Nitrogen

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Effects of Drought Stress on Red Clover-Grass Mixed Stands Compared to Grass Monoculture Stands in Nitrogen-Deficient Systems

Chathuranga De Silva [1], Pramod Rathor [1], Hari P. Poudel [2] and Malinda S. Thilakarathna [1,*]

[1] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB T6G 2P5, Canada; batapola@ualberta.ca (C.D.S.); prathor@ualberta.ca (P.R.)
[2] Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, AB T1J 4B1, Canada; hari.poudel@agr.gc.ca
* Correspondence: malinda.thilakarathna@ualberta.ca

Abstract: Drought stress is one of the major abiotic stress factors affecting forage production; thus, it is essential to obtain a better understanding of how forage responds to drought. The main objective of this study was to evaluate how legume-grass mixed forage stands respond to drought stress when compared to grass monoculture. A greenhouse pot experiment was conducted using a red clover (*Trifolium pratense* L.)—timothy grass (*Phleum pratense* L.) mixed stand and a timothy monoculture stand, where plants were subjected to severe drought (20% field capacity—FC), moderate drought (40% FC), and well-watered (80% FC) conditions for four weeks and subsequently allowed to recover for another four weeks by adjusting moisture back to 80% FC. Both moderate and severe droughts significantly reduced the shoot biomass of the mixed stand, while no difference was exhibited in the timothy monoculture. The shoot biomass and nitrogen fixation capacity of red clover were reduced under drought stress. However, red clover plants subjected to moderate drought were able to recover shoot growth and nitrogen fixation capacity during the recovery phase, allowing more biologically fixed nitrogen and shoot nitrogen production similar to the plants growing under well-watered conditions. Overall, the results demonstrate that the inclusion of legumes in forage mixtures enhances resilience to moderate drought stress.

Keywords: forage legumes; timothy; red clover; nitrogen fixation; drought

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1. Introduction

Legume-grass mixtures consistently produce greater biomass yields and offer a balanced feed source for ruminants compared to their monocultures [1,2]. Incorporating legumes into forage mixtures enhances the efficient utilization of natural resources while decreasing the need for external nitrogen fertilizers through biological nitrogen fixation [3]. A mixture of species has positive effects on biomass production through (i) the complementarity effect—positive interaction between different species or improved efficiency in acquiring and utilizing available resources, (ii) the sampling effect—based on the greater possibility of the most productive species that dominate the community of a mixture-containing species pool, and (iii) the insurance effect—where mixtures with more species guarantee that some species continue functioning when others fail [4–6]. Therefore, the utilization of crop mixtures is a promising strategy for achieving sustainable intensification.

However, legume-grass mixtures frequently encounter significant challenges due to biotic and abiotic stresses that substantially impede the successful establishment and productivity of such mixtures. Forage production relies heavily on sufficient moisture availability [7–9]. With the advent of climate change, it is anticipated that moisture availability will become scarcer in both semiarid and temperate climates, primarily due to the heightened likelihood of droughts [10]. Additionally, as temperatures continue to rise, the probability of drought incidences is expected to grow further [11].

Due to the adverse impacts of drought stress on plant growth and development, the agriculture sector, including forage production, will be significantly influenced by these alterations [12,13]. Drought stress impedes plant growth through nutrient imbalance, production and accumulation of reactive oxygen species (ROS), and inactivation of enzymes that are involved in different metabolic pathways of plant growth and development [14–16]. The duration and severity of drought stress determine plant survival and crop yield losses [17]. One of the most well-documented physiological responses to drought stress is the closure of the stomata, which helps to prevent water loss through transpiration [18]. However, the stomata closure reduces carbon assimilation, establishing a close link between carbon uptake and water loss [19]. Carbon isotope discrimination (CID) in carbon assimilation exhibits an inverse relationship with water-use efficiency (WUE) and, therefore, is regarded as an indirect indicator for assessing the yield of C3 plants like legumes and cool-season grasses [20,21]. In addition, drought often induces a carbon/nitrogen imbalance [22], involving complex mechanisms that coordinate carbon assimilation and the nitrogen metabolism [23]. Drought stress is also known to have detrimental effects on nitrogen fixation in legumes [24]. This also affects the carbon and nitrogen dynamics between legumes and grasses [25], which remains largely unexplored in various ecological systems, such as mixed grass-legume forage combinations.

Several studies have consistently reported that plant mixtures comprising diverse functional groups not only integrate tolerance and resilience in response to reduced precipitation but also lead to higher productivity compared to their monocultures [2,26–29]. Red clover (*Trifolium pratense* L.) is an important forage legume species in pastoral systems with high nitrogen-fixing capacities [30], and timothy grass (*Phleum pratense* L.) is a commonly cultivated forage species in cool and humid areas [31]. Red clover and timothy are commonly grown in combination to produce pasture and silage [32]. Forage legumes are rich in protein due to their nitrogen-fixing capabilities, complementing the lower protein content of grasses. Additionally, in the legume-grass mixtures, the grass benefits from the belowground nitrogen transfer from the legumes, contributing significantly to the increased overall biomass [33]. This synergy improves the nutritional quality of forage mixtures while reducing external fertilizer inputs [34]. The grass component in legume-grass mixed stands also helps mitigate issues of weed encroachment and legume lodging [35].

