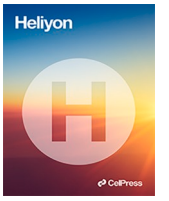
Heliyon 10 (2024) e28975

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Assessing the growth dynamics of alfalfa varieties (*Medicago sativa* cv. Bilensoy 80 and Nimet) response to varied carbon dioxide (CO_2) concentrations

Tefide Kizildeniz

Niğde Ömer Halisdemir University, Faculty of Agricultural Sciences and Technologies, Biosystems Engineering Department, 51240 Niğde, Turkey

ARTICLE INFO

Keywords:
Alfalfa growth
Medicago sativa
Bilensoy 80
Nimet
Carbon dioxide concentration
Climate change impact

ABSTRACT

Rising atmospheric CO_2 levels drive greenhouse effects, elevating temperatures, and diminishing water accessibility in semi-arid regions, affecting agriculture. Alfalfa contributes to climate change mitigation by sequestering carbon, enhancing soil fertility and carbon storage, reducing synthetic nitrogen fertilizer use, preventing soil erosion, supplying high-quality livestock feed, and serving as a bioenergy source. This research examined the effects of elevated CO_2 levels in climate change scenarios (600, 800, and 1000 ppm, with control at 400 ppm) on two alfalfa varieties, *Medicago sativa* cv. Nimet and Bilensoy-80. The experiments were conducted in specialized Climate Change Simulation Greenhouses, allowing control of CO_2, water, and temperature variables. Results revealed a positive relationship between higher CO_2 concentrations and increased photosynthesis ($P \leq 0.001$), promoting the plant growth leaf area ($P \leq 0.001$), yields and both leaf ($P \leq 0.05$) and stem dry biomass ($P \leq 0.001$). At 1000 ppm CO_2, a saturation point was reached, halting further photosynthesis. This down-regulation was linked to decreased intercellular CO_2 levels, which expedited chlorophyll and breakdown and potentially induced leaf senescence. High CO_2 levels led to greater biomass, as anticipated. However, total protein levels, a forage quality indicator, initially decreased with high CO_2 concentrations (up to 1000 ppm) due to an inverse relationship with shoot yield. Surprisingly, the 1000 ppm CO_2 concentration mitigated this protein reduction in both alfalfa varieties.

1. Introduction

Climate is one of the most crucial aspects of agricultural output [1–3]. Climate patterns are altering as a consequence of a rise in greenhouse gas levels in the atmosphere [4,5]. The amount of CO_2 has steadily been increasing with a huge acceleration, especially in the past 100 years [6,7]. Global warming occurs because of the rising levels of CO_2 in the atmosphere, which traps heat and causes a rise in global temperature day by day [8]. Global warming alters the earth's climatic conditions, and the quantity of CO_2 in the atmosphere can influence agricultural productivity [9–11]. Due to changes in CO_2 levels, agricultural productivity was impacted in some locations [12], especially in the Mediterranean climatic zone [13–15] and continental climatic zones [16,17]. On the other hand, high CO_2 concentrations have a significant effect on both growth and response their harsh environments [18], which that is required to address research directions that focus on enhancing overall productivity via new technological advances in artificial intelligence [19].

Alfalfa, known for its resilience and versatility [20,21], plays a significant role in mitigating the effects of climate change for several

E-mail address: tkizildeniz@ohu.edu.tr.

https://doi.org/10.1016/j.heliyon.2024.e28975
Received 17 November 2023; Received in revised form 28 February 2024; Accepted 27 March 2024
Available online 5 April 2024

reasons [22]. It is a perennial legume crop that has the ability to sequester CO_2 [23] from the atmosphere through photosynthesis. It captures and stores carbon in its roots and soil helping to offset carbon emissions and reduce overall atmospheric CO_2 levels [24]. Thanks to its deep roots that enhance soil structure and organic matter content, which improves soil fertility [25] and allows for greater carbon storage in the soil, contributing to carbon sequestration and aiding in climate change mitigation [26,27]. It is also a commonly cultivated nutritious crop for livestock [28] that has a symbiotic relationship with nitrogen-fixing bacteria in its root nodules [29,30] to transform atmospheric nitrogen into a form that can be used by plants. This mitigates the demand for nitrogen-based synthetic fertilizers, which require an extensive amount of energy to make and contribute to greenhouse gas emissions, and is able to be utilized by other crops in a specific rotation.

By promoting the growth of alfalfa and incorporating it into livestock diets, it will allow us to improve both livestock productivity [31,32] and sustainable agriculture that reduces the environmental footprint of livestock farming, which is a significant source of greenhouse gas emissions [33]. By reducing reliance on carbon-emitting energy sources and contributing to a more sustainable energy mix, alfalfa can also be utilized as a source of bioenergy or biomass [34,35], providing an alternative to fossil fuels.

In C3 plants, a rise in short-term photosynthetic rate [36] and a recent enhancement in plant production seem to be the major consequences of escalating CO_2 levels in the atmosphere [37]. In fact, plants exhibit significant variability in their reaction to photosynthetic acclimation, which is influenced by their native ecological habitat distribution [38]. However, this reaction of plants to the increment in atmospheric CO_2 does not persist over time, and photosynthesis gradually falls [39,40,41], and this is called "*photosynthesis acclimation*" or "*photosynthetic down-regulation*". Studies with alfalfa have shown that down regulation of this photosynthesis is associated with reduced photosynthetic rate [42,43]. The ability of balancing carbon (C) fixation with the plant's carbon requirement has been described as an important process that conditions photosynthetic performance under high atmospheric CO_2 conditions [44–46]. These results suggest that plants reduce their photosynthesis process to equalize C source engagement and precipitation capability when growth occurs under high CO_2 circumstances that cause an asymmetry between C fixation and C needs [47]. Nevertheless, the ability of CO_2 to increment growth in the atmosphere is contingent upon the lack of productivity constraints due to factors commonly present in natural surroundings, like nutrient insufficiency, low temperatures, or water scarcity. In accordance with this, Aranjuelo et al. [40] observed that the simulative effect of CO_2 on alfalfa growth was only evident in well-watered plants, with no CO_2-induced enhancement in biomass production observed under suboptimal water conditions. It is well-known that high CO_2 concentrations have the potential to mitigate the effects of drought stress, thereby aiding in the enhancement of crop productivity [48]. Nitrogen fixation, on the other hand, may become drought tolerant when CO_2 concentrations rise. Assessing the growth responses of these alfalfa cultivars to various CO_2 levels is critical in the context of today's agricultural challenges. The previous studies were examined from 350 ppm up to varying from 550 ppm [49] to 700 ppm atmospheric CO_2 concentration effects [47,50–52] in greenhouse experiments and simulated in the models reached a maximum CO_2 level of 650 ppm [53]. To the best of our knowledge, no research has been done to determine the different high levels of atmospheric CO_2 starting from 400 ppm up to 1000 ppm on the growth and biomass partitioning of alfalfa plants. The knowledge gained from the experiment matters not just for optimizing alfalfa growing practices, but also for guiding broader agricultural plans to ensure food security in the face of changing environmental challenges. Ultimately, this study adds to our joint efforts to prevent the adverse consequences of climate change on agriculture and ensure sustainable food production for future generations. Therefore, the aim of this experiment is to evaluate the growth dynamics of two unique alfalfa types, Bilensoy 80 and Nimet, when exposed to more extreme and varying amounts of CO_2 concentration in their surrounding environment. Thus the idea of this research is to shed light on the vital subject of how these alfalfa cultivars' growth dynamics respond to changes in atmospheric CO_2 levels, with an emphasis on comprehending the consequences of climate change on the agricultural sector.

2. Materials and methods

2.1. Plant materials

The dormancy value is vital for plant adaptability and survival throughout the winter [54]. To assess the appropriateness of the varieties for the region, the dormancy rate should be known. In alfalfa, dormancy is defined as a slowing growth period during autumn with decreasing temperature and day length [55]. The Bilensoy-80 alfalfa variety (*Medicago sativa* L. cv Bilensoy-80) is a dormant (dormancy degree, FD: 4) and has a high degree of winter resistance as it grows slowly with stem elongation after cutting in autumn [56] and is a productive local alfalfa variety grown in the Central Anatolia region, registered by the Field Crop Central Research Institute in 1984 [57]. It is particularly well-adapted to the climatic conditions of Central Anatolia and the transition regions. Depending on the ecological conditions, it takes 4–6 harvests in a vegetation period, and the plant height varies between 70 and 80 cm. When grown in these regions, it has an average yield of 1 ton, and 1.5 tons of hay when grown in the hot coastal belt. When it is harvested during the flowering period, the dry grass contains 16–18% total protein, 1–1.5% crude oil, 9–11% solid mineral content remaining, 25–30% crude fiber, 34–36% core materials without nitrogen, and 5–6% water. It is also a very disease-resistant variety. Between 25 and 30 kg of seeds are planted per hectare [57]. *Medicago sativa* cv. Bilensoy-80 exceptionally excels at the above-mentioned topics, it is a disease, drought, and cold-resistant plant, and surprisingly, it has satisfactory properties [57]. Thus, it can be concluded that the Bilensoy-80 variety is well adapted to continental climate conditions. Although the current conditions for alfalfa are satisfactory, there is still room for improvement to fully optimize its potential.

On the other hand, another variety of *Medicago sativa* L. is the Nimet variety, and is also used as forage. The Nimet variety is needed in coastal regions to adapt to the hot temperature and can be used for grass production in the field of agriculture. The Nimet alfalfa variety is highly productive, and non-dormant (degree of dormancy, FD: 8). It grows vigorously in fall, forming long rigid shoots, and

continues rapid shoot lengthening after fall harvest [56]. It is a coastal type that is suitable for coastal areas and can be used for grass production in field agriculture. This variety was developed by the Eastern Mediterranean Agricultural Research Institute [58] and was registered in 2011. It has taken its place in the national varieties list, with a plant height varying between 90 and 100 cm. There is a yield of 8–10 tons da^{-1} of fresh grass and 2 tons da^{-1} of dry grass, for a total of 7 forms per year. In sowing, 20 kg of seeds and 250 kg of DAP fertilizer are implemented per hectare. The total protein rate in dry grass is 18–20%, ADF rate is 32–36%, and the NDF rate is 40–44% [58]. In Mediterranean climate conditions, the Nimet variety has the highest dry matter yield and total protein yield when compared with different varieties [59]. When the Nimet and Bilensoy-80 varieties are compared according to their germination, root length, and vigor index, they have close results compared to the other varieties of alfalfa under the same circumstances of stress [60].

2.2. Experimental design

In order to simulate climate change scenarios for two different varieties of *Medicago sativa* (L.) cv. Nimet and Bilensoy-80, this research was conducted in the Climate Change Simulation Greenhouses (CCSGs), installed at Malatya Turgut Özal University Faculty of Agriculture (38.27°N, 38.21°E) in Malatya, Türkiye. The experiment was set up with a completely randomized plot design with five replications. Under the CCSGs conditions, the research was conducted in four separate automated greenhouses, each with a 25 m^2 area. Each of the greenhouses was pumped with approximately ca. 400 as control, ca. 600, ca. 800 and ca. 1000 ppm carbon dioxide. The CCSGs were set at 26/15 °C air temperature (day/night), 60/80% relative humidity (RH), the light intensity in the greenhouses ranging from 3000 to 5000 lux (56–93 (μmol m^{-2} s^{-1}) Photosynthetic Photon Flux Density) without providing additional light source, and 14 h of daytime photoperiod, with other climatic conditions being the same in all the CCSGs [61,62].

