SOIL MOISTURE IMPACT ON BIOMASS PARTITIONING AND RELATIVE CHLOROPHYLL CONTENT FOR LEGUME GRASS MIXTURES IN A CONTROLLED ENVIRONMENT

BAI, Y.[1,2] – YAN, R.[3] – DAI, J.[4] – WEI, Z.[5] – XIA, J.[1] – YANG, H.[1] – SCHELLENBERG, M. P.[2*]

[1]*Shandong Key Laboratory of Eco-Environmental Science for Yellow River Delta, Binzhou University, Binzhou 256603, China*

[2]*Swift Current Research and Development Centre, Agriculture and Agri-Food Canada, 1 Airport Rd, Box 1030, Swift Current, Saskatchewan S9H 3X2, Canada*

[3]*Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China*

[4]*College of Agriculture and Forestry Engineering and Planning, Tongren University, Tongren 554300, China*

[5]*College of Grassland, Resources and Environment/Key Laboratory of Grassland Resources of the Ministry of Education/Inner Mongolia Key Laboratory of Grassland Management and Utilization/Key Laboratory of Forage Cultivation, Processing and High Efficient Utilization of the Ministry of Agriculture, Inner Mongolia Agricultural University, No. 29 Erdos Street, Hohhot 010011, Inner Mongolia, China*

[*]*Corresponding author*
e-mail: mpschell2020@gmail.com

(Received 24th Aug 2022; accepted 11th Nov 2022)

Abstract. Drought is a widespread abiotic stress that impacts plant growth, productivity and survival. A randomized complete block design pot experiment was conducted to determine the effects of drought on above- ground biomass, root biomass, root/shoot (R/S) and relative leaf chlorophyll content (SPAD) of monoculture and legume-grass mixtures at the Swift Current Research and Development Centre (SCRDC) of Agriculture and Agri-Food Canada (AAFC). The legumes were Canadian milk-vetch (*Astragalus canadensis*) and white prairie-clover (*Dalea candida*). The grasses were northern wheatgrass (*Elymus lanceolatus*) and side-oats grama (*Bouteloua curtipendula*). Three water treatments (40%, 60% and 80% field capacity) and three cuts were the abiotic factors applied. Except for monoculture side-oats grama, multiple-species forage mixtures were more adaptable than a simple grass–legume mixture or monoculture in a water-limiting environment. The forage mixtures of Canadian milk-vetch and northern wheatgrass tolerated lower moisture levels than the other mixtures. Decreased soil moisture resulted in decreased total biomass and altered biomass allocation to roots resulting in higher R/S ratios in stressed seedlings. The SPAD value of Canadian milk -vetch mixture decreased with water stress, and white prairie-clover+ northern wheatgrass and white prairie-clover+ northern wheatgrass+ Canadian milk-vetch were better adapted to low soil moisture than the monocultures.
Keywords: *water stress, forage mixtures, biomass, R/S ratio, relative chlorophyll content*

Introduction

Plants continuously face harsh environments due to varying abiotic stresses. Stresses may be characterized in two ways, biotic stresses and abiotic stresses (Singh et al., 2015). Drought is a widespread abiotic stress that elicits responses including altered metabolism, growth and development of plants (Golldack et al., 2014). The episodic nature of precipitation in grasslands produces moisture stress at multiple scales, from decade-long

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2101_439450

reductions in precipitation, seasonal dry periods, and forage drought stress induced at mid-day on a daily basis (Tucker et al., 2011). With projected increases in temperature and reduced water availability during the growing season for many grassland areas, drought is likely to increase in importance as a driver of future grassland community structure (Shinoda et al., 2010). Aboveground primary production in North American grasslands is characterized by the highest inter-annual variability among different biomes; it is also affected by moderate precipitation variability and the potential for accumulated plant responses to drought. Understanding native plants' tolerance to drought is therefore of fundamental importance and a major research topic (Shinoda et al., 2010; Pinheiro and Chaves, 2011; Habermann et al., 2019).