However, as many forage species are susceptible to drought and are likely to be impacted by the water scarcity that is predicted to occur in the future, it is crucial to investigate the interactions of legume-grass mixtures under drought conditions. We hypothesized that grass grown with forage legumes has better resilience compared to the grass monoculture. Therefore, this study aimed to assess how plant growth, symbiotic nitrogen fixation, forage nitrogen production, carbon and nitrogen dynamics, and WUE are impacted under different drought-severity conditions and after a recovery phase following drought in red clover-timothy mixed stands vs. grass monocultures.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The experiment was conducted under greenhouse conditions at the University of Alberta. Seeds of timothy grass (variety: Alma) and red clover (variety: Marino) were surface-sterilized using 70% ethanol for two minutes and subsequently soaked in 4% sodium hypochlorite for three minutes, followed by six rinses using sterile distilled water. Seeds were pre-germinated on moistened sterile filter papers, placed in petri dishes, and kept in the dark for three days at room temperature. After three days of growth, uniform seedlings were selected and transferred to pots (volume—6.5 L, height—21.7 cm, diameter—22 cm) containing a mixture of a professional growing mix (Sun Gro Horticultural Canada, Ltd., Vilna, AB, Canada) and sand (Target Products, Ltd., Crippsdale, AB, Canada) homogenized in a 3:1 ratio (*v:v*), maintaining low available soil nitrogen. Nine pre-germinated seedlings were planted in each pot, where the monoculture (N pots = 36) stand included nine seedlings of timothy grass alone and the mixed culture stand (N pots = 36) included six seedlings of timothy grass and three seedlings of red clover. Pots were arranged in a completely randomized design and maintained at 24 °C ± 4 during the day and 18 °C ± 4 at night under 16 h light/8 h dark photoperiods and light intensity of 500 µmol photons $m^{-2}s^{-1}$.

After one week of growth, each red clover seedling was inoculated with 1 mL of *Rhizobium leguminosarum* bv. *trifolii* inoculum, with rhizobial density adjusted to OD_{600} = 0.1. One week after the first inoculation, the same process of inoculation was repeated to ensure successful nodulation. Plants were labelled with 50 mL of 0.5 mM $K^{15}NO_3$ solution (10 atom% ^{15}N; 348481-25G; Sigma Aldrich, Oakville, ON, Canada) two and three weeks after planting to measure symbiotic nitrogen fixation. Each week, plants were supplied with 200 mL of quarter-strength N-free Hoagland's nutrient solution (HOP03-50LT, Caisson Labs, Smithfield, UT, USA). Each pot was watered every other day for eight weeks to maintain 80% field capacity (FC) using a gravimetric method [36], and their locations were randomly re-assigned at watering time throughout the experiment.

2.2. Drought Stress and Data Collection

After eight weeks of growth, pots from each monoculture and mixed culture stand were randomly assigned to three soil moisture levels in a randomized complete block design comprising 12 pots. The three soil moisture levels included 80% FC (well-watered), 40% FC (moderate drought), and 20% FC (severe drought) for four weeks by withholding water until the pots reached the described FC levels. At the end of the drought phase, half of the pots from each soil moisture level (n = 6) were harvested, while the remaining set of halves was subjected to a recovery phase for another four weeks by adjusting and maintaining FC of growing media at 80% FC (Figure 1). Aboveground plant tissues from respective pots were harvested after each drought and recovery phase, sorted into separate plant species, and then dried in a hot-air oven set at 60 °C for three days. The dry weight of the shoots was measured for each species in different plant stands.

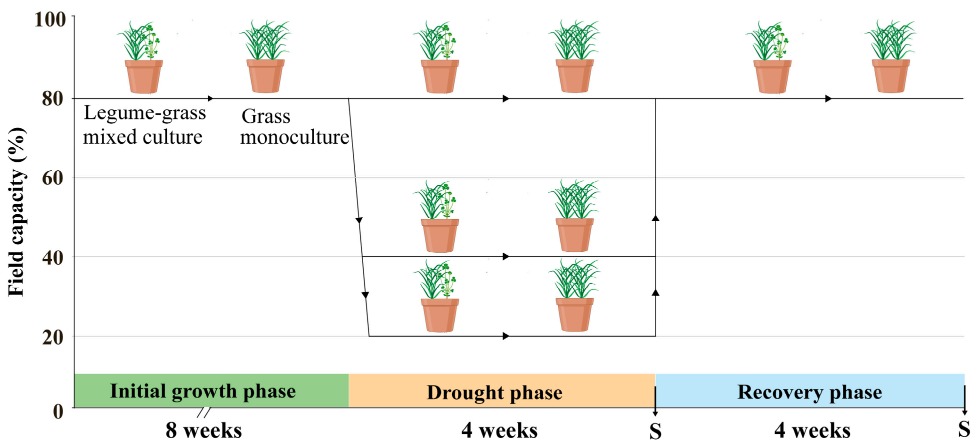

Figure 1. Schematic representation of the soil moisture treatments maintained during the initial growth, drought, and recovery phases. Drought stress was imposed by maintaining the moisture content until the field capacity (FC) reached 40% FC (moderate drought) or 20% FC (severe drought), and the plants were maintained at specific FCs for four weeks. Pots were re-watered and maintained at 80% FC in the recovery phase, following the drought phase. Plants were harvested after four weeks of drought and four weeks of post-drought recovery (depicted by letter S). Each treatment consisted of six replicate pots.

