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Original Research

The Responses of Stomatal Parameters and SPAD Value in Asian Tobacco Exposed to Chromium

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Abstract

In this study, the responses of Asian tobacco varieties to chromium stress were investigated. To determine the responses arising from genotypic differences, Basma and Dubek varities were used. Basma was suitable for removing Cr from soil and was more tolerant than Dubek to chromium stress. A significant variation in stomatal characters, except for stomatal width, was observed in both varieties. In parallel with the increasing doses of Cr, stomata density significantly increased in both genotypes. Also, it was observed that stomatal length decreased with increases in Cr dosage. The increase in Cr concentration leads to decrease in SPAD value. It was observed that the SPAD value of Basma was more than Dubek in control plants. The correlation of stomatal parameters with each other and SPAD values were also calculated. A negative correlation was found between the SPAD value and stoma density in both varieties. Also, a positive correlation was observed between SPAD value and stomatal length. There was a significant negative correlation between stoma density and stomatal length.

Keywords: chromium, tobacco, stomata, SPAD value, phytoremediation

Introduction

Environmental pollution caused by heavy metals is a problem all over the world and, especially since the beginning of this century, occurring heavy metals have reached a critical point in terms of living organisms [1-4]. One of the techniques to overcome this problem is phytoremediation. The removal of environmental pollutants using plants as green technology in phytoremediation is less costly than other technologies. Chromium (Cr) as heavy metal is an important environmental pollutant due to its widespread industrial use [5]. Cr micro molar range, which is highly toxic, has severe phytotoxic symptoms (membrane damage, structural changes in organelles, metabolic activity, distortion), and can cause growth inhibition [6]. Much research, applying different concentration levels of Cr, have reported that it causes inhibition of plant growth [7-10]. Cr stress in plants depends on plant species and Cr concentrations [10], and the phytoremediation technique can be used to remove Cr from soil. So, it is needed to investigate the morphological and physiological mechanisms.

Stomata play an important role in regulation of plant water balance and gas exchange. This regulation depends on specific environmental conditions [11] and is controlled by internal and external factors [12]. Some changes can be seen in stomata density (SD) and size, depending on stressful conditions. The responses of guard cell size and stomatal number to environmental variables clearly depend on a time scale from milliseconds to millions of years [13]. Stomatal parameters can be used as stressful condition signs [14-16]. By using the stomatal parameters as stressful condition signals, the breeders can improve new genotypes tolerant to stress conditions. Improving regulation capacity in stomatal traits by breeding selection and/or genetic methods would enable plants to acclimate to environmental stresses such as drought [17]. Some research indicates

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changes in the number of stomata in various stressful conditions such as drought [18, 19] and decrease in stomatal size [20, 21]. Several reports have demonstrated that an excess of heavy metals (Cd, Pb, Ni, and Mn) induce changes in leaf anatomy [22, 23], including stomatal parameters [24]. Unfortunately, studies describing how SD and guard cell size respond to different heavy metal stresses are limited. Also, there are limited studies about the changes in stomatal parameters of tolerant and sensitive varieties in response to heavy metals stress. Actually, the physiological mechanisms of stomatal response are very complex and not yet fully understood [25, 26].

Chlorophyll content is one of the factors that determine photosynthesis. Like its usage as criteria in anatomical and chemical examinations, chlorophyll can be used as criteria in stressful conditions related to photosynthesis [27-29], and the SPAD (soil plant analysis development) value can be used as an indicator of chlorophyll content [30]. Chlorophyll level changes depend on stressful conditions, like being affected by different photochemical events. Determining the level of chlorophyll using chlorophyllmeter is the main determinant method for various stresses [31-33]. Also, the chlorophyll level in leaf may change under heavy metal stress [34]. There are a few studies about chlorophyll content changes of sensitive and tolerant genotype under stress. For instance, Kuo et al. [35] in their study on Chinese cabbage reported that the chlorophyll level in tolerant varieties was higher than intolerant varieties. However, limited investigations have addressed the relationship between stomatal parameters and chlorophyll content, and behavior of sensitive and tolerance genotypes. Most of the works on heavy metal stress gave minor attention to the functional link between morphological and physiological traits as potential targets to improve tolerance. Moreover, a clear picture of overall correlation between stomatal parameters and chlorophyll content has not been fully appreciated. Therefore, in this study the relationship between stomatal parameters and SPAD value of variation arising from genotypic differences of oriental tobacco varieties under Cr stress was investigated.