Mixtures of grass and legume are common in western Canada. In a forage mixture, legumes provide N to the pasture system through N fixation, which reduces N fertilizer requirements (Biligetu et al., 2014). An ideal forage mixture composed of grasses and legume has the potential to adapt to a greater range of environmental conditions and may provide a more reliable forage yield and nutritional quality under different environmental conditions (Schellenberg et al., 2012a; Serajchi et al., 2017). The majority of introduced Eurasian cool-season grasses are highly productive, with some species having invasive characteristics and becoming a serious threat to native grasslands (Otfinowski et al., 2007; Biligetu et al., 2014). Forage mixtures of warm-season and cool-season species have the potential to provide higher forage quality than a monoculture or simple cool-season mixtures (Oliveira et al., 2011). In the Canadian Prairies in southwestern Saskatchewan, some legumes have been observed to be short lived and not well adapted to the semiarid conditions, while native perennial grasses are often the dominant plants due to superior stress tolerance and thus preserve the productivity and stability of the plant community (Hooper et al., 2005; Picasso et al., 2008). However, few studies have explored the drought tolerance of grass and legume mixtures.

Based on previous studies, potentially highly productive native grass and legume species were selected for evaluation in monoculture and binary mixtures. In this study, we selected forage species, including two grasses and two legumes found in the semiarid region of Saskatchewan, Canada, to plant in monoculture and in mixtures. The objectives of this study were to (i) evaluate forage yield and quality of species in monoculture and determine the relationship between forage mixtures and productivity; (ii) assess the persistence of less competitive species in the mixtures in order to understand the effects of functional group diversity on forage productivity and quality.

Materials and methods

Experimental design

A pot study was conducted to determine optimal mixtures of legumes and grasses in the greenhouse (16 h photoperiod, 8 h dark; 19-22°C during the day and 17-20°C during the night) at the Swift Current Research and Development Centre (SCRDC) of Agriculture and Agri-Food Canada (AAFC). The study was implemented from 15th January to 4th April 2018 and repeated from 10th April to 30th June 2019. Supplemental day light was turned on when natural light energy was less than 500 Wm^2, and turned off if accumulated light reached 3620 Wm^2 hr from 7 am to 11 pm every day. The experimental soil was collected at SCRDC, and was an Orthic Brown Chernozem type. The experiment was carried out as a split-plot design based on randomized complete block design with 39 treatments and three replicates. The main plots were three water

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DOI: http://dx.doi.org/10.15666/aeer/2101_439450

treatments with fixed moisture levels: 80% water treatment (i.e., 80% of field capacity), 60% water treatment (i.e., 60% of field capacity) and 40% water treatment (i.e., 40% of field capacity); The legume species studied were: Canadian milk-vetch (*Astragalus canadensis* L.) and white prairie-clover (*Dalea candida (Michx.) Willd*); and the grasses were northern wheatgrass (*Elymus lanceolatus (Scribn. & Sm) Gould ssp. lanceolatus var. lanceolatus*) and side-oats grama (*Bouteloua curtipendula (Michx.) Torr. var.curtipendla*). The subplot monocultures and mixtures were: (i) Canadian milkvetch (CMV), (ii) white prairie-clover (WPC), (iii) northern wheatgrass (NWG), (iv) side-oats grama (SOG), (v) NWG + CMV, (vi) SOG + CMV, (vii) NWG + WPC, (viii) SOG+ WPC, (ix) NWG + CMV+ WPC, (x) SOG + CMV+ WPC, (xi) NWG +SOG + CMV, (xii) NWG +SOG + WPC and (xiii) NWG +SOG + CMV+ WPC. Each 17.5 cm (2 L) clay pot contained 2.5 kg of soil. Establishment and growth of the plants in the pots at different growth stages were visually observed. Each pot was planted six plants，were equal distribution according to different treatments. Water was applied after three weeks and everyday water was supplemented using an Economy Soil Moisture Tester (Spectrum Technologies, Inc.).

