GAS EXCHANGE PARAMETERS IN RED COVER (*TRIFOLIUM PRATENSE* L.) AND FESTULOLIUM (*FESTULOLIUM BRAUNII* (K. RICHT) A. CAMUS) UNDER DROUGHT STRESS

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Abstract. Water deficit in the soil is one of the factors that limit the yield of crops, causing great damage to agricultural production. This is the result of genotypic expression as modulated by interaction with the environment. The effect of water deficit on gas exchange parameters of *Festulolium* hybrid and red clover grown in pure stand and in mixture were studied. Two-factor pot experiment was performed in the completely randomized block method, with four replications. Objects were evaluated at two soil moisture levels: well-watered conditions and drought stress. The studies have shown that all the measured parameters were affected by drought stress. Net photosynthetic rate, transpiration rate and stomatal conductance were significantly lower under drought stress than under well-watered conditions in all treatments. Red clover grown in a pure stand responded to stress the most, while *Festulolium* hybrid – the least. It was also found that the highest water use efficiency index (WUE) was observed in *Festulolium* which proves a more economical water management compared to red clover. The mixtures showed smaller yield losses under drought stress compared to red clover grown in pure stand, which indicates their higher suitability to be grown in areas with less rainfall.
Keywords: *photosynthesis, transpiration, stomatal conductance, water-use efficiency*

Introduction

The frequency of extreme climatic events has increased due to global climate change. Environmental stresses are limiting factors for production of important agricultural crops worldwide. According to Li et al. (2011), 25% of the world's agricultural land is under the influence of drought stress, limiting growth, development and productivity of many forage crops. It is argued that breeding of forage species should aim to improve plant strategies to cope with relevant abiotic stresses and optimize growth and phenology to new seasonal variation, and that plant diversity at all levels is a good adaptation strategy (Ergon et al., 2018).

Agriculture is based mainly on rainwater, which is why frequent rainfall is a real threat to agricultural production. Water shortage causes the inhibition of plant growth and development processes as well as disrupts physiological processes, including photosynthesis. It also disrupts the transport and distribution of assimilates (Starck, 2010). Stress leads to disturbance of water balance of the plant, through the decrease of water potential in cells and accumulation of secondary metabolites, which on the one hand, reduce the osmotic potential, but on the other hand, protect the cellular structures. The prolonged stress damages the thylakoid membranes and degradation of lipids

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stabilizing protein complexes, which consequently leads to damage to photosystems, mainly PSII. The photosynthetic apparatus exhibits a particular sensitivity to stress factors, which is why it is the first to react to any changes in the environment. Drought inhibits the intensity of photosynthesis, which is probably caused by a decrease in RuBisCo activity and a decrease in diffusion conductivity, which in turn, limits the availability of CO_2 in intercellular spaces (Hura et al., 2007). Under optimal cultivation conditions, the yield of plants depends on the intensity of the photosynthesis and is reduced by the loss of biomass due to respiration process. According to Lawlor (1995), nearly 90% of the accumulated biomass depends on the intensity of the photosynthesis.

The response of individual species to abiotic stresses may vary considerably. First and foremost, this is determined by genetic determinants, but also by a number of external factors, such as: light intensity, temperature, water availability, oxygen and carbon dioxide concentration in the air; and internal factors, such as: leaf structure, chlorophyll content, enzyme activity, or mineral supply (Starck, 2010). From the point of view of yield biology, the plant is resistant to stress, when under unfavourable environmental conditions, it can yield a little less than under optimal conditions (Dziadczyk, 2002). According to Blum (2009), this may be associated with the formation of a larger mass of roots, longer roots, or with the increased root permeability. Olszewska (2008) reported that, the intensity of gas exchange processes also depends on the type of plant cultivation. Compared to monotypic sowing, mixtures are generally less susceptible to adverse environmental conditions due to varied environmental requirements of individual components, different development rhythm, and differences in root system morphology. This makes mixtures yield better and be more reliable in cultivation (Lucero et al., 1999; Hakala and Jauhiainen, 2007).

Red clover (*Trifolium pratense* L.) is an important forage legume to world agriculture because of their environmental and agricultural benefits. It is an assort-lived perennial legume, meaning that it is only productive for two to three years and harvested three to five times a year (OMAFRA, 2011; Tucak et al., 2016). The short persistence of red clover grown in pure stand is mainly due to poor overwintering which can be caused by physical damage, e.g. low temperature and ice cover and pathogens, especially root rot and clover root for which deep snow cover provides ideal conditions (Hakala and Jauhiainen, 2007). Red clover needs at least 500-600 mm of annual precipitation to properly develop, including 300-400 mm during vegetation period (Rojek, 1986). The greatest demand for water is in the period of intensive growth, that is, in the phase of forming main shoots and branches and developing inflorescences. Red clover grows best in a humid, moderately cold climate, under frequent and evenly distributed rainfall during the growing season (OMAFRA, 2011). Several multi-year studies showed that red clover biomass weight was lower in years with less than average precipitation, suggesting that red clover may be susceptible to drought stress. Grasses have high water requirements as well. Their daily demand varies from 0.5 to 3.0 $dm^3 \cdot m^{-2}$, and the amount of water transpired from 1 m^2 of grass sward per year can amount up to 1000 dm^3 (Thomas, 1994). According to Łabędzki (2006), drought during the summer, accompanied by high temperature, can cause a decrease in grass yield by about 30%.

Growing perennial legume-grass mixtures has many benefits. In addition to being valuable, balanced feed for ruminants, mixtures reduce weeds, protect the soil from erosion by rain and wind, increase soil organic matter and soil fertility, improve soil water-holding capacity, and improve to soil structure and yield stability (Gaudin et al.,

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2013). Mixtures allow improved resource utilisation and beneficial biological interactions between the crops. Successful mixtures relies on the component crops having complementary rather than competing traits and thus using resources more efficiently than pure crops. They also require smaller doses of mineral nitrogen than grasses grown in pure sowing, due to ability of legumes to fix atmospheric N, utilizing a symbiotic relationship with *Rhizobium trifolii*. Red clover contributes a large amount of naturally produced nitrogen to the soil for use by companion grasses (Queen et al., 2009). It provides them with a unique advantage compared to other plant species. With the increase of energy cost, N-fertiliser has become more expensive, a trend that is expected to continue in the future, which will likely further increase the need of legume production, including red clover (Jensen and Hauggaard-Nielsen, 2003).

Knowledge about the physiological and genetic responses of grasses and legumes to drought stress is insufficient. It is predicted that climate change may increase the risk of local droughts, with severe consequences for agricultural practises (Lipiec et al., 2013). This indicates a need to conduct research aimed at recognizing and understanding the responses of various crop species to adverse environmental factors and their possibilities of adapting and acclimatizing to changing conditions. This will allow a bigger use of drought-resistant species resistant, which can effectively use habitat resources and exhibit good water management. High hopes are placed on interspecific and intergeneric hybrids, which combine beneficial traits of parental species in their genomes. They constitute a valuable source of variability for increasing resistance to abiotic and biotic stresses. One of such hybrids is *Festulolium* (*Festulolium braunii* (K. Richt) A. Camus), the effect of the crossing of italian ryegrass (*Lolium multiflorum* Lam.) and meadow fescue (*Festuca pratensis* Huds.) (Østrem et al., 2013). According to Kryszak et al. (2002) *Festulolium* give higher yields by about 20% in comparison to red clover. For this reason, it is used in intensive feed production and sown on arable land and temporary grasslands, both in pure sowing and in mixtures with legumes (Olszewska, 2008). *Festulolium* is one of the most used species in Denmark (Elgersma and Søegaard, 2018).