The alfalfa seeds were planted directly into 9 L pots containing a mixture of peat and mixture (v/v, 3:1). Since peat is considered an organic nutritional source, no additional nutrient solution was provided. According to T.C. Tarım ve Orman Bakanlığı [57], the seed intensity for both varieties varied between 20 and 30 kg of seeds per hectare. This was done to simulate the real-time planting method, where seeds were equally planted on the top of 9 L pots. Plants received irrigation straight away as required (approximate two times per week). The experiments lasted approximately 3 months until the first alfalfa flowers appeared.

When the photosynthetic acclimation phenomenon occurs with the impacts of climate change, it is expected that plants exposed to high CO_2 conditions will show differences in their biomass as a result of changes in their photosynthetic activities in the short and long term. Considering the physiological stages of the alfalfa plant, it is generally expected to increase its photosynthetic activities depending on the variety with their genetic heritage, in the short period after exposure to high CO_2.

After this short-term increase in photosynthetic activities, plants are expected to begin to acclimatize, and a decrease in these photosynthetic values is expected. Thus, the sampling was conducted in 3 stages: (i) 1st stage (Pre-acclimation phase), 2nd stage (initial acclimatization phase); and 3rd stage (final acclimatization phase) these are monitored based on the various physiological stages of alfalfa crops, specifically in the context of climate change conditions.

2.3. Plant growth parameters

With the goal of evaluating biomass partitioning, vegetative growth was measured as leaves, stems and roots. Plant materials from each sample were separated into leaves, stems, and roots with the goal of determining biomass allocation. The plant was weighed to assess the fresh weight. To determine the dry weight of these plant organs, oven-dried plant matter at 80 °C for 48 h was measured.

Leaf area (LA) was measured via the automatic portable leaf surface area meter (LAI-2200, Plant Canopy Analyzer, LICOR®, NE, U.S.A). Specific Leaf Area (SLA) was counted up according to bisecting the LA by Leaf DW. The stem to leaf ratio (stem/leaf ratio DW) was calculated dividing the DW of the stem by leaf DW as an alfalfa forage quality parameter. The root to vegetative ratio was calculated by dividing the dry weight of the root by the sum of the total dry weight of the vegetative above-ground organs (the total dry weight of the stems and leaves combined).

2.4. Gas exchange parameters and chlorophyll content index

During the greenhouse testing, gas exchange parameters were evaluated on four youth totally evolved leaves from each treatment utilizing a portable photosynthesis meter (LI-6400XT System, LICOR®, NE, U.S.A). This was done on three sampling dates on a sunny day during 3 h of morning starting from 9.00 a.m. to until 12.00 p.m. at local time.

To determine stomatal closure phenomena, the intercellular CO_2 concentration was measured as an indicator of stomatal limitation [42,63]. Net photosynthesis and intercellular CO_2 concentration were assessed under atmospheric CO_2 conditions (approximately 400 ppm) and high CO_2 concentrations (600 ppm, 800 ppm and 1000 ppm) applied in each greenhouse, with a white LED light intensity of 1400 mol m^2 s^{-1}, and air cuvette temperatures ranging from 25 to 30 °C for both plants grown at ambient and enhanced CO_2. Since stomatal conductance is utilized as a stress-related to water indicator [64,65], and it was not included as a treatment in this study, this parameter was not measured. Examinations of plants cultivated at current and increased CO_2 and assessed at the same CO_2 amount, either ambient or high, show photosynthetic acclimatization.

The chlorophyll content (Chl) was estimated using the SPAD-502 Plus Chl meter (Konica Minolta Optics, Japan) which measures the relative leaf Chl concentration in an easy-to-use, rapid, and nondestructive method. The meter is firmly attached to a leaf, and the light absorbance throughthe leaf at 650 and 940 nm is measured. The 650 nm wavelength corresponds to the spectroscopic area corresponding to the highest Chl activity, but the 940 nm absorbance is utilized as a standard to account for characteristics such as leaf water content and thicknesses. The device produces three-digit SPAD readings, which are assumed to be proportional to the leaf's

chlorophyll concentration [66]. The sensor analyzes the ratio of two wavelengths to determine chlorophyll content index, not the actual value of chlorophyll *a*, *b* or a+b [67].

2.5. Biochemical analyses

The total protein and solid mineral content remaining (SMCR) of Nimet and Bilensoy 80 alfalfa varieties were determined using oven-dried samples in the Malatya University lab. The samples were pounded to pass through a 1 mm sieve for biochemical analysis. The SMCR was assessed by oven-dried samples at 105 °C overnight and usinga muffled furnace to burn the samples for 6-h at 550 °C for [68]. The total protein content of the feds was assessed by multiplying the resulting nitrogen value [69] by a factor of 6.25.

2.6. Data analysis

The data was assessed using a two-way ANOVA (with two factors: (i) CO_2 concentration and (ii) variety) and four levels to assess the impacts and possible interactions of applications, under CO_2 conditions: ca. 400 ppm, 600 ppm, 800 ppm, and 1000 ppm. Bilensoy-80 and Nimet varieties were tested. Differences between groups will be examined first with Fisher's Least Significant Differences (LSD) post-hoc test. This analysis is beneficial when main impacts or interactions are important. Results are paid attention to statistically significant at $p \leq 0.05$. Data was showed as mean ± standard error (SE). Both absolute values and values relative to control (Bilensoy-80, grows at 400 ppm CO_2; set to 1) are used. All these statistical analyze was run with SPSS® v. 15.0 statistical software.

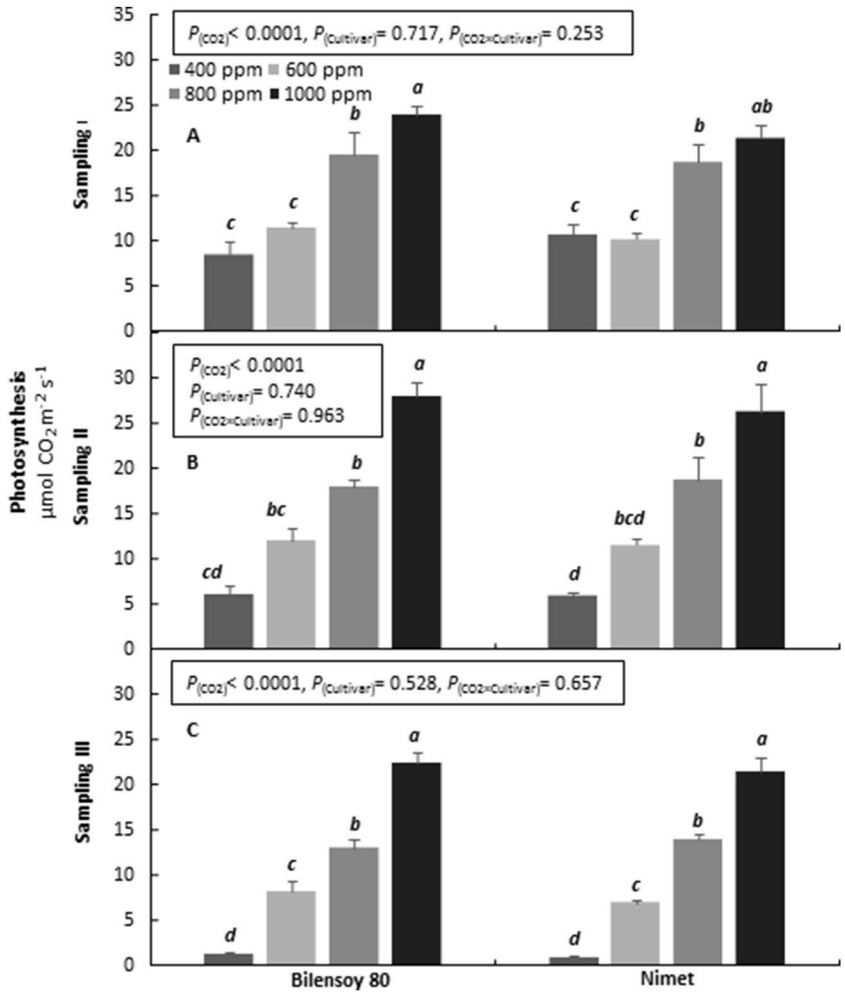


Fig. 1. Photosynthesis at three sampling physiological stages (I, pre-acclimation, A; II, initial acclimation, B; III, final acclimation, C; see Fig. S7) in Bilensoy 80 and Nimet varieties (*Medicago sativa* L.) plants cultivated under varied CO_2 levels (ambient (400 ppm), or high (600 ppm, 800 ppm or 1000 ppm). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per the LSD test (n = 4).

3. Results

3.1. Different high CO_2 levels influence on gas exchange parameters and chlorophyll content index of alfalfa

The photosynthetic rate was measured in alfalfa crops grown under different CO_2 concentrations (Fig. 1). In the first sampling, it was observed that the maximum photosynthesis rate occurred in the treatment with 1000 ppm CO_2 in Bilensoy 80. Both 1000 ppm and 800 ppm CO_2 levels resulted in a statistically significant increase in photosynthesis for Bilensoy 80 and Nimet varieties (Fig. 1A). Nonetheless, there was no statistically significant change observed between the 800 ppm and 1000 ppm CO_2 treatments for the Nimet variety (Fig. 1A). Moving on to the second sampling, it was found that the maximum photosynthesis rate was observed in both treatments. There was a tendency towards increased net photosynthesis with the 600 ppm and 800 ppm CO_2 treatments; however, this increase was not statistically significant for both Bilensoy 80 and Nimet varieties (Fig. 1B). In the third sampling, it was observed that the maximum photosynthesis rate occurred at all CO_2 levels for both Bilensoy 80 and Nimet varieties (Fig. 1C). CO_2 as a factor had a highly significant influence on the net photosynthesis rate in all stages of sampling ($P_{(CO2)} < 0.0001$) (Fig. 1A–C).

The intercellular CO_2 concentration otherwise known as sub-stomatal CO_2 concentration (C_i) was measured in alfalfa crops under various CO_2 concentrations as an indicator of stomatal limitation (Fig. 2). For Sampling I, which represents the first form of alfalfa in Bilensoy-80, the highest C_i efficiency parameter was observed at 1000 ppm CO_2 level, while the lowest value was recorded at 400 ppm CO_2 level. This indicates that the Bilensoy-80 varieties exhibit a higher C_i at higher CO_2 levels, specifically at 1000 ppm. While the Nimet variety has the lowest C_i value at the 600 ppm CO_2 level, there was not significant difference among other CO_2 levels. Lastly, for Sampling III, which represents the third form of alfalfa, there are no significant differences among treatments (Fig. 2C). Although CO_2, Cultivar, and the interaction between CO_2 and cultivar were all considered significant factors at the beginning ($P_{(CO2)} < 0.0001$,

