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Short-term exposure to elevated temperature and CO_2 alters phytoestrogen production in red clover

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Phytoestrogens are plant-produced secondary compounds that mimic the animal sex hormone estrogen. Several legumes, including red clover, produce phytoestrogens as stress defense molecules, and climate change-driven increases in atmospheric temperature and CO_2 may intensify their production. We conducted a growth chamber study to determine the effects of short-term exposure to elevated temperature (*e*T) and CO_2 (*e*CO_2), both alone and in combination, on phytoestrogen concentrations in red clover and cowpea. Plants were grown in ambient conditions (24/18 °C, day/night, and ~400 ppm CO_2) and then exposed to *e*T (35/26 °C, day/night), *e*CO_2 (750 ± 50 ppm), or both factors for 10 days. Phytoestrogen concentrations in cowpea vegetative tissues were below the level of detection under all conditions. In red clover, exposure to *e*T reduced total phytoestrogen concentration by 50%, from 3.9 to 1.9 mg/g dry matter. Most of this decrease was driven by reduced concentrations of the isoflavones formononetin and biochanin A. Elevated CO_2 did not influence total phytoestrogen levels in red clover but reduced daidzein concentration by 43%. Plant physiological variables measured concurrently with phytoestrogens were weakly correlated with concentrations of individual phytoestrogen compounds and total phytoestrogens in red clover.

Keywords Climate change, CO_2 enrichment, Heat stress, Isoflavone, *Trifolium pratense*, *Vigna unguiculata*

Red clover (*Trifolium pratense L.*) and cowpea (*Vigna unguiculata* L. Walp) are two agriculturally important legumes grown as animal feed[1,2]. Red clover is widely grown as a forage crop in temperate regions throughout the world, while cowpea, a common food and forage crop in Africa and Asia, is increasingly being grown in portions of the United States, especially as a summer annual forage[3,4]. While widely grown as forages for livestock, some legume species can produce phytoestrogens, which are secondary metabolites that can structurally and functionally mimic the mammalian sex hormone estrogen[5]. Excess consumption of phytoestrogen-rich forage legumes may impair animal reproductive performance[6–8]. Further, phytoestrogens ingested by dairy cows can be transferred to their milk and consequently enter into the human food chain[9,10].

Previous research has shown that the isoflavones formononetin and biochanin A are the primary phytoestrogens in red clover, with concentrations ranging from 0.6 to 17 mg/g of dry matter (DM) depending on the cultivar and growing conditions[11,12]. In contrast, cowpea seeds have been shown to contain low concentrations of phytoestrogens (< 0.02 mg/g of DM), mainly as the isoflavones daidzein, genistein, and biochanin A[13,14]. However, to the best of our knowledge there is no information on the concentrations of phytoestrogens in the vegetative tissues of cowpea that would be consumed as forage.

A number of phytoestrogenic legume species, including red clover, have been observed to upregulate their phytoestrogen production in response to environmental stress factors such as drought, ozone (O_3) level, and fungal diseases[15,16]. Some physiological responses to excess heat are similar to other phytoestrogen-inducing environmental stressors, but it is unclear if phytoestrogen production is associated with temperature in forage legumes[17]. In portions of the northeastern United States, such as New England, the average annual temperature has increased by 1.7 °C or more since 1901, and much of this increase has occurred during the summer months when forages are actively being grazed or harvested for stored feed such as silage and hay[18,19]. By 2035, the

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Northeast United States is expected to experience more frequent and severe heatwaves and an increase in average temperature of over 2 °C from preindustrial levels[19]. These changes in temperature are driven, in part, by increases in atmospheric CO_2 concentration. At present, atmospheric CO_2 concentrations (425 ppm) are nearly double what they were in 1880 (280 ppm), and by the end of the century, concentrations could reach 700 ppm[20,21]. Both atmospheric CO_2 levels and temperature play important roles in regulating plant physiological and biochemical processes[22,23]; however, their individual and interactive effects on phytoestrogen production are not well understood.

Increasing temperature generally elicits an unimodal growth response in plants; growth rate increases as increased stomatal conductance, respiration, and transpiration support higher photosynthetic rates, and then declines as plants experience environmental (e.g. water deficits) and physiological limitations from temperature stress[24,25]. Increased concentrations of CO_2 can counter some of the negative effects of temperature stress by decreasing stomatal conductance and increasing water use efficiency[26,27]. Further, changes in CO_2 can alter plant chemical composition, including changing concentrations of proteins and minerals like iron and zinc[28–30].

Whether similar compensatory physiological and biochemical processes mediate the stress response and phytoestrogen production of forage legumes under *e*T and eCO_2 remains unclear. Additionally, while cool season red clover and warm season cowpea are both C3 legumes, they have different temperature preferences and therefore may be expected to respond differently to elevated temperature (*e*T) and elevated CO_2 (eCO_2).

The goal of this research was to understand how *e*T and eCO_2 associated with climate change may influence the accumulation of phytoestrogens in the vegetative tissues of two important C3 forage legumes with differing temperature optima. Hence, our objectives in this study were to: (i) quantify the effects of *e*T and eCO_2 individually and in combination on phytoestrogen concentrations in red clover and cowpea, two species expected to respond differently to these stress factors, and (ii) assess the relationship between phytoestrogen concentrations and physiological indicators of plant stress. Our rationale for the second objective was two-fold. First, physiological indicators of plant stress would help point to potential mechanisms mediating the response of phytoestrogens to treatments. Second, phytoestrogens are labor intensive and expensive to measure; thus, physiological indicators that are correlated with phytoestrogen levels in legumes could be used to develop farmer decision support tools for determining whether such legumes are "safe" to graze or harvest.