2.3. Determination of C:N Ratio and Carbon Isotope Discrimination (CID)

Dry plant materials mentioned in the previous section were ground to fine powder using a coffee grinder. A subsample from each ground sample was further ground in a 2 mL microcentrifuge tube along with two steel beads in a bead beater homogenizer (OMNI International, Kennesaw, GA, USA). Afterward, 5 mg of the finely powdered plant tissues was measured into a small tin capsule (8 mm × 5 mm, D1008, Isomass Scientific Inc., Calgary, AB, Canada) using a microbalance (Sartorius Quintix35-1S, Goettingen, Germany). A tiny pellet was made by enveloping and compressing the sample, ensuring no air remained. The tin capsules were placed in a 96-well plate and sent to the Stable Isotope Facility, Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, to analyze ^{13}C and total C%. Samples were measured using an Isotope Ratio Mass Spectrometer (IRMS) fitted with a Flash 2000 Elemental Analyzer (Thermo Fisher Scientific, Voltaweg, The Netherlands) and Conflo IV (Thermo Fisher Scientific, Bremen, Germany) interface between the IRMS. Subsequently, we measured the deviation between the carbon isotope compositions of these samples and the isotopic composition of the Pee Dee Belemnite (PDB) standard, following the methodology outlined by [37]:

$$\delta[‰] = \frac{Rp - Rs}{Rs} \tag{1}$$

where Rp is the isotopic abundance in the plant and Rs is the abundance ratio $^{13}C/^{12}C$ of the standard, for which a fossil from the Pee Dee Formation (Pee Dee Belemnite, PDB) was used. CID was calculated as follows:

$$CID[‰] = \frac{\delta a - \delta p}{1 + \delta p} \times 1000 \tag{2}$$

where δa is the isotopic composition of the atmosphere (approximately −8‰) and δp is the isotopic composition of the plant sample [37].

2.4. Determination of Nitrogen Fixation-Related Parameters

Similar to the above method, ^{15}N and total N% were analyzed [34] and the percentage of nitrogen derived from the atmosphere (%Ndfa) in red clover was calculated according to the isotope dilution technique using the following formula [38]:

$$\%Ndfa = \left(1 - \frac{\text{atom\% }^{15}\text{N excess}_{(\text{redclover})}}{\text{atom\% }^{15}\text{N excess}_{(\text{timothygrass})}}\right) \times 100 \tag{3}$$

where atom% ^{15}N excess = atom% ^{15}N (red clover or timothy grass) − 0.3663.

The amount of fixed nitrogen in shoot was calculated based on the total aboveground nitrogen content and %Ndfa (total shoot N content of red clover × %Ndfa/100).

2.5. Statistical Analysis

All data were tested for normal distribution (using Shapiro-Wilk test function "shapiro.test" in base R), and with the assumption that the responses were from normal population distribution, data were subjected to a two-way analysis of variance ($\alpha < 0.05$) by testing the effects of soil moisture levels, forage-stand types, and their interactions on aboveground plant biomass, C:N ratio, carbon isotope discrimination (CID), shoot N concentration, and nitrogen fixation. Differences between means were determined for two-way interaction with a post hoc Fisher's least significant difference test ($\alpha < 0.05$). Each treatment comprised six replicates. All analyses were conducted using R Studio (version 4.3.1).

3. Results

3.1. Effects of Drought Stress on Aboveground Biomass

The aboveground dry biomass of the plants grown in both mono- and mixed stands was recorded following the exposure to drought stress and recovery phases (Figure 2A,B). After the drought phase, the total aboveground biomass of the mixed stand was reduced by 23% under moderate drought (40% FC) and 48% under severe drought (20% FC) compared to the plants maintained under well-watered conditions (80% FC) ($p < 0.001$; Figure 2C). When legumes and grass were grown in a mixed stand, drought stress significantly reduced the biomass of the legumes (by 40% in moderate and 65% in severe drought), whereas no significant difference was recorded in grass biomass compared to the plants grown under well-watered conditions (Figure 2C). When grass was grown in the monoculture stand, no significant difference was observed in biomass at different drought levels, as compared to the well-watered plants (Figure 2C). Following four weeks of recovery phase after drought stress, the total aboveground biomass of the mixed stand was 38% lower under severe drought stress compared to the well-watered controls. However, no significant difference was observed in the total biomass of the mixed stand under moderate drought stress compared to the plants grown under well-watered conditions (Figure 2D). Similar to the drought phase, no significant difference was observed following the recovery phase in grass biomass either in the monoculture or the mixed stand at different drought levels compared to the well-watered plants (Figure 2D).