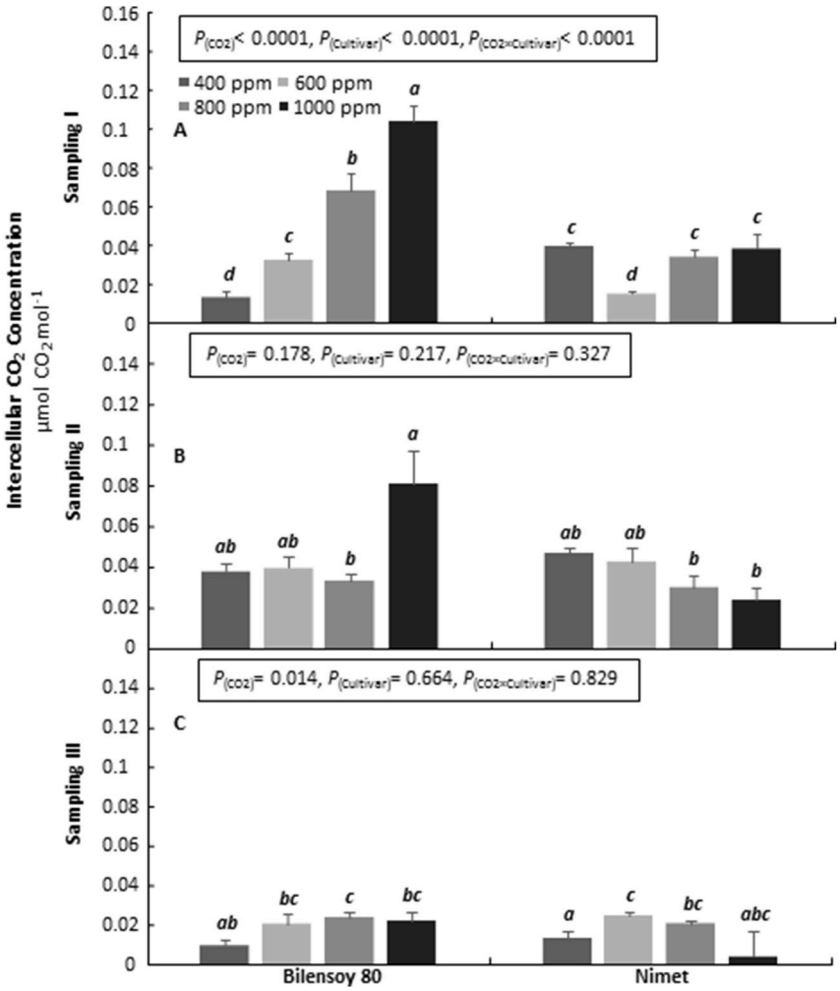


Fig. 2. Intercellular CO_2 concentration at three sampling physiological stages (I, pre-acclimation, A; II, initial acclimation, B; III, final acclimation, C; see Fig. S7) in Bilensoy 80 and Nimet varieties (*Medicago sativa* L.) plants cultivated under varied CO_2 levels (ambient (400 ppm), or high (600 ppm, 800 ppm or 1000 ppm). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per the LSD test (n = 4).

$P_{(Cultivar)} < 0.0001$, $P_{(CO2XCultivar)} < 0.0001$), only CO_2 ($P_{(CO2)} = 0.014$; Fig. 2A) remained a significant influence on intercellular CO_2 concentration by the end of the growing stage (Fig. 2C).

Chlorophyll content index value is a measurement used to evaluate plant health and photosynthesis activity by measuring the amount of chlorophyll in the leaves of plants. In Sampling I, the analysis of the chlorophyll content index parameters in the Bilensoy 80 and Nimet cultivars of alfalfa plants at different CO_2 levels. It showed no statistically noticeable variations compared to the samples (Fig. 3A). The fact that the highest chlorophyll content index for both Bilensoy 80 and Nimet was at the 600 ppm CO_2 level indicates that photosynthesis activity is higher than other CO_2 levels (Fig. 3A). Comparing the two cultivars, it can be observed that the Bilensoy 80 cultivar generally exhibited lower chlorophyll content indexvalues compared to the Nimet cultivar at all CO_2 levels (Fig. 3A).

In the case of Sampling II, the highest chlorophyll content indexparameter in Bilensoy 80 was obtained at 800 ppm, while the lowest value was observed at 1000 ppm (Fig. 3B). As for Nimet, the lowest chlorophyll content index parameter was observed at 600 ppm, and the highest chlorophyll content indexparameter was observed at 400 ppm CO_2 level (Fig. 3B). This difference between the data implies that CO_2 levels do not contribute significantly in both Bilensoy 80 and Nimet varieties on the chlorophyll content index (Fig. 3B). Lastly, for Sampling III, the highest chlorophyll content indexparameter was found at 800 ppm CO_2, whereas the lowest value was recorded at 1000 ppm CO_2 (Fig. 3C). This significant variation between the two high CO_2 values suggests that an enhancement in CO_2 levels from 800 ppm to 1000 ppm negatively influences the chlorophyll content index parameter in the alfalfa plants in the Bilensoy 80 variety (Fig. 3C). For the Nimet variety, the highest SPAD-502 chlorophyll index was observed at the 600 ppm CO_2 level, while the lowest was measured at 1000 ppm (Fig. 3C). CO_2 has significant influence at all growing stages on the chlorophyll index parameter ($P_{(CO2)} = 0.022$, Fig. 3A; $P_{(CO2)} = 0.001$, Fig. 3B; $P_{(CO2)} = 0.001$, Fig. 3C), while the cultivar had an impact at the beginning of growing stages ($P_{(Cultivar)} < 0.0001$, Fig. 3A; $P_{(Cultivar)} < 0.0001$, Fig. 3B) it did not remain significant at the end ($P_{(Cultivar)} = 0.560$, Fig. 3C).

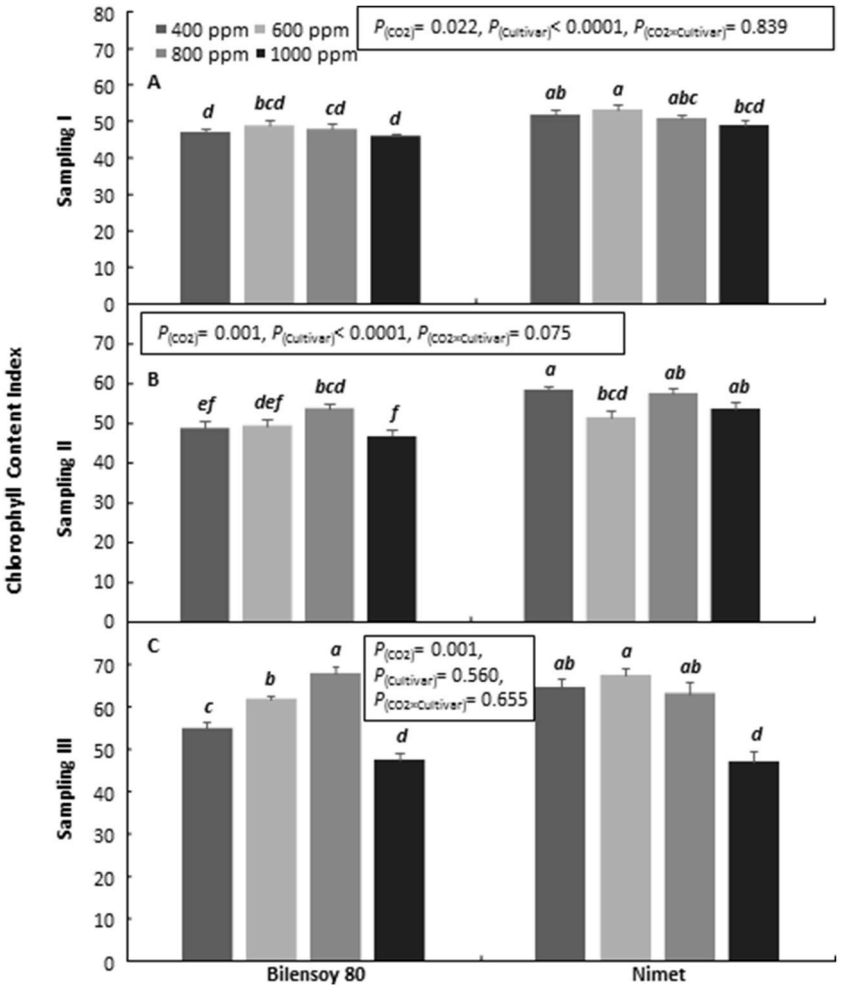


Fig. 3. Chlorophyll content indexat three sampling physiological stages (I, pre-acclimation, A; II, initial acclimation, B; III, final acclimation, C; see Fig. S7) in Bilensoy 80 and Nimet varieties (*Medicago sativa* L.) plants cultivated under varied CO_2 levels (ambient (400 ppm), or high (600 ppm, 800 ppm or 1000 ppm). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per the LSD test (n = 4).

3.2. Different high CO_2 levels influence on alfalfa growth

The root to vegetative biomass ratio of alfalfa crops was assessed (Fig. 4). The root-to-vegetative biomass ratio at the initial growing stage was observed at both 400 and 800 ppm at Bilensoy 80 and at 400 ppm at Nimet variety (Fig. 4A), while the lowest data was recorded at 600 ppm and 1000 ppm CO_2 level at Bilensoy 80 and 600 ppm and 800 ppm CO_2 level at Nimet variety (Fig. 4A). On the contrary, as the CO_2 level increases for the Nimet variety, the root-to-vegetative ratio decreases significantly ($P_{(CO2)} < 0.0001$; Fig. 4A) and interactions between cultivar and CO_2 has significant influence at the beginning of growing stage ($P_{(CO2XCultivar)} = 0.009$, Fig. 4A). The highest data at second sampling was observed at 600 ppm CO_2 level in both varieties, while the other CO_2 levels are demonstrating the lowest root to vegetative ratio (Fig. 4B). At the final sampling stage, root to vegetative ratio was demonstrated the lowest value at 1000 ppm CO_2 level on Bilensoy 80 variety, while the rest of the CO_2 levels show an equally bigger value (Fig. 4C), indicating that the highest CO_2 level provoked more vegetative part than root. On the other hand, Nimet variety's root to vegetative ratio is decreased, while CO_2 levels are increased (Fig. 4C), demonstrating that all high CO_2 levels provoked more vegetative parts than root. This also indicates the influence of CO_2, cultivar and their interactions on root to vegetative ratio at harvesting stage ($P_{(CO2)} = 0.004$; $P_{(Cultivar)} = 0.006$; $P_{(CO2XCultivar)} = 0.030$, Fig. 4C).

The stem to leaf ratio of alfalfa crops was measured (Fig. 5). For Sampling I, the highest stem to leaf ratio efficiency was observed at 400 ppm in Bilensoy 80 and the lowest data was obtained at 1000 ppm CO_2 concentration for Bilensoy 80 variety and 400 ppm CO_2 concentration for Nimet variety (Fig. 5A). This indicates that increasing the CO_2 concentration to 1000 ppm affects the stem to leaf ratio efficiency positively for Bilensoy 80, while it negatively affects it for Nimet (Fig. 5A). Moving on to Sampling II, the highest efficiency was observed at 1000 ppm CO_2 level in Bilensoy 80 and 800 ppm in Nimet variety (Fig. 5B). The minimum efficiency is 400 ppm for Bilensoy 80 (Fig. 5B). This implies that a higher CO_2 concentration of 1000 ppm has a positive effect on the stem to leaf ratio