Materials and Methods

Chromium Treatments

The experiments were conducted under laboratory conditions to evaluate differences in the Cr stress tolerance of the tobacco at seedling stage. As a conclusion of previous experiments on the varieties of oriental tobacco, we noticed that the Basma variety is effective in removing Cr from soil and is more tolerant than Dubek to Cr stress. As only this variety has a hyperaccumulation mechanism, it led us to consider it as a suitable tool for genetic studies. Seeds of Basma and Dubek as Asian tobacco varieties (*Nicotiana tabacum* L.) were obtained from the Aegean Agricultural Research Institute (İzmir, Turkey) and germinated on Murashige and Skoog medium (MS) containing 0, 10, 100, 150, and 200 μM of hexavalent chromium [Cr (VI)]. The Cr concentration level was chosen based on Samantaray [8]. Potassium dichromate ($K_2Cr_2O_7$) was used as a source of Cr. Tobacco seeds were surface-sterilized for 1 min in 70% (v/v) ethanol and then soaked in 20% (v/v) commercial bleach (commercial bleach contains about 5% v/v sodium hypochlorite) for 10 minutes. Seeds were rinsed 3 times in sterile distilled water. After 45 days, the seedlings were acclimatized into external conditions. All seedlings were grown at 22ºC and 16h light/8h dark photoperiod under fluorescent lights. In this study, the experiments were repeated three times, every treatment had three replicates, and each treatment included 10 seedlings.

Measurements of Traits

Stomatal Parameters

Micro-morphological observations were carried out 45 days after sowing (DAS) using a bright-field light microscope. The SD, stomatal length (SL), and stomatal width (SW) were determined from the underside of each leaf using prints made with nail varnish. SL was measured between the junctions of the guard cells at each end of the stoma as defined by two research groups [36, 37]. The SW was measured perpendicularly to maximum width, which represents the maximum potential opening of the stomatal pore, but not the aperture of opening that actually occurs. SD (number of stomata per mm^2) was determined as described by a previous study [38]. Stomatal surface (SS), stomatal shape coefficient (SSC), potential conductance index (PCI) and relative stomatal surface (RSS) was obtained using equation numbers 1 to 4 (equations from Wang et al. [39], with some modifications).

$$SS = \frac{SL * SW * \pi}{4} \tag{1}$$

$$SSC = 100 * \frac{SW}{SL} \tag{2}$$

$$PCI = (SL)^2 * SD * 10^{-4} \tag{3}$$

$$RSS = SPS * SD * 100 \tag{4}$$

The SL and SW values are measured as micrometers (μm), SS in (μm^2), and RSS in percentages.

SPAD Value

Chlorophyll content was determined from intact leaves using a portable chlorophyllmeter SPAD-502 (Minolta, Ltd., Osaka, Japan). Reading of SPAD values were done on the 60th day after treatment. Three measurements were made per plant, three leaves were chosen from each plant (lower, middle and upper leaves of plant), and three different regions of each leaf (middle and two ends of leave) were used for tests. The chlorophyllmeter was used to estimate the nitrogen status of crops. The instrument measures transmission of red light at 650 nm, at which chlorophyll

Table 1. Results of the ANOVA for the effects of Cr concentrations on SD and SL, SW, SS, SSC, PCI, RSS, and SPAD value of Basma and Dubek varieties.

Source of variation	df	SD	SL	SW	SS	SSC	PCI	RSS	SPAD
Varieties (V)	1	**	**	ns	*	*	**	**	*
Cr concentration (Cr)	4	**	**	ns	**	**	**	ns	**
V × Cr	4	ns	ns	ns	ns	ns	ns	ns	ns

Significant at: *p = 0.05; **p = 0.01; ns – not significant.

absorbs light, and transmission of infrared light at 940 nm, at which no absorption occurs. On the basis of these two transmission values the instrument calculates a SPAD value that is quite well correlated with chlorophyll content [40, 41]. Chlorophyllmeter readings (SPAD values) were repeatedly taken at the center of the leaves. Air temperature was maintained at 18±20ºC in all experiments.

Statistical Analysis

The data were subjected to analysis of variance (ANOVA) using factorial randomized complete plots designed with three replications, and least significant difference (LSD 0.05) was used to determine significant differences between treatment means. All graphs were created using SPSS version 15 for Window (SPSS Inc., Chicago, IL).