The aim of the study was to compare gas exchange parameters of *Festulolium* and red clover grown both in pure stands and in mixture under conditions of optimal soil moisture and long-term drought stress.

Materials and methods

Plant material and growth conditions

The research was based on a two-factors pot experiment carried out in 2012-2014, in a greenhouse of the Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy, in Poland [51° 24′ 59″ N, 21° 58′ 9″ E] in the completely randomized block method, with four replications. *Festulolium* hybrid cultivar Agula and red clover tetraploid cultivar Bona were grown in pure stands and in mixture (50% grass + 50% legume). Objects were assessed at two soil moisture levels: 70% field water capacity (FWC) as optimum moisture content and 40% FWC as drought stress. Soil moisture content was differentiated 8 weeks after sowing, in the second and third year of growing – 2 weeks after starting of vegetation. In order to maintain the appropriate soil moisture, water losses were made up on a daily basis, to achieve a specified weight of the pot with soil. The treatments were stop after last cut and plants were watered as needed. During the day pots where standing outside and during the night inside the

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greenhouse. Temperature of the air in greenhouse was similar to the temperature outside. Average temperature in Puławy, for the years 2012-2014 during vegetation months (IV-X) was respectively: 9.7, 15.2, 17.8, 20.8, 19.0, 13.7, 9.1 °C. In winters pots were in greenhouse, where the lowest temperature did not fall under 0 °C. The pots were cuts every 4-5 weeks in the flowering stage of red clover, on the height of 4-5 cm.

The Mitcherlich pots were filled with 7 kg of lessive soil from arable layer (0-30 cm). It was characterized by a neutral reaction (pH in 1 M KCl 7.4), the available nutrient content of the soil was as follows (mg·100 g^{-1} soil): phosphorus 24.8, potassium 14.2 and magnesium 2.2.

The seeds were sown in 11 of April 2012, three seeds at 15 points in each pot. After emergence poorly developed seedlings were removed, leaving 8 plants per pot (in mixture – 4 units of *Festulolium* and 4 units of red clover). This proportion lasted over three years. The soil material was fortified with mineral fertilizers at doses (g·pot^{-1}): 0.5 N, 1.0 P, 1.5 K, 0.5 Mg in the form of solutions: NH_4NO_3, KH_2PO_4, K_2SO_4 and $MgSO_4 \times 7\ H_2O$ before sowing and 0.5 N after each cut of *Festulolium* in pure stand and half of this dose after each cut of mixture. Red clover in pure stand was not fertilized by nitrogen (except of start dose).

Methods and measurements

Gas exchange parameters and dry mass yield (DMY) were evaluated. Physiological plants parameters: net photosynthetic rate (P_N), transpiration rate (E) and stomatal conductance (g_s), were measured with an apparatus CIRAS-2 Portable Photosynthesis System (USA), a 1-2 days before each cut. Leaf gas exchange parameters were measured between 7.00–11.00 am, on the second fully exposed leaf in six replications, at a concentration of 390 ppm CO_2, 1000–1200 PAR [$\mu mol \cdot m^{-2} \cdot s^{-1}$] and 17–25 °C. Water use efficiency (WUE) was calculated based on the quotient of instantaneous values of photosynthesis and transpiration (P_N/E). Plant material for the research was collected three times during the first growing season, and four times during second and third vegetation year.

The agricultural drought index, determining crop reductions due to water shortage in the soil, was calculated according to the formula (Łabędzki, 2006):

$$YR = 1 - (Y_{re} \cdot Y_p^{-1})$$

where:
YR - agricultural drought index, quantizing the yield reduction,
Y_{re} – yield reduced due soil water deficit,
Y_p – potential yield under optimal soil moisture.

Statistical analysis

The date presented are the mean values from the years 2012-2014, as a result of a similar reaction of the plant examined to different soil moisture uncovered during three study years. The results were statistically analysed with the use of the analysis of variance for the completely randomized design, using Statistica v.10.0 program. Tukey’s multiple comparison test was used to compare differences between the means for main effects (factors), while confidence intervals for the means of LSD ($\alpha = 0.05$) were used to compare the means from the subclasses (interactions).

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Results

The effect of the main factors soil moisture (SM) and treatment (T) as well as that of the interactions SM × T on P_N, E, g_s, WUE were in most cases significant ($p < 0.05$) in all regrowth and average value. Drought stress led to significant decrease in photosynthesis and transpiration rate, stomatal conductance and increased in water use efficiency of red clover and *Festulolium* grown in pure stand and in mixture.

An important factor shaping the level of plant yield is the intensity of leaf gas exchange processes. An analysis of research results showed that red clover and *Festulolium* grown in pure and mixed stand reacted differently to stress. In all the years of research, in the optimal moisture conditions, the red clover cultivated in pure stand assimilated carbon dioxide the most effectively (*Table 1*). Under stress caused by the limit of moisture in the soil, the tested species responded with a significant reduction in the rate of photosynthesis (on average by 12.6% in the first, by 20.1% in the second, and 22.7% in the third year of vegetation). The strongest response to the water deficit in the first and third year of vegetation was noted for red clover cultivated in pure stand, where the average photosynthesis rate decreased by 16.8 and 34.6% respectively, while the red clover cultivated in the mixture demonstrated the highest rate of this process (9.48 and 16.15 μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), compared to the optimally moistened treatments. In the second year of vegetation, the decrease in the rate of photosynthesis under drought stress was the biggest in *Festulolium*. Moreover, this hybrid cultivated in mixture showed significantly higher rate of photosynthesis under drought stress in relations to its cultivation in pure stand. Analysing individual regrowths, it was found that the largest assimilation of carbon dioxide by plants in the first year of vegetation occurred in the third regrowth, while in the second and third year - in the first, second and third regrowth. The calculated values of determination coefficients (R^2 = 58% in the first, R^2 = 49% in the second, and R^2 = 69% in the third year of vegetation) and regression equations showed a significant, positive relationship between the photosynthetic rate and dry mass of the species tested in all years of research (*Fig. 1*). This dependency was directly proportional, which indicates a highly significant influence of the intensity of this process on the yield of the tested species.

