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PHYSIOLOGICAL RESPONSE OF SEVERAL ALFALFA GENOTYPES TO DROUGHT STRESS

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ABSTRACT

Alfalfa is generally considered resistant to drought, but its yield may fluctuate appreciably under drought conditions. The strategies for sustainable use of water and drought resistance improvement based on the physiological traits are important and physiological approaches should be integrated in conventional breeding. Research was performed on 74 alfalfa genotypes under vegetation house conditions at two watering levels. The objective was to identify the available genetic variation and to establish efficient testing methods for characters which might positively influence alfalfa performance under drought conditions. Our research focused on plant height, number of shoots, stomatal resistance and biomass yield. The results showed that for drought tolerant genotypes the plant height and number of shoots were reduced less than in sensitive ones. The stomatal resistance was higher for sensitive genotypes than for resistant ones, but there were some sensitive genotypes with low stomatal resistance and vice versa, which opens new opportunities to improve drought resistance. The results showed that the stomatal resistance of young plants was correlated with yield both under optimal conditions and stress conditions, indicating that stomatal resistance has a very significant impact on production both under stress and optimum conditions.

Keywords: alfalfa, drought susceptibility index, morpho-physiological traits, stomatal resistance.

INTRODUCTION

Global climate change is the main cause of increased drought frequency, and climate forecasts for Romania show the increase of average temperatures and of droughts frequency.

Yield under water stress has been and continues to be the main index of selection used in many breeding programs to improve the drought tolerance of crops. Now, however, when it comes to a so-called "plateau" of production, the emphasis is on looking for those secondary traits that can ensure good progress. These traits should be hereditary, correlated with stress tolerance and easy to evaluate.

Compared to other crop species, little is known about mechanisms by which genetic and physiological factors contribute to drought tolerance in alfalfa. So, improving the water use efficiency could be an effective strategy for the development the varieties with green mass production during drought periods. Among the traits related to improving water use efficiency, we studied: plant height, number of shoots and stomatal resistance.

Under drought conditions, plants reduce stem growth and foliar surface to minimize transpiration, but often increase root biomass production to exploit soil water (Erice et al., 2010).

Another mechanism of drought tolerance is by regulating stomatal resistance/conductance. Stomatal conductance regulates absorbed CO_2 and water losses by plants through stomata. Drought-tolerant plants can adjust their stomatal conductance under drought conditions to limit water loss (Rodriguez et al., 2016).

The aim of this work was to assess the response of several alfalfa (*Medicago sativa* L.) varieties under two water levels during different vegetation stages. This will help us to identify the genetic variation and to establish efficient testing methods for traits which might positively influence alfalfa

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performance under drought, in order to select drought resistant genotypes as components of alfalfa synthetic cultivars.

MATERIAL AND METHODS

Plant material

Experiment 1. The experiment was conducted in greenhouse of the National Agriculture Research and Development Institute (NARDI), Fundulea, Romania from September 2015 to August 2017. Twenty four alfalfa synthetic cultivars were grown in Mitcherlich pots (24 kg capacity) filled with a soil-sand mixture (3:1). A single plant was left in each pot and four replicate pots were used per cultivar and treatments. Pots were maintained at the field capacity of soil until middle of November, when the above ground parts were harvested to ensure an adequate build-up of energy reserves for survival through the winter.

Experiment 2. Fifty alfalfa genotypes (in the first stages of selection and breeding at NARDI Fundulea) were studied. The experiments were conducted in a vegetation house using plastic pots filled with a soil-sand mixture (3:1).

Control and drought treatments

The control plots were continuously watered and the soil water content remained at the optimal moisture, corresponding to 70% from soil water capacity.

For the first experiment, drought treatments were imposed in the second year of vegetation before flowering (45-day-old plants) by maintaining soil moisture at 40% from soil water capacity. Water stress duration was 14 days. In order to avoid nutrient differences between control and stressed plants, 100 ml Hoagland nutrient solution was added to each pot one week before drought treatment. At the nutritional level, this Hoangland nutrient solution is adequate to allow correct alfalfa growth (normal leaf colour and development) for control and drought treatments. For the second harvest, the same drought treatment was applied.

For the second experiment, the drought (soil moisture at 40% from soil water capacity) was applied to 30 days old plants. Water stress duration was 12 days.

Analyses

1. *The height of plants*, *the number of shoots* and *the biomass* were determined by measuring with ruler, counting and gravimetric weighing.