Results and Discussion

Stomatal Parameters

Significant variation for stomatal parameters except for SW was observed between the two cultivars and also between different Cr concentration levels (Table 1). The SD of Basma was more than Dubek in control plants. The stomatal parameter values of genotypes according to changes in dosages of Cr are shown in Fig. 1. In parallel with increase in Cr dosage, SD significantly increased in both genotypes. These values are in line with increases in SD during stress, which were determined by Htay et al. [42] on bean, Kuo et al. [43] on cabbage, and Labate et al. [44] on tomato. Especially in response to heavy metal stress, the SD increased which was agreeing with SHI and CAI [24] report. The change in SD depending on Cr stress in Basma (19.7%) was relatively less than Dubek (24.2%). Under stress conditions, the SD and stomatal size are different in tolerant (sensitive) and intolerant plants [45]. Our results are consistent with Moriana and Fereres [46], which showed that the stable plants are more tolerant to drought. In term of determining stomatal size, Dubek has had longer SL than Basma. Also, it was observed that SL decreases with the increase in Cr dosage in both genotypes, which is in agreement with the results of Quarrie and Jones [20], Yang et al. [47], and Meng et al. [48]. Although the SL decreased with the increase in Cr dosage, there was no significant difference in SW in both varieties. However, Zhang et al. [19] reported that SL increases under limited irrigation conditions, whereas its width decreases. Nevertheless, different effects of abiotic factors on stomatal size may depend on plant species/varieties [37, 49].

The SS parameters obtained from equations, showed that there were significant differences between varieties and also between different Cr concentration levels. The SS of Dubek was more than Basma. As the plants were exposed to increasing Cr dosages, the decrease in SS occurred. The SSC values indicate that by increasing the Cr stress, the SS becomes more circular in both varieties. As a result of this observation it can be said that the more circular stomata can be beneficial for alleviating Cr stress. By increasing the Cr stress the increase in PCI values was observed. The PCI value itself, is related to SD and SL. So it can be concluded that the decrease in SL was more effective than the increase of SD in PCI value changes. The RSS value of Basma was more than Dubek, and the RSS values did not change by increasing in Cr dosage. This means that decreases in SS were balanced with increases in SD when Cr stress increased. By determining SS, SSC, and RSS values, the differences between stomatal parameters of varieties became clearer. As a result, it was observed that Cr stress resulted in changes in stomatal parameters depending on their tolerance response levels to heavy metal.

SPAD Value

SPAD value as an expresser for chlorophyll content was determined on 60th DAS. In terms of SPAD value there was a significant difference between varieties (Table 1). The changes in SPAD value of genotypes exposed to different levels of Cr concentration are shown in Fig. 1. It was observed that SPAD value of Basma (34.75) was more than Dubek (24.45) in control plants. The increase in Cr concentration led to a decrease in SPAD value, which is in accordance with the results of other research [30, 50]. Although the decrease in SPAD value was seen in both varieties, the decrease in Dubek was slight and not statistically significant compared with Basma, which showed a marked decline. In Basma variety, the decrease in SPAD value as a result of increase in Cr concentration was more than Dubek, which is in contrast with its more tolerant property to Cr stress. This contrast shows that the photosynthetic rate does not just depend on chlorophyll content. The observed decrease of photosynthetic rate in Cd-stressed plants could be partly attributed to lower chlorophyll content, as expressed by SPAD value [30].