A significant variability was recorded in the intensity of water transpiration from the leaf surface in red clover and *Festulolium* grown under limited and optimal soil moisture. In all vegetation years, the highest rate of transpiration was noted for red clover cultivated in pure stand (*Table 2*). Both species responded to water deficiency in soil with a significant reduction in the rate of this process (averagely by 29.5% in the first, 21.2% in the second and 31.5% in the third year of vegetation), compared to the optimally moistened treatments. Species cultivated in pure stand limited the evaporation of water from their leaf surface under stress conditions more than under mixed stand, whereas the smallest difference was found in the *Festulolium* hybrid grown in mixture. A statistical analysis of the results showed a highly significant, positive relationship between the rate of transpiration and the dry mass yield of red clover and *Festulolium* in all the years of the research, as evidenced by high values of determination coefficients (R^2 = 74%, R^2 = 65% and R^2 = 87%, respectively) (*Fig. 2*).

Under stress conditions, the studied species had significantly lower values of stomatal conductance (on average by 27.8% in the first, by 43.8% in the second, and by 51.6% in the third year of vegetation) compared to optimal conditions (*Table 3*). Irrespective of the method of cultivation and the level of soil moisture, red clover showed higher values of this parameter in the first and third year of vegetation, while

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the *Festulolium* hybrid - in the second year of vegetation. Significantly higher values of stomatal conductance, regardless of the level of soil moisture, were recorded in the second and third year of vegetation than in the first. The calculated values of determination coefficients and regression equations showed a significant, positive relationship between stomatal conductance and dry mass yield of red clover and *Festulolium* in all the years of the study (R^2 = 63%, R^2 = 68% and R^2 = 87%, respectively) (*Fig. 3*). This dependency was directly proportional, which shows a highly significant effect of this process on the yields of the tested species.

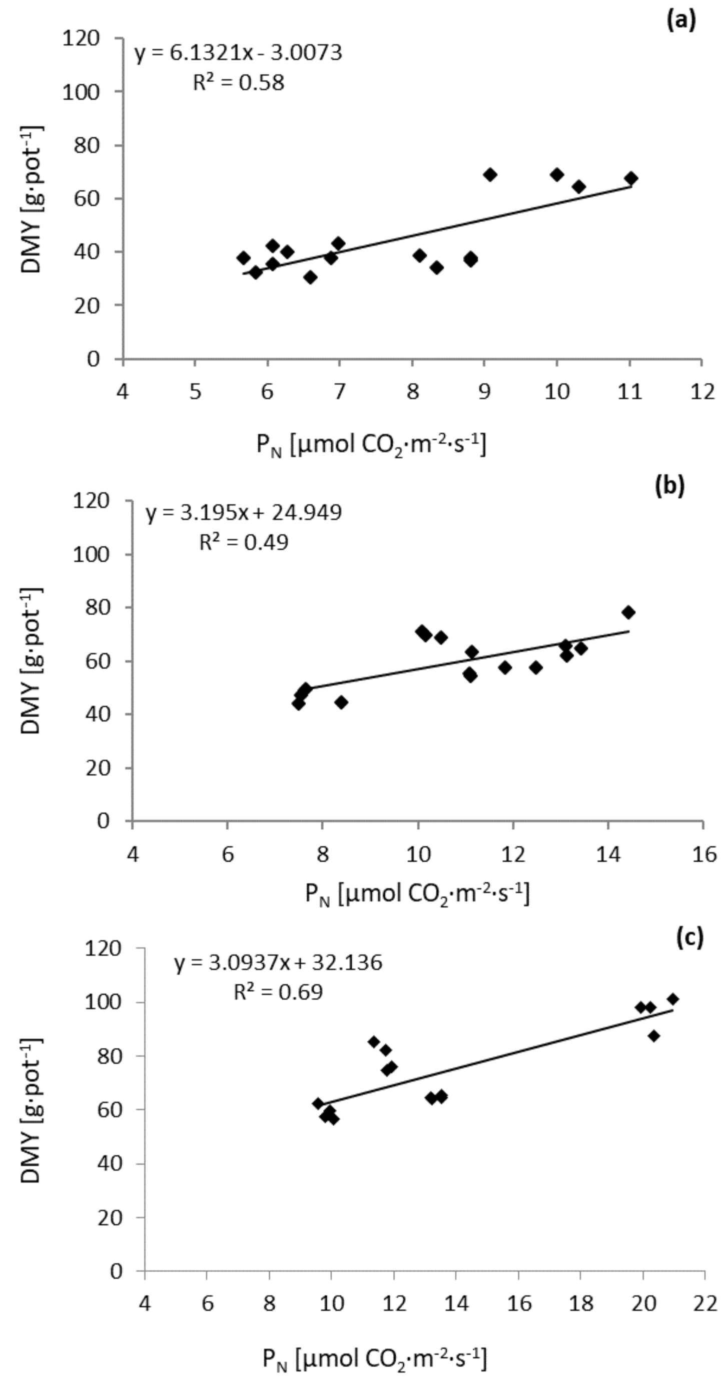

Figure 1. *Relationship between net photosynthetic rate (P_N) and dry mass yield (DMY) in the years: 2012 (a), 2013 (b), 2014 (c)*

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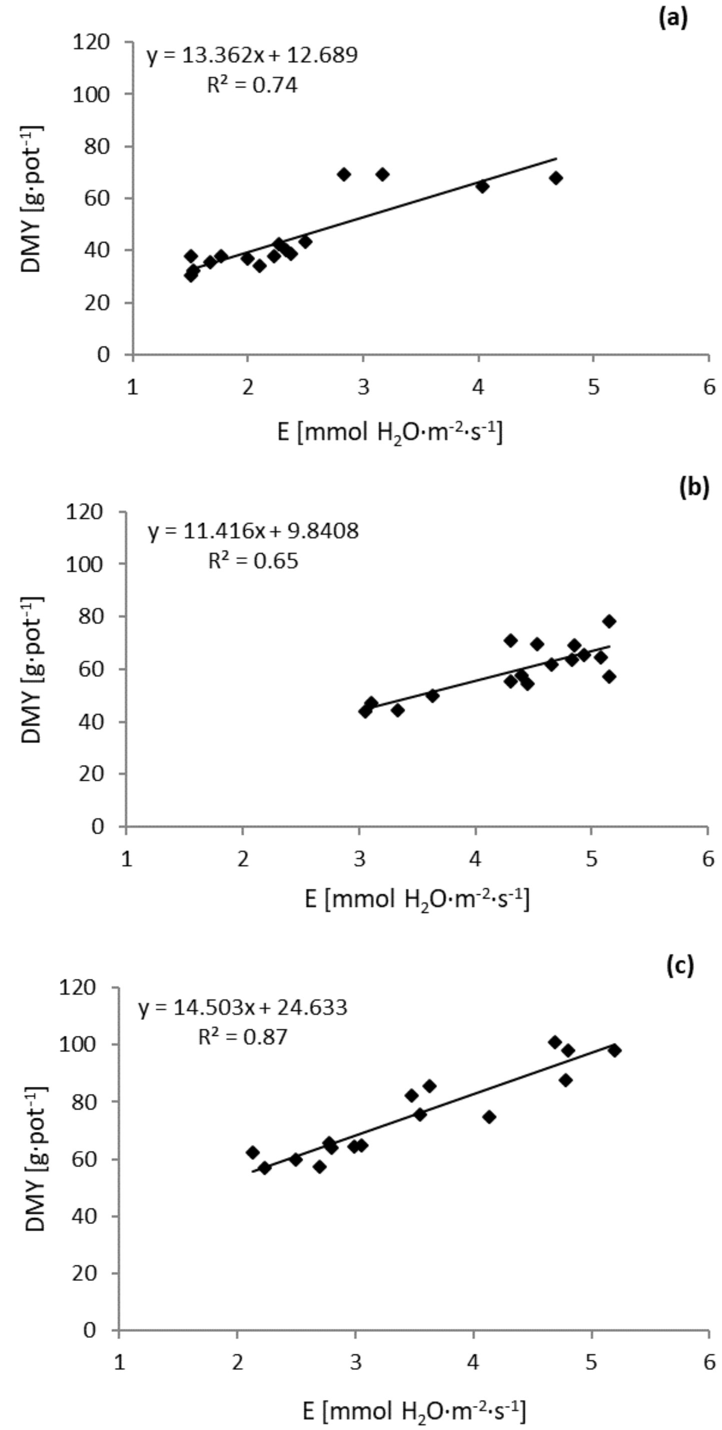