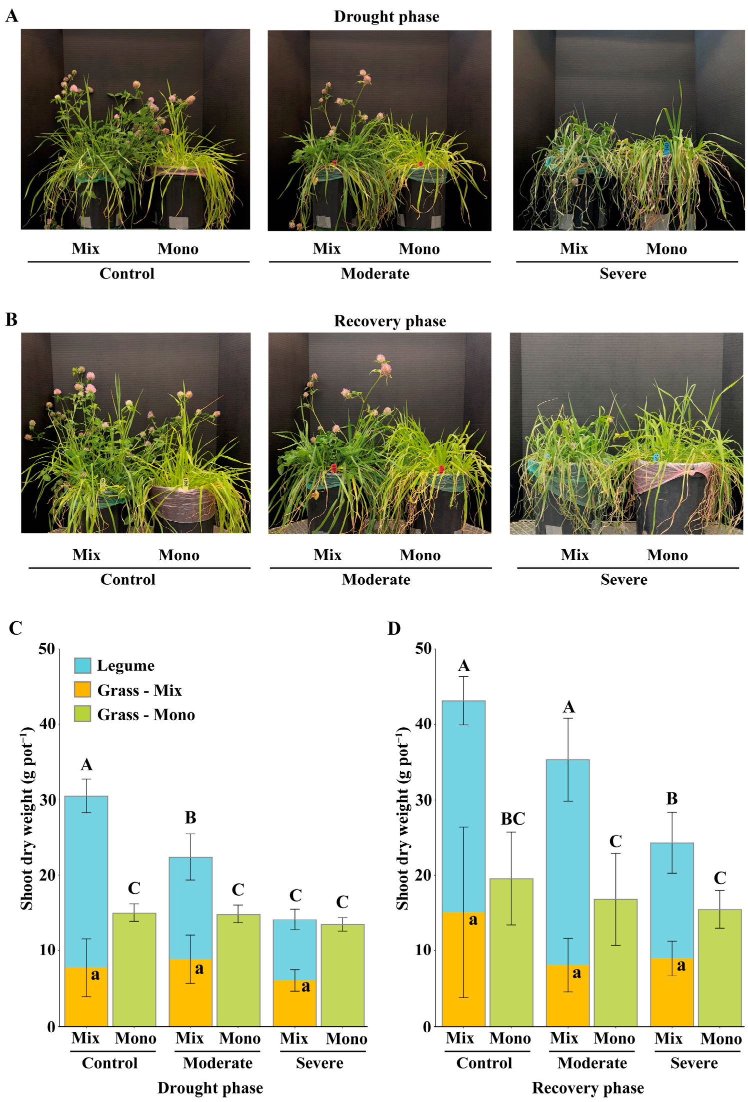

Figure 2. Photographs and bar graphs of shoot-growth biomass of timothy-red clover mixed culture and timothy monoculture grown under four weeks of moderate (40% field capacity) and severe drought stress (20% field capacity) and following four weeks of recovery. Control plants were maintained at 80% field capacity. (**A**,**C**) Drought phase. (**B**,**D**) Recovery phase. Different uppercase letters indicate significant differences between total aboveground biomasses, while different lowercase letters indicate significant differences in the aboveground biomass of timothy in the mixture according to the Fisher LSD test at $p < 0.05$. Each treatment comprised six replicates. Values correspond to the means ± SE ($n = 6$). Means and SE followed by the same letter are not significantly different.

3.2. Effects of Drought Stress on C:N Ratio and Carbon Isotope Discrimination

The C:N ratio of the shoots was determined based on carbon and nitrogen concentrations in the aboveground tissues (Figure 3). There was no significant difference in the C:N ratios of red clover in the mixed stand at different drought levels in the drought phase (Figure 3A). A similar pattern was recorded after four weeks of recovery phase (Figure 3B). Similar to red clover, there was no significant difference in the C:N ratio of grass when grown in a mixed stand at different soil moisture levels (Figure 3A). However, in the recovery phase, the C:N ratio of the grass in the mixed stand was significantly reduced (35%) under severe drought stress compared to well-watered plants ($p < 0.001$; Figure 3B). At the end of the drought phase, the C:N ratio of the grass in the monoculture stand was significantly higher (18%) under moderate drought conditions, wherein severe drought reduced the C:N ratio (28%) compared to the well-watered controls ($p < 0.001$; Figure 3A). When grass was grown in a monoculture stand, the C:N ratio was significantly reduced under both moderate (16%) and severe drought (24%) stress conditions, as compared to the plants maintained under well-watered conditions in the recovery phase ($p < 0.001$; Figure 3B).

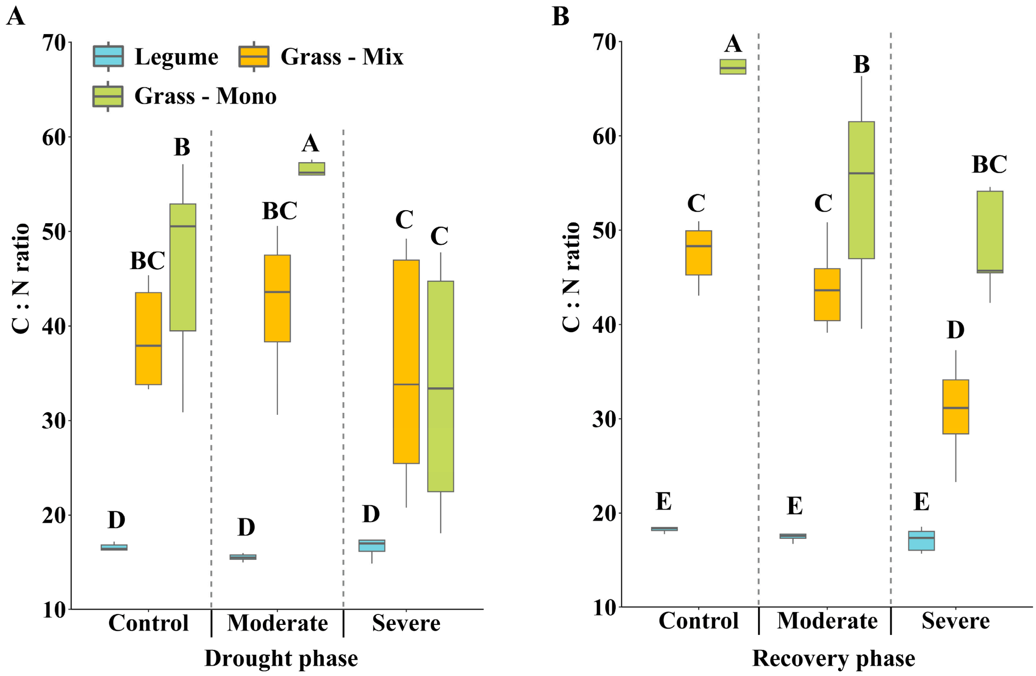

Figure 3. Boxplots of shoot C:N ratio showing the median values and variability of timothy-red clover mixed culture and timothy monoculture grown under moderate (40% field capacity) and severe drought stress (20% field capacity) conditions compared to the well-watered controls (80% field capacity). (**A**) Drought phase. (**B**) Recovery phase. Bold horizontal lines inside boxes represent median values. The lower and upper ends of the boxes represent the first and third quartiles, respectively; vertical lines extend to the most extreme data points. Different letters above the boxes represent significant differences according to the Fisher LSD test ($p \leq 0.05$). Each treatment comprised six replicates. Means followed by the same letter are not significantly different.

