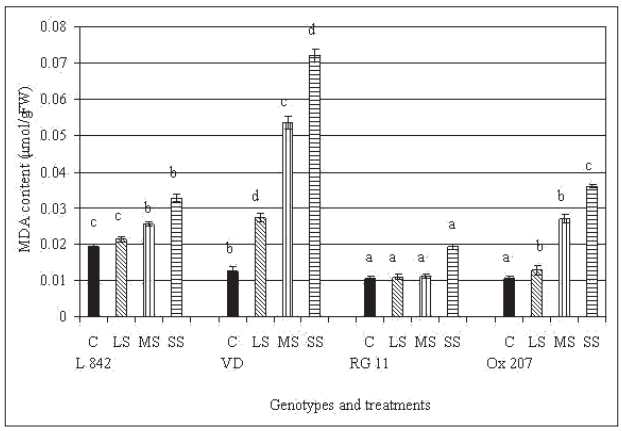

Fig. 3. Effect of water-deficit stress on MDA content in leaves of four Virginia (flue-cured) tobacco genotypes. Each column indicates the mean±SE obtained from four replicates of at least two independent experiments; different letters indicate significant differences between the genotypes under various treatment options assessed by Fisher LSD test (P≤0.05) after performing ANOVA multifactor analysis.

As shown in Fig. 3, significant differences in the levels of MDA caused by water deficit were detected among the four tobacco genotypes. Irrespective of experimental conditions, MDA levels were lower in RG11 than in the L 842, Ox 207 and VD genotypes. MDA content increased significantly in the leaves of the drought-treated plants in cv VD, as an increase of almost 6-fold was evident under severe stress conditions. Also, a gradual increase in the lipid peroxidation level of L 842 was found. In fact, L 842 line accumulated more MDA than RG11; a small 'water-deficit-dependent' increase in lipid peroxidation level in the leaves of Ox 207 line became apparent after moderate and severe stress, which was not statistically significant.

DISCUSSION

Flue-cured tobacco, an important industrial crop cultivated in Bulgaria, frequently suffers from water-deficit stress during the growing season from late April to September. The present study was aimed at better

understanding the relationships between water-deficit stress and Virginia tobacco genotypes. We conducted this study to examine the responses of different tobacco genotypes to water deficit, especially the antioxidant defenses they use to adapt to the stressful conditions. During the experiment, four irrigation regimes were applied to induce water-deficit stress. In situations of water deprivation, the magnitude of oxidative stress increased in parallel with the severity of water-stress treatment.

It is known that the accumulation of proline under stress protects cells by balancing the osmotic strength of cytosol with that of vacuole and external environment [39]. Also, proline accumulation might have a scavenger function [40,41]. Thus, it was suggested that an increased proline concentration can cause, or at least contribute substantially to, the plant defense mechanisms against environmental stress [29,42-44]. In this study, the proline content increased dramatically as the differences in the level of proline among genotypes were not substantial. Among the genotypes tested, line RG11 was the most tolerant to water-deficit-induced oxidative stress with regard to free proline content (Fig. 1). There is evidence that the plants' ability to accumulate proline under severe stress correlates positively with their drought-resistant rating [45- 49].

The levels of lipid peroxidation were measured on the basis of the accumulation of MDA, a major product of lipid peroxidation [50]. Our results revealed that exposure to low, moderate and severe water deficit led to differential increases in the MDA content of the four tobacco genotypes (Fig. 3). Moreover, some of the effects were very large in magnitude; e. g. there was a 6-fold increase in MDA content in cv VD under severe stress conditions compared with increase in the other genotypes. The increase of MDA content, *a priori*, suggested that the water deficit was associated with lipid peroxidation mechanisms. The marked difference between the tobacco genotypes in responding to water stress indicated that genotype could participate as a significant component in the mechanism of adaptation to abiotic stress conditions in plant cells. Recent studies showed that at different levels of water stress, each tobacco genotype behaved differently according to its genetic makeup [29]. These results are in agreement with the findings of several authors [17,48,51-54], who also showed that MDA content increased with increase in the degree of stress in wheat, bean, barley and sunflower.