***Figure 2**. Relationship between transpiration rate (E) and dry mass yield (DMY) in the years: 2012 (a), 2013 (b), 2014 (c)*

Water use efficiency (WUE index) in red clover and *Festulolium* was generally higher under stress conditions caused by water deficit in the soil than under optimal moisture conditions (averagely for treatments by 22.1% in the first, by 5.2% in the second, and by 13.0% in the third year of vegetation) (*Table 4*). In the first year of vegetation significant differences were found in those species, which were grown in

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pure stand, in the third year - in red clover cultivated in a mixture and *Festulolium* in pure stand. In the second year of vegetation, there was no significant difference in the water use efficiency among particular species, the way in their cultivation and the level of soil moisture, but only the tendency to more economical water management for crops grown in long-term drought conditions.

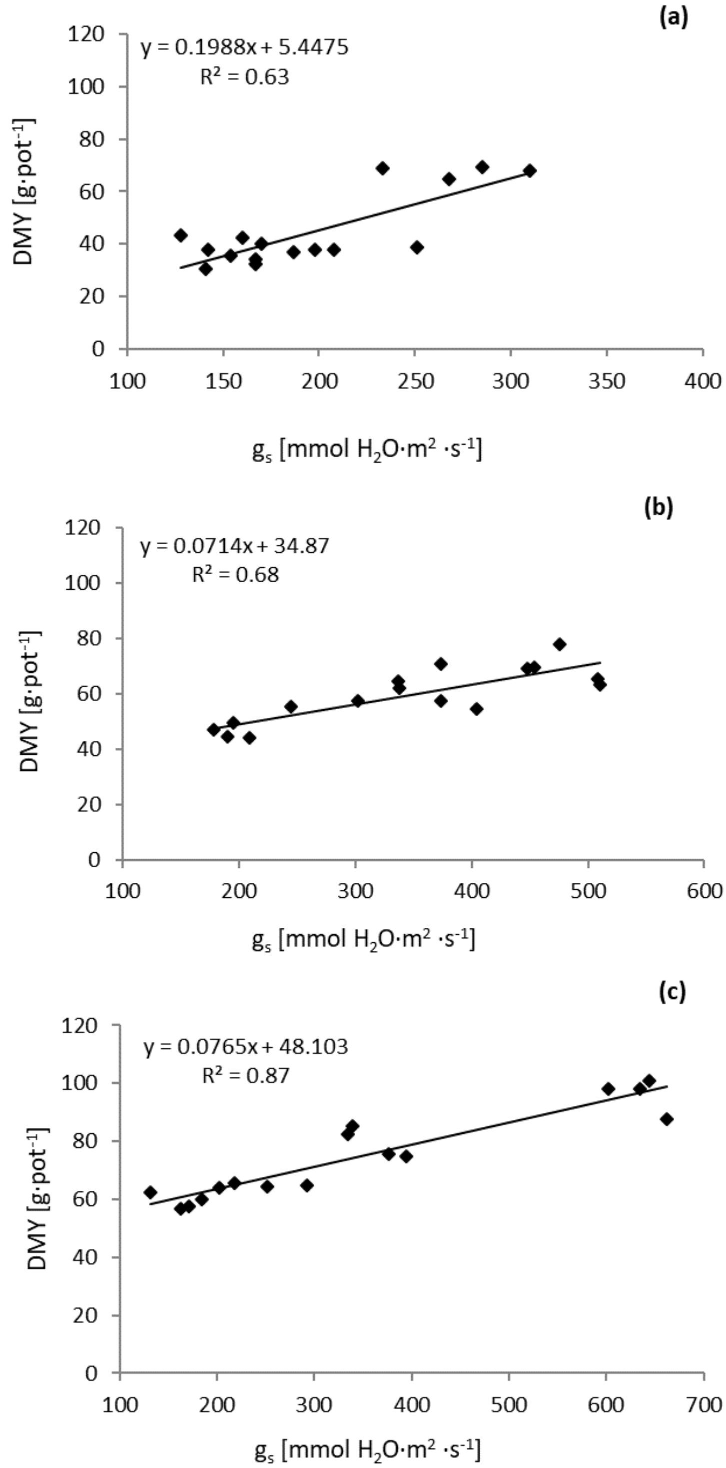

Figure 3. *Relationship between stomatal conductance (g_s) and dry mass yield (DMY) in the years: 2012 (a), 2013 (b), 2014 (c)*

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Table 1. *Net photosynthetic rate (P_N) of red clover and Festulolium grown in pure stand and in mixture under optimal and drought stress conditions [μmol CO_2 m^{-2} s^{-1}] (mean ± standard deviation, n = 6)*