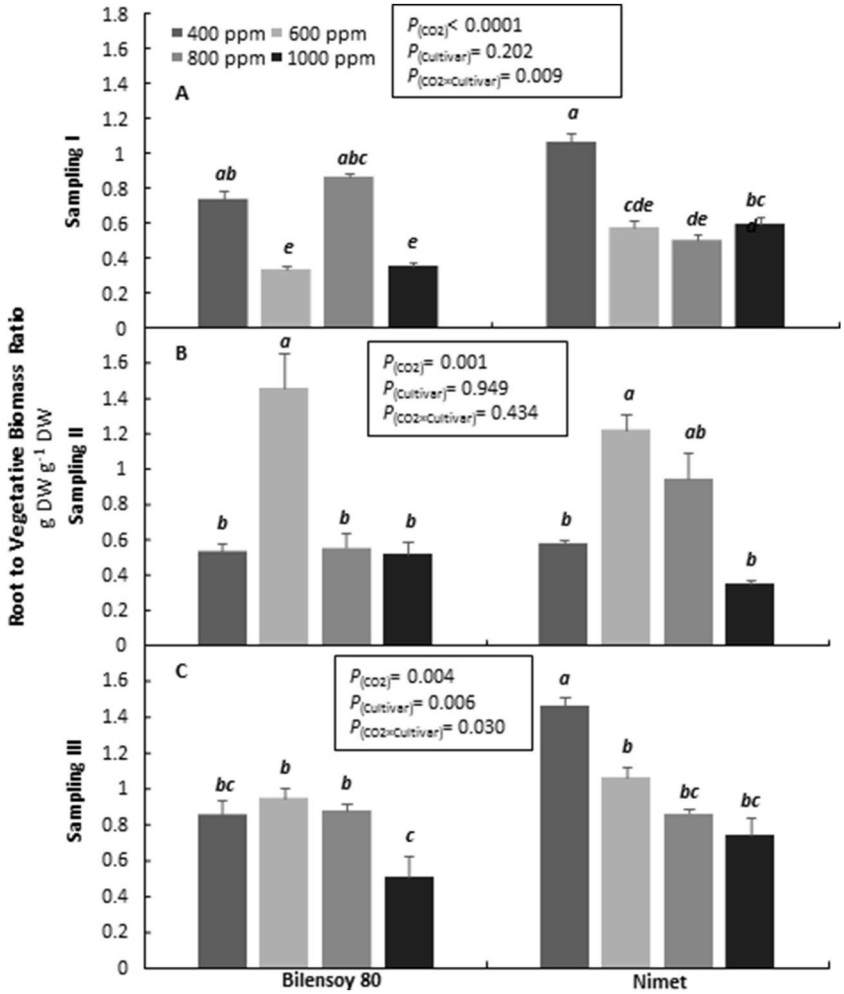


Fig. 4. Root to Vegetative Biomass Ratio at three sampling physiological stages (I, pre-acclimation, A; II, initial acclimation, B; III, final acclimation, C; see Fig. S7) in Bilensoy 80 and Nimet varieties (*Medicago sativa* L.) plants cultivated under varied CO_2 concentrations (ambient (400 ppm), or high (600 ppm, 800 ppm or 1000 ppm). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per LSD test (n = 4).

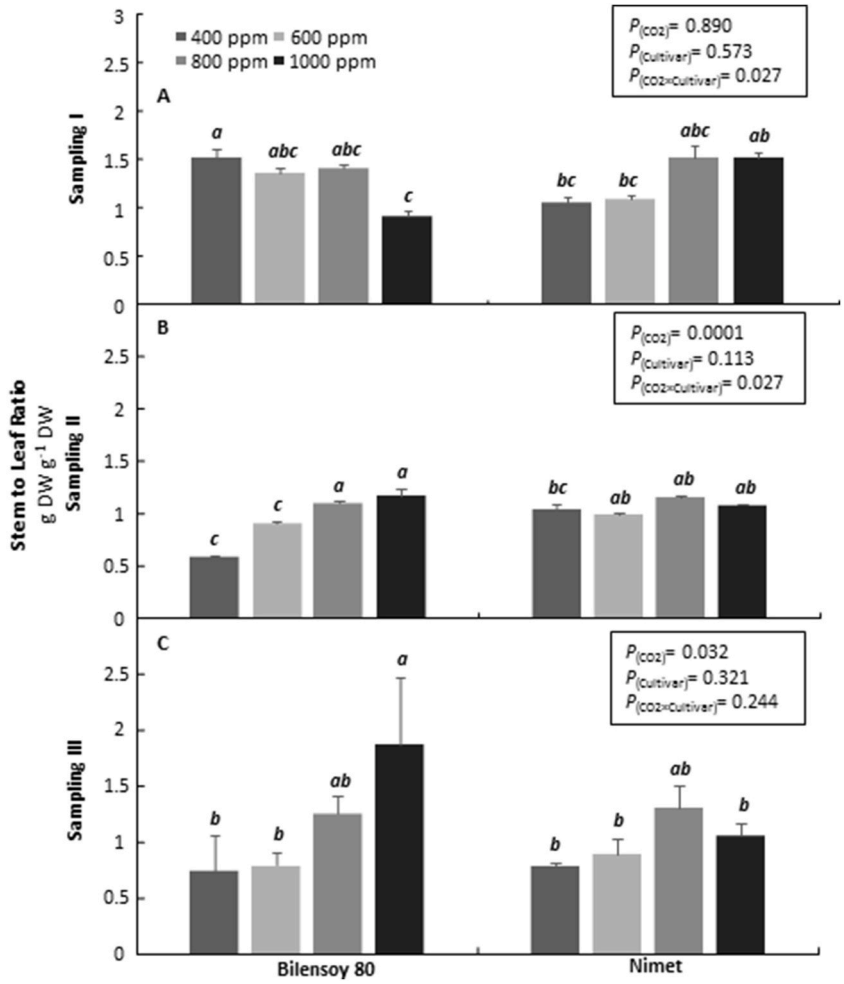


Fig. 5. Stem to Leaf Ratio at three sampling physiological stages (I, pre-acclimation, A; II, initial acclimation, B; III, final acclimation, C; see Fig. S7) in Bilensoy 80 and Nimet varieties (*Medicago sativa* L.) plants cultivated under varied CO_2 concentrations (ambient (400 ppm), or high (600 ppm, 800 ppm or 1000 ppm). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per LSD test (n = 4).

efficiency in Bilensoy 80 and the least significant CO_2 concentration of 400 ppm has a negative effect (Fig. 5B). For the Nimet variety, no significant increase or decrease on CO_2 and stem leaf ratio efficiency was observed. Lastly, for Sampling III, the highest efficiency was observed at 1000 ppm CO_2 level in Bilensoy 80 variety and Nimet variety has the lowest value at 400 ppm (Fig. 5C). This significant increase shows that there is a direct proportion between CO_2 and efficiency (Fig. 5C). Although the Nimet variety had the highest yield at 800 ppm CO_2 level and the lowest yield at 400 ppm CO_2 level, no significant increase or decrease was detected between CO_2 and stem to leaf ratio efficiency (Fig. 5C).

Specific leaf area is reducing significantly with all high CO_2 exposures in Bilensoy 80, while no significant changes were observed for the Nimet variety at the beginning of the growing stage. However, when cultivar and CO_2 factors have interactions, there was significant influence on specific leaf area ($P_{(CO2XCultivar)} \leq 0.01$; Table 1). While leaf area at same growing stage had an impact for all factors ($P_{(CO2)} \leq 0.001$, $P_{(Cultivar)} \leq 0.01$, $P_{(CO2XCultivar)} \leq 0.05$; Table 1). At second harvest, the cultivar is not significant for leaf area, meanwhile CO_2 and its interaction with cultivar significantly impacts to leaf area ($P_{(CO2)} \leq 0.001$, $P_{(CO2XCultivar)} \leq 0.05$; Table 1), which is clearly observed at 800 and 1000 ppm CO_2 influence resulting high leaf area as well as high specific leaf area on Bilensoy 80 variety ($P_{(CO2XCultivar)} \leq 0.01$; Table 1). At the end of final harvest, CO_2 still had an impact on leaf area ($P_{(CO2)} \leq 0.001$, Table 1).

On the other hand, while specific leaf area is impacted by CO_2 and cultivar interaction at the beginning of the growing stages ($P_{(CO2XCultivar)} \leq 0.01$ at 1st and 2nd samplings), this influence did not remain at the final harvest. All high CO_2 levels demonstrated high leaf area in Bilensoy 80 variety, yet it was not very clear for Nimet. Comparing both varieties Bilensoy 80 shows somewhat more specific leaf area ($P_{(Cultivar)} \leq 0.05$; Table 1).

3.3. *Different high CO_2 levels influence on alfalfa biochemical change*

The protein content within the alfalfa plant constitutes a pivotal parameter in assessing plant quality (Fig. 6B). The results indicate

Table 1
Effects of high CO_2 in different concentrations (400, 600, 800 and 1000 ppm) on *Medicago sativa* L. cv Nimet and Bilensoy 80 varieties leaf area and specific leaf area. Data were plotted as mean ± standard error (n = 4). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per LSD test (n = 4). ANOVA: n.s. not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Sampling #	Cultivar	CO_2 Level	Leaf Area	Specific Leaf Area
			cm^2	cm^2 leaf area g^{-1} leaf DW
I	Nimet	400	49.26 ± 3 cd	233.82 ± 16 bc
		600	31.58 ± 3 d	172.96 ± 11 c
		800	106.67 ± 16 a	355.61 ± 26 ab
		1000	80.35 ± 12 b	315.12 ± 27 bc
	Bilensoy 80	400	35.19 ± 3 cd	633.67 ± 61 a
		600	32.5 ± 4 d	319.73 ± 22 bc
		800	60.51 ± 2 bc	196.96 ± 12 bc
		1000	74.82 ± 11 b	145.19 ± 12 c
	$P_{(CO2)}$		***	n.s.
	$P_{(Cultivar)}$		**	n.s.
	$P_{(CO2\ x\ Cultivar)}$		*	**
II	Nimet	400	88.75 ± 5 bc	901.82 ± 62 c
		600	109.53 ± 15 ab	2099.80 ± 148 a
		800	110.87 ± 12 ab	1207.17 ± 80 bc
		1000	120.2 ± 17 ab	1326.00 ± 63 bc
	Bilensoy 80	400	54.6 ± 6 c	825.38 ± 51 c
		600	49.9 ± 8 c	926.25 ± 63 c
		800	107.5 ± 10 a	1677.23 ± 97 bc
		1000	136.4 ± 20 a	1971.35 ± 215 ab
	$P_{(CO2)}$		***	n.s.
	$P_{(Cultivar)}$		n.s.	n.s.
	$P_{(CO2\ x\ Cultivar)}$		*	**
III	Nimet	400	475.28 ± 27 bcd	826.48 ± 95 c
		600	443.35 ± 55 cd	1304.08 ± 193 bc
		800	758.62 ± 56 ab	976.41 ± 46 bc
		1000	719.62 ± 187 abc	1168.92 ± 59 bc
	Bilensoy 80	400	290.75 ± 19 d	996.44 ± 96 bc
		600	602.52 ± 55 bc	2180.54 ± 164 a
		800	751.52 ± 56 ab	1076.83 ± 78 bc
		1000	942.47 ± 192 a	1601.72 ± 221 ab
	$P_{(CO2)}$		***	**
	$P_{(Cultivar)}$		n.s.	*
	$P_{(CO2\ x\ Cultivar)}$		n.s.	n.s.

that there is a significant interaction between the CO_2 levels and the cultivars in terms of protein content ($P_{(CO2XCultivar)} = 0.003$), and individual factors are significant ($P_{(CO2)} < 0.0001$; $P_{(Cultivar)} = 0.001$). The highest protein efficiency was observed at 400 ppm, while the lowest efficiency was recorded at 800 ppm (Fig. 6B). This significant finding suggests that an 800 ppm CO_2 concentration, which is a high CO_2 level negatively, influences protein efficiency in both Bilensoy 80 and Nimet varieties (Fig. 6B). The protein level is decreasing with increasing the atmospheric CO_2 up to 800 ppm level. However, when the CO_2 level reached 1000 ppm, this effect becomes reverse, and the protein content increases again (Fig. 6B).

As a solid mineral content remaining (SMCR) was measured (Fig. 6). Upon analyzing the data, it is evident that the CO_2 concentrations have a significant influence on the SMCR content parameters of both cultivars ($P_{(CO2)} < 0.0001$; Fig. 6A) and there is a significant interaction between the CO_2 levels and the cultivars in terms of SMCR value ($P_{(CO2XCultivar)} < 0.0001$). For Bilensoy 80, the highest SMCR content parameter was obtained at 800 and 1000 ppm, while the lowest was observed at 400 ppm (Fig. 6A). This suggests that higher CO_2 levels positively influence the SMCR content of Bilensoy 80 cultivar in alfalfa plants (Fig. 6A). On the other hand, for the Nimet cultivar, the highest SMCR content was observed at 400 ppm, whereas the lowest was recorded at 800 ppm (Fig. 6A). This indicates that the optimum CO_2 level for maximizing SMCR content in Nimet cultivar is 400 ppm (Fig. 6A). These significant findings suggest that higher CO_2 levels (particularly 800 and 1000 ppm), for Bilensoy 80, and the lowest CO_2 level (400 ppm) for Nimet, can enhance the SMCR content in alfalfa plants (Fig. 6A).