2. *Drought susceptibility index*

Plants were harvested by cutting at the soil line for measuring biomass yield. Fresh weights of stressed and unstressed plants were used to determine the drought susceptibility index (DSI), also called drought resistance index. DSI is the ratio of yield reduction due to stress in a given genotype as compared to the mean reduction over all genotypes in a given test. DSI is an improved parameter for estimating stress response over the simple expression of yield under stress as percent of yield under non-stress conditions. The formula of DSI (Fischer and Maurer, 1978) was as follow:

$$DSI = (1 - Yd/Yw)/D,$$

where:

Yd = mean yield of genotype under drought;

Yw = mean yield of genotype under well-watered conditions;

D (environmental stress intensity) = 1 - (mean yield of all genotypes under drought/mean yield of all genotypes under well-watered conditions).

3. *Stomatal resistance*

Stomatal resistance was measured on leaves using a Delta T porometer. The calibration curve was made each time before starting measurements.

The estimates of simple correlation coefficients among all studied characters were computed using Excel facilities.

RESULTS AND DISCUSSION

1. Height of plant and relationships with drought susceptibility index

The analysis of variance of the height of plants indicated a significant effect of treatment and genotype at the 0.1% level of significance. The interaction between genotypes and treatments was significant at the 1% level of significance, and reveals the existence of differences in response to water stress among tested cultivars. The variation due to water stress was higher (78-88%) than that due to the genotype and interactions of the factors, both for first and second harvests (Table 1).

Table 1. Analysis of variance for shoot height of alfalfa genotypes

Source of variance	DF	First cutting		Second cutting	
		Mean square	F value	Mean square	F value
A Factor: treatments	1	1406.25 (78.41%)	29.65***	1161(88.31%)	138.79***
Error A	2	47.42 (2.64%)		8.37 (0.64%)	
B Factor: genotype	23	256.15 (14.28%)	13.24***	91.29 (6.94%)	5.26***
Interaction AxB	23	64.32 (3.59%)	3.32**	36.70 (2.79%)	2.12**
Error B	92	19.35 (1.08%)		17.37 (0.32%)	

*** significant for p<0.01%; ** significant for p<0.1.

Assessment of performance of alfalfa cultivars for height of plants under two water levels (interaction between water levels and tested cultivars) are presented in Table 2. Considerable variation for drought tolerance was observed among the cultivars used. Generally, the water stress decreased height plants of all tested cultivars. The height of plants under optimum water supply conditions was on average 81 cm and 52 cm at the first and second cutting respectively, as compared with 75 and 46 cm under water stress.

Table 2. The effect of water stress on height of plants (average of two years)

Genotype	Height of plants (cm)				Drought susceptibility index	
	First cutting		Second cutting		First cutting	Second cutting
	Control	Water stress	Control	Water stress		
F 1543-03	74	72	47	45	0.13	0.92
F 1712-04	79	79	57	52	0.13	1.16
F 1414-02	75	74	60	43	0.16	0.99
F 1412-02	90	88	57	56	0.25	0.98
F 1715-05	87	87	49	41	0.35	1.01
Daniela	83	76	49	46	0.42	0.84
F 1535-03	76	76	47	46	0.43	1.03
Roxana	90	87	49	49	0.57	0.89
F 1608-04	86	84	47	46	0.58	0.85
F 1413-02	83	81	52	44	0.67	1.11
F 1913-07	90	77	50	49	0.88	0.89
F 1615-04	79	67	51	51	1.05	1.09
F 1821-06	82	70	52	40	1.11	0.99
F 1814-07	88	82	55	42	1.14	1.08
F 1710-04	75	75	52	41	1.19	0.99
F 1610-04	75	70	47	40	1.28	0.96
F 2007-08	76	75	51	50	1.33	1.11
F 1914-07	81	75	56	54	1.35	0.97
F 2010-08	80	65	52	42	1.43	0.85
F 1918-07	87	71	50	45	1.49	1.22
Magnat	82	67	62	56	1.56	1.08
F 1916 -07	62	60	53	44	1.62	1.01
F 1711-05	85	62	55	44	1.86	0.98
F 1543-03	74	72	47	45	0.13	0.92
Average	*81*	*75*	*52*	*46*	*0.95*	*1.00*
Relationship with DSI	r=-0.24	**r=-0.68*****	r=0.37	r=0.17		

All cultivars responded in same manner to water stress. However, the response to stress varied with the cultivars. Reductions due to stress were generally between 7% and 11%, but there were also genotypes less or not affected by water stress (Roxana, F 1412-02, F 1412-02, F 1535-03, F 2007-08, F 2017-08), (Table 2).

Drought susceptibility index (DSI), for the studied genotypes ranged from 0.35 (F 1715-05) to 1.86 (F 1711-05) for first cut and from 0.84 (Daniela) to 1.22 (F 918-07) for second cut.