The carbon isotope discrimination (CID) of red clover was significantly reduced under both moderate (12%) and severe (15%) drought conditions compared to the plants maintained under well-watered conditions in the drought phase ($p < 0.001$; Figure 4A). However, the CID was reduced in grass grown in the mixed stand (12%) only at the severe drought level ($p < 0.001$; Figure 4A). Similarly, a significant reduction (15%) in CID was found in grass grown in the monoculture stand under severe drought stress ($p < 0.001$; Figure 4A). When plants were subjected to the recovery phase for four weeks following the drought phase, the CID of the legume was significantly reduced (5%) under severe drought stress compared to the well-watered plants ($p < 0.001$; Figure 4B). The CID of grass grown in both mixed and mono-stands was significantly reduced under moderate (6% and 6%) and severe (20% and 10%) drought levels compared to the well-watered conditions (Figure 4B).

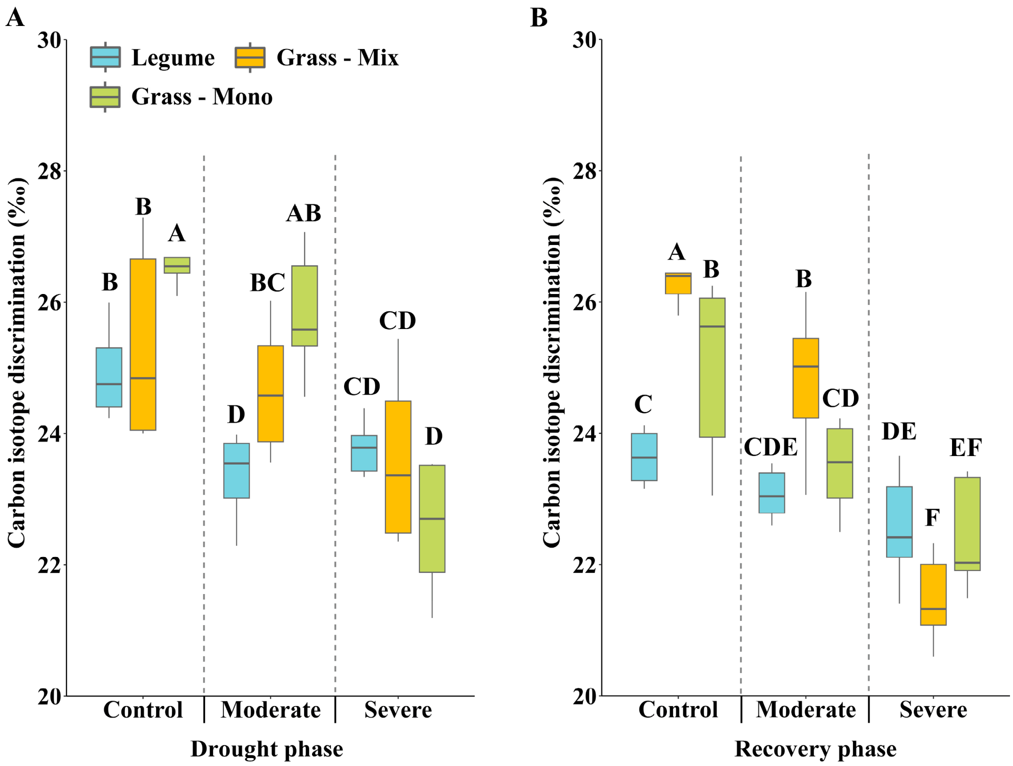

Figure 4. Boxplots of carbon isotope discrimination (CID) showing the median values and variability of timothy-red clover mixed culture and timothy monoculture grown under moderate (40% field capacity) and severe drought stress (20% field capacity) conditions compared to the well-watered controls (80% field capacity). (**A**) Drought phase. (**B**) Recovery phase. Bold horizontal lines inside boxes represent median values. The lower and upper ends of the boxes represent the first and third quartiles, respectively; vertical lines extend to the most extreme data points. Different letters above the boxes represent significant differences according to the Fisher LSD test ($p \leq 0.05$). Each treatment comprised six replicates. Means followed by the same letter are not significantly different.

3.3. Effects of Drought on Shoot N Concentration and Symbiotic Nitrogen Fixation

The total shoot N concentrations of red clover or grass in the mixed stand did not exhibit significant differences at different moisture levels in the drought phase (Figure 5A). Grass grown in the mono-stand showed a significant increase (39%) in total N concentration under severe drought stress, whereas grass in the mixed stand showed a 17% increase in total N concentration under severe drought stress, although it was not significant (Figure 5A). In the recovery phase, the shoot N concentration of the legumes was found to be increased under severe drought (10%) conditions ($p < 0.001$; Figure 5B). The total shoot N concentration of the grass in the mixed stand showed a significant increase (31%) under severe drought stress, whereas no significant difference was found under moderate drought conditions (Figure 5B). Similarly, grass grown in the monoculture stand showed a significant increase (57%) in shoot N concentration under severe drought stress, whereas no significant difference was found under moderate drought stress compared to the well-watered plants (Figure 5B). The total shoot N content of red clover showed a significant reduction under both moderate (38%) and severe (64%) drought stress conditions in the drought phase compared to the well-watered controls (Figure 5C). However, grass grown in the monoculture and mixed stands did not exhibit significant differences in total shoot N content at different soil moisture levels (Figure 5C). At the end of the recovery phase, the total shoot N content of the red clover was significantly lower (40%) under severe drought stress, whereas no significant difference was observed under moderate drought stress compared to the well-watered controls (Figure 5D). Similar to the drought phase, no significant differences were observed for the total shoot N content in the recovery phase when the grass was grown in either monoculture or mixed stands under both moderate and severe drought stress conditions (Figure 5D).