It was established that hydrogen peroxide, as a reactive oxygen species (ROS), directly correlated with specific plants' responses to a variety of abiotic stressors that are dependent on peroxide accumulation mediated by calcium ion movement and mitogen-activated protein kinase cascades [55]. In our experiments, an overproduction of H_2O_2 was observed in plants exposed to a number of water-deficit stress conditions, especially remarkable in cv. VD, followed by L 842 (Fig. 2). The lower levels of H_2O_2 and MDA in RG11 correlated with the absence or low number of yellow leaves in this genotype under all the stress conditions (Table 2). On the whole, the response of the tobacco genotypes to water-deficit-induced stress with respect to H_2O_2 level revealed a similar trend to that of free proline content and MDA (Fig. 3). The apparent increase in the level of leaf H_2O_2 in the tested tobacco plants may be attributable to the stress-induced membrane damage. It was revealed that the generation of H_2O_2 as a part of plant defense systems is a signal for the activation of specific responses to biotic and abiotic stressors [56]. The current results are in accordance with reports suggesting a correlation between environmental stress and the rapid synthesis of H_2O_2 in cell organelles and in the apoplast [57,58].

In the present study, the drought-response difference was clearly expressed among the tested genotypes with respect to two traits: number of yellow and wilting leaves (accelerated aging of leaves) and total number of leaves formed during the treatment period. According to Chinchev [26], the development of a great number of leaves is genetically determined. In general, cv VD was the most susceptible after exposure to water-deficit where the total number of leaves, including the yellowing ones, decreased in parallel with the severity of the stress. The number of leaves decreased non-significantly in L 842 but the number of yellow leaves increased in parallel with the severity of stress treatment. The observed decrease in RWC, especially, under moderate and severe stress in L 842 and VD correlated with the plant growth reduction, number of yellowed leaves, and also with subsequent withering of the plants due to dehydration of the tissue. In addition, after low and moderate stress all the leaves of cv RG11 remained green while under severe stress 50% of them became yellow.

These results supported the suggestion that tobacco cv RG11 was drought-tolerant, while both L 842 and Ox 207 genotypes were moderately tolerant; cv VD was drought-sensitive.

In conclusion, the presented data strongly suggest that a number of physiological and biochemical features of tobacco plants such as fresh weight and height of the aboveground parts, number, color and area (leaf area index) of developed leaves before treatment, and proline, H_2O_2 and MDA concentrations, are directly affected by water-deficit-induced stress. Although the four genotypes had similar responses to the water stress, the cv RG11 was less affected by various parameters as compared to the three other genotypes. Results from this study indicated that both L 842 and Ox 207 genotypes were moderately tolerant to water-deficit stress, while cv VD seemed to be drought-sensitive. In agreement with the observation of Celik and Atak [29], who reported that the drought response of tobacco was strongly affected by genetic factors, this study indicated a genotype-depending water-deficit stress response. Genotypic differences in drought tolerance could be, at least in part, attributed to the ability of plants to acclimate and induce different defense mechanisms under severe water stress.

If we also consider the wider implications of this work, it is clear that water-deficit stress dramatically alters plant development and changes a range of antioxidants. The results indicate that drought-induced oxidative-stress tolerance is mostly dependent on the genetic potential. This initial study of water-deficit stress in *N. tabacum* plants could serve as a valuable test system for screening drought-tolerant genotypes at the early stages of plant growth and development.

Authors' contribution: Lydia Shtereva and Elisaveta Stoimenova designed and organized the study. Elisaveta Stoimenova and Bistra Michailova conducted plant growth and plant development experiments under laboratory condition. Lydia Shtereva and Tanja Kartzeva performed biochemical experiments. Roumiana Vassilevska-Ivanova and Lydia Shtereva wrote the manuscript, edited the article and bear the basic responsibility for its final content. Marina Drumeva-Yoncheva technically supported the plant growth in field conditions.

Conflict of interest disclosure: The abovementioned manuscript has not been published before and is not under consideration for publication anywhere else. The publication of this article was approved by all authors.