3.4. Different high CO_2 levels influence on alfalfa biomass

Alfalfa crop biomass related data were represented in Table 2. The highest leaf fresh (FW) weight of leaf at first sampling was observed in both treatments of 800 ppm and 1000 ppm in both varieties (Table 2), whilst the lowest rate is obtained in the treatments of 400 and 600 ppm in both varieties (Table 2). Therefore, CO_2 has a relatively smaller influence at leaf FW at the first growing stage ($P_{(CO2)} \leq 0.05$). In second sampling of leaf FW, the tendency that was observed in the previous sampling remains for Bilensoy 80 variety at 800 and 1000 ppm treatments as CO_2 factor demonstrates influence ($P_{(CO2)} \leq 0.01$) while all CO_2 treatments did not make significant changes on Nimet variety (Table 2). On the other hand, the influence of CO_2 factor is reduced ($P_{(CO2)} \leq 0.05$) in last sampling of leaf FW and overall, there is not any significant change (Table 2). As observed on leaf FW, there is the tendency observed in

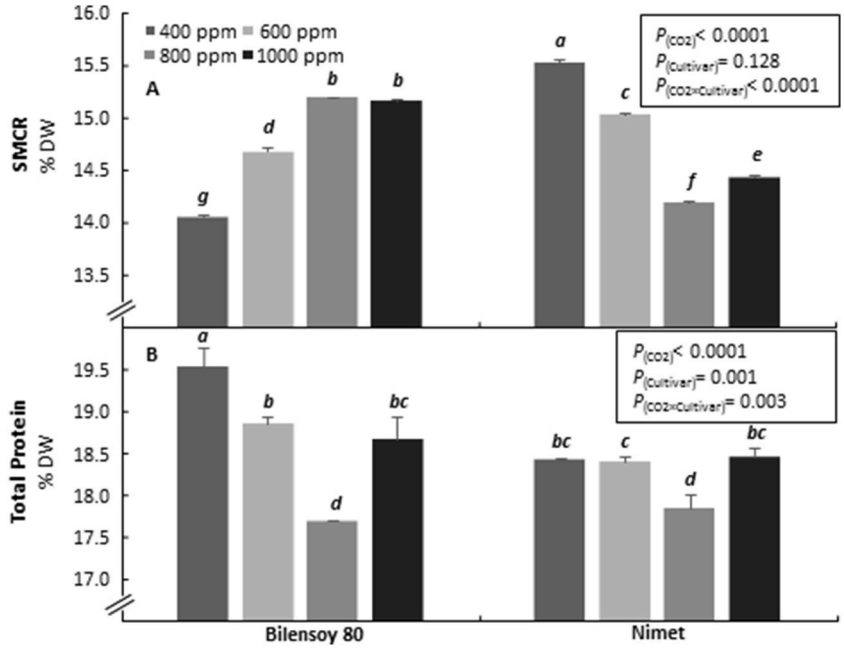


Fig. 6. The solid mineral content remaining (SMCR) (A) and total protein (B) in vegetative areal parts at maturity (sampling III, see Fig. S7) in Bilensoy 80 and Nimet varieties (*Medicago sativa* L.) plants cultivated under varied CO_2 concentrations (ambient (400 ppm), or high (600 ppm, 800 ppm or 1000 ppm). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per LSD test (n = 4).

the first and second sampling for Bilensoy 80 variety at 800 and 1000 ppm effects are similar for stem FW as well (Table 2) as also indicates the significant effects of CO_2 ($P_{(CO2)} \leq 0.001$). At the end of the final sampling, the control CO_2 treatment demonstrates the lowest stem FW in both varieties while 1000 ppm CO_2 treatment shows clear influence on Bilensoy 80 (Table 2) as also observed CO_2 ($P_{(CO2)} \leq 0.001$) and its interaction with cultivar ($P_{(CO2XCultivar)} \leq 0.05$).

At the beginning of sampling, the root FW does not demonstrate any significant change ($P_{(CO2)}$ = n.s, $P_{(Cultivar)}$ = n.s, $P_{(CO2XCultivar)}$ = n.s.; Table 2). While the growing process continues at second sampling, the previously mentioned tendency of 800 and 1000 ppm CO_2 influence of leaf and stem FW in Bilensoy 80 was also observed on root FW as well, and this influence is similar for Nimet's root FW (Table 2). These clear effects also reflect CO_2 ($P_{(CO2)} \leq 0.001$), cultivar ($P_{(Cultivar)} \leq 0.05$), and their interaction ($P_{(CO2XCultivar)} \leq 0.05$; Table 2). However, this tendency does not remain at the harvesting of Bilensoy 80 variety's root FW, but Nimet's root FW demonstrates the highest value under the influence of 1000 ppm CO_2 concentration (Table 2). The highest dry weight (DW) of leaf at the beginning of growing stage was observed at 1000 ppm of CO_2 level in Bilensoy 80 variety, while Nimet's DW is highest in both 800 and 1000 ppm CO_2 levels (Table 2), as CO_2 factor demonstrates significant influence on leaf DW ($P_{(CO2)} \leq 0.001$), as well as its interaction with cultivar ($P_{(CO2XCultivar)} \leq 0.01$, Table 2).

As observed on the leaf and stem FW, the tendency also remains for Bilensoy 80's DW of leaf and stem observed in the first and second sampling (even more clearly) at 800 and 1000 ppm. Additionally this kind of tendency was observed for Nimet's leaf and stem DW, as seen in the root FW of Nimet where leaf and stem DW reduces at 600 ppm and then 1000 ppm of CO_2 is recovering this reduction (Table 2) which also indicates the significant effects of CO_2 in both leaf ($P_{(CO2)} \leq 0.001$) and stem DW ($P_{(CO2)} \leq 0.001$). However, this clear tendency was not clearly observed on both varieties' leaf DW at the last harvest for both varieties ($P_{(CO2)} \leq 0.05$), while in stem DW of Bilensoy 80 continues to be influenced at 800 and 1000 ppm ($P_{(CO2)} \leq 0.001$; $P_{(CO2XCultivar)} \leq 0.01$, Table 2).

4. Discussion

4.1. *Different high CO_2 levels influence on gas exchange parameters and chlorophyll content index of alfalfa*

In this study, atmospheric CO_2 in four different concentrations as related to the main effect of climate change has been investigated. The primary effects of escalating CO_2 levels in the atmosphere in C3 plants is an increase photosynthetic rate and plant production in short-term [37]. Then, photosynthesis gradually declines in long term, which is called as the "*photosynthesis acclimation*" or "*photosyntetic down-regulation*" [39,40,43]. Studies with alfalfa have shown that downregulation of this photosynthesis is associated with reduced photosynthetic rate [42,43]. In our study, comparing the three sampling stages, the photosynthetic rate is reduced (Fig. 1). All high CO_2 treatments significantly increased photosynthesis in both Nimet and Bilensoy-80 varieties (Fig. 1) as indicated by Erice et al. [51] and De Luis et al. [70]. These findings suggest that higher CO_2 levels, particularly 800 ppm and 1000 ppm, have a positive impact on photosynthesis efficiency in Bilensoy 80 and Nimet alfalfa varieties. However, the significance of this increase varied between the two varieties, with Bilensoy 80 showing a more pronounced response. CO_2 as factor has significant influence during all growth stages

Table 2
Effects of high CO_2 in different concentrations (400, 600, 800 and 1000 ppm) on *Medicago sativa* L. cv Nimet and Bilensoy 80 varieties fresh (FW) and dry weight (DW) of leaf, stem, and root. Data were plotted as mean ± standard error (n = 4). The various letters represent substantial variations in treatment ($P \leq 0.05$) as per LSD test (n = 4). ANOVA: n.s. not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Sampling #	Cultivar	CO_2	Leaf FW	Stem FW	Root FW	Leaf DW	Stem DW	Root DW
			g FW plant^{-1}			g DW plant^{-1}		
I	Nimet	400	0.58 ± 0.1 ab	0.41 ± 0.1 b	0.4 ± 0.1 ab	0.22 ± 0.02 cd	0.22 ± 0.02 bcd	0.46 ± 0.04 ab
		600	0.33 ± 0.1 b	0.26 ± 0.1 b	0.28 ± 0.1 ab	0.19 ± 0.02 de	0.20 ± 0.03 cd	0.23 ± 0.04 cde
		800	0.84 ± 0.3 a	0.87 ± 0.3 a	0.96 ± 0.2 a	0.29 ± 0.03 bc	0.42 ± 0.05 ab	0.36 ± 0.07 bc
		1000	0.69 ± 0.1 ab	0.69 ± 0.1 ab	0.25 ± 0.0 ab	0.32 ± 0.04 b	0.58 ± 0.18 a	0.52 ± 0.04 ab
	Bilensoy 80	400	0.38 ± 0.1 b	0.29 ± 0.0 b	0.17 ± 0.0 b	0.08 ± 0.01 f	0.13 ± 0.02 d	0.18 ± 0.06 de
		600	0.38 ± 0.1 b	0.3 ± 0.1 b	0.59 ± 0.2 ab	0.11 ± 0.02 ef	0.16 ± 0.02 d	0.09 ± 0.01 e
		800	0.62 ± 0.0 ab	0.62 ± 0.0 ab	0.37 ± 0.1 ab	0.27 ± 0.02 bcd	0.38 ± 0.03 abc	0.56 ± 0.05 a
		1000	0.86 ± 0.2 a	0.91 ± 0.3 a	0.33 ± 0.1 ab	0,45 ± 0.06 a	0.40 ± 0.05 abc	0.25 ± 0.06 cd
	$P_{(CO2)}$		*	**	n.s.	***	***	***
	$P_{(Cultivar)}$		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	$P_{(CO2\ x\ Cultivar)}$		n.s.	n.s.	n.s.	**	n.s.	***
II	Nimet	400	0.98 ± 0.1 ab	0.88 ± 0.0 c	1.17 ± 0.1 a	0.10 ± 0.01 a	0.10 ± 0.02 a	0.11 ± 0.01 bc
		600	1.06 ± 0.2 ab	1.02 ± 0.1 bc	0.42 ± 0.1 b	0.05 ± 0.01 d	0.05 ± 0.004 b	0.12 ± 0.02 abc
		800	1.39 ± 0.3 ab	1.68 ± 0.2 ab	1.23 ± 0.2 a	0.10 ± 0.01 a	0.12 ± 0.02 a	0.21 ± 0.07 a
		1000	1.73 ± 0.5 a	1.84 ± 0.4 a	1.48 ± 0.2 a	0.10 ± 0.01 a	0.11 ± 0.01 a	0.08 ± 0.01 bc
	Bilensoy 80	400	0.66 ± 0.1 b	0.56 ± 0.1 c	0.43 ± 0.0 b	0.07 ± 0.01 bcd	0.04 ± 0.003 b	0.05 ± 0.01 c
		600	0.68 ± 0.2 b	0.77 ± 0.1 c	0.25 ± 0.0 b	0.06 ± 0.004 cd	0.05 ± 0.01 b	0.16 ± 0.04 ab
		800	1.52 ± 0.3 a	1.91 ± 0.4 a	1.41 ± 0.1 a	0.09 ± 0.01 ab	0.10 ± 0.01 a	0.10 ± 0.02 bc
		1000	1.68 ± 0.2 a	2.33 ± 0.4 a	1.28 ± 0.3 a	0.08 ± 0.01 abc	0.10 ± 0.01 a	0.10 ± 0.03 bc
	$P_{(CO2)}$		**	***	***	***	***	n.s.
	$P_{(Cultivar)}$		n.s.	n.s.	*	n.s.	*	n.s.
	$P_{(CO2\ x\ Cultivar)}$		n.s.	n.s.	*	n.s.	*	n.s.
III	Nimet	400	1.73 ± 0.3 b	1.78 ± 0.2 d	3.19 ± 0.3 c	0.66 ± 0.12 abcd	0.52 ± 0.11 bc	1.59 ± 0.06 a
		600	1.24 ± 0.3 a	1.05 ± 0.2 a	1.48 ± 0.2 c	0.42 ± 0.12 bcd	0.34 ± 0.06 cd	0.79 ± 0.08 bc
		800	2.01 ± 0.4 ab	2.20 ± 0.3 cd	2.24 ± 0.3 c	0.81 ± 0.12 a	0.99 ± 0.08 a	1.58 ± 0.34 a
		1000	1.83 ± 0.2 a	1.60 ± 0.3 bc	3.45 ± 0.8 a	0.62 ± 0.17 abcd	0.61 ± 0.10 b	0.95 ± 0.26 abc
	Bilensoy 80	400	1.38 ± 0.2 ab	1.04 ± 0.2 cd	1.34 ± 0.5 c	0.33 ± 0.06 cd	0.26 ± 0.06 d	0.44 ± 0.06 c
		600	0.98 ± 0.1 a	0.70 ± 0.1 b	1.16 ± 0.1 ab	0.30 ± 0.08 d	0.22 ± 0.02 d	0.50 ± 0.05 c
		800	1.81 ± 0.3 ab	1.91 ± 0.3 bc	2.80 ± 0.3 ab	0.73 ± 0.16 ab	0.88 ± 0.07 a	1.43 ± 0.14 ab
		1000	1.81 ± 0.3 a	2.83 ± 0.4 ab	1.19 ± 0.1 bc	0.69 ± 0.19 abc	1.01 ± 0.12 a	0.93 ± 0.40 abc
	$P_{(CO2)}$		*	***	**	*	***	*
	$P_{(Cultivar)}$		n.s.	n.s.	n.s.	n.s.	n.s.	*
	$P_{(CO2\ x\ Cultivar)}$		n.s.	*	**	n.s.	**	n.s.