Results

Phytoestrogen concentrations

Phytoestrogen concentrations in cowpea were below our method's quantification level (~ 1.2 µg/ml) regardless of stress treatment (data not shown). In contrast, phytoestrogen concentrations in red clover were well above the detection level and varied by treatment ($p < 0.01$; Fig. 1). Compared to the ambient control (3.9 mg/g), *e*T, when applied alone and in combination with eCO_2, resulted in a 50% (2.0 mg/g) and 53% (1.8 mg/g) reduction in total phytoestrogen concentration, respectively. However, total phytoestrogen concentration in red clover did not differ compared to the control when eCO_2 was applied alone. A representative chromatogram of red clover and cowpea is shown in Supplementary Figure S1.

Individual phytoestrogen compounds in red clover varied in their responses to the treatments (Fig. 2). Compared to the control, concentration of formononetin was 49% and 47% lower, respectively, in the *e*T and $eT + eCO_2$ conditions ($p < 0.05$, Fig. 2a). Similarly, compared to the control, biochanin A concentration was 63% lower in $eT + eCO_2$ ($p < 0.05$, Fig. 2b). In contrast, daidzein concentration was influenced more by CO_2 level than temperature and was reduced by 43% in eCO_2 and 42% in $eT + eCO_2$ ($p < 0.01$, Fig. 2c). The concentrations of genistein, glycitein, and prunetin were not influenced by the treatments.

Physical, physiological, and total phenolic responses

Effects of the treatments on physical responses were visually apparent in both legume species (Fig. 3). Temperature and CO_2 treatments affected aboveground biomass, plant height, and leaf area in both cowpea and red clover; however, for each species, the nature of these effects varied depending on the treatment (Table 1). In cowpea, biomass ($p < 0.01$) and leaf area ($p < 0.05$) were greater in eCO_2 compared to the ambient control. Leaf area of cowpea in the $eT + eCO_2$ treatment was also greater compared to the control, but did not differ from eCO_2. In contrast, the height of cowpea was reduced in the $eT + eCO_2$ compared to the eCO_2 treatment, but neither treatment differed from the ambient control ($p < 0.01$). Relative to the ambient control, *e*T alone did not affect any of the four physical variables measured on cowpea. In red clover, none of the treatments resulted in growth differences compared to the ambient control. However, aboveground biomass ($p < 0.05$), plant height ($p < 0.01$), and leaf area ($p < 0.01$) were all greater in the eCO_2 treatment compared to the treatments with elevated temperature, either alone (*e*T) or in combination with elevated CO_2 ($eT + eCO_2$; Table 1).

In general, cowpea was physiologically responsive to the treatments (Table 1). Elevated temperature alone altered all physiological variables relative to the ambient control except for net photosynthetic rate. Elevated CO_2 alone altered the same variables as did *e*T, except for SPAD, net photosynthetic rate, and total phenolics. In contrast, the only physiological variables measured that differed from the control under the combination of *e*T and eCO_2 were vapor pressure deficit, water use efficiency, and total phenolics (Table 1).

Red clover was less physiologically responsive to the treatments (Table 1). The only physiological variable that differed under *e*T compared to the ambient control was vapor pressure deficit ($p < 0.01$), which was greater in all treatments compared to the control (Table 1). Relative to the control, elevated CO_2 alone and in combination with elevated temperature ($eT + eCO_2$) resulted in decreased stomatal conductance ($p < 0.01$), net photosynthetic rate ($p < 0.01$), and transpiration rate ($p < 0.01$), but did not affect any of the other physiological variables measured on red clover. The combination of the two factors ($eT + eCO_2$) was the only treatment that affected SPAD values relative to the control ($p < 0.01$, Table 1).