Data collection

Plant biomass was harvested every 15 days for a total of three cuts after 3 weeks of growth (*Figure 1*). For the first two cuts, plants were clipped to a height of 5 cm and dry matter was weighted, while last cut was at the soil surface. At the end of the third harvest, root samples were washed and weighed to obtain the root biomass. The plant material was dried at 70°C in an oven for 48 h, and the plant roots were washed and oven dried for one week before root biomass determination. Plant biomass was measured as the accumulation of whole plants per pot at every cut. Total biomass was the accumulated above-ground biomass of three cuts. The root/shoot ratio (R/S) was determined by the root biomass of all plants (R) divided by shoot biomass at the last cutting (S). Relative leaf chlorophyll content was represented by the soil–plant analysis development (SPAD value with the measured using a handheld Minolta SPAD 502 Chlorophyll Meter (Minolta Camera Co., Ltd., Japan) (Viciedo et al., 2021). The SPAD value of 10 leaves was measured in each pot before every cutting. Monitoring soil moisture content at three sites in one pot was desirable，and get the average. Monitoring soil moisture content at three sites in one pot was desirable，and get the average. Available water in the stressed treatments were 80%, 60% and 40%, being calculated based on the field capacity (23 g [100 g dry soil]$^{-1}$; soil water potential, -0.03 MPa) and permanent wilting point (11 g [100 g dry soil]$^{-1}$; soil water potential, -1.5 MPa) determined by pressure plate. The plants were irrigated daily (total of 117 times). On each occasion, the pots were weighed and watered to reach the treatment moisture levels and the added amounts were recorded. Total water use was calculated as the sum of water added to each pot.

Statistical analysis

All statistical analyses were conducted using SPSS 20.0 software (SPSS statistical package, Chicago, IL, United States). Two-way ANOVA was used to determine the effects of water, species and their interactions on above-ground biomass, root biomass, R/S ratio, and SPAD value. Mean comparisons (Duncan’s Studentized Range Test) were conducted to evaluate the above-ground biomass, root biomass, R/S ratio, and SPAD value among the different water levels, and under the three cutting treatments at the same

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DOI: http://dx.doi.org/10.15666/aeer/2101_439450

water level. Prior to analysis, we confirmed that our response variables met normality and homogenous variance assumptions. An alpha of 0.05 was used to test for statistical significance. If parameters were significant, an *LSD* (*α≤0.05*) was applied.

Figure 1. *The photo of experiment site and taking sample*

Results

Above-ground biomass

The effects of water treatments were statistically significant for each cut and for total biomass, as were water and species interactions (*Table 1*). Due to the interaction in water- and species- treatments, we integrated 39 combinations of species and water for overall analysis (*Figure 2*). Except for NWG, WPC, CMV, SOG+ CMV+ WPC and NWG+ SOG+ WPC, the other combinations showed significant differences among different water treatments ($P < 0.05$). As expected, total forage mixture biomass was higher than under the non-drought conditions (i.e. 60% and 80% water). Interestingly, the biomass of NWG+ CMV under the 60% water treatments was significantly higher than in the 40% and 80% treatments ($P < 0.05$). While the combinations of NWG+CMV+WPC and NWG+CMV+SOG under 60% water treatment was lower than that under 80% water treatment ($P < 0.05$), and there were no significant differences between drought environment (40%) and well-watered environment (80%) ($P > 0.05$). The three highest biomass producing treatments were the four-species mixture (80%), NWG+SOG+ CMV (80%), and SOG+CMV (60%). Compared with other monocultures, SOG produced greater biomass under the 80% water treatments.

Root biomass and R/S ratio

There were significant interactions between water treatments and species for root biomass and R/S ratio ($P < 0.05$, *Table 1*). Compared to the 40% and 60% water treatments, most treatments receiving 80% water treatment had higher root biomass values. The highest five values were observed in SOG+CMV (80%), WPC (80%), SOG+ WPC (80%), SOG (80%), and SOG+ WPC (60%), which were shown with red stars in *Figure 3*. The root biomass of well-watered (80%) SOG+CMV and WPC were significantly higher than for other mixtures ($P < 0.05$). However, the R/S ratio of SOG+CMV and WPC under the 80% water treatment, were lower than in other treatments

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(*P < 0.05*). The four highest significant R/S ratio values were observed for CMV (80%), NWG (80%), CMV (60%), and NWG+WPC (60%) (*P< 0.05*).