on photosynthesis ($P_{(CO2)} < 0.0001$; Fig. 1A–C).

In order to determine stomatal closure phenomena, the intercellular CO_2 concentration was measured as an indicator of stomatal limitation [42,63]; [71]. Intercellular CO_2 concentration or sub-stomatal CO_2 concentration measurements (Fig. 2) showedthat adjustments in photosynthesis could not be explained by stomatal limitations [42] as an indicated by Ref. [63,71]. In this study, intercellular CO_2 concentrations were analyzed in alfalfa plants of Bilensoy 80 and Nimet varieties at various CO_2 concentrations (400 ppm, 600 ppm, 800 ppm, 1000 ppm). In the present study, the observed down-regulation was as a consequence of decreased intercellular CO_2 concentration due to stomatal closure [42]; [45] because during all samplings, plants cultivated at high CO_2 demonstrated lower C_i (Fig. 2) than those cultivated at current CO_2.

The chlorophyll concentration of the leaf is frequently used to forecast its physiological status as impacted by numerous natural and man-made factors. It is a valuable indicator of plant stress and, therefore, the capacity for plant CO_2 absorption and development. Final sampling is also applied as yield, allowing us to compare with other studies. Therefore, for Sampling III, the highest chlorophyll content index parameter was found at 800 ppm CO_2, whereas the lowest value was recorded at 1000 ppm CO_2 (Fig. 3C), which is in line with Erice et al.[52]. This significant variation between the two high CO_2 values suggests that an enhancement in CO_2 level from 800 ppm to 1000 ppm negatively influences the chlorophyll content index parameter in the alfalfa plants in Bilensoy 80 alfalfa variety (Fig. 3C). For the Nimet alfalfa variety, the highest chlorophyll content index was observed at 600 ppm CO_2 level, while the lowest was measured at 1000 ppm (Fig. 3C), which is also in line with Erice et al. [52]. CO_2 as a factor has significant influence at all growing stages on chlorophyll concentration ($P_{(CO2)} = 0.022$, Fig. 3A; $P_{(CO2)} = 0.001$, Fig. 3B; $P_{(CO2)} = 0.001$, Fig. 3C), while cultivar has impact in the beginning of growing stages ($P_{(Cultivar)} < 0.0001$, Fig. 3A; $P_{(Cultivar)} < 0.0001$, Fig. 3B); however it does not remain at the end ($P_{(Cultivar)} = 0.560$, Fig. 3C). Similar findings were reported by Munns [72] and Chen et al. [73], indicating that increased CO_2 levels expedite chlorophyll breakdown and potentially promote leaf senescence [74].

4.2. *Different high CO_2 levels influence on alfalfa growth*

In previous research [43,47,50–52] high CO_2 levels enhanced drought resilience in alfalfa plants by augmenting the root to shoot ratio. This augmentation facilitated better exploration of water and mineral resources in the soil, ultimately leading to improved water

utilization efficiency. However, in our study the plants were not exposed to any stress factors such as drought as in the previous studies. Therefore the root to vegetative biomass ratio was decreased, so that Bilensoy 80 alfalfa variety got the maximum benefit, producing more leaf and stem at 1000 ppm CO_2 concentration while Nimet alfalfa variety maximized this benefit at all CO_2 levels at the end of harvesting stage (Fig. 4C).

Our data demonstrated a decrease in the stem to leaf ratio under elevated CO_2 conditions, consistent with previous reports by [43, 75–78]; [79]. The interaction between CO_2 level and cultivar significantly influenced the stem to leaf ratio at first and second growing stages ($P_{(CO2XCultivar)} = 0.027$ at both samplings; Fig. 5A and B). However, this impact was not observed at final harvesting stage (Fig. 6C), while the impact of CO_2 initiated at second and last growing stages ($P_{(CO2)} = 0.0001$, Fig. 5B; $P_{(CO2)} = 0.032$, Fig. 5C).

Specific leaf area significantly decreased with all high CO_2 exposures in Bilensoy 80, while no significant changes were observed for Nimet variety at the beginning of the growing stage. At the harvesting stage, CO_2 maintained its impact on leaf area ($P_{(CO2)} \leq 0.001$, Table 1). Our results also confirm the previous studies, which have already reported that subjecting plants to extended CO_2 enrichment generally results in an augmentation of plant biomass [43,52] (as shown in Table 2), total leaf area (Table 1), and changes in net rates for photosynthesis (Fig. 1).

4.3. Different high CO_2 levels influence on alfalfa biochemical change

The solid mineral content remaining is a substance of forage crop that remains after burning it [80]. It is a simple indicator of the total mineral content of forage. Depending on the genetic variability, the solid mineral content remaining in alfalfa genotypes varied between 10.3 and 11.5 with an average of 11.1 % DW [81]. The optimum CO_2 level for maximizing the SMCR in Nimet cultivar is 400 ppm (Fig. 6A). These significant findings suggest that higher CO_2 levels (particularly 800 and 1000 ppm), for Bilensoy 80 variety, and the lowest CO_2 level (400 ppm) for Nimet variety, can enhance the SMCR in alfalfa plants.

The protein content serves as an indicator of forage quality [79]. The protein content within the alfalfa plant constitutes a pivotal parameter in assessing plant quality. The highest protein efficiency was observed at 400 ppm, while the lowest efficiency was recorded at 800 ppm (Fig. 6B) as indicated by the fact that total protein was negatively related to shoot yield [79] (Table 2) This aligns with the concept of the 'dilution effect'. This effect has been widely discussed in plant nutrition and soil fertility studies, indicating that changes in environmental conditions such as temperature, fertilizer application, or, in this case, CO_2 concentration, can lead to decreases in the concentration of certain elements in plant tissues [82,83]. In our study, significant increases in shoot yield were accompanied by reductions in protein concentrations, reflecting the impact of elevated CO_2 levels on protein efficiency.

This significant finding suggests that 800 ppm CO_2 concentration, which is a high CO_2 level negatively, influences protein efficiency in both Bilensoy 80 and Nimet varieties (Fig. 6B). This is most probably due to the negative relationship between total proteins and shoot yield as mentioned above [79]. The protein level is decreasing with increasing the atmospheric CO_2 up to 800 ppm level. However, when CO_2 level reached 1000 ppm, this effect becomes reversed, and the protein content increases again (Fig. 6B), as similarly observed for the SMCR of Nimet variety (Fig. 6A). This is due to high CO_2 reducing alfalfa forage quality through a reduction in total protein (Fig. 6B) and an increase of fiber content [79]; Fig. 6A) up to 800 ppm atmospheric CO_2 concentration. Photosynthetic acclimation of plants grown in high CO_2 was an outcome of reduced carboxylation effectiveness as a consequence of decreased protein content [42]. However, 1000 ppm CO_2 concentration recovers this reduction in protein content of both varieties (Fig. 6B). Up to now, there has not been any study or report such a recovery process in protein levels with a 1000 ppm CO_2 concentration level.

4.4. Different high CO_2 levels influence on alfalfa biomass

CO_2 restricts photosynthesis and production of biomass in C3 plants at current atmospheric levels, with the highest CO_2 fixation rate ranging from 700 to 1000 ppm CO_2 [84,85]. Several investigations have demonstrated that raising CO_2 content has a positive impact on alfalfa growth [47] as in line with our study. Nonetheless, it is widely acknowledged that the high CO_2 impact is dependent on CO_2 concentration as well, and the effects of different CO_2 levels during alfalfa growth have not been studied in and of themselves. The immediate impact of high CO_2 on growth, comparing 350–700 mmol mol^{-1}, is the augmentation of plant biomass [86], which can clearly also be observed at the harvesting stage of sampling in both varieties FW and DW parameters (Table 1). In addition to that, the storage behavior of the shoot under high CO_2 differs from that of the root during this timeframe [52]. The shoots exhibited characteristics of a temporary photosynthetic store, unlike the roots which functioned as long-term storage organs [43].

5. Conclusions

Elevated CO_2 levels enhance photosynthesis in plants at the beginning of the growing stages due to the fact that CO_2 is a key reactant in the photosynthesis process. There was a positive relation between high CO_2 and photosynthesis resulting in increased plant growth and yields. However, concentrations of 1000 ppm CO_2 cause a saturation point where further photosynthesis does not increase, and there is clear photosynthetic acclimation in this study. It is possible to conclude that the observed down-regulation in photosynthesis resulted in decreased intercellular CO_2 concentration due to stomatal closure. Interestingly, an increase of 1000 ppm CO_2 level expedites chlorophyll breakdown and potentially promotes leaf senescence. The Bilensoy 80 alfalfa variety demonstrates a threshold at 1000 ppm CO_2 level to reduce the root to vegetative biomass ratio whereas the Nimet variety reduced the root to vegetative biomass ratio once CO_2 increases, starting from 600 ppm CO_2 level. In this study, extended CO_2 concentration generally resulted in increased biomass as expected. On the other hand, the quality of alfalfa has vital importance for animal forage. The total protein content serves as an indicator of forage quality. An 800 ppm CO_2 concentration, which is a high CO_2 level, negatively

influences protein efficiency in both Bilensoy 80 and Nimet varieties due to a negative relationship between total proteins and shoot yield. Photosynthetic acclimation of plants grown in high CO_2 is an outcome of reduced carboxylation effectiveness as a consequence of decreased protein content. However, a 1000 ppm CO_2 concentration recovers this reduction in protein in both varieties. This indicates another CO_2 level threshold for protein content. So, it has been concluded that alfalfa, as a C3 crop, shows a positive response under elevated CO_2 levels; however, there are thresholds.