REFERENCES

1. Ashraf M. Inducing drought tolerance in plants: Recent advances. Biotechnol Adv. 2010; 28:169-83.
2. Tuteja N, Gill SS, Tuteja R. Improving crop productivity in sustainable agriculture. Wiley-Blackwell; 2012. 536 p.
3. Bray EA, Bailey-Serre J, Weretylnik E. Responses to abiotic stresses. In: Buchanan B, Gruissem W, Jones R, editors. Biochemistry and molecular biology of plants. Rockville: American society of plant physiologists; 2000. p. 1158-203.
4. Chaves MM, Maroco JP, Pereira JS. Understanding plant responses to drought - from genes to the whole plant. Funct Plant Biol. 2003;30(3):239-64.
5. Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 2003;218:1-14.
6. Farooq M. Wahid A, Kobayashi N, Fujita D, Basra SMA. Plant drought stress: effects mechanisms and management. Agron Sustainable Dev.2009;29:185-212.
7. Alscher RG, Donahue JL, Cramer CL. Reactive oxygen species and antioxidants: relationships in green cells. Physiol Plant 1997;100:224-33.
8. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 2002;7:405-10.
9. Neill S, Desikan R, Hancock J. Hydrogen peroxide signaling. Curr Opin Plant Biol. 2002;5:388-95.
10. Imlay JA. Pathways of oxidative damage. Annu Rev Microbiol. 2003;57:395-418.
11. Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 2010;33:453-67.
12. Sakamoto A, Murata N. The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant Cell Environ. 2002;25:163-71.
13. Luna C, Garcia-Seffino L, Arias C, Taleisnik E. Oxidative stress indicators as selection tools for salt tolerance. Plant Breed. 2000;119(4):341-45.
14. Hernandez JA, Almansa MS. Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. Physiol Plant. 2002;115:251-57.
15. Meloni DA, Oliva MA, Martinez CA, Cambraia J. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Env Exp Bot. 2003; 49(1):69-76.
16. Filippou P, Bouchagier P, Skotti E, Fotopoulos V. Proline and reactive oxygen/nitrogen species metabolism is involved in the tolerant response of the invasive plant species *Ailanthus altissima* to drought and salinity. Env Exp Bot. 2014;97 (1):1-10
17. Sairam RK, Srivastava GC. Water stress tolerance of wheat (*Triticum aestivum* L.): variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. J Agron Crop Sci. 2001;186(1):63-70.
18. Sparrow GN, Miles JD, Stansell R. Irrigation of flue-cured tobacco. Transactions of the ASAE. 1966;9(1):132-34.
19. McNee P, Warrell LA, van den Muyzenberg EWB. Influence of water stress on yield and quality of flue-cured tobacco. Aust J Exp Agr Anim Husbandry. 1978;18:726-31.