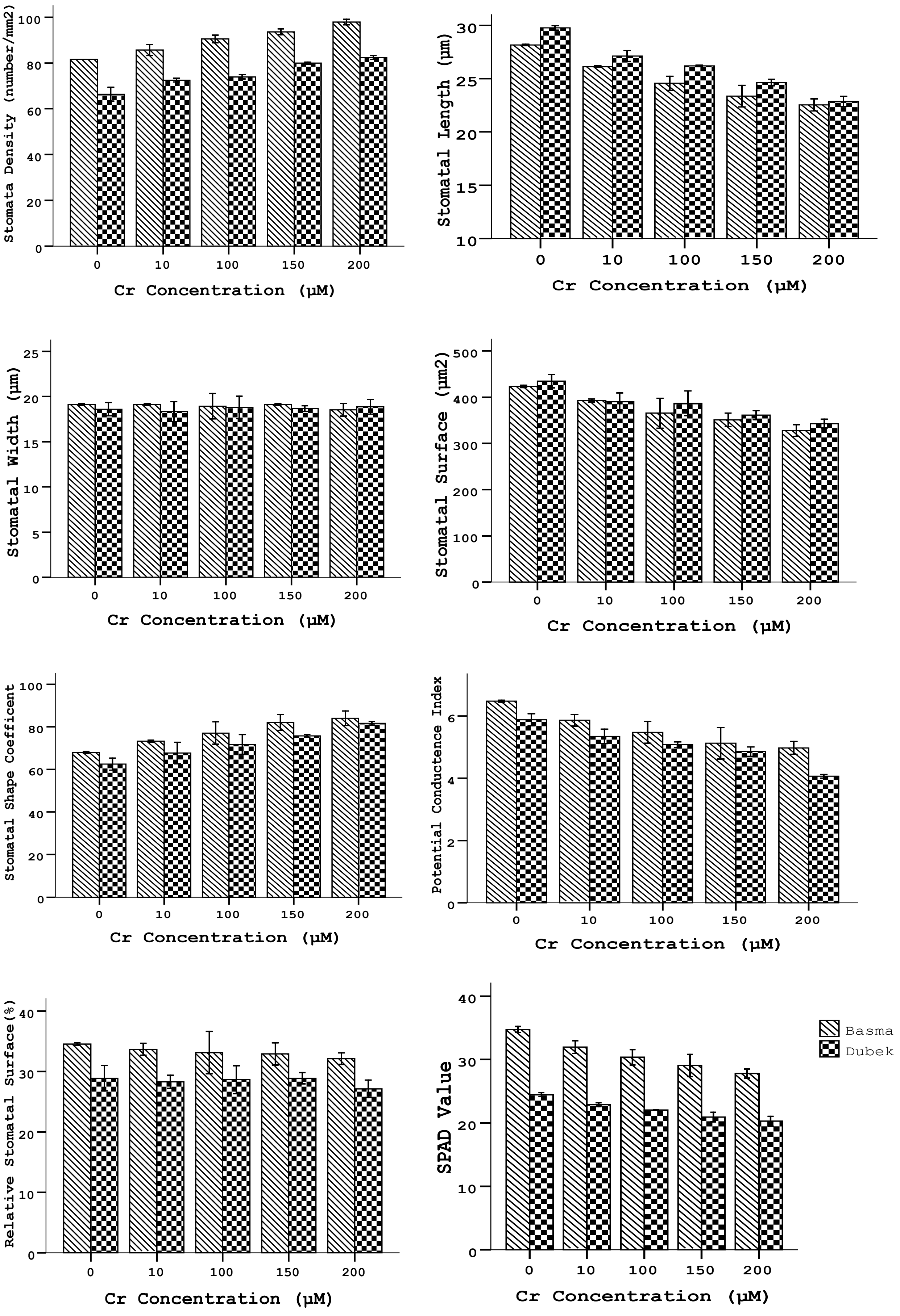

Fig. 1. Changes in SD, SL, SW, SS, SSC, PCI, RSS, and SPAD value of Basma and Dubek varieties with increasing Cr concentrations. Error bars represent mean of standard error.

Table 2. Correlation matrix of the SD, SL, SW, and SPAD values for Basma and Dubek varieties.

	Basma				Dubek			
	SPAD	SD	SL	SW	SPAD	SD	SL	SW
SPAD	1				1			
SD	-0.772**	1			-0.741**	1		
SL	0.879**	-0.935**	1		0.836**	-0.930**	1	
SW	0.217	0.430	0.434	1	0.000	0.023	0.067	1

Significant at: *p = 0.05; **p = 0.01.

To further understand the relationship between stomatal parameters (morphologic) and SPAD values (physiologic) of genotypic differences arising from the responses to Cr stress, a correlation analysis was performed (Table 2). A negative correlation was found between the SPAD value and SD in both varieties (-0.772 and -0.741 for Basma and Dubek, respectively), and it is in line with results of Karipcin [51] studies. However, a positive correlation was observed between SPAD value and SL of both varieties (0.879 and 0.836 for Basma and Dubek, respectively). The correlation between SD and SL was negatively high. Other researchers reported that in other stressful conditions such as drought, SD negatively correlates with SL in some *Jujube* leaves [49] and *Platanus acerifolia* leaves [52]. Also, during plant evolution the same trend can be observed [53]. Martinez et al. [54] reported that throughout an adaptation to drought, increases in SD and a decrease in cell size under water stress could occur. The correlation between stomatal parameters and SPAD value was similar in Basma and Dubek. This means that the tolerance value of varieties did not reflect in the relationship between investigated parameters.

There may be some changes in correlation between some parameters that were not investigated in this research. Therefore, further detailed research at the cell development levels are still needed to infer the differing responses between guard cells and epidermal cells in terms of water status.

Conclusions

In Basma and Dubek, the increase in Cr stress led to a decrease in SPAD value, SL, SS, PCI, and RSS, but increases in SD and SSC. The differences of Basma and Dubek in response to Cr stress are related to the differences in intensity of changes that occurred in stomatal parameters and SPAD value. By the way that all the changes in measured parameters had the same way in two varieties, significance and insignificancy of the values were different in Basma and Dubek. The stomatal parameters and SPAD values are suitable indicators of stress evaluation. The differences seen between the responses of Basma and Dubek toward Cr stress make them useful tools for determining Cr stress. Contrary to Cr tolerance, the correlation between evaluated parameters in Basma and Dubek was similar. So it can be suggested to combine these parameters with other uninvestigated parameters to clarify this contrast and obtain more details.

Abbreviations

Stomatal density (SD), stomatal length (SL), stomatal width (SW), stomatal surface (SS), days after sowing (DAS), stomatal shape coefficient (SSC), potential conductance index (PCI), and relative stomatal surface (RSS).

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