Ethical approval

Ethical approval was not required for this study.

Funding

This research received no external funding.

Data availability statement

Relevant data are available from the corresponding author upon reasonable request.

Patents

It is not applicable.

Consent to participate

Consent to participate is not applicable.

Disclaimer/Publisher's Note

The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

CRediT authorship contribution statement

Tefide Kizildeniz: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The author appreciated the support provided by BSc student of Ahmet Fatih Akansu, and MSc student of Kadriye Yurtaslan, and thank to Prof. Dr. Mustafa Avcı from Niğde Ömer Halisdemir University for providing the seeds and sharing his precious knowledge about these alfalfa varieties. The experiment was conducted in Turgut Özal University, Faculty of Agriculture, and Department of Plant Production. Special thanks for the facilitation support in conducting the experiment and analyses that were provided by Prof. Dr. Nihat Tursun and kind support received from Doç. Dr. Ayşe Özlem Tursun and PhD candidate, Yücel Karaman from Turgut Özal University. The author also acknowledges the invaluable climate change research techniques taught by their PhD supervisors, Dr. Juan Jose Irigoyen from the Plant Stress Physiology Group at the Universidad de Navarra and Dr. Fermin Morales from the Instituto de Agrobiotecnología, CSIC.

References

[1] D.T. Loi, L.V. Huong, P.A. Tuan, N.T. Hong Nhung, T.T. Quynh Huong, B.T. Hoa Man, An assessment of agricultural vulnerability in the context of global climate change: a case study in ha tinh province, vietnam, Sustainability 14 (3) (2022) 1282.

[2] M. Getnet, K. Descheemaeker, M.K. van Ittersum, H. Hengsdijk, Narrowing crop yield gaps in Ethiopia under current and future climate: a model-based exploration of intensification options and their trade-offs with the water balance, Field Crops Res. 278 (2022) 108442.

[3] M.A. Ali, M. Hassan, M. Mehmood, D.H. Kazmi, F.A. Chishtie, I. Shahid, The potential impact of climate extremes on cotton and wheat crops in Southern Punjab, Pakistan, Sustainability 14 (3) (2022) 1609.

[4] S. Naz, Z. Fatima, P. Iqbal, A. Khan, I. Zakir, H. Ullah, S. Ahmad, An introduction to climate change phenomenon. Building Climate Resilience in Agriculture: Theory, Practice and Future Perspective, 2022, pp. 3–16.
[5] X. Jie, Greenhouse effects on the world agriculture—based on computable general equilibrium model analysis, in: 2010 International Conference on Mechanic Automation and Control Engineering, IEEE, 2010, pp. 2082–2085. June.
[6] W. Steffen, W. Broadgate, L. Deutsch, O. Gaffney, C. Ludwig, The trajectory of the Anthropocene: the great acceleration, Anthropocene Rev. 2 (1) (2015) 81–98.
[7] D. Toporov, P. Bocian, P. Heil, A. Kellermann, H. Stadler, S. Tschunko, R. Kneer, Detailed investigation of a pulverized fuel swirl flame in CO2/O2 atmosphere, Combust. Flame 155 (4) (2008) 605–618.
[8] C.H. Lim, B.S. Lim, A.R. Kim, D.U. Kim, J.W. Seol, J.H. Pi, C.S. Lee, Climate change adaptation through ecological restoration, in: Natural Resources Conservation and Advances for Sustainability, Elsevier, 2022, pp. 151–172.
[9] Z. Fatima, S. Naz, P. Iqbal, A. Khan, H. Ullah, G. Abbas, S. Ahmad, Field crops and climate change. Building Climate Resilience in Agriculture: Theory, Practice and Future Perspective, 2022, pp. 83–94.
[10] H. Elbasiouny, H. El-Ramady, F. Elbehiry, V.D. Rajput, T. Minkina, S. Mandzhieva, Plant nutrition under climate change and soil carbon sequestration, Sustainability 14 (2) (2022) 914.
[11] A.O. Siddiqui, A. Yazlık, K. Jabran, Weed management and climate change. Building Climate Resilience in Agriculture: Theory, Practice and Future Perspective, 2022, pp. 211–223.
[12] R.M. Adams, B.H. Hurd, S. Lenhart, N. Leary, Effects of global climate change on agriculture: an interpretative review, Clim. Res. 11 (1) (1998) 19–30.
[13] D. Cammarano, S. Ceccarelli, S. Grando, I. Romagosa, A. Benbelkacem, T. Akar, D. Ronga, The impact of climate change on barley yield in the Mediterranean basin, Eur. J. Agron. 106 (2019) 1–11.
[14] F.N. Tubiello, M. Donatelli, C. Rosenzweig, C.O. Stockle, Effects of climate change and elevated CO2 on cropping systems: model predictions at two Italian locations, Eur. J. Agron. 13 (2–3) (2000) 179–189.
[15] T. Yano, M. Aydin, T. Haraguchi, Impact of climate change on irrigation demand and crop growth in a Mediterranean environment of Turkey, Sensors 7 (10) (2007) 2297–2315.
[16] G. Maracchi, O. Sirotenko, M. Bindi, Impacts of present and future climate variability on agriculture and forestry in the temperate regions: Europe, Climatic Change 70 (1–2) (2005) 117–135.
[17] J.E. Olesen, M. Bindi, Consequences of climate change for European agricultural productivity, land use and policy, Eur. J. Agron. 16 (4) (2002) 239–262.
[18] S. Jian, S. Li, F. Liu, S. Liu, L. Gong, Y. Jiang, X. Li, Elevated atmospheric CO2 concentration changes the eco-physiological response of barley to polystyrene nanoplastics, Chem. Eng. J. 457 (2023) 141135.
[19] Z. Ünal, T. Kizildeniz, M. Özden, H. Aktaş, Ö. Karagöz, Detection of bruises on red apples using deep learning models, Sci. Hortic. 329 (2024) 113021.
[20] A. Latif, Y. Sun, A. Noman, Herbaceous Alfalfa plant as a multipurpose crop and predominant forage specie in Pakistan, Front. Sustain. Food Syst. 7 (2023) 1126151.
[21] A. Martsa, Exploring and Screening Rhizobia Nodulating Forage Legumes of Alfalfa (Medicago Sativa) and Vetch (Vicia Villosa) Growing in Ethiopia (Doctoral Dissertation, Arba Minch University, 2023.
[22] T. Li, S. Wan, G. Chen, Z. Cui, J. Wang, Z. Fan, Y. Zhai, Effects of planting Density and nitrogen application on soil greenhouse gas fluxes in the Jujube–alfalfa intercropping System in arid areas, Agronomy 14 (2) (2024) 273.
[23] M.C. Mortenson, G.E. Schuman, L.J. Ingram, Carbon sequestration in rangelands interseeded with yellow-flowering alfalfa (Medicago sativa ssp. falcata), Environ. Manag. 33 (2004) S475–S481.
[24] Y.Y. Duan, J. Zhang, Z. Jiang, X.X. Wei, Z.G. Guo, H.X. Liu, Partial root-zone drying (PRD) leads to lower carbon retention in the soil-plant systems of alfalfa, Irrigat. Sci. 42 (1) (2024) 45–56.
[25] J. Qi, D. Fu, X. Wang, F. Zhang, C. Ma, The effect of alfalfa cultivation on improving physicochemical properties soil microorganisms' community structure of grey desert soil, Sci. Rep. 13 (1) (2023) 13747.
[26] T.L. Anthony, D.J. Szutu, J.G. Verfaillie, D.D. Baldocchi, W.L. Silver, Carbon-sink potential of continuous alfalfa agriculture lowered by short-term nitrous oxide emission events, Nat. Commun. 14 (1) (2023) 1926.
[27] L. Deng, K.B. Wang, J.P. Li, Z.P. Shangguan, S. Sweeney, Carbon storage dynamics in alfalfa (Medicago sativa) fields in the Hilly-Gully region of the loess plateau, China, Clean: Soil, Air, Water 42 (9) (2014) 1253–1262.
[28] M. Farshadfar, M. Kakaei, Y. Salehabadi, Z. Baghaeifar, A.A. Jaffari, Study of diversity for yield and quality traits in alfalfa (Medicago sativa L.) and determination of the best population for cultivation in dryland farming in Iran, J. Rangeland Sci. 12 (1) (2022) 87–101.
[29] M.X. Zhang, L.Y. Zhao, Y.Y. He, J.P. Hu, G.W. Hu, Y. Zhu, J.L. Zhang, Potential roles of iron nanomaterials in enhancing growth and nitrogen fixation and modulating rhizomicrobiome in alfalfa (Medicago sativa L.), Bioresour. Technol. 391 (2024) 129987.
[30] R.F. Zhu, F.L. Tang, J.L. Liu, F.Q. Liu, X.Y. Deng, J.S. Chen, Co-inoculation of arbuscular mycorrhizae and nitrogen fixing bacteria enhance alfalfa yield under saline conditions, Pakistan J. Bot. 48 (2) (2016) 763–769.
[31] A. Guerchi, W. Mnafgui, A. Mengoni, M. Badri, Alfalfa (Medicago sativa L.)/Crops intercropping provides a feasible way to improve productivity under environmental constraints, J. Oasis Agric. Sustain. Dev. 5 (2) (2023) 38–47.
[32] K.A. Beauchemin, B.I. Farr, L.M. Rode, G.B. Schaalje, Effects of alfalfa silage chop length and supplementary long hay on chewing and milk production of dairy cows, J. Dairy Sci. 77 (5) (1994) 1326–1339.
[33] M.U. Ghani, M. Kamran, I. Ahmad, A. Arshad, C. Zhang, W. Zhu, F. Hou, Alfalfa-grass mixtures reduce greenhouse gas emissions and net global warming potential while maintaining yield advantages over monocultures, Sci. Total Environ. 849 (2022) 157765.
[34] R. Parajuli, M.T. Knudsen, S.N. Djomo, A. Corona, M. Birkved, T. Dalgaard, Environmental life cycle assessment of producing willow, alfalfa and straw from spring barley as feedstocks for bioenergy or biorefinery systems, Sci. Total Environ. 586 (2017) 226–240.
[35] P.A. Vadas, K.H. Barnett, D.J. Undersander, Economics and energy of ethanol production from alfalfa, corn, and switchgrass in the Upper Midwest, USA, Bioenergy Res. 1 (2008) 44–55.
[36] A. Gessler, R. Zweifel, Beyond source and sink control–toward an integrated approach to understand the carbon balance in plants, New Phytol. (2024).
[37] D.W. Lawlor, G. Cornic, Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants, Plant Cell Environ. 25 (2) (2002) 275–294.
[38] J.M. Muino, C. Großmann, T. Kleine, K. Kaufmann, Natural genetic variation in GLK1-mediated photosynthetic acclimation in response to light, BMC Plant Biol. 24 (1) (2024) 87.
[39] E.A. Ainsworth, A. Rogers, The response of photosynthesis and stomatal conductance to rising [CO2]: mechanisms and environmental interactions, Plant Cell Environ. 30 (3) (2007) 258–270.
[40] I. Aranjuelo, J.J. Irigoyen, S. Nogués, M. Sánchez-Díaz, Elevated CO2 and water-availability effect on gas exchange and nodule development in N2-fixing alfalfa plants, Environ. Exp. Bot. 65 (1) (2009) 18–26.
[41] G. Erice, I. Aranjuelo, J.J. Irigoyen, M. Sánchez-Díaz, Effect of elevated CO2, temperature and limited water supply on antioxidant status during regrowth of nodulated alfalfa, Physiol. Plantarum 130 (1) (2007) 33–45.
[42] I. Aranjuelo, P. Pérez, L. Hernández, J.J. Irigoyen, G. Zita, R. Martínez-Carrasco, M. Sánchez-Díaz, The response of nodulated alfalfa to water supply, temperature and elevated CO2: photosynthetic downregulation, Physiol. Plantarum 123 (3) (2005) 348–358.
[43] G. Erice, J.J. Irigoyen, M. Sánchez-Díaz, J.C. Avice, A. Ourry, Effect of drought, elevated CO2 and temperature on accumulation of N and vegetative storage proteins (VSP) in taproot of nodulated alfalfa before and after cutting, Plant Sci. 172 (5) (2007) 903–912.
[44] E.A. Ainsworth, A. Rogers, H. Blum, J. NoËsberger, S.P. Long, Variation in acclimation of photosynthesis in Trifolium repens after eight years of exposure to Free Air CO2 Enrichment (FACE), J. Exp. Bot. 54 (393) (2003) 2769–2774.
[45] Á. Sanz-Sáez, G. Erice, I. Aranjuelo, S. Nogués, J.J. Irigoyen, M. Sánchez-Díaz, Photosynthetic down-regulation under elevated CO2 exposure can be prevented by nitrogen supply in nodulated alfalfa, J. Plant Physiol. 167 (18) (2010) 1558–1565.