Genotypes with low DSI values (less than 1) can be considered to be drought resistant (Mustățea et al., 2009), because they exhibited smaller yield reductions under water stress compared with well-watered conditions than the mean of all genotypes. Analysing the values of the drought sensitivity index we can see that some of the genotypes noted by a satisfactory DSI (values <1) at first cut maintained this quality. These were Daniela, Roxana, F 1412-02, F 1413-02 and F 1608-04 (Table 2).

The height of plants is a physiological trait that was relatively correlated with the degree of tolerance to drought irrespective of the stage of plant development. Thus, in the case of analyses carried out on young alfalfa plants, the results showed that in drought-tolerant genotypes (those with a subunit drought sensitivity index) the plant size was reduced by no more than 25%, while in sensitive genotypes the reductions were up to 50%. There were also three exceptions, sensitive genotypes F 2507/16, F 68015/11 and F 2506-16 had 25% and only 5% reductions, which opens new opportunities for improvement (Figure 1).

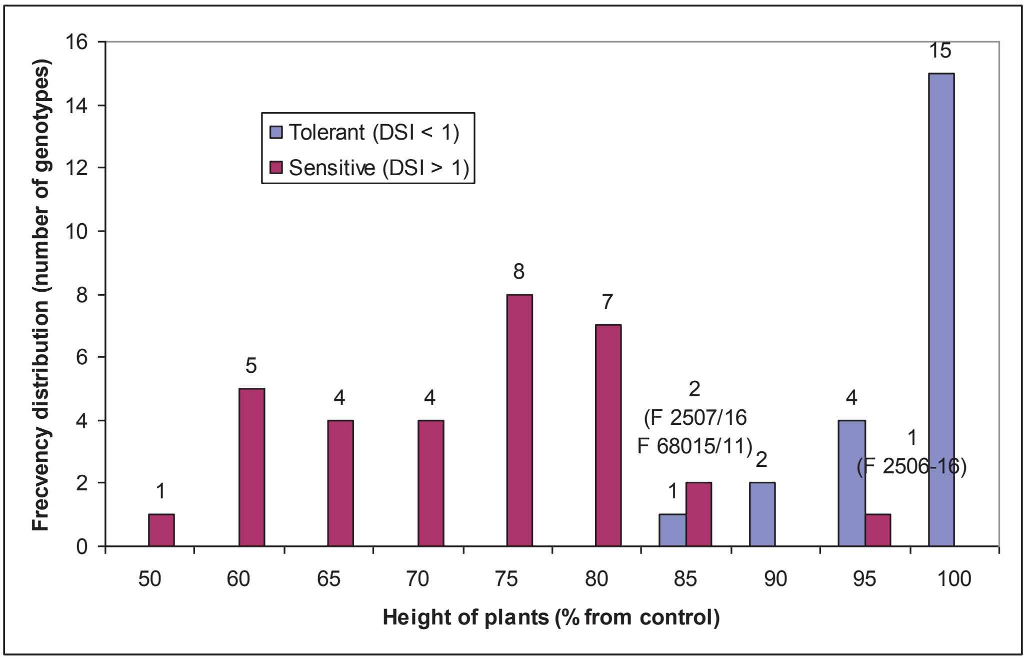

Figure 1. Frequency distribution for the height of studied alfalfa genotypes

Correlation studies between characters are very important in plant breeding for indirect selection and have also been of great value in the determination of the most effective breeding procedures. The results of correlation presented in Table 2 and Figure 2 show that, plant heights under stress had the highest negative correlation with drought susceptibility index (at the first harvest of mature plants, coefficient of correlation r=-0.68*** and for young plants r=-0.52***), while, height of plants under control showed the lowest correlation with DSI.