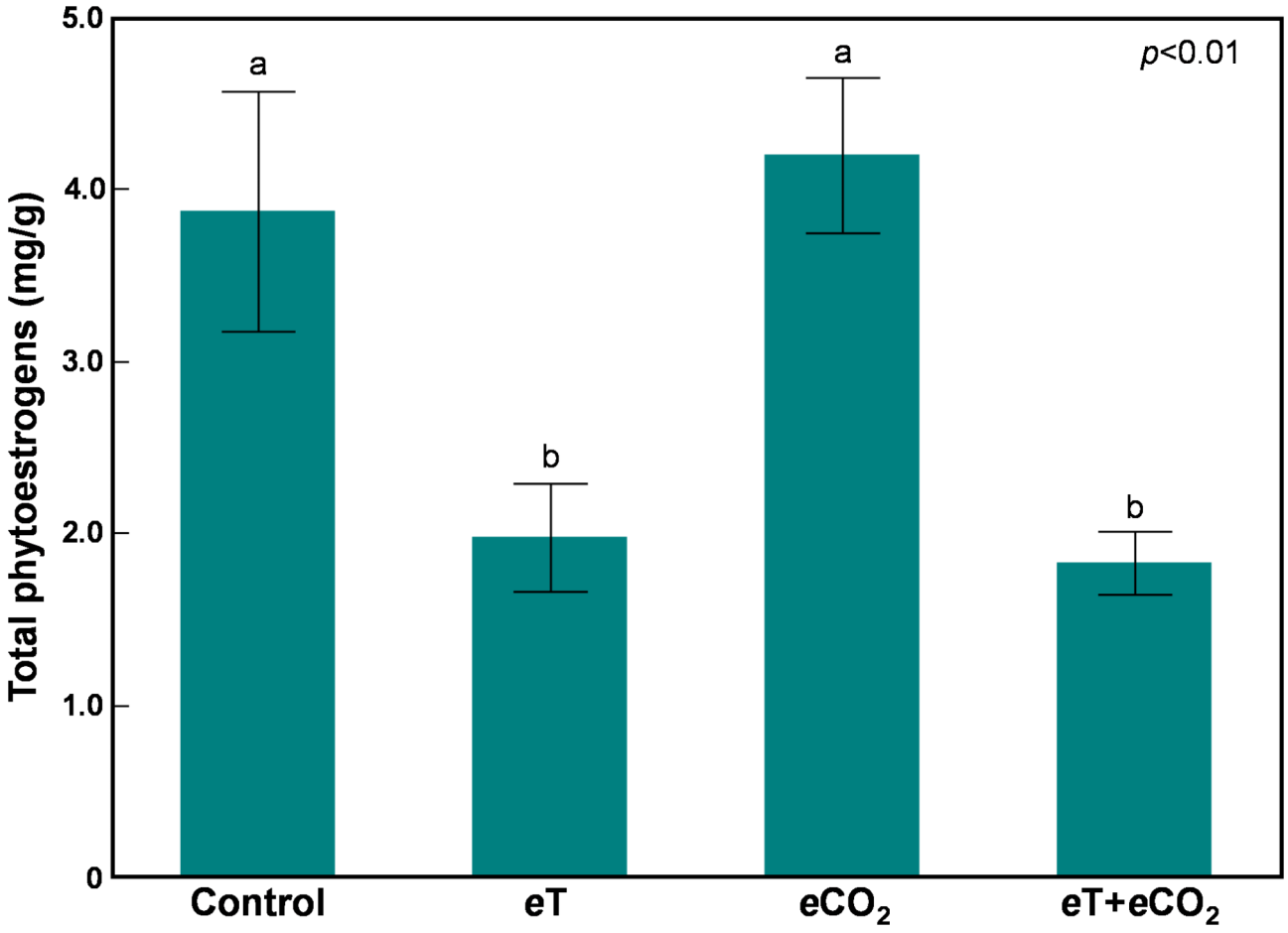

Fig. 1. Total phytoestrogen concentration in red clover (mg/g of dry matter) after 10 days of exposure to elevated temperature (eT) and CO_2 (eCO_2). Bars indicate standard errors of the mean ($n = 4$). Treatments sharing the same letter are not significantly different ($\alpha = 0.05$) as determined by Tukey's HSD test.

Correlation between phytoestrogen concentration and physiological responses

We calculated Spearman correlation coefficients to investigate relationships between physiological responses and concentrations of individual phytoestrogen compounds in red clover (Fig. 4). Of the two most abundant phytoestrogen compounds in red clover, only biochanin A was correlated with a physiological variable, specifically a negative correlation ($p < 0.05$) with vapor pressure deficit. In contrast, daidzein concentration was positively correlated with sub-stomatal CO_2 concentration, stomatal conductance, transpiration rate, and net photosynthetic rate, and negatively correlated with SPAD. The only other correlations observed were between genistein concentration and water use efficiency and between prunetin and total phenolic content.

We used partial least-squares regression (PLSR) to model the relationship between total phytoestrogen concentration and the physiological variables measured on red clover. The initial PLSR model included all seven physiological variables and total phenolics as predictor variables. This model explained 93.7% of the variation in the predictor variables and 54.4% of the variation in total phytoestrogen concentration across four factors. A reduced model was then fit using only the predictor variables with a Variable Importance of Projection (VIP) > 0.8. This reduced model accounted for 51% of the variation in total phytoestrogen concentration across two factors. Physiological variables retained in this reduced model were vapor pressure deficit, water use efficiency, SPAD, and total phenolic content. Variables loading most strongly on PLSR factor 1 ($R^2 = 44.1\%$) were vapor pressure deficit and SPAD while total phenolic content and SPAD loaded most strongly on PLSR factor 2 ($R^2 = 7.1\%$) (Fig. 5).

Discussion

We exposed the forage legumes cowpea and red clover to elevated temperature (eT) and elevated CO_2 (eCO_2) conditions for 10 days in a growth chamber to determine the individual and interactive effect of these two potential stress factors on the production of phytoestrogens. Previous research has demonstrated that cowpea seeds contain low concentrations of phytoestrogens[13,31], and that concurrent application of eT and eCO_2 can decrease isoflavone concentrations in seeds of other legumes such as soybean[32]. We found phytoestrogen concentrations in cowpea vegetative tissues to be below the level of detection of our method. To our knowledge, ours is the first study to document the lack of phytoestrogens in cowpea vegetative tissues. Importantly, the