20. Çakir R, Cebi U. Growth and dry matter accumulation dynamics of flue-cured tobacco under different soil moisture regimes. J Agron. 2006;5:79-86.
21. Maw BW, Stansell JR, Mullinix BG. Soil-plant-water relationships for flue-cured tobacco. University of Georgia. Res Bull Georgia Agricul Exp Station. 2009;427:1-40.
22. Biglouei MH, Assimi MH, Akbarzadeh A. Effect of water stress at different growth stages on quantity and quality traits of Virginia (flue-cured) tobacco type. Plant Soil Environ. 2010;56(2):67-75.
23. Wilkinson CA, Reed TD, Johns JL. Flue-cured tobacco variety information for 2002. Virginia: Polytechnic Institute and State University Press; 2002. 447 p.
24. Çakir R, Cebi U. Yield, water use and yield response factor of flue-cured tobacco under different levels of water supply at various growth stages. Irrig Drain. 2010;59:453-64.
25. Yuan YB, Chen DY, Shao XH, Li YH, Ding F, Kwizera C. Effects of different irrigation quantities on plant growth and photosynthesis characters of flue-cured tobacco. J Food Agric Environ. 2012;10(2):1160-63.
26. Chinchev B. Study of introduced and new varieties of tobacco Virginia. [dissertation].[Plovdiv]: Faculty of Agriculture, University of Plovdiv;1989. 297 p
27. Riga P, Vartanian N. Sequential expression of adaptive mechanisms is responsible for drought resistance in tobacco. Aust J Plant Physiol. 1999;26:211-20.
28. Tsai YJ, Maw BW. Effects of water deficit on tobacco growth: a modeling approach. ASAE. St. Joseph, Mich. 1988;19.
29. Celik O, Atak C. The effect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties. Turk J Biol. 2012;36:339-56.
30. Yonchev Y. Reaction of introduced tobacco varieties type Virginia to PVY and TMV – economically important virus diseases. Tobacco (Prilep, Macedonia). 2008;58(1-2):41-5.
31. Yonchev Y, Stoimenova E. Monitoring of viruses causing economically important diseases in tobacco. Plant Protect (Macedonia). 2009;20:111-6.
32. Hunt R. Growth analysis, individual plants. In: Thomas B, Murphy DJ, Murray D, editors. Encyclopaedia of applied plant sciences. London: Academic Press; 2003. p. 579-88.
33. Moustakas NK, Ntzanis H. Estimating flue-cured tobacco leaf area from linear measurements under Mediterranean conditions. Agric Med. 1998;128:226-31.
34. Ritchie SW., Nguyen HT, Holaday AS. Leaf water content and gas exchange parameters of two wheat genotypes differing in drought resistance. Crop Sci. 1990;30:105-11.
35. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil. 1973;39:205-7.
36. Alexieva V, Sergiev I, Mapelli S, Karanov E. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ. 2001;24(12):1337-44.
37. Cakmak I, Horst WJ. Effect of aluminum on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). Physiol Plant.1991;83:463-8.
38. Quan LJ, Zhang B, Shi WW, Li HY. Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. J Integrat Plant Biol. 2008;50:2-18.
39. Aspinall D, Paleg LG. Proline accumulation: physiological aspects. In: L. Paleg, D. Aspinall editors. The Physiology and Biochemistry of Drought Resistance in Plants. Sidney: Academic Press: 1981. p. 215-28.
40. Jaleel CA, Gopi R, Manivannan P, Gomathinayagam M, Riadh K, Ines J, Chang-Xing Z, Hong-Bo S, Panneerselvam R. Antioxidant defense responses: Physiological plasticity in higher plants under abiotic constraints. Acta Physiol Plant. 2009;31(3):427-36.
41. Liang X, Zhang L, Natarajan SK, Becker DF. Proline mechanisms of stress survival. Antioxidants Redox Signaling. 2013;19(9):998-1011.
42. Heuer B. Osmoregulatory role of proline in water and salt-stressed plants. In: Pessarakli, M. editor. Handbook of Plant and Crop Stress. New York: 1994. p. 363-81.
43. Sankar B, Jaleel CA, Manivannan P, Kishorekumar A, Somasundaram R, Panneerselvam R. Drought-induced biochemical modifications and proline metabolism in *Abelmoschus esculentus* (L.) Moench. Acta Bot Croat. 2007;61:43-56.
44. Szabados L, Savoure A. Proline: a multifunctional amino acid. Review. Trends Plant Sci. 2009;15(2):89-97.
45. Zaifnejad M, Clark RB, Sullivan CY. Aluminum and water stress effects on growth and proline of Sorghum. J Plant Physiol. 1997;150:338-44.
46. Hanson AD, Nelsen CE, Evanson EH. Evaluation of free proline accumulation as an index of drought resistance using two contrasting barley cultivars. Crop Sci. 1977;17:720-6.
47. Al-Karaki GN, Clark RB, Sullivan CY. Phosphorus nutrition and water stress effects on proline in sorghum and bean. J Plant Physiol. 1996;148:745-51.
48. Türkan I, Bor M, Ozdemir F, Koca H. Differential response of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius Gray* and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Sci. 2005;168:223-31.
49. Dobra J, Vankova R, Havlova M, Burman AJ, Libus T, Storchova H. Tobacco leaves and roots differ in the expression of proline metabolism-related genes in the course of drought stress and subsequent recovery. J Plant Physiol. 2011;168:1588-97.
50. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4- hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med. 1991;11:81-128.
51. Tatar O, Gevrek MN. Influence of water stress on proline accumulation, lipid peroxidation and water content of wheat. Asian J Plant Sci. 2008;7(4):409-12.
52. Pandey HC, Baig MJ, Chandra A, Bhatt RK. Drought stress induced changes in lipid peroxidation and antioxidant system in genus *Avena*. Environ Biol. 2010;31:435-40.

53. Wu GQ, Zhang LN, Wang YY. Response of growth and antioxidant enzymes to osmotic stress in two different wheat (*Triticum aestivum* L.) cultivars seedlings. Plant Soil Environ. 2012;58(12):534-9.
54. Baloğlu MC, Kavas M, Aydın G, Öktem HA, Yücel AM. Antioxidative and physiological responses of two sunflower (*Helianthus annuus*) cultivars under PEG-mediated drought stress. Turk J Bot. 2012;36:707-14.
55. Khanna-Chopra R, Selote D. Acclimation to drought stress generates oxidative stress tolerance in drought-resistant than -susceptible wheat cultivar under field conditions. Environ Exp Bot. 2007;60(2):276-83.
56. Prasad TK, Anderson MD, Martin BA, Stewart CR. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell. 1994;6:65-74.
57. Foyer C, Lorez-Delgao H, Dat J, Scott I. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. Physiol Plant. 1997;100:241-54.
58. Foyer CH, Noctor G. Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 2005;29:1056-7.