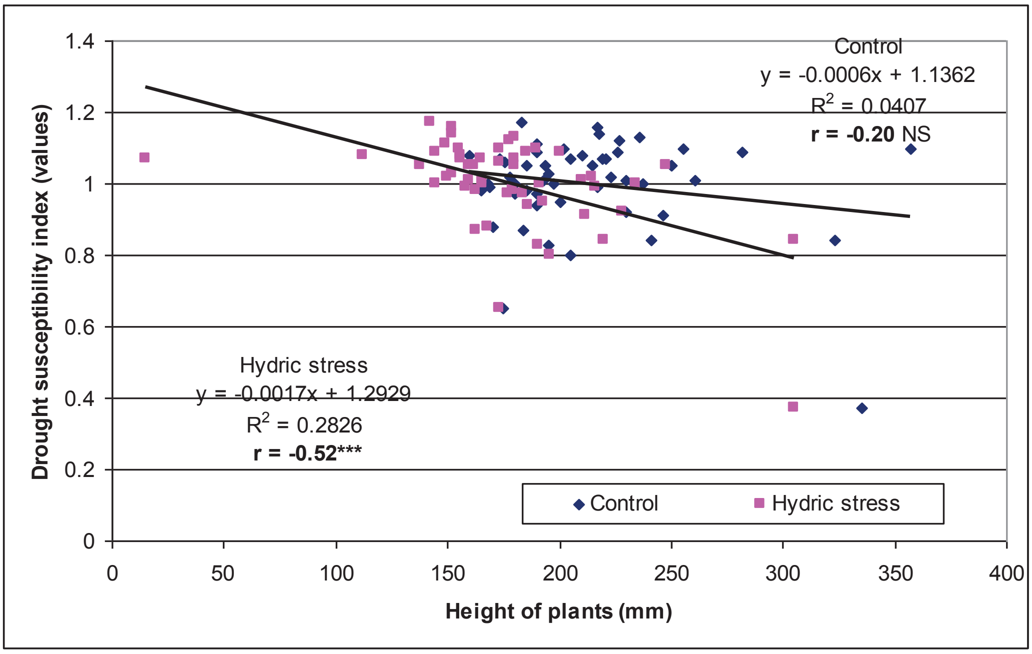

Figure 2. Relationship between plant heights and drought susceptibility index

2. The number of shoots and relationships with drought susceptibility index

Our results highlight the very significant effect of water stress on the number of shoots and the different ability of the genotypes studied in the production of shoots. The variation due to the treatment was greater than the variance due to the genotypes, and with regard to the interaction between the factors only the second cutting showed a very significant effect (Table 3).

Table 3. Analysis of variance for number of shoot

Source of variance	DF	First cutting		Second cutting	
		Mean square	F value and significance	Mean square	F value and significance
A Factor: treatments (control, water stress)	1	235.11(86.44%)	53.65***	2558 (95.2%)	3802.9***
Error A	2	4.38 (1.61%)		0.67 (0.03%)	
B Factor: genotype	23	18.2 (6.69%)	2.71**	76.06 (2.83%)	6.84***
Interaction AxB	23	7.57 (2.79%)	1.12	41.05 (1.53%)	3.69***
Error B	92	6.72 (2.47%)		11.12 (0.41%)	

The water stress impact on shoots number was genotype dependent. The number of shoots was reduced in most genotypes under water stress conditions, with an average reduction of 14% and 28% for first and second cutting respectively. After the second cut, there was an almost double increase in the number of shoots both for optimal supply and water stress conditions. The genotypes F 1414-02, F 1711-05, F 1715-05, F 2010-08 were among the best performing cultivars under both conditions (Table 4).

In case of young alfalfa plants, under water stress conditions the drought-tolerant genotypes had 30% fewer shoots than under optimum conditions, while for sensitive genotypes the reductions were up to 55%. There were also four exceptions, sensitive genotypes F 2510-16, F 68015/11, Magnat and F 66039/5 had only 5% to 15% reductions, which opens new opportunities for breeding (Figure 3).

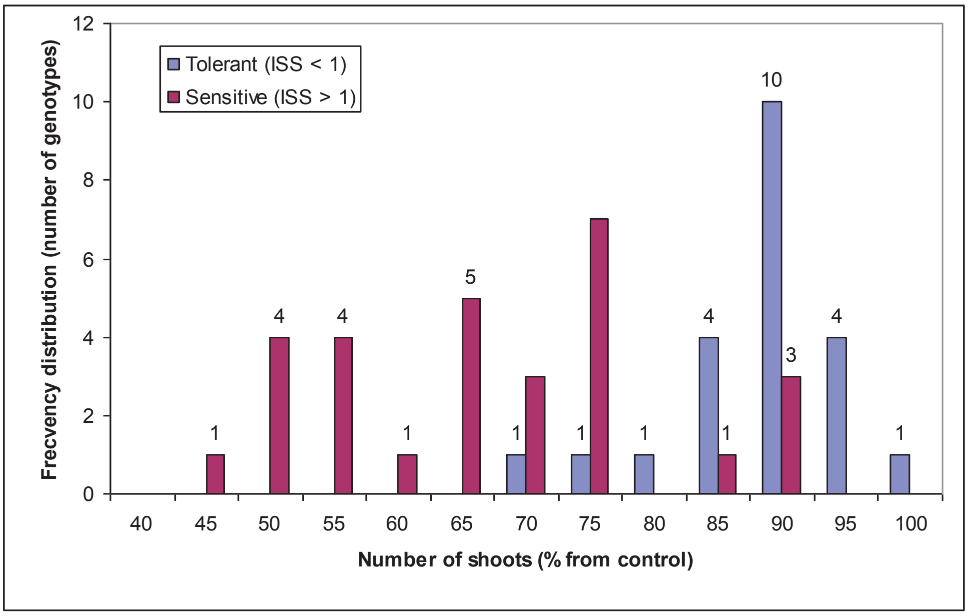

Figure 3. Frequency distribution for the number of shoots of studied alfalfa genotypes