Treatment	I regrowth		II regrowth		III regrowth		IV regrowth		Average[3]	
	Soil moisture conditions									
	Optimum	Stress	Optimum	Stress	Optimum	Stress	Optimum	Stress	Optimum	Stress
2012										
Red clover PS[1]	4.62±0.63	3.30±1.11	6.70±2.27	4.33±0.73	19.7±2.26	18.2±1.40	-	-	10.3±1.49	8.60±0.59
Red clover MX[2]	7.80±1.86	4.58±1.27	3.98±0.68	3.83±0.76	20.4±2.79	20.0±1.47	-	-	10.7±1.20	9.48±0.99
Festulolium PS	6.83±1.04	6.57±1.26	3.47±0.52	2.50±0.51	9.63±1.92	9.15±1.20	-	-	6.65±0.72	6.07±0.56
Festulolium MX	5.27±0.92	4.98±1.19	2.77±0.63	2.53±0.48	7.00±1.98	5.78±1.12	-	-	5.01±0.71	4.43±0.53
LSD (α=0.05)	1.88		1.54		n.s.[4]				n.s.	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	-	-	f-ratio	p
Soil moisture SM	13.19	<0.001	10.47	0.002	2.89	0.097	-	-	15.39	<0.001
Treatment T	12.08	<0.001	19.98	<0.001	165.59	<0.001	-	-	93.67	<0.001
SM × T	3.93	0.015	3.19	0.034	0.26	0.855	-	-	1.15	0.341
2013										
Red clover PS	11.2±2.37	8.92±1.55	15.0±1.64	14.8±0.67	18.7±1.64	13.6±1.12	8.43±0.86	7.05±1.76	13.3±1.03	11.1±1.03
Red clover MX	12.1±2.33	8.70±2.46	6.88±1.00	6.00±0.95	9.73±0.96	9.07±1.37	7.12±1.53	6.00±2.31	8.97±0.67	7.44±0.57
Festulolium PS	9.22±1.69	8.10±1.76	9.27±1.18	9.85±0.89	14.7±1.78	8.63±1.58	7.88±1.09	5.67±1.07	10.3±0.59	8.06±0.93
Festulolium MX	11.9±3.90	10.2±3.78	16.2±4.11	11.4±1.94	16.5±2.62	11.8±2.15	8.57±1.10	6.87±1.62	13.3±0.59	10.1±1.73
LSD (α=0.05)	n.s.		2.93		2.68		n.s.		1.49	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	7.80	0.008	4.02	0.051	68.28	<0.001	13.90	<0.001	63.72	<0.001
Treatment T	1.78	0.166	72.70	<0.001	34.93	<0.001	2.08	0.118	57.29	<0.001
SM × T	0.43	0.733	14.28	<0.001	5.65	0.002	0.30	0.823	3.47	0.024
2014										
Red clover PS	22.7±1.22	12.3±1.63	23.2±1.59	15.6±1.40	22.8±0.70	16.0±1.12	12.9±0.97	9.40±1.38	20.4±0.54	13.3±0.25
Red clover MX	20.2±3.44	17.5±1.86	22.7±2.85	15.7±4.44	21.4±1.27	20.9±0.54	11.1±0.96	10.5±2.37	18.8±1.14	16.2±0.55
Festulolium PS	15.2±0.93	12.4±1.08	12.5±1.30	10.8±0.58	11.2±1.25	10.6±0.63	7.83±0.76	5.80±1.44	11.7±0.29	9.91±0.52
Festulolium MX	12.8±1.86	11.7±1.60	11.7±0.72	11.0±1.30	13.7±1.10	9.13±1.00	9.47±0.79	4.77±0.95	11.9±0.61	9.16±0.29
LSD (α=0.05)	2.87		3.31		1.54		2.02		0.92	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	61.90	<0.001	47.09	<0.001	117.5	<0.001	51.54	<0.001	432.0	<0.001
Treatment T	32.77	<0.001	52.85	<0.001	345.8	<0.001	37.89	<0.001	481.3	<0.001
SM × T	15.16	<0.001	8.17	<0.001	28.84	<0.001	5.62	0.002	47.56	<0.001

[1]PS – species grown in pure stand; [2]MX – species grown in mixture; [3]average over the three (2012) or four regrowth (2013, 2014); [4]n.s. – not significant

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Table 2. *Transpiration rate (E) of red clover and Festulolium grown in pure stand and in mixture under optimal and drought stress conditions [mmol H_2O $m^{-2}\cdot s^{-1}$] (mean ± standard deviation, n = 6)*

Treatment	**I regrowth**		**II regrowth**		**III regrowth**		**IV regrowth**		**Average**[3]	
	Soil moisture conditions									
	Optimum	**Stress**	**Optimum**	**Stress**	**Optimum**	**Stress**	**Optimum**	**Stress**	**Optimum**	**Stress**
2012										
Red clover PS[1]	3.85±0.93	1.38±0.23	4.77±1.36	2.82±0.51	2.40±0.20	1.90±0.26	-	-	3.67±0.69	2.04±0.22
Red clover MX[2]	3.63±0.36	2.72±0.89	2.92±0.60	2.27±0.72	2.57±0.38	2.40±0.35	-	-	3.04±0.30	2.46±0.47
Festulolium PS	1.88±0.18	1.68±0.25	3.82±0.56	1.75±0.36	1.58±0.45	1.25±0.24	-	-	2.43±0.24	1.56±0.08
Festulolium MX	1.57±0.51	1.38±0.23	1.87±0.31	2.10±0.37	1.33±0.36	1.07±0.10	-	-	1.59±0.17	1.52±0.06
LSD (α=0.05)	0.81		1.05		n.s.[4]				0.53	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	-	-	f-ratio	p
Soil moisture SM	37.52	<0.001	31.87	<0.001	12.08	0.001	-	-	62.60	<0.001
Treatment T	25.50	<0.001	14.65	<0.001	44.01	<0.001	-	-	38.94	<0.001
SM × T	12.17	<0.001	7.86	<0.001	0.59	0.624	-	-	10.76	<0.001
2013										
Red clover PS	3.88±1.01	2.97±0.35	3.92±0.97	3.72±0.85	5.87±0.77	4.97±1.04	7.00±1.46	5.73±1.75	5.17±0.50	4.35±0.35
Red clover MX	4.82±1.75	3.85±1.73	4.13±1.24	2.73±1.40	3.87±0.45	3.15±0.36	4.42±0.84	3.20±1.0	4.31±0.72	3.24±0.39
Festulolium PS	4.30±1.12	2.48±0.62	3.73±0.84	3.47±0.63	5.67±0.56	3.08±0.60	6.05±0.66	4.40±0.51	4.94±0.26	3.36±0.25
Festulolium MX	4.90±1.97	3.47±1.06	5.25±1.01	4.33±0.74	5.40±1.42	5.28±0.59	4.52±0.36	4.38±0.67	5.02±0.48	4.37±0.35
LSD (α=0.05)	n.s.		1.54		1.23		n.s.		0.75	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	11.56	0.003	17.28	<0.001	27.46	<0.001	13.23	<0.001	74.73	<0.001
Treatment T	1.79	0.061	20.78	<0.001	17.88	<0.001	14.09	<0.001	21.23	<0.001
SM × T	0.37	0.828	12.38	<0.001	4.07	0.013	1.23	0.310	3.06	0.005
2014										
Red clover PS	4.10±0.33	2.10±0.40	6.37±1.05	3.72±0.36	2.63±0.29	1.73±0.16	6.15±0.66	4.23±0.68	4.81±0.33	2.95±0.12
Red clover MX	2.53±0.52	2.15±0.16	5.92±0.71	3.85±0.72	3.30±0.89	2.83±0.58	5.98±0.67	4.28±0.13	4.44±0.29	3.28±0.24
Festulolium PS	3.08±0.23	2.40±0.26	4.55±0.72	3.70±0.56	2.40±0.78	1.40±0.10	4.48±0.50	2.12±0.47	3.63±0.25	2.41±0.26
Festulolium MX	2.03±0.28	1.77±0.29	4.07±0.24	3.53±0.28	2.13±0.36	1.17±0.21	3.83±0.45	2.47±0.83	3.02±0.18	2.23±0.18
LSD (α=0.05)	0.51		0.99		n.s.		n.s.		0.38	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	77.10	<0.001	68.12	<0.001	32.09	<0.001	117.20	<0.001	316.7	<0.001
Treatment T	29.67	<0.001	10.40	<0.001	17.62	<0.001	43.58	<0.001	78.14	<0.001
SM × T	17.65	<0.001	7.32	<0.001	0.71	0.551	1.52	0.223	10.06	<0.001