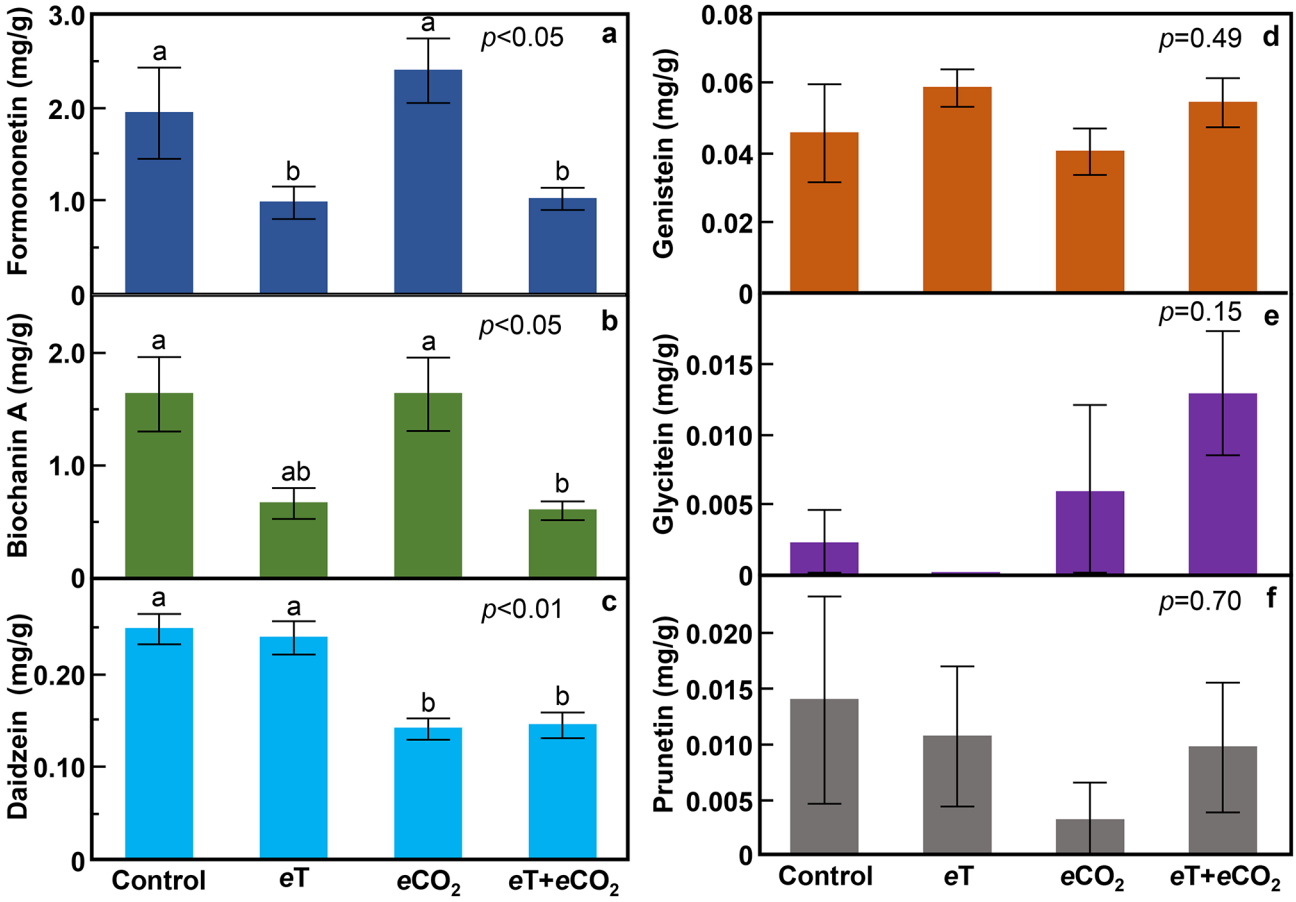

Fig. 2. Effects of 10 days of exposure to elevated temperature (*e*T) and CO_2 (eCO_2) on concentrations (mg/g of dry matter) of individual isoflavones formononetin (**a**), biochanin A (**b**), daidzein (**c**), genistein (**d**), glycitein (**e**), and prunetin (**f**) in red clover. Bars indicate standard errors of the mean ($n = 4$). Treatments sharing the same letter are not significantly different ($\alpha = 0.05$) as determined by Tukey's HSD test.

apparent lack of phytoestrogen accumulation in cowpea vegetative tissues was independent of the observed changes in physiological and growth parameters such as stomatal conductance, transpiration, and photosynthetic rate in response to *e*T and eCO_2 treatments. The physiological and growth responses we observed in cowpea are largely consistent with other research[33]. In contrast to our results, however, Singh et al.[34] observed an increase in net photosynthesis rate in cowpea cultivars after eight days of exposure to a combination of elevated temperature and CO_2, levels of which differed from those used in our study. Whether cowpea might upregulate phytoestrogen concentrations in vegetative tissues under other types of environmental stress, such as drought or pest damage, or how *e*T or eCO_2 may influence phytoestrogen concentrations in later plant developmental stages or in cowpea seeds, remains unknown.

Formononetin and biochanin A were the most abundant phytoestrogen compounds in red clover, together accounting for 83–96% of the total phytoestrogens measured, depending on treatment, which is in line with previous studies[17,35]. Among the phytoestrogens present in forage legumes, formononetin has received substantial attention from researchers because it is metabolized by the ruminal microbiota of large and small ruminants to equol, a more potent estrogenic compound[36]. Previous research has shown that formononetin concentrations of > 3 mg/g of DM can cause infertility in sheep[37]; however, the threshold levels for other ruminant species are largely unknown. The concentration of formononetin in red clover in our ambient control treatment (1.9 mg/g of DM) was below this critical level and was reduced further by *e*T (1.0 mg/g of DM in both the *e*T and *e*T + eCO_2 treatments). Baseline formononetin concentrations in red clover can vary depending on a range of factors, including cultivar, plant maturity and developmental stage, and growing conditions[11,12,17,32,38]. Formononetin concentrations in red clover grown under our ambient control treatment conditions were lower than those observed in a previous greenhouse study conducted using the same cultivar at similar maturity (3.2 mg/g of DM)[17], possibly due to differences in the ambient growing conditions in the climate-controlled growth chamber compared to those in a greenhouse[32].