***Table 1.** Results (P-values) of a two-way ANOVA on the effects of water and mixture treatments, and their interactions on total biomass, root biomass, R/S ratio and chlorophyll content in mixture treatments with four native forages. Four native forages were northern wheatgrass (NWG), side-oats grama (SOG), Canadian milk-vetch (CMV) and white prairie-clover (WPC)*

Treatments	**Biomass (g)**	**Root biomass (g)**	**R/S ratio**	**Chlorophyll content**			
				NWG	**SOG**	**CMV**	**WPC**
Main plot (water)							
Cut1	<.0001	—	—	0.003	0.349	0.051	0.600
Cut2	0.003	—	—	0.355	0.906	0.685	0.141
Cut3	<.0001	—	—	<.0001	0.051	<.0001	<.0001
Total	<.0001	<.0001	0.028	—	—	—	—
Subplot							
Cut1							
Mixture	<.0001	—	—	0.047	0.600	<.0001	0.001
Water*Mixture	0.075	—	—	0.143	0.988	0.61	0.306
Cut2							
Mixture	<.0001	—	—	0.475	0.063	0.001	0.105
Water*Mixture	0.001	—	—	0.705	0.896	0.598	0.565
Cut3							
Mixture	<.0001	—	—	<.0001	<.0001	<.0001	<.0001
Water*Mixture	<.0001	—	—	<.0001	<.0001	<.0001	<.0001
Total							
Mixture	<.0001	<.0001	<.0001	—	—	—	—
Water*Mixture	0.002	<.0001	<.0001	—	—	—	—

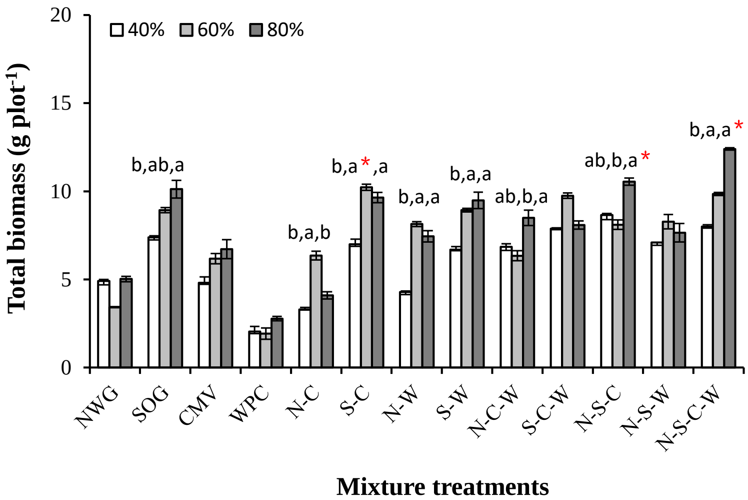

***Figure 2.** The effects of total biomass of 13 subplot combinations with different water levels and mixture treatments. The vertical bars represent the standard errors. Different letters denote the significant different effect of different water treatments on forage mixture combinations (P<0.05). The red stars denote the three highest treatments (P> 0.05). Four native forages were northern wheatgrass (NWG), side-oats grama (SOG), Canadian milk-vetch (CMV) and white prairie-clover (WPC)*

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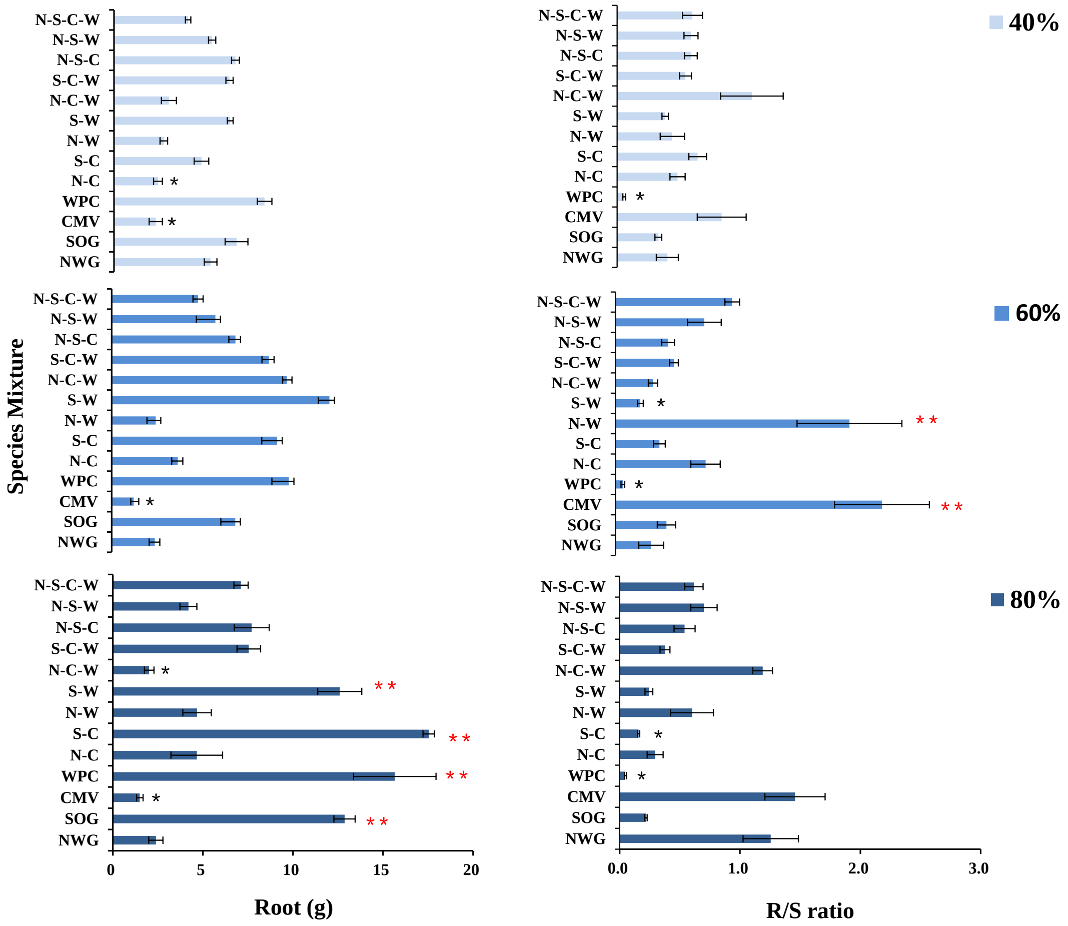