[1]PS – species grown in pure stand; [2]MX – species grown in mixture; [3]average over the three (2012) or four regrowth (2013, 2014); [4] n.s. – not significant

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Table 3. *Stomatal conductance (g_s) of red clover and Festulolium grown in pure stand and in mixture under optimal and drought stress conditions [mmol H_2O m^{-2} s^{-1}] (mean ± standard deviation, n = 6)*

Treatment	I regrowth		II regrowth		III regrowth		IV regrowth		Average[3]	
	Soil moisture conditions									
	Optimum	Stress	Optimum	Stress	Optimum	Stress	Optimum	Stress	Optimum	Stress
2012										
Red clover PS[1]	208 ± 85	47 ± 9	224 ± 34	149 ± 26	398 ± 55	427 ± 123	-	-	276 ± 28	207 ± 44
Red clover MX[2]	132 ± 28	75 ± 28	162 ± 25	166 ± 20	643 ± 191	448 ± 105	-	-	312 ± 61	230 ± 36
Festulolium PS	53 ± 4	40 ± 10	183 ± 22	149 ± 16	349 ± 161	267 ± 76	-	-	195 ± 54	152 ± 21
Festulolium MX	48 ± 17	40 ± 19	153 ± 24	172 ± 11	294 ± 162	216 ± 40	-	-	165 ± 57	143 ± 17
LSD (α=0.05)	54.4		36.5		n.s.[4]				n.s.	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	-	-	f-ratio	p
Soil moisture SM	34.51	<0.001	10.06	0.003	5.04	0.030	-	-	18.96	<0.001
Treatment T	16.88	<0.001	2.65	0.062	12.47	<0.001	-	-	19.71	<0.001
SM × T	12.26	<0.001	9.44	<0.001	1.59	0.206	-	-	1.17	0.333
2013										
Red clover PS	529 ± 113	281 ± 51	323 ± 36	296 ± 106	347 ± 175	308 ± 106	424 ± 210	375 ± 208	406 ± 74	315 ± 62
Red clover MX	406 ± 242	271 ± 159	160 ± 64	81 ± 27	332 ± 74	154 ± 20	527 ± 84	197 ± 86	356 ± 65	176 ± 26
Festulolium PS	316 ± 180	203 ± 72	207 ± 75	181 ± 17	564 ± 82	131 ± 34	773 ± 96	285 ± 71	465 ± 64	200 ± 15
Festulolium MX	431 ± 353	219 ± 114	412 ± 28	150 ± 39	502 ± 190	373 ± 136	753 ± 83	446 ± 172	525 ± 109	297 ± 83
LSD (α=0.05)	n.s.		88.0		182.7		214.5		106.2	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	10.96	0.002	38.25	<0.001	32.56	<0.001	53.85	<0.001	93.62	<0.001
Treatment T	1.24	0.307	29.02	<0.001	5.48	0.003	7.69	<0.001	9.48	<0.001
SM × T	0.35	0.786	11.38	<0.001	6.14	0.001	5.17	0.004	3.59	0.022
2014										
Red clover PS	481 ± 62	146 ± 31	602 ± 105	190 ± 44	785 ± 96	318 ± 110	642 ± 64	323 ± 112	628 ± 27	244 ± 31
Red clover MX	361 ± 98	198 ± 26	597 ± 93	219 ± 89	795 ± 142	757 ± 62	614 ± 137	284 ± 52	592 ± 41	364 ± 25
Festulolium PS	264 ± 49	147 ± 26	313 ± 96	201 ± 39	448 ± 153	191 ± 39	418 ± 63	115 ± 39	361 ± 38	164 ± 22
Festulolium MX	292 ± 55	147 ± 50	297 ± 55	198 ± 34	580 ± 183	208 ± 80	348 ± 43	154 ± 87	379 ± 52	177 ± 23
LSD (α=0.05)	84.6		115.8		182.2		n.s.		53.3	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	144.60	<0.001	134.32	<0.001	69.40	<0.001	147.16	<0.001	643.46	<0.001
Treatment T	10.28	<0.001	16.17	<0.001	35.23	<0.001	26.08	<0.001	120.86	<0.001
SM × T	9.75	<0.001	15.06	<0.001	7.34	<0.001	1.79	0.165	19.67	<0.001

[1]PS – species grown in pure stand; [2]MX – species grown in mixture; [3]average over the three (2012) or four regrowth (2013, 2014); [4] n.s. – not significant

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Table 4. *Water use efficiency (WUE) of red clover and Festulolium grown in pure stand and in mixture under optimal and drought stress conditions [$\mu mol\ CO_2 \cdot md^{-1}$ of air] (mean ± standard deviation, n = 6)*

Treatment	I regrowth		II regrowth		III regrowth		IV regrowth		Average[3]	
	Soil moisture conditions									
	Optimum	Stress	Optimum	Stress	Optimum	Stress	Optimum	Stress	Optimum	Stress
2012										
Red clover PS[1]	1.26±0.39	2.45±0.99	1.41±0.27	1.57±0.33	8.23±0.86	9.72±1.51	-	-	2.86±0.37	4.27±0.52
Red clover MX[2]	2.16±0.55	1.82±0.76	1.39±0.22	1.77±0.37	8.11±1.48	8.50±1.48	-	-	3.56±0.49	3.92±0.54
Festulolium PS	3.64±0.54	3.90±0.41	0.91±0.11	1.46±0.27	6.43±1.72	7.72±2.60	-	-	2.77±0.45	3.91±0.49
Festulolium MX	3.29±0.57	3.65±0.90	1.49±0.30	1.20±0.05	5.24±0.17	6.12±1.08	-	-	3.11±0.42	2.92±0.32
LSD (α=0.05)	n.s.[4]		0.41		n.s.				0.71	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	-	-	f-ratio	p
Soil moisture SM	3.49	0.069	6.78	0.013	3.73	0.060	-	-	26.23	<0.001
Treatment T	25.68	<0.001	5.13	0.004	13.25	<0.001	-	-	5.45	0.003
SM × T	2.59	0.066	5.62	0.002	0.53	0.661	-	-	7.65	<0.001
2013										
Red clover PS	3.12±0.45	3.01±0.42	3.85±0.62	3.86±0.61	3.23±0.38	2.87±0.71	1.24±0.20	1.28±0.29	2.62±0.19	2.58±0.12
Red clover MX	2.70±0.68	2.56±0.89	1.72±0.27	2.58±0.97	2.54±0.24	2.88±0.21	1.63±0.37	1.85±0.29	2.11±0.21	2.33±0.35
Festulolium PS	2.55±0.83	3.32±0.51	2.54±0.41	2.89±0.39	2.60±0.24	2.81±0.25	1.31±0.16	1.29±0.15	2.12±0.22	2.40±0.21
Festulolium MX	2.42±0.65	3.01±0.79	2.26±0.68	2.65±0.34	2.87±0.35	2.25±0.47	1.90±0.25	1.58±0.37	2.32±0.24	2.31±0.39
LSD (α=0.05)	n.s.		n.s.		0.61		n.s.		n.s.	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	2.00	0.164	5.68	0.022	0.85	0.362	0.07	0.795	2.26	0.140
Treatment T	1.04	0.386	19.75	<0.001	3.32	0.029	11.39	<0.001	5.07	0.004
SM × T	1.43	0.247	1.09	0.363	4.05	0.013	1.95	0.137	1.17	0.334
2014										
Red clover PS	5.55±0.36	5.97±0.97	3.71±0.50	4.21±0.30	8.81±1.26	9.38±1.29	2.12±0.22	2.24±0.24	4.25±0.26	4.54±0.23
Red clover MX	8.13±1.50	8.18±1.01	3.90±0.85	4.04±0.57	6.87±1.75	7.69±1.72	1.86±0.08	2.46±0.60	4.26±0.40	4.95±0.39
Festulolium PS	4.93±0.31	5.20±0.34	2.78±0.29	2.97±0.38	5.06±1.56	7.61±0.99	1.76±0.22	2.75±0.50	3.23±0.20	4.15±0.34
Festulolium MX	6.51±1.76	6.69±0.60	2.89±0.27	3.13±0.46	6.52±0.54	8.09±1.27	2.50±0.37	2.01±0.38	3.96±0.30	4.12±0.25
LSD (α=0.05)	n.s.		n.s.		n.s.		0.57		0.47	
ANOVA summary	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p	f-ratio	p
Soil moisture SM	0.63	0.431	3.46	0.070	12.38	0.001	8.27	0.006	33.08	<0.001
Treatment T	20.98	<0.001	17.30	<0.001	8.67	<0.001	0.23	0.873	20.57	<0.001
SM × T	0.07	0.974	0.34	0.800	1.28	0.293	9.15	<0.001	3.94	0.014