Contrary to our hypothesis, we found that *e*T depressed total phytoestrogen production in red clover vegetative tissues. This result is congruent with previous research examining the effects of temperature on phytoestrogen contents of soybean seeds and pods[32,39,40]. For example, compared to when soybean seed development occurred under 18 °C, isoflavone concentration in seeds developing under high temperature (28 °C) was decreased by

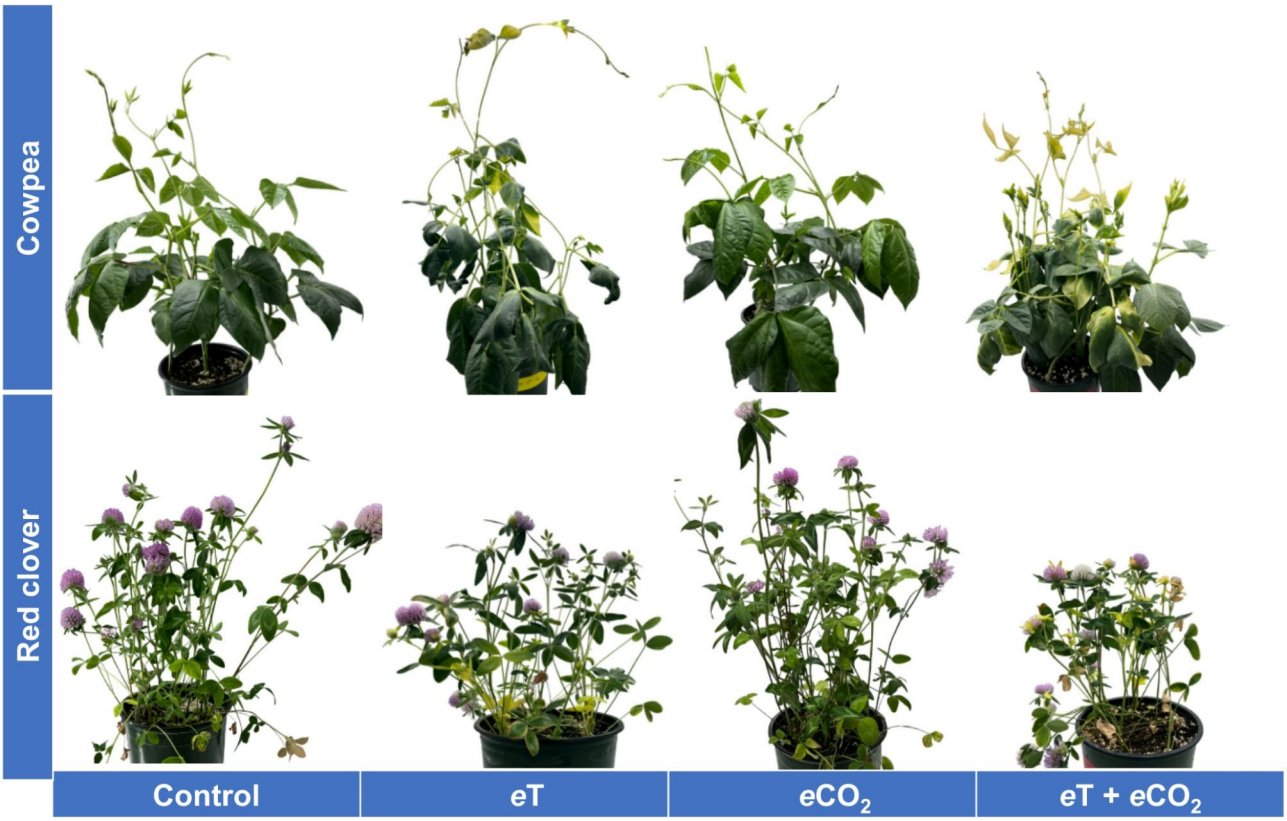

Fig. 3. Representative photographs of cowpea in the vegetative stage (upper row) and red clover in the flowering stage (lower row) in each stress treatment following 10 days of exposure to elevated temperature (*e*T) and CO_2 (eCO_2).