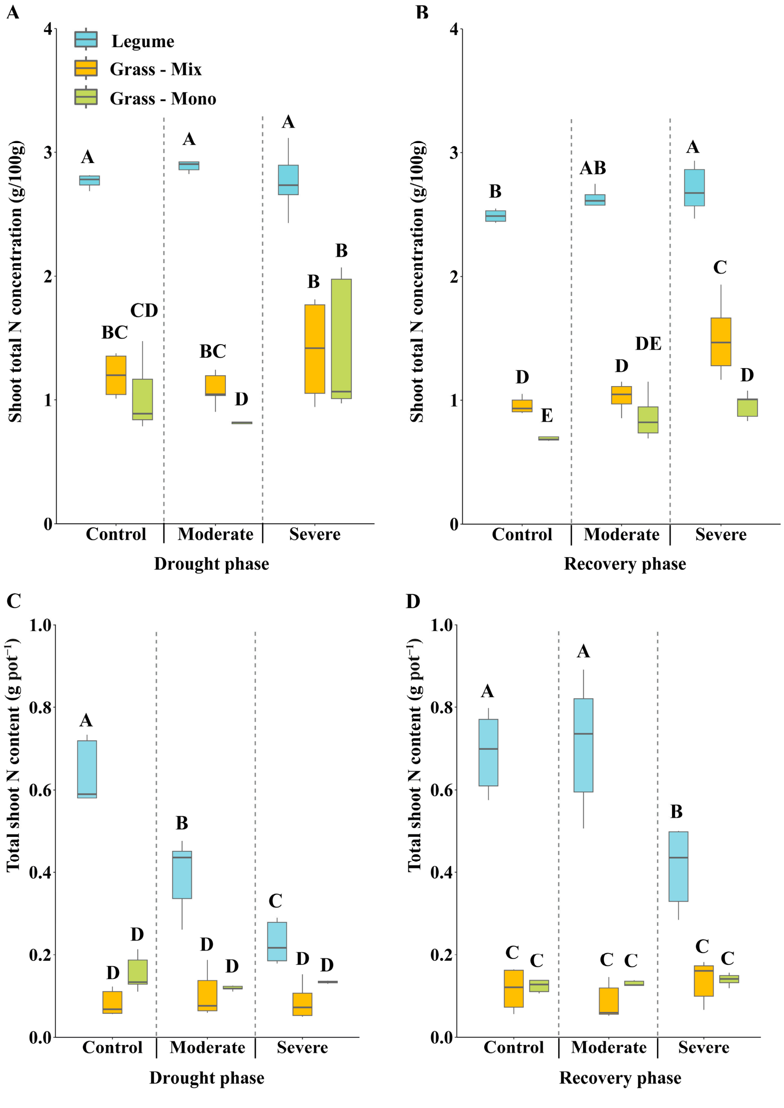

Figure 5. Boxplots of total shoot nitrogen concentration and total shoot nitrogen content showing the median values and variability of timothy-red clover mixed culture and timothy monoculture grown under moderate (40% field capacity) and severe drought stress (20% field capacity) conditions compared to the well-watered controls (80% field capacity). (**A**) Total shoot nitrogen concentrations in the drought phase. (**B**) Total shoot nitrogen concentrations in the recovery phase. (**C**) Total shoot nitrogen content in the drought phase. (**D**) Total shoot nitrogen content in the recovery phase. Bold horizontal lines inside boxes represent median values. The lower and upper ends of the boxes represent the first and third quartiles, respectively; vertical lines extend to the most extreme data points. Different letters above the boxes represent significant differences according to the Fisher LSD test ($p \leq 0.05$). Each treatment comprised six replicates. Means followed by the same letter are not significantly different.

Red clover exposed to drought stress in mixtures with timothy exhibited a 10% reduction in %Ndfa under severe drought conditions ($p < 0.01$; Figure 6A) in the drought phase. A similar pattern was observed in the recovery phase four weeks after the drought phase (Figure 6A). In the drought phase, a significant reduction in the total fixed N was observed under moderate (42%) and severe (66%) drought conditions, compared to the well-watered plants ($p < 0.01$; Figure 6B). However, following the recovery phase of four weeks, the total fixed N was significantly lower (34%) under severe drought stress compared to the well-watered controls ($p < 0.01$; Figure 6B). There was no significant difference in the total fixed N of red clover under moderate drought and well-watered conditions (Figure 6B).