Figure 3. *Total root biomass and R/S ratio of 13 subplot combinations under water treatments. The vertical bars represent the standard errors. The red stars denote the first five highest treatments (P>0.05). Two red stars denote the significantly higher treatments (P<0.05), and dark stars denote the five lowest treatments than other treatments (P>0.05). Four native forages were northern wheatgrass (NWG), side-oats grama (SOG), Canadian milk-vetch (CMV) and white prairie-clover (WPC)*

Relative leaf chlorophyll content

At first cut, the relative leaf chlorophyll content (SPAD value) of all NWG treatments under 80% water treatment were lower than other water treatments (*P<0.05*; *Table 1*, *Fig. 4*). Water treatments did not significantly affect the SPAD values for SOG, CMV or WPC (*P > 0.05*). The SPAD value of NWG four-species (CMV+ NWG+ SOG+ WPC) was significantly lower than NWG monoculture, CMV+ NWG, and CMV+ NWG+ WPC at first cut (*P < 0.05, Fig. 5-A*). The SPAD value of CMV monoculture was significantly higher than SOG+ CMV at first cut (*P < 0.05, Fig. 5-B*). The SPAD value of WPC monoculture was significantly higher than other species mixtures at first cut (*P < 0.05, Fig. 5-C*). At first and second cut, the higher SPAD values for CMV were found with CMV in monoculture, CMV+ NWG, CMV+ NWG+ WPC and CMV+ NWG+ SOG+ WPC. Different water treatments and species mixtures significantly affected SPAD value for third cut (*P<0.05; Table 1, Fig. 6*). The SPAD value of NWG in the four-species mixture was significantly higher than for other treatments at the third cut (*P < 0.05, Fig. 7-A*). The SPAD values of SOG in SOG+ CMV under 40% and 60% water treatments,

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DOI: http://dx.doi.org/10.15666/aeer/2101_439450

were all higher than for other mixtures at the third cut (*P < 0.05*, *Fig. 7-B*). At third cut, the SPAD value of monoculture and mixture were all affected significantly by water treatments (*P < 0.05*). The higher SPAD values were observed for CMV+ NWG (60% and 80%), CMV+ NWG+ WPC (60% and 80%), and CMV+ NWG+ SOG+ WPC (80%) (*P<0.05, Fig. 7-C*). At third cut, the SPAD values of WPC (80%), WPC+ NWG (40% and 80%), and WPC+ NWG+ CMV (40% and 80%) were significantly higher than for other treatments (*P<0.05, Fig. 7-D*).