[1]PS – species grown in pure stand; [2]MX – species grown in mixture; [3]average over the three (2012) or four regrowth (2013, 2014); [4] n.s. – not significant

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Soil moisture was an important factor shaping the level of dry matter yield of red clover and *Festulolium* cultivated in pure and mixed stands. Regardless of the method of cultivation, both species generally responded with yield reduction under long-term stress (*Table 5*). The highest decrease in total yield was noted in red clover cultivated in pure stand (PS) - by 34.9% in the first, by 33.7% in the second, and by 22.7% in the third year of vegetation, whereas this decrease was smaller in the mixture (by 10.1, 26.9, and 19.3%, respectively). *Festulolium* hybrid grown in pure stand turned out to be the least sensitive to drought. In the first year of vegetation (1st and 2nd regrowth) and in the second and third year of vegetation (1st regrowth), it showed a higher relative dry matter yield under soil deficit than under optimal water conditions. The reduction in total dry matter yield of red clover and *Festulolium*, expressed as an agricultural drought index (YR), ranged from - 0.061 to 0.349 in the first, from 0.212 to 0.337 in the second, and from 0.104 to 0.227 in the third year of vegetation (*Fig. 4*). The highest values of the YR index were recorded in the pure stand of red clover, while the smallest – for *Festulolium* hybrid. The yield reduction in mixed crops was on an average level.

Discussion

Water is one of the most important environmental resources determining plant productivity. Plants' response to water deficit is complex. It includes adaptation changes and harmful effects of water stress, as a result of which there occurs disturbance in fundamental life processes. One of the effects of these changes is reduction or inhibition of growth. It results from the reduction of the intensity of gas exchange processes, a decrease in the export of photosynthesis products from leaves to other organs of the plants, and the disruption in transport and distribution of assimilates. Water shortage also inhibits the growth of plants, including leaf blades, which due to their reduced surface, receive less light and absorb less carbon dioxide (Starck, 2010). Our studies showed that red clover and *Festulolium* yielded lower in drought stress conditions. The biggest decline in the total yield was recorded for red clover grown in pure stand (on average by 30.2% for three years), lower in a mixture (by 19.3%), and the smallest in *Festulolium* in pure stand (by 9.5%). Adopting the level of agricultural yield decrease as a criterion of crop resistance to stress, allowed to conclude that *Festulolium* hybrid grown in pure stand was the most resistant to the effects of long-term stress. Red clover grown in pure stand showed the greatest sensitivity to drought, while in the mixture with *Festulolium*, its yield loss was smaller by 10.9%. Similar results were obtained by the authors in studies on the response of alfalfa (*Medicago* × *varia* Martyn) to water deficit in the soil depending on the type of sowing (Staniak et al., 2018). Under stress conditions, the yield decrease of alfalfa grown in pure stand was by 13% higher compared to the cultivation in a mixture with *Festulolium*. Studies of Küchenmeister et al. (2013) showed that yellow alfalfa (*Medicago falcata* L.), white clover (*Trifolium repens* L.) and birdsfoot trefoil (*Lotus uliginosus* Schkuhr) grown in a mixture with perennial ryegrass (*Lolium perenne* L.) responded to stress with a much lower yield decrease than in pure stand. Tucak et al. (2016) showed high sensibility of red clover grown in pure stand to stressful conditions caused by drought resulted in low yields in 23 cultivars and populations. Also Gaudin et al. (2013) reported about the high sensibility of red clover in pure stand to drought stress. Studies of AbdElgawad et al. (2015) showed that water deficit reduced biomass of legumes (black medic (*Medicago lupulina* L.) and birdsfoot trefoil) and grasses (meadow bluegrass (*Poa pratensis* L.)

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and perennial ryegrass) at the dry weight and fresh weight levels, but this effect was stronger in the legumes species.

Table 5. *Relative [%] dry mass yield (DMY) of red clover and Festulolium grown in pure stand and in mixture under drought stress in relation to optimal conditions*

Treatment	**Regrowth**				**Total DMY[3]**
	I	**II**	**III**	**IV**	
			2012		
Red clover PS[1]	61.7	65.7	74.9	-	65.1
Festulolium PS	128.7	106.4	86.9	-	109.5
Red clover + *Festulolium* MX[2]	98.3	83.8	81.9	-	89.9
			2013		
Red clover PS[1]	67.0	63.6	63.3	71.8	66.3
Festulolium PS	85.3	81.3	71.3	73.3	79.0
Red clover + *Festulolium* MX[2]	70.1	77.2	67.3	83.5	73.1
			2014		
Red clover PS[1]	96.4	75.0	59.1	70.2	77.3
Festulolium PS	103.3	84.8	85.3	84.4	89.6
Red clover + *Festulolium* MX[2]	93.0	80.5	78.6	76.2	83.4
			Sum of three years		
Red clover PS[1]	73.0	68.9	64.1	71.1	69.8
Festulolium PS	103.1	90.3	81.6	78.2	90.5
Red clover + *Festulolium* MX[2]	84.2	80.5	74.8	79.9	80.7