	Cowpea					Red clover				
Variables [a]	Control	*e*T	eCO_2	*e*T + eCO_2	ANOVA	Control	*e*T	eCO_2	*e*T + eCO_2	ANOVA
AGB	15.3 ± 1.7 b	13.9 ± 0.8 b	21.3 ± 1.2 a	17.6 ± 0.6 ab	$p < 0.01$	4.5 ± 0.9 ab	2.6 ± 0.5 b	6.3 ± 0.1 a	2.8 ± 0.2 b	*$p < 0.05$
PH	92.1 ± 5.2 ab	107.4 ± 3.4 a	104.8 ± 4.8 a	79.6 ± 6.6 b	$p < 0.01$	44.4 ± 1.7 ab	31.9 ± 1.0 b	50.3 ± 2.5 a	30.6 ± 1.3 b	$p < 0.01$
L/P	12.0 ± 2.1	14.0 ± 0.7	12.6 ± 0.8	12.9 ± 0.6	$p > 0.05$	22.5 ± 3.6	18.9 ± 1.9	28.1 ± 1.3	19.3 ± 2.9	*$p > 0.05$
LA/P	1047.2 ± 106.7 b	1161.9 ± 107.5 ab	1454.9 ± 96.9 a	1443.1 ± 56.0 a	$p < 0.05$	149.5 ± 22.6 ab	94.5 ± 5.3 b	203.9 ± 17.2 a	86.3 ± 12.0 b	$p < 0.01$
SPAD	53.4 ± 1.9 b	70.8 ± 1.8 a	59.0 ± 2.2 b	60.1 ± 0.6 b	$p < 0.01$	54.1 ± 0.6 b	54.5 ± 1.6 b	58.5 ± 1.0 ab	63.3 ± 2.0 a	$p < 0.01$
Ci	154.6 ± 12.3 c	257.5 ± 11.1 a	241.2 ± 8.6 ab	194.8 ± 14.02 bc	$p < 0.01$	272.8 ± 2.5	257.8 ± 6.6	195.3 ± 37.9	236.0 ± 13.2	$p > 0.05$
gs	147.6 ± 14.0 c	307.7 ± 39.9 b	418.0 ± 23.7 a	151.9 ± 18.6 c	$p < 0.01$	709.0 ± 49.1 a	588.9 ± 114.8 ab	251.9 ± 91.9 bc	202.0 ± 24.8 c	$p < 0.01$
A	17.8 ± 1.8 abc	16.5 ± 1.6 bc	25.6 ± 1.2 ab	13.9 ± 1.5 c	$p < 0.01$	28.4 ± 1.3 a	23.9 ± 3.5 a	13.7 ± 1.6 b	14.0 ± 1.5 b	$p < 0.01$
E	4.1 ± 0.4 b	8.8 ± 0.9 a	8.5 ± 0.5 a	5.0 ± 0.5 b	$p < 0.01$	11.1 ± 0.3 a	11.8 ± 1.7 a	5.4 ± 1.6 b	5.5 ± 0.7 b	$p < 0.01$
VPD	2.9 ± 0.1 b	3.2 ± 0.1 a	2.3 ± 0.1 c	3.5 ± 0.04 a	$p < 0.01$	2.0 ± 0.1 b	2.8 ± 0.2 a	2.7 ± 0.1 a	3.0 ± 0.1 a	$p < 0.01$
WUE	4.3 ± 0.2 a	1.9 ± 0.2 c	3.0 ± 0.2 b	2.9 ± 0.2 b	$p < 0.01$	2.6 ± 0.1	2.1 ± 0.1	3.7 ± 0.8	2.6 ± 0.2	* $p > 0.05$
TP	16.6 ± 0.9 b	30.2 ± 0.9 a	12.8 ± 0.8 b	28.0 ± 1.78 a	$p < 0.01$	14.5 ± 1.78	14.1 ± 1.1	14.2 ± 1.4	13.2 ± 0.1	$p > 0.05$

Table 1. Results of ANOVA (or Kruskal–Wallis test*) conducted on physical, physiological, and biochemical responses (treatment means ± se, $n = 4$) of red clover (cv 'Freedom!') and Cowpea (cv 'Red Ripper') after 10 days of exposure to elevated temperature (*e*T) and CO_2 (eCO_2). For each species and response variable, treatments sharing the same letter are not significantly different according to Tukey HSD test, $p < 0.05$. [a] Abbreviations: variables are AGB, aboveground biomass per plant (g); PH, plant height (cm); L/P, number of leaves per plant; LA/P, leaf area per plant (cm^2); SPAD, chlorophyll content; Ci, sub-stomatal CO_2 concentration (μmol mol^{-1}); gs, stomatal conductance (mmol H_2O m^{-2} s^{-1}); A, net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}); E, transpiration rate (mmol H_2O m^{-2} s^{-1}); VPD, vapor pressure deficit (kPa); WUE, water use efficiency (mmol CO_2 mol H_2O); and TP, Total phenolics (mg/g dry matter). The asterisk (*) represents Kruskal–Wallis test where did not satisfy variance and distributional assumptions of ANOVA.