Between the number of shoots of the alfalfa plants under control and under water stress respectively and the drought sensitivity index there was a negative correlation only for the second cutting (r=-0.53***, r=-0.69***), and for the young plants there were also significant correlations (r=-0.41*** and r=-0.69*** (Table 4 and Figure 4).

Table 4. The effect of water stress on number of shoots (average of two years)

Genotype	Number of shoots (no)				Relative reduction (% from control)	
	First cutting		Second cutting			
	Control	Water stress	Control	Water stress	First cutting	Second cutting
Magnat	12	12	25	13	100	52
Daniela	18	14	29	28	78	97
Roxana	18	12	28	25	67	89
F 1412-02	14	12	29	22	86	76
F 1413-02	16	12	26	21	75	81
F 1414-02	13	13	24	23	100	96
F 1543-03	16	14	35	28	88	80
F 1535-03	15	14	35	20	93	57
F 1608-04	16	13	36	30	81	83
F 1610-04	12	8	33	18	67	55
F 1615-04	12	12	31	20	100	65
F 1710-04	12	12	32	26	100	81
F 1711-05	12	9	23	23	75	100
F 1712-04	13	7	23	12	54	52
F 1715-05	15	12	23	22	80	96
F 1814-07	15	10	29	19	67	66
F 1821-06	12	11	30	16	92	53
F 1913-07	13	10	28	19	77	68
F 1914-07	15	14	32	20	93	63
F 1916 -07	11	9	27	19	82	70
F 1918-07	15	13	21	14	87	67
F 2007-08	13	11	31	23	85	74
F 2010-08	14	12	32	29	86	91
F 2017-08	15	8	39	19	53	49
Average	14	12	29	21	83	73
Relationship with DSI	r=-0.45	r=-0.35	r=-0.53***	r=-0.69***		

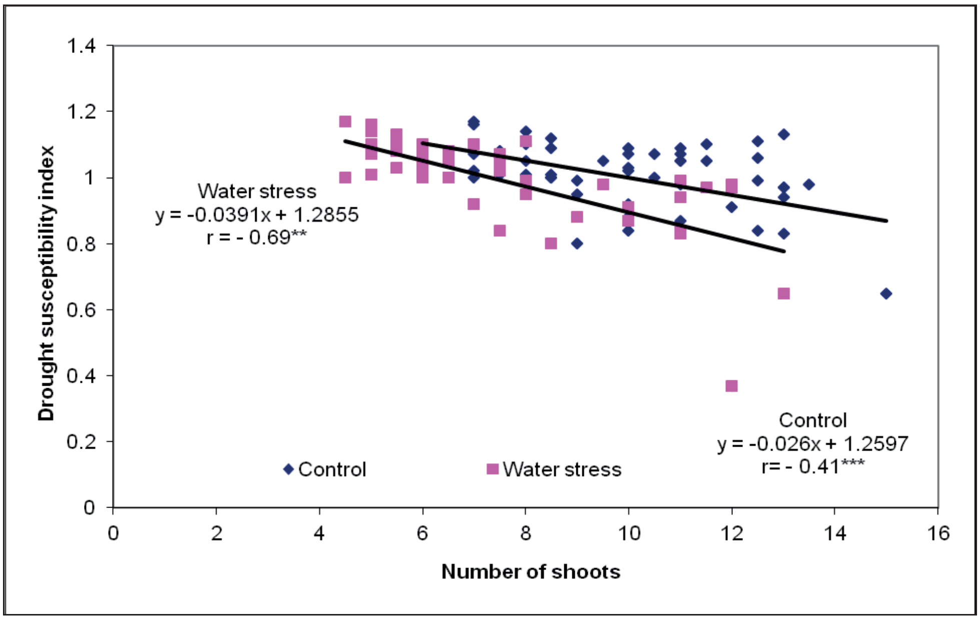

Figure 4. The relationships between drought susceptibility index and number of shoots of alfalfa genotypes (Test with young plants)

3. Stomatal resistance and relationships with drought susceptibility index

The ANOVA analysis regarding stomatal resistance shows a very significant influence of the treatment, genotype and their interaction. The variance of treatments was higher than the variance due to genotypes (Table 5).