[1]PS – species grown in pure stand
[2]MX – species grown in mixture
[3]Total DMY is the sum of dry mass yield over the three (2012) or four regrowth (2013, 2014)

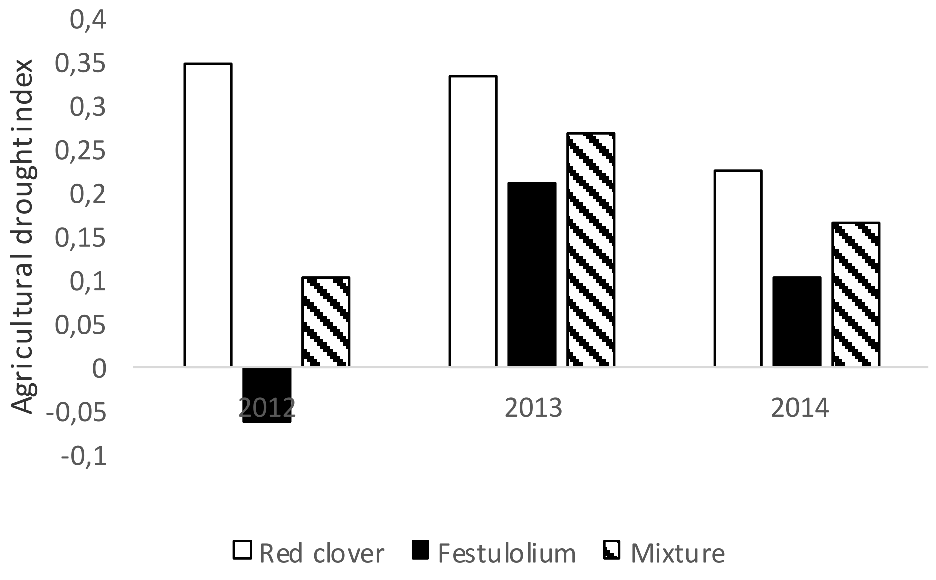

Figure 4. *Agricultural drought index for red clover, Festulolium and mixture under drought stress conditions*

Inhibition of plant growth in drought conditions is a defensive response of plants. Water deficit in the soil affects the reduction of water potential of plant shoots and stimulates the growth of the root. As a result, the plant changes its distribution of assimilates, which in turn causes a lower yield of the aboveground mass (Lucero et al., 1999). According to Lazzarotto et al. (2009) as well as Skinner and Comas (2010) higher resistance in grasses than legumes species to drought could possibly be related to an increased root/shoot ratio under stress conditions, improving water and nutrient use. Lucero et al. (1999) observed that increased soil water deficit decreased root dry matter yield for perennial ryegrass grown in pure stand, but in mixture with white clover root yield decrease was smaller. Many authors underline also that the productivity of mixed crops is influenced by the competition of the plants and their mutual impact on each other. According to Frankow-Lindgerg (1986), an intraspecific competition more strongly affects the productivity of red clover, while the interspecific one - of grasses. Hence, growing red clover together with grasses is a very good practice.

Photosynthesis is one of the most stress-sensitive life processes. Factors limiting the photosynthetic activity of plants include: water, carbon dioxide, temperature, and light. The influence of water is not direct, as the water content in chloroplasts is sufficient for photosynthesis. Water, however, strongly affects CO_2 assimilation, as under water deficit, stomata close and CO_2 input gets cut off, which reduces its concentration in the intercellular spaces and hence, photosynthesis is inhibited (Córdobaa et al., 2015). In grasses, a big decrease of photosynthesis can result from the increased participation of photorespiration in gas exchange, which often intensifies under stress, as one of the mechanisms of energy dissipation (Flexas and Medrano, 2002). Our studies have shown that drought stress caused a significant reduction in the rate of photosynthesis and transpiration in the tested plants, regardless of the species and way of cultivation, whereas the highest sensitivity to stress was recorded for red clover grown in pure stand, while the smallest – for hybrid *Festulolium* cultivated in the mixture. Studies of Olszewska (2008) showed a higher rate of photosynthesis and transpiration in *Festulolium* hybrid grown in a mixture with white clover (respectively by 49 and 57%) and birdsfoot trefoil (respectively by 24 and 34%) compared to the pure stand. In turn, the studies AbdElgawad et al. (2015) showed that legumes (black medic and birdfoot trefoil) exhibit a significantly a higher photosynthesis rate under optimal conditions, but under drought conditions, they limited this process stronger than grasses (perennial ryegrass and meadow bluegrass). According to Dziadczyk (2002), the plant is resistant to stress if it can keep the intensity of the most important life processes on the least changed level in environmental conditions significantly deviating from the optimum.

The survival of plants in drought conditions depends on efficient water management, which is well expressed by water use efficiency index (WUE). It is dependent on the intensity of the photosynthesis and transpiration processes, but also reflects the influence of environmental factors. Our studies have shown that values of WUE index in the tested plant species were higher under stress conditions than under optimal soil moisture in the first and third year of vegetation, especially in the case *Festulolium* hybrids. Studies of Olszewska (2008) showed a significantly higher value of WUE index in *Festulolium* grown in a mixture with white clover and birdsfoot trefoil, but only in the first of the three years of vegetation. This proves that *Festulolium* has a more economical water management and greater resistance to drought stress compared to clover. According to Hall (1990), a high value of WUE index allowed to maintain a high coefficient of crop yields even during drought. In turn, Blum (2009) believes that

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WUE index is not associated with drought resistance, as some species resistant to drought show lower WUE values, which is associated with a deeper root system and higher use of water. According to this author, the increase in crop yields under drought conditions is closely connected with the growth and development of the root system, especially at the beginning of the growing season.

Conclusions

The present experiment indicates, that a long-term stress caused by water deficit in the soil significantly reduced the yield of dry mass of red clover and *Festulolium*, regardless of the method of cultivation. Mixtures showed smaller yield losses under drought stress compared to red clover grown in pure stand, which indicates their higher suitability to be grown in areas with less rainfall. Photosynthetic effectiveness of red clover was higher than *Festulolium*, regardless of the level of soil moisture. In terms of long-term drought, the photosynthesis and transpiration rate in the tested species were lower than in the treatments optimally moisturized. Red clover grown in pure stand responded to stress the most, while *Festulolium* hybrid cultivated in the mixture - the least. The water use efficiency index (WUE) of the tested plant species was higher under stress caused by water deficit in the soil than under optimal conditions, whereas significant differences were reported in the first and third year of vegetation. The biggest differences were noted in *Festulolium*, which proves a more economical water management of this hybrid compared to red clover. The selection of crop with high water use efficiency (WUE) traits would be one of the adaptation measures for climate change.

Our studies have shown, that the cultivation of red clover in a mixture with *Festulolium* hybrid is more favorable than in pure stand, especially in areas with less rainfall, due to better yields and higher durability resulting from, i.e. a more efficient water management. It is associated with course of the main physiological processes. Red clover reacted to soil water deficit with a significant decrease of the rate of photosynthesis and transpiration but stronger reaction was observed in pure sowing than in mixture.

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