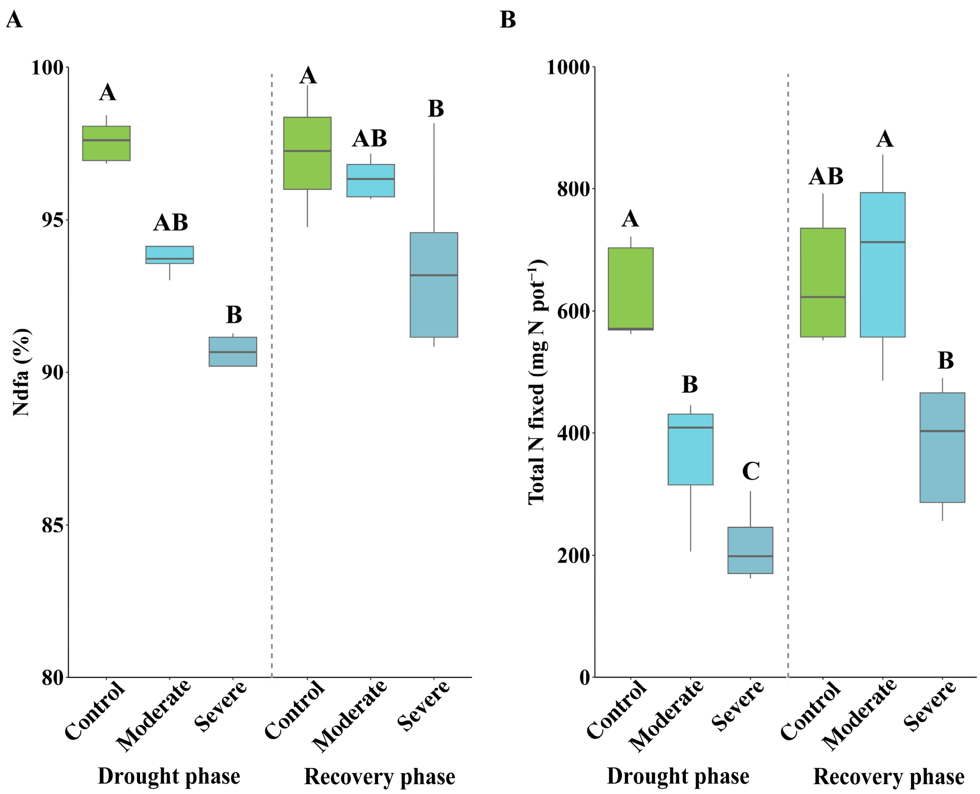

Figure 6. Boxplots of the percentage of nitrogen derived from the atmosphere (%Ndfa) and total fixed nitrogen showing the median values and variability of red clover grown in mixed cultures with timothy under both moderate (40% field capacity) and severe drought stress (20% field capacity) conditions and recovery phases compared to the well-watered controls (80% field capacity). (**A**) Percentage nitrogen derived from the atmosphere (%Ndfa). (**B**) Total shoot fixed nitrogen. Bold horizontal lines inside boxes represent median values. The lower and upper ends of the boxes represent the first and third quartiles, respectively; vertical lines extend to the most extreme data points. Different letters above the boxes represent significant differences according to the Fisher LSD test ($p \leq 0.05$). Each treatment comprised six replicates. Means followed by the same letter are not significantly different.

4. Discussion

Drought stress causes substantial reductions in crop yields [17,39–41] by negatively affecting plant growth and development. The degree of yield loss and plant survival depends upon the duration and severity of the drought stress [42]. In general, moderate and severe drought stress conditions result in 40% and 70% reductions in forage yield, respectively [9]. The greenhouse study conducted here aimed to evaluate the impact of a brief drought episode followed by a recovery phase on both mixed culture and monoculture forage systems. Overall, the results indicate that drought exerts detrimental effects on various aspects, including aboveground plant biomass, shoot C:N ratio, shoot N concentration, WUE, and symbiotic nitrogen fixation (SNF).

Drought stress leads to a reduction in the soil moisture content and water potential in aerial parts of the plant, such as stems and leaves. Low water availability in the root zone can significantly reduce the nutrient transfer and bioavailability in soil, resulting in nutrient imbalance, thus hampering plant growth, quality, and overall performance [43]. This study revealed a significant reduction in the biomass of red clover under drought stress, while no such effect was observed in the grass. This aligns with a previous study, which also showed that clover exhibited higher sensitivity to drought stress when compared to timothy [44]. A significant difference between the aboveground biomasses of mixed culture and monoculture stands during both drought and recovery phases was observed (Figure 2A–D). Mixing grasses and legumes has been shown to increase dry matter [1,45] with no negative effects on nutritive value [46]. The increase in biomass was found to be mainly associated with the red clover. During the recovery phase, the plants subjected to drought stress showed a substantial increase in total aboveground biomass compared to the end of the drought phase, mainly due to an increase in red clover biomass (Figure 2A–D). This indicates the resilience provided by red clover within the grass-legume mixture. Interestingly, the aboveground biomass of timothy grass, which is known for its drought sensitivity [39,47] did not exhibit a significant reduction in biomass in both mixed and monoculture settings during the drought phase or the recovery phase (Figure 2A–D). This could be attributed to the timing and duration of the drought, which may not have been prolonged enough to yield a noticeable difference, as well as the adaptability of the timothy cultivar to exhibit a certain degree of tolerance.