[46] L.H. Ziska, Rising atmospheric carbon dioxide and plant biology: the overlooked paradigm, DNA Cell Biol. 27 (4) (2008) 165–172.
[47] I. Aranjuelo, J.J. Irigoyen, P. Perez, R. Martinez-Carrasco, M. Sanchez-Diaz, Response of nodulated alfalfa to water supply, temperature and elevated CO2: productivity and water relations, Environ. Exp. Bot. 55 (1–2) (2006) 130–141.
[48] S. Li, X. Li, Z. Wei, F. Liu, ABA-mediated modulation of elevated CO2 on stomatal response to drought, Curr. Opin. Plant Biol. 56 (2020) 174–180.
[49] J.P. Castaño-Sánchez, C.A. Rotz, H.D. Karsten, A.R. Kemanian, Elevated atmospheric carbon dioxide effects on maize and alfalfa in the Northeast US: a comparison of model predictions and observed data, Agric. For. Meteorol. 291 (2020) 108093.
[50] I. Aranjuelo, J.J. Irigoyen, P. Perez, R. Martinez-Carrasco, M. Sanchez-Díaz, The use of temperature gradient tunnels for studying the combined effect of CO2, temperature and water availability in N2 fixing alfalfa plants, Ann. Appl. Biol. 146 (1) (2005) 51–60.
[51] G. Erice, J.J. Irigoyen, P. Pérez, R. Martínez-Carrasco, M. Sánchez-Díaz, Effect of elevated CO2, temperature and drought on dry matter partitioning and photosynthesis before and after cutting of nodulated alfalfa, Plant Sci. 170 (6) (2006) 1059–1067.
[52] G. Erice, J.J. Irigoyen, P. Pérez, R. Martínez-Carrasco, M. Sánchez-Díaz, Effect of elevated CO2, temperature and drought on photosynthesis of nodulated alfalfa during a cutting regrowth cycle, Physiol. Plantarum 126 (3) (2006) 458–468.
[53] Q. Jing, B. Qian, G. Bélanger, A. VanderZaag, G. Jégo, W. Smith, G. Hoogenboom, Simulating alfalfa regrowth and biomass in eastern Canada using the CSM-CROPGRO-perennial forage model, Eur. J. Agron. 113 (2020) 125971.
[54] C. Wang, B.L. Ma, X. Yan, J. Han, Y. Guo, Y. Wang, P. Li, Yields of alfalfa varieties with different fall-dormancy levels in a temperate environment, Agron. J. 101 (5) (2009) 1146–1152.
[55] D.P. Malinowski, W.E. Pinchak, B.A. Kramp, H. Zuo, T.J. Butler, Supplemental irrigation and fall dormancy effects on alfalfa productivity in a semiarid, subtropical climate with a bimodal precipitation pattern, Agron. J. 99 (3) (2007) 621–629.
[56] Öten M., S. Albayrak, Yüksek dormansi grubunda bulunan yonca (Medicago sativa L.) Genotiplerinin Verim ve Kalite Özellikleri, Anadolu Tarım Bilimleri Dergisi 36 (2) (2021) 293–300.
[57] T.C. Tarım ve Orman Bakanlığı1, Bilensoy 80 (Yonca), Available online: https://arastirma.tarimorman.gov.tr/tarlabitkileri/Menu/114/Yonca, 2020 (accessed on 30.November.2022).
[58] T.C. Tarım ve Orman Bakanlığı, Yonca Islahı Araştırmaları, Available online: https://arastirma.tarimorman.gov.tr/cukurovataem/Belgeler/Yay%C4%B1mlar/yonca.pdf, 2020 (accessed on 30.November.2022).
[59] M. Avci, R. Hatipoglu, S. Çinar, N. Kiliçalp, Assessment of yield and quality characteristics of alfalfa (Medicago sativa L.) cultivars with different fall dormancy rating, Legume Res.- Int. J. 41 (3) (2018) 369–373.
[60] M. Özkurt, İ. Saygili, K.Ö. Dirik, Bazı yonca (Medicago sativa L.) çeşitlerinin erken gelişme dönemindeki tuz toleransının belirlenmesi, Int. J. Agric. Nat. Sci. 1 (3) (2018) 251–258.
[61] A. Yasar, Y. Karaman, I. Gokbulut, A.O. Tursun, N. Tursun, I. Uremis, M. Arslan, Chemical composition and herbicidal activities of essential oil from aerial parts of Origanum hybrids grown in different global climate scenarios on seed germination of Amaranthus palmeri, J. Essential Oil-Bear. Plants 24 (3) (2021) 603–616.
[62] A.O. Tursun, I. Telci, The effects of carbon dioxide and temperature on essential oil composition of purple basil (Ocimum basilicum L.), J. Essential Oil-Bearing Plants 23 (2) (2020) 255–265.
[63] M. Sánchez-Díaz, J.J. Irigoyen, N. Gómez-Casanovas, A. Pardo, J. Azcón-Bieto, El cambio climático global. Efecto previsible del CO2 sobre los vegetales, in: La Ecofisiología Vegetal. Una Ciencia de Síntesis, Universidad de Vigo Vigo, Spain, 2004, pp. 1111–1140.
[64] T. Kizildeniz, I. Pascual, J.J. Irigoyen, F. Morales, Future CO2, warming and water deficit impact white and red Tempranillo grapevine: photosynthetic acclimation to elevated CO2 and biomass allocation, Physiol. Plantarum 172 (3) (2021) 1779–1794.
[65] H. Medrano, J.M. Escalona, J. Bota, J. Gulías, J. Flexas, Regulation of photosynthesis of C3 plants in response to progressive drought: stomatal conductance as a reference parameter, Ann. Bot. 89 (7) (2002) 895–905.
[66] T.G. Shibaeva, A.V. Mamaev, E.G. Sherudilo, Evaluation of a SPAD-502 plus chlorophyll meter to estimate chlorophyll content in leaves with interveinal chlorosis, Russ. J. Plant Physiol. 67 (2020) 690–696.
[67] M.B. Hani, Y.A. Othman, M.G. Al-Ajlouni, T.S. A'saf, Deep planting improved stem root growth, flower yield and quality of Lilium cultivars, Hortic. Bras. 40 (2022) 143–150.
[68] AOAC (Association Official Analytic Chemists), Official Methods of Analysis, fifteenth ed., AOAC. Inc. Anc. Arlington, Virginia, U.S.A., 1990, p. 1298.
[69] J. Kjeldahl, New method for the determination of nitrogen, Chem. News 48 (1240) (1883) 101–102.
[70] I. De Luis, J.J. Irigoyen, M. Sánchez-Díaz, Elevated CO2 enhances plant growth in droughted N2-fixing alfalfa without improving water status, Physiol. Plantarum 107 (1) (1999) 84–89.
[71] M.C. Antolín, M. Sánchez-Díaz, Effects of temporary droughts on photosynthesis of alfalfa plants, J. Exp. Bot. 44 (8) (1993) 1341–1349.
[72] R. Munns, Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses, Plant Cell Environ. 16 (1) (1993) 15–24.
[73] K. Chen, G. Hu, N. Keutgen, M. Blanke, F. Lenz, Effects of CO2 concentration on strawberry. II. Leaf photosynthetic function, Angew. Bot. 71 (5–6) (1997) 173–178.
[74] K. Chen, G. Hu, N. Keutgen, M.J. Janssens, F. Lenz, Effects of NaCl salinity and CO2 enrichment on pepino (Solanum muricatum Ait.): II. Leaf photosynthetic properties and gas exchange, Sci. Hortic. 81 (1) (1999) 43–56.
[75] T. Kizildeniz, Effects of Climate Change on Nodulated Alfalfa Plants: Physiology, Biochemical and Molecular Analyses, Master Thesis, Mediterranean Agronomic Institute of Zaragoza-University of Lleida, Spain, 2013.
[76] I.D. Luis, J.J. Irigoyen, M. Sánchez-Díaz, Low vapour pressure deficit reduces the beneficial effect of elevated CO2 on growth of N2-fixing alfalfa plants, Physiol. Plantarum 116 (4) (2002) 497–502.
[77] S.C. Wong, Elevated atmospheric partial pressure of CO 2 and plant growth: II. Non-structural carbohydrate content in cotton plants and its effect on growth parameters, Photosynth. Res. 23 (1990) 171–180.
[78] G.A. Finn, W.A. Brun, Effect of atmospheric CO2 enrichment on growth, nonstructural carbohydrate content, and root nodule activity in soybean, Plant Physiol. 69 (2) (1982) 327–331.
[79] Á. Sanz-Sáez, G. Erice, J. Aguirreolea, F. Muñoz, M. Sánchez-Díaz, J.J. Irigoyen, Alfalfa forage digestibility, quality and yield under future climate change scenarios vary with Sinorhizobium meliloti strain, J. Plant Physiol. 169 (8) (2012) 782–788.
[80] R.K. Momin, V.B. Kadam, Determination of ash values of some medicinal plants of genus Sesbania of Marathwada region in Maharashtra, J. Phytol. 3 (12) (2011).
[81] G. Kebede, G. Assefa, F. Feyissa, T. Tekletsadik, M. Minta, Tesfaye, M. Yield and quality of alfalfa (Medicago sativa), in: G. Assefa, F. Feyissa, A. Lakew (Eds.), Results of Livestock Research 2015, Ethiopian Institute of Agricultural Research: Holetta, Ethiopia, 2018, pp. 147–161. ISSN: 9789994466559.
[82] D.I. Leskovar, Y.A. Othman, Direct Seeding and transplanting influence root dynamics, morpho-physiology, yield, and head quality of globe artichoke, Plants 10 (5) (2021) 899.
[83] W.M. Jarrell, R.B. Beverly, The dilution effect in plant nutrition studies, Adv. Agron. 34 (1981) 197–224.
[84] D.W. Lawlor, R.A.C. Mitchell, The effects of increasing CO2 on crop photosynthesis and productivity: a review of field studies, Plant Cell Environ. 14 (8) (1991) 807–818.
[85] K.E. Idso, S.B. Idso, Plant responses to atmospheric CO2 enrichment in the face of environmental constraints: a review of the past 10 years' research, Agric. For. Meteorol. 69 (3–4) (1994) 153–203.
[86] B.A. Kimball, Carbon dioxide and agricultural yield: an assemblage and analysis of 430 prior observations 1, Agron. J. 75 (5) (1983) 779–788.