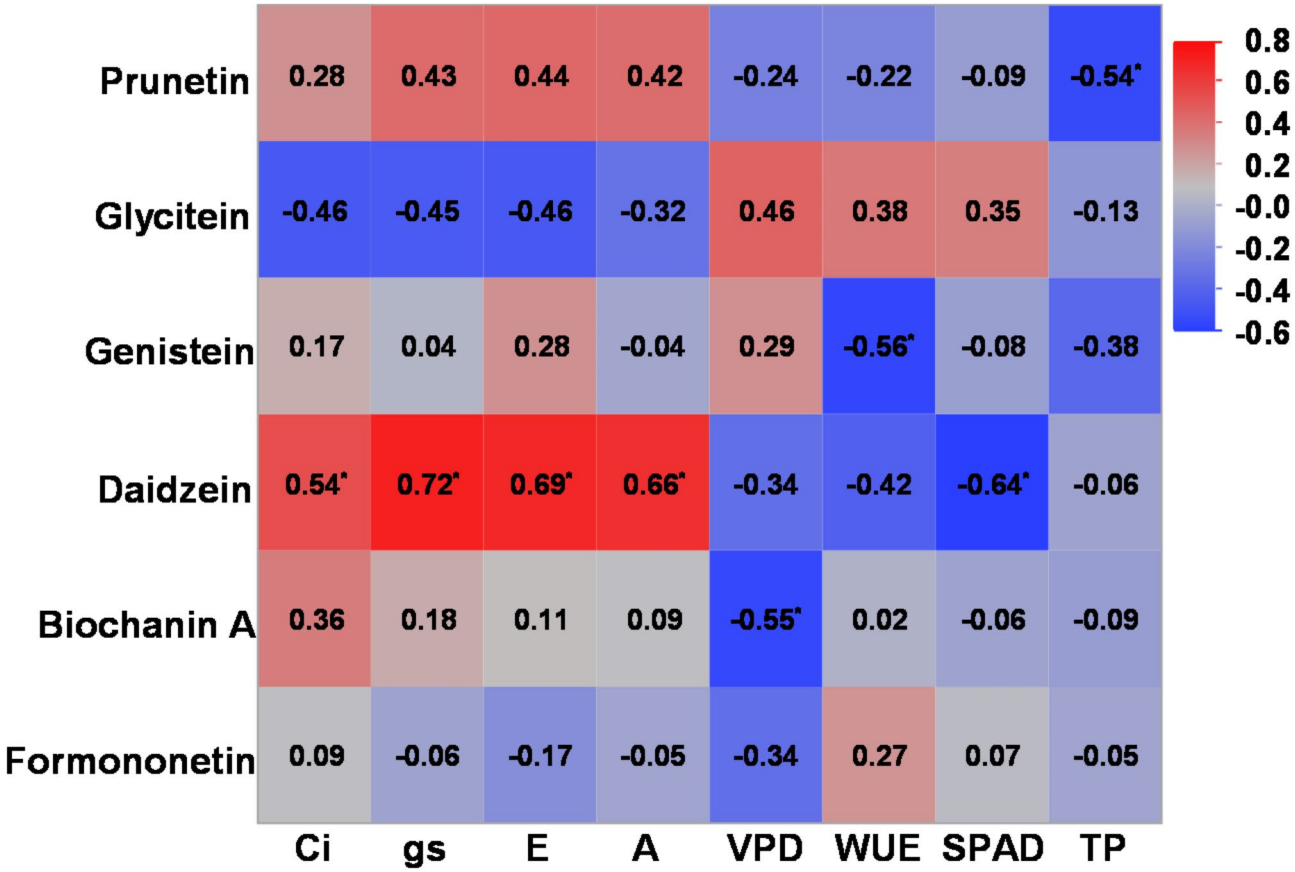

Fig. 4. Heatmap matrix showing Spearman correlation coefficients between physiological responses and concentrations of individual phytoestrogen compounds in red clover. Positive correlations are indicated in red and negative correlations are indicated in blue, while the strength of the correlation (value in box) is indicated by color intensity. The asterisk (*) indicates a significant correlation ($p < 0.05$) between variables. Physiological variables are Ci, sub-stomatal CO_2 concentration; gs, stomatal conductance; E, transpiration rate; A, net photosynthetic rate; VPD, vapor pressure deficit; WUE, water use efficiency; and SPAD, chlorophyll content. TP is total phenolic content.

nearly 90%[32]. The decrease in isoflavone synthesis in some legumes under heat stress may have several potential explanations. Plants may prioritize use of carbon in producing primary metabolites to sustain plant growth or shift the precursor metabolites of isoflavones toward synthesizing other stress response secondary metabolites like flavonols, tannins, or glyceollins[41]. Alternatively, heat stress may reduce the activity or stability of key enzymes involved in isoflavone synthase and change the gene expression, limiting the accumulation of these compounds[42,43]. Lastly, plants may follow stress induced resource allocation, where they prioritize resources to synthesize heat shock proteins and to activate other protective mechanisms at the expense of isoflavone production[44]. However, determining which of these mechanisms may explain our observations in red clover requires further research.

Isoflavone synthesis through the phenylpropanoid pathway requires carbon, with about 20% of photosynthetic carbon going toward making phenolic compounds, including flavonoids[45,46]. In soybean, increased levels of CO_2 have been shown to increase isoflavone concentrations in seeds and this can offset the declines in concentrations due to high temperature[32,39,40]. Our observation that red clover exposed to eCO_2 either alone or in combination with eT did not increase formononetin and biochanin A concentrations suggests that synthesis of these compounds was not carbon limited despite stomatal conductance and net photosynthetic rates being negatively affected by our treatments.

We found that daidzein concentration in red clover responded to the treatments differently than did formononetin and biochanin A, and it was influenced only by eCO_2. Daidzein is biosynthesized along the phenylpropanoid pathway, but is also a substrate for the synthesis of glyceollins[47]. Hence, whether eCO_2 reduced daidzein concentrations by reducing the activity of specific enzymes involved in daidzein synthesis or by stimulating the conversion of daidzein to glyceollins is unclear. Regardless of mechanism, the observed reduction in daidzein concentration we observed under eCO_2 and lack of response to eT contrasts with what has been observed in soybeans, where high-temperature stress during either the vegetative or seed development stage led to increased daidzein concentrations in the seeds and the effects of CO_2 enrichment on soybean physiological performance were found to be temperature dependent[48].