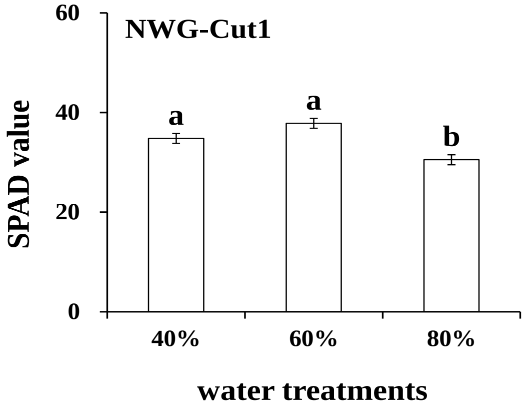

Figure 4. *Species SPAD value of NWG for main plots (water treatment) is shown. The vertical bars represent the standard errors. Different lower case letters indicate significant difference between treatments according LSD test at P < 0.05*

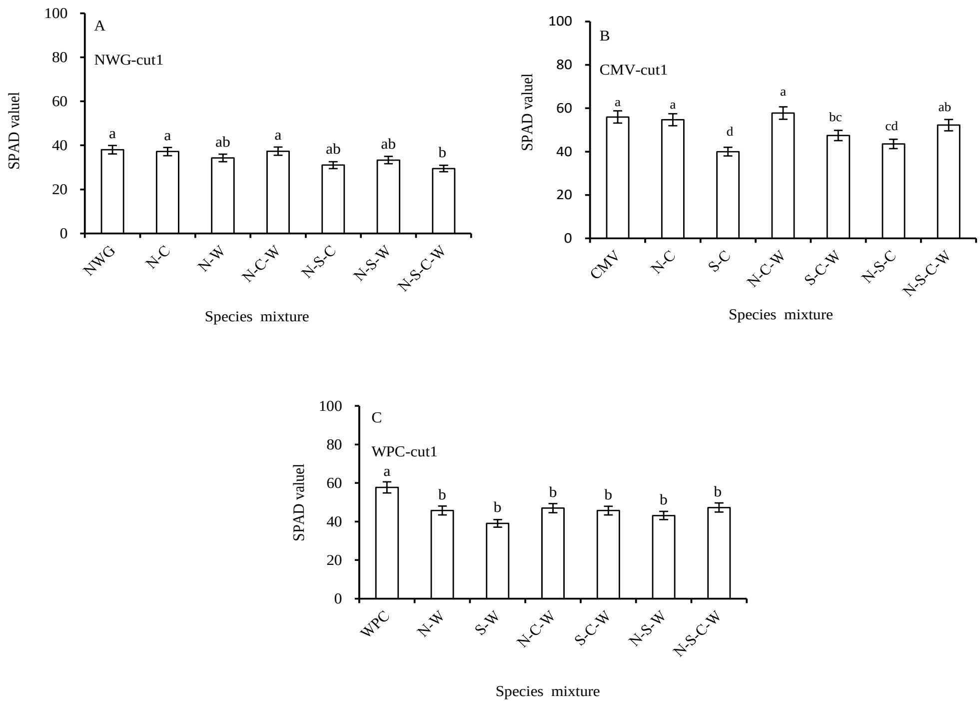

Figure 5. *Species SPAD value of 13 subplot combinations under water treatments of cut 1. Different lower case letters indicate significant difference between treatments according LSD test at P < 0.05. Four native forages were northern wheatgrass (NWG), side-oats grama (SOG), Canadian milk-vetch (CMV) and white prairie-clover (WPC)*

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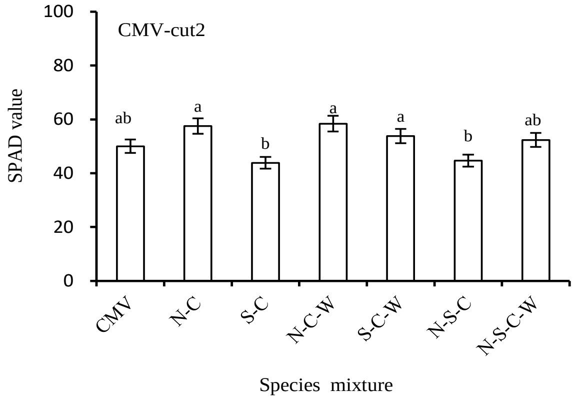