Carbon and nitrogen are vital for plant cellular functions, and their sufficient supply is crucial for plant growth, development, and stress resilience [48,49]. Drought typically leads to an imbalance in carbon and nitrogen [22], and the stoichiometry of C and N in plants mirrors their metabolic balance and can be a predictor of plant growth and development [50]. In general, legumes have a lower C:N ratio compared to grasses because of the SNF in legumes. It is worth noting that the red clover exhibited a significantly lower C:N ratio in both the drought and recovery phases when compared to its grass counterpart (Figure 3A,B), which corroborates previous findings [51,52]. A low C:N ratio plays a significant role in the decomposition of legume litter and contributes to increased nitrogen input. This leads to the accelerated breakdown of legume residues within legume-grass mixtures, resulting in a greater release of nitrogen from litter when compared to grass monocultures [53]. In our study, a marked difference was observed in C:N ratio between mixed culture and monoculture grass under both moderate and severe drought conditions (Figure 3B). Although the nitrogen transfer from the legumes to the grass was not measured in this study, the results suggest nitrogen rhizodeposition by the red clover, leading to an increased availability of nitrogen in the growing media and subsequent absorption by the timothy in the recovery phase. This also aligns with a recent finding showing increased available soil nitrogen following severe drought stress in red clover [54]. In addition, our study shows a significant increase in the shoot nitrogen concentration (Figure 5B) of grass in a mixed culture subjected to severe drought, suggesting a potential nitrogen transfer from the legumes to the grass in the mixed stand during the recovery phase.

CID is widely accepted as an indirect method for estimating WUE in C3 plants, such as forage legumes [55] and cool-season grasses [56]. CID has a negative correlation with WUE, while it exhibits a positive correlation with yield [54]. CID decreases under drought stress as a result of a reduction in stomatal function and the water-saving mode adopted by plants [57]. Our results show that after four weeks of drought, CID was lower in both mixed culture and monoculture grass under severe drought when compared to well-watered plants, indicating higher WUE (Figure 4A). In red clover, a similar pattern was observed under both moderate and severe drought stress conditions, suggesting higher WUE in red clover under moderate and severe drought conditions. During the recovery phase, a noteworthy contrast between mixed culture and monoculture became apparent in grass subjected to moderate drought, with the highest value observed in the mixed culture, indicating a lower WUE. Although a high WUE is considered to maintain a high plant yield [58], it is not always associated with drought resistance and higher yields under drought conditions [59].

Drought stress stands out as a significant factor that impacts the different steps of nitrogen fixation in the legume-rhizobium symbiosis [13,60,61]. Various mechanisms have been proposed to explain the adverse impacts of drought on nodulation and inhibition of nitrogen fixation in legumes. These mechanisms include carbon deficiency, nitrogen feedback, insufficient oxygen supply, and the influence of oxidative stress [62–65]. In this study, it was found that severe drought stress significantly reduced the %Ndfa (Figure 6A), which is consistent with findings from a recently published study [54]. No significant reduction was observed in the %Ndfa under moderate drought stress, and this could be associated with red clover's capacity to demonstrate resilience under moderate drought conditions, although not in extreme droughts [66]. It was observed that the total nitrogen fixed by red clover per pot under both moderate and severe drought conditions was significantly reduced (Figure 6B). Our findings distinctly demonstrate that under moderate drought conditions, fixed nitrogen allocation did not prioritize biomass production, highlighting the trade-off between plant resource allocation for drought tolerance and survival (Figure 6B). This decreased above-ground biomass was also demonstrated in previous studies as well [67,68]. A similar trend in the %Ndfa was observed in both the drought and recovery phases (Figure 6A), although a distinct difference in the total fixed nitrogen was observed during the recovery phase (Figure 6B). Red clover plants subjected to moderate drought exhibited a notable rise in total fixed nitrogen during the recovery phase. These results imply that red clover can withstand drought stress to a certain extent by displaying resilience during the post-drought recovery phase. An earlier study demonstrated that despite a notable reduction in biomass during drought stress, red clover rapidly regains vitality once the drought conditions are alleviated [69]. On the other hand, the increased nitrogen content observed in the mixed grass is an indication of elevated crude protein content and forage quality. The increase in crude protein content in companion grass is attributed to the greater availability of soil nitrogen due to the association of legumes, as reported previously [70]. The resilience of red clover to endure drought stress to a certain extent, as evidenced by its recovery during the post-drought phase, along with its capacity to overcome nitrogen limitations through nitrogen fixation, suggests that legume-grass mixtures exhibit resilience during the post-drought recovery phase. These insights will provide better guidance for farmers in the management of nitrogen in post-drought conditions, aiming to optimize productivity and forage quality while minimizing environmental impact.

The %Ndfa values of red clover were above 90% in this study, even in severe drought. The %Ndfa values are highly dependent on the reference plant [71], and timothy was used as the reference plant in this study. It could have been ideal to use a non-nodulating red clover as the reference plant in this study, since phenology, rooting depth, and nitrogen uptake patterns in the grass are different compared to those of legumes and can affect the %Ndfa values.

5. Conclusions

While plant responses to drought stress have been extensively studied, there has been comparatively less focus on post-drought recovery, which is crucial for understanding stress resilience. Nonetheless, our study brings novel insight by highlighting the findings in post-drought recovery and the resilience of legume-grass mixed stands. This study revealed that in both moderate and severe drought stress conditions, the mixed cultures of red clover and timothy grass, rather than the grass monocultures, resulted in positive outcomes, especially with respect to biomass production, the C:N ratio of the grass, nitrogen fixation, and total shoot nitrogen content. Timothy grass, which is known to be drought-sensitive exhibited minimal to no response to the period of four weeks of drought in terms of biomass reduction, though improved shoot N content and lower C:N ratio in mixed culture were observed. This was also true for the post-drought recovery phase, demonstrating the potential benefits of incorporating legumes with grass in mixed culture stands for enhancing the overall forage quality and resilience of red clover in the face of water scarcity. Collectively, these findings enhance our understanding of forage legume-grass responses to drought stress and recovery.

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