Table 5. Analysis of variance for stomatal resistance (average of two year, first cutting)

Source of variation	Sum of squares	DF	Mean square	F value and significance
Treatment	26030	1	26030	13569.41***
Genotypes	4124	22	187	97.72094***
Interaction	3520	22	160	83.416***
Error	176	92	1.918	

Stomatal resistance of stressed plants was significantly higher (values > 10 s/cm) than that of non-stressed plants (values < 10 s/cm), which reflects the injury to the photosynthesis process, higher values reflecting a higher degree of plant suffering (Figures 5 and 6).

Stomatal resistance of not stressed plants and drought susceptibility index were not correlated (Figure 5) but, between stomatal resistance of stressed plants and drought susceptibility index there was a very significant correlation (Figure 6). This indicates that stomatal resistance has a very important impact on production under stress conditions.

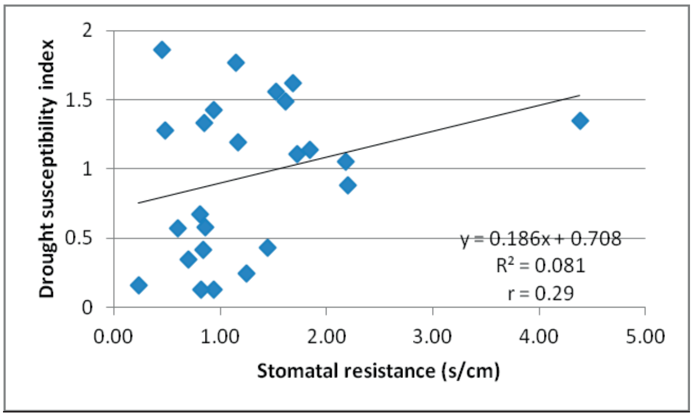

Figure 5. Relationship between stomatal resistance of not stressed plants and drought susceptibility index

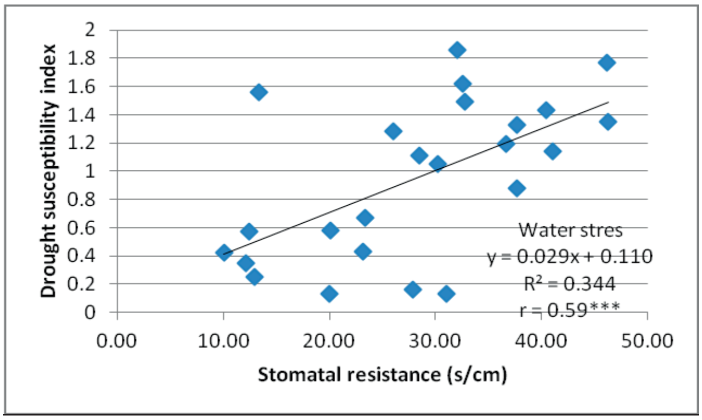

Figure 6. Relationship between stomatal resistance of stressed plants and drought susceptibility index

Stomatal resistance of young alfalfa plants varied from 12 to 62 s/cm under water stress, with an average of 27.90 for "*tolerant*" genotypes and 43.13 for "*sensitive*" ones. Generally the stomatal resistance was higher for sensitive genotypes than for resistant ones, but there were some sensitive genotypes with low stomatal resistance and vice versa, which opens new opportunities to improve drought resistance (Figure 7).

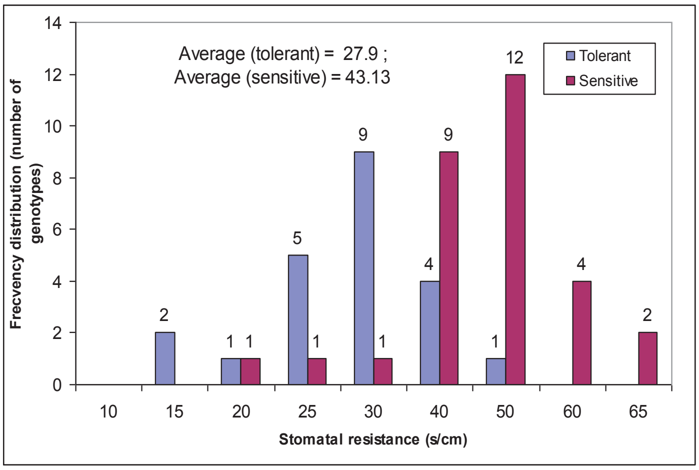

Figure 7. Frequency distribution for stomatal resistance in studied alfalfa genotypes with different drought resistance

The stomatal resistance of young plants was correlated with DSI, both under optimal and under stress conditions (Figure 8). This indicates that stomatal resistance has a very important impact on production both under stress and under optimum conditions.