Figure 6. *Species SPAD value of 13 subplot combinations under water treatments of cut 2. Different lower case letters indicate significant difference between treatments according LSD test at P < 0.05. Four native forages were northern wheatgrass (NWG), side-oats grama (SOG), Canadian milk-vetch (CMV) and white prairie-clover (WPC)*

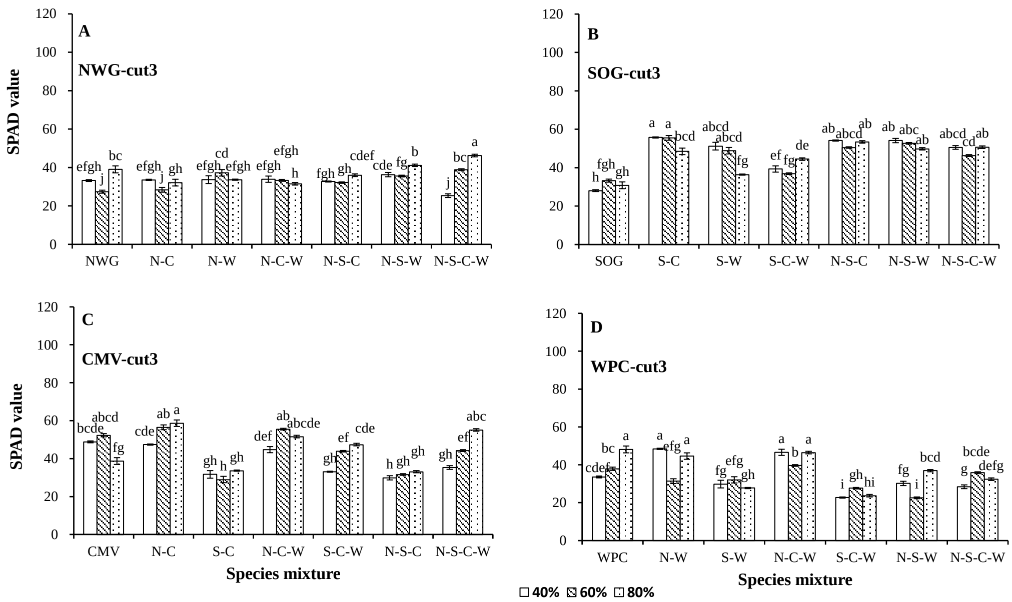

Figure 7. *Species SPAD value of 13 subplot combinations under water treatments of cut 3. Different lower case letters indicate significant difference between treatments according LSD test at P < 0.05. Four native forages were northern wheatgrass (NWG), side-oats grama (SOG), Canadian milk-vetch (CMV) and white prairie-clover (WPC)*

Discussion

Above-ground biomass of forage mixture response to drought of forage mixtures

As expected, most forage mixtures produced more biomass, especially total biomass, under well-watered (80% water) conditions. The three highest biomass mixtures were SOG+ CMV, NWG+ SOG+ CMV, and NWG+ SOG + CMV+ WPC, which all contained SOG. The monoculture SOG treatment had the highest total biomass of 39 treatments. The reason might be that side-oats grama is a perennial grass, with the following recognised attributes are recognized attributes (Mafakheri et al., 2010).

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The biomass of NWG+ CMV+ WPC and NWG+ SOG+ CMV under drought condition (40%) was significantly higher than that under the 60% water treatment. The biomass of NWG+ CMV under 60% water treatment was significantly higher than under other water treatments. Other studies have found that grasses were able to maintain above-ground biomass under severe moisture limited treatment, while legume-grass mixtures performed better than legume monocultures (Bahrani et al., 2010). In our study, we found that NWG+ CMV might be an optimal mixture for drought tolerance. Mild water deficits will induce partial stomatal closure of Canadian milk-vetch, which can result in improvements in water-use efficiency because of the nonlinear relationship between net photosynthetic rate and stomatal conductance (Xu et al., 2006, 2010). The drought tolerance of Canadian milk-vetch based on total biomass was likely due to a more sensitive stomatal response under water-stress than the response of other mixtures, and a greater ability of the mesophyll cells to utilize intercellular CO_2 that was more directly related to photosynthesis than to stomatal aperture (Singh, 2003; Basu et al., 2016).