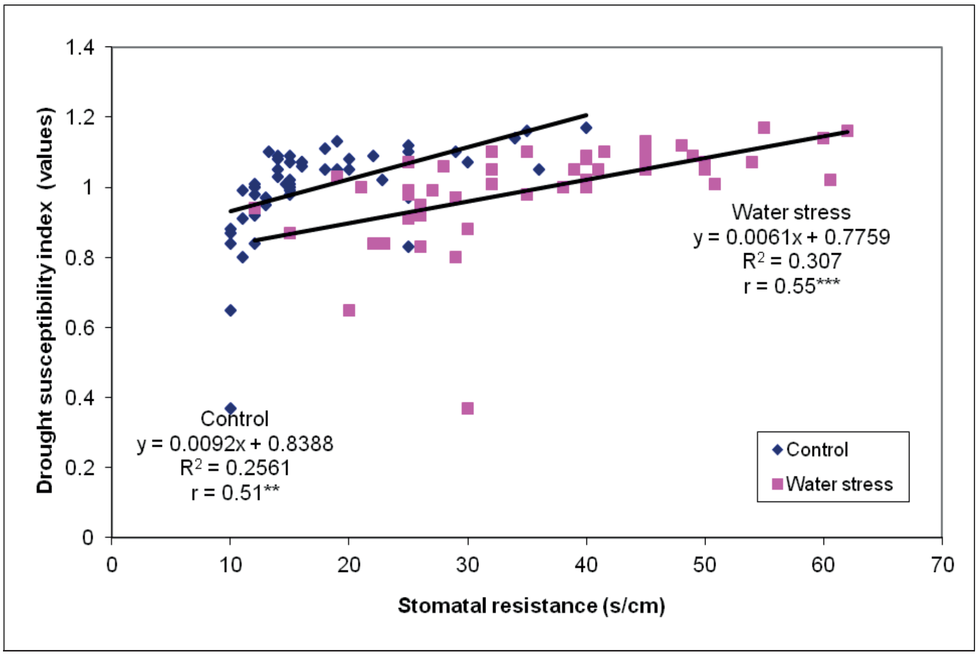

Figure 8. Relationships between drought susceptibility index and stomatal resistance

DISCUSSIONS

Alfalfa (*Medicago sativa* L.), the most economically important forage crop in Europe (and in Romania, too), produces highly nutritious hay, silage, pasture and contributes to sustainable agricultural systems. Use of varieties with wide adaptability for contrasting environmental conditions and water supply can reduce the risks of decreased yields in unfavourable years. If many physiological traits are involved in the determination of a complex character, as is the case of drought resistance, deliberate selection in order to combine more physiological traits could be more efficient and lead to obtaining results in a shorter period than if a more empirical strategy is used. The first condition for a successful introduction of physiological traits in a breeding program is to determine the degree of genetic variability for the traits of interest. In this paper we have addressed some physiological traits more and less explored in alfalfa. The first of them is based on the relationship between the plant growth (height of plant and number of shoots) and productivity (drought susceptibility index based on biomass production). This study clearly showed that alfalfa genotypes tested showed a reduction in stem elongation under water stress conditions. This was correlated with a reduction in biomass production under water stress. Differences in response to water stress were noticeable among different genotypes. *Plant* height, leaf size, and the *stem* diameter were significantly reduced under the *water* limiting conditions in maize (Khan et al., 2015) and *sunflower* (Petcu et al., 2001). In soybean, the stem length was also decreased under water deficit conditions (Specht et al., 2001). The plant height was reduced up to 25% in water stressed citrus seedlings (Wu et al., 2008). Stem length was significantly affected under water stress in potato (Heuer and Nadler, 1995), alfalfa (Anower et al., 2017), soybean (Zhang et al., 2004) and parsley (*Petroselinum crispum*) (Petropoulos et al., 2008). The reduction in plant height was associated with a decline in the cell enlargement and more leaf senescence in *A. esculentus* under water stress (Bhatt and Srinivasa Rao, 2005). Growth of alfalfa (*Medicago sativa* L.) was negatively affected by drought stress and the height of the alfalfa plants at the first cutting was generally higher compared to the one achieved in the following cuttings (both in the optimal and drought conditions), (Misar et al., 2015).

Our results showed that after the second cut of alfalfa, there was an almost double increase in the number of shoots, both for optimal and water stress conditions. The results from the literature have shown an increase in the number of shoots with the aging process, and in summer cuts, the number of shoots decreases due to heat and drought conditions, (Sengul, 2002; Saeed and El-Nadi, 1997).

Lower length and number of shoots per plants under water stress could be a strategy of plants to reduce the area of transpiration in order to limit the water loss under water stress conditions. Correlations with drought susceptibility index (calculated on basis of biomass production) suggest that a progress in alfalfa breeding for drought resistance could be achieved if the synthetic alfalfa cultivars will be formed with genotypes which have a high number of shoots and which do not reduce too much height under water stress conditions.

Another physiological trait which was studied is linked to the stomatal apparatus. Stomatal resistance (stomatal closure) and leaf growth inhibitions were among the earliest responses to drought and protected plants from extensive water loss, which might result in cell dehydration and death (Hasheminasab et al., 2012). Variation in stomatal response to environment is usually ascribed to processes external to the guard cells, such as osmotic adjustment in leaf mesophyll cells. So, the increase in stomatal conductance under water stress was ascribed to biomass production being reduced less by drought than water use or to net CO_2 assimilation rate being decreased less by water stress than transpiration rate (Brodribb and Holbrook, 2005). The studies performed by Dong et al. (2014) and Yusuf et al. (2010) evidenced that under stress conditions, higher leaf water retention beside a reduction in stomatal conductance and transpiration rate represent mechanisms to drought resistance for *Arabidopsis* and alfalfa. More research is necessary to understand the stomatal or photosynthetic responses of different species or cultivars with differing drought-resistance, perhaps simultaneously with changes in root hydraulic conductivity and morphology under constant drought, (Montague et al., 2008). This understanding will be very useful to illustrate the mechanism(s) of resistance of plants to water stress.

In very few cases, genetic variability for stomatal traits has been demonstrated within the same species, although affected traits were mostly related to stomatal size and density (Jones et al., 1987).

In our case, genetic variability for stomatal resistance existed, and most resistant genotypes had a lower stomatal resistance (this means higher stomatal conductance). This confirms the hypothesis that higher-stomatal conductance species have better adaptation to drought. However, the existence of a susceptible genotype with lower stomatal resistance and vice versa shows the complexity of genetic control of stomatal properties and opens new perspectives for breeders. We consider that the concept is especially important to agriculture, as water availability often limits production, and stomatal traits are major determinants of water use efficiency. Identification of genetic variability for stomatal properties would provide a new tool for plant breeders to improve crop adaptation to stressful environments, even more for improved varieties of alfalfa synthetic cultivars, that usually are obtained through three or four generations of open pollinated reproduction of polycross seeds.

The obtained results confirm that varieties performance under drought depends on many traits and none of them can explain alone the observed differences among genotypes. But each of them can improve performance under drought and for this, it is necessary to know the available variability for each of these traits. The hypothesis is that the use of physiological traits related to water use efficiency under water stress could be used to develop alfalfa genotypes with improved survival capacity, early vigour, and recovery after drought. We can emphasize some genotypes with better response under water stress for height of plants (F 1715-05, Roxana, F 1608-04), number of shoots (F 1615-04, F 1710-04, F 1914-07), for stomatal resistance (Daniela and Roxana), or for DSI (F 1712-04, F 1414-02) and also genotypes which combined good values both for DSI and stress performances such as F 1535 and F 1412-02.

CONCLUSIONS

The results obtained from the present study show that there were significant differences among genotypes for all physiological traits when grown under drought stress condition. Lower length and number of shoots per plants under water stress is a strategy of plants to reduce the area of transpiration in order to limit the water loss under drought. Correlations with drought susceptibility index (calculated on basis of biomass production) suggest that a progress in alfalfa breeding for drought resistance could be achieved if the synthetic alfalfa cultivars will be formed with genotypes which have a high number of shoots and which do not reduce too much height under water stress.

Genetic variability for stomatal resistance has been highlighted for young plants, both under optimal conditions and under stress conditions. Genotypes that are susceptible to drought (according to the drought sensitivity index) shut down their stomata (their stomatal resistance was higher than in tolerant genotypes), leading to a decline in photosynthesis. The existence of drought sensitive genotypes with low stomatal resistance and vice versa, creates new possibilities for improving drought tolerance based on this physiological trait.

The evaluation of physiological variability under water stress offers the possibility of characterization and selection of parents for the production of tolerant to drought synthetic cultivars.

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