Root biomass and R/ S ratio e response to drought of forage mixtures

Side-oats grama, a perennial sod-forming grass with short, scaly rhizomes, is widely used for drought land reclamation (Schellenberg et al., 2012b). In this study, we found that the root biomass of side-oats grama was not significantly different in the 40% and 60% water treatments, which is consistent with its drought tolerance (Heitschmidt et al., 2005). However, we detected a significant difference among water treatments of the shoot- and root- biomass of northern wheatgrass and side-oats grama. The literature indicates side-oats grama is a warm-season perennial plant and adapted to drier conditions compared to northern wheatgrass (Vassilev et al., 2012). We also found that the root biomass of white prairie-clover was significantly higher than that of Canadian milk-vetch. The cool season legumes, such as Canadian milk-vetch, are more likely to be exposed to transient drought in a longer water deficit condition (Blessing et al., 2018). In our study, SOG+ CMV root biomass was significantly higher than other treatments. The higher mixture SOG +CMV was higher might be that warm-season grasses are more resilient during dry weather in their zones of adaptation, the cool legumes and warm grasses used in temperate pasture systems have a higher critical P requirements for maximum productivity than the grasses with which they are grown(Biligetu et al., 2014; Yang et al., 2017).

Plants can adapt to water stress by a number of mechanisms, including the ability to maintain viability at water potentials below the turgor loss point with rapid root growth to exploit restricted soil water resources, which leads to a high R/S ratio (Kathiresan et al., 2006). Our results were consistent with other studies on different plant species, in which water-stress decreased the total biomass of WPC and altered biomass allocation to roots (Fotelli et al., 2001; Li et al., 2008). We observed that the total biomass of NWG+ WPC and CMV under the 60% water treatment were not significantly higher than in other treatments, but their R/S ratio increased significantly. This may be because water-stress decreased total biomass and altered biomass allocation to roots resulting in higher R/S ratios in stressed seedlings compared to well-watered plants (Xu et al., 2010).

Relative leaf chlorophyll content response to drought

The direct use of leaf chlorophyll content has a physiological basis, due to the inherent dependence of photosynthesis on chlorophyll molecules as the primary means of harvesting light energy to drive electron transport reactions (Croft et al., 2017; Zhang et

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al., 2018). We used relative leaf chlorophyll content (SPAD value) as a proxy for leaf photosynthetic capacity (Zhang et al., 2018). We found positive effects of species mixture for NWG and SOG on SPAD value, especially in the third cut, indicating that leaf photosynthetic capacity is an important mechanism of leaf regrowth for defoliation tolerance (Nicotra, 2010; Sanaullah, 2012). Moreover, the SPAD value of CMV after the third cut showed a decreasing trend with water stress. This may be because plant cutting removed the old and dead tissues, which have lower leaf chlorophyll content. Then the negative effect of drought stress on leaf chlorophyll content was highlighted with the remnant leaves regrowth. (Zhao et al., 2008; Zhang et al., 2018). The disadvantage of white prairie-clover for above ground biomass production under drought conditions is well known (Hui et al., 2018). In our study, we found that the mixtures WPC+ NWG and WPC+ NWG+ CMV were better adapted to a water limited environment than the WPC monoculture. This illustrates that species mixture and plant cut attenuated the impact of drought on leaf chlorophyll content of WPC.

Conclusions

In this study, we found that multiple forage species planted in mixtures were more adaptable than a simple grass-legume mixture or monoculture in a drought environment except for the monoculture side-oats grama. The forage mixture Canadian milk-vetch and northern wheatgrass was observed to have greater drought tolerance than the other mixtures. Under 60% water treatment, the total above-ground biomass of northern wheatgrass+ white prairie-clover and Canadian milk-vetch were not significantly higher than other treatments, but the R/S ratio significantly increased. After three cuts, the SPAD value of Canadian milk-vetch combination decreased with water stress, and WPC+ NWG and WPC+ NWG+ CMV were better adapted to imposed drier environmental conditions than monocultures.

Acknowledgments. This experiment was supported financially by AAFC Growing Forward 2, the Beef Science Cluster, Shandong Key Laboratory of Eco-Environmental Science for Yellow River Delta, Inner Mongolia Key Lab of Grassland Management and Utilization, and Innovative Team of Grassland Resources from the Ministry of Education of China (IRT_17R59). Thanks to L. Fast, K. Hagman, S. Bender, S. Quilichini for their assistance in this greenhouse study.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2101_439450

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