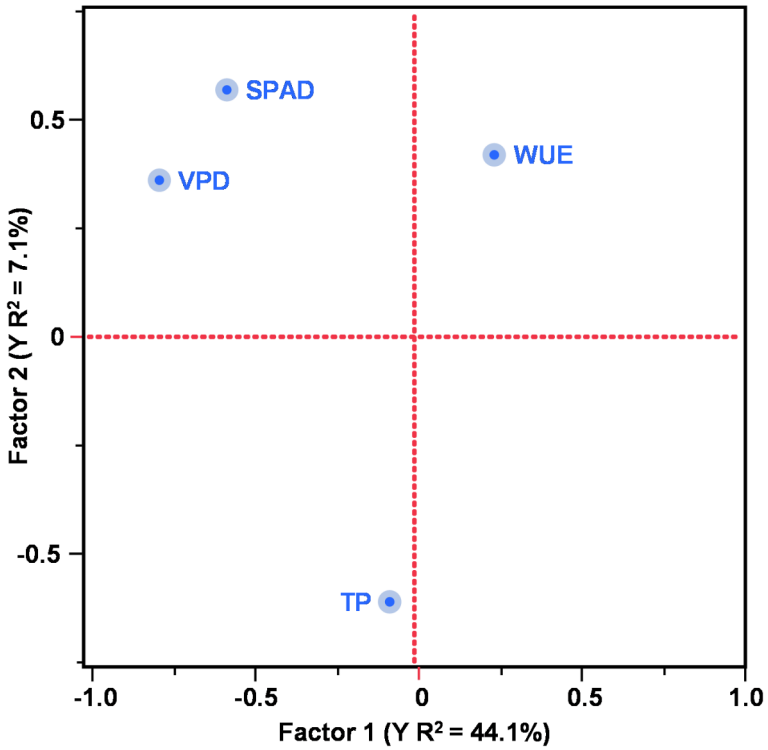

Fig. 5. Factor-loading scatter loading plot for the final reduced partial least-squares regression model predicting total phytoestrogen concentration in red clover. Predictor variables include VPD, vapor pressure deficit; WUE, water use efficiency; SPAD, chlorophyll content; and TP, total phenolic content.

Identification of relatively easy-to-measure physiological indicators of phytoestrogen status would aid in the development of diagnostic tools that could help farmers limit livestock consumption of potentially problematic forages. Only one of the individual physiological variables measured, vapor pressure deficit, was significantly correlated with biochanin A, one of the most abundant phytoestrogens in red clover. Biochanin A is receiving increasing attention among livestock researchers due to evidence that it may improve weight gain in beef cattle and reduce N_2O emission and ammonia volatilization from animal waste[49–51]. In a previous greenhouse study with red clover, a model including vapor pressure deficit, along with leaf water potential and total phenolic content, accounted for 56% of the variation in biochanin A concentration in plants subjected to different levels of water stress[17]. This is congruent with previous research on other plant species that has shown that artificially altering vapor pressure deficit can lead to changes in their foliar metabolite profiles[43,44].

Although none of the individual variables were correlated with formononetin, several physiological variables, including SPAD, which would be the most convenient variable for a farmer to assess, were negatively correlated with daidzein. Daidzein, while a minor component of the overall phytoestrogen profile of red clover is one of the most abundant phytoestrogens produced by soybean, along with genistein and glycitein[52]. Determining whether these variables could be useful indicators of phytoestrogen content in soybean requires further research. In addition, future studies could explore expression analyses of key biosynthetic genes, such as isoflavone synthase (IFS1), to directly assess how environmental stressors influence the transcriptional control of phytoestrogen synthesis[53].

Despite individual physiological variables being relatively weak correlates with many of the individual phytoestrogen compounds, the combination of four variables, vapor pressure deficit, SPAD, total phenolics, and water use efficiency, accounted for over 50% of the variation in total phytoestrogen concentration, the majority of which was formononetin and biochanin A. Formononetin concentration was also correlated with total phytoestrogen concentration (Pearson $R^2 = 0.85$, $p < 0.0001$), suggesting that diagnostic tools accounting for these variables in combination may have utility for estimating both total phytoestrogen levels and formononetin concentrations in red clover. It is perhaps not surprising that combinations of physiological indicators are better predictors of phytoestrogen content in red clover than are single indicators given the complexity of the biochemical pathways responsible for phytoestrogen synthesis and because of the multiple roles that individual phytoestrogens likely play in regulating plant metabolic functions[54]. In an earlier study conducted on red clover, leaf water potential, either alone or in combination with another physiological indicator, accounted for substantial variation (38–54%) in concentrations of individual and total phytoestrogen compounds in plants subjected to varying levels of moisture stress[17]. Since we did not measure leaf water potential in this study, it is unknown whether it would have had a similar predictive value for plants subjected to heat stress, which could be explored in future research.

Several limitations of the study warrant caution if attempting to extrapolate these results beyond the experimental conditions of the growth chambers. First, the temperature and CO_2 treatments were not applied independent of the two individual chambers and, therefore, it is possible that other factors contributed to the observed responses. While this explanation is unlikely, as the two growth chambers were identical except for their temperature and CO_2 parameters, it cannot be conclusively ruled out. Second, under field conditions, other stress factors are likely to co-occur over periods of elevated temperature, such as soil moisture or nutrient limitation, and these may interact to influence phytoestrogen concentration. Similarly, under natural conditions plants experience CO_2 at a relatively constant level rather than as a 10-day pulse as was performed in this study and which may have limited how these two factors interact to influence phytoestrogen responses. Longer-term, multigenerational experiments are needed to better understand future impacts of elevated CO_2.

These caveats aside, our findings suggest that short-term increases in atmospheric temperature (i.e., heatwaves) associated with climate change may lead to overall reductions in total phytoestrogen concentration in red clover that are driven largely by decreases in the compounds formononetin and biochanin A. The physiological variables measured in this study, either when assessed alone or in combination, had relatively low explanatory power and, therefore, may have limited practical value as part of an eventual farmer decision support tool for estimating phytoestrogen risk in red clover. Our study provides the only evidence we are aware of that cowpea utilized as forage may not be a significant source of phytoestrogen exposure to livestock.

Materials and methods

Experimental setup and design

We conducted a pot experiment in growth chambers (CMP6050, Conviron Inc., Canada) at the Macfarlane Research Greenhouse Facility at the University of New Hampshire, United States, from June to October 2023. Legume species, red clover, cv 'Freedom!' and cowpea, cv 'Red Ripper' were grown in ambient or *e*T and *e*CO_2 stress treatment. We applied the four treatments to both legumes in two batches due to limitations in the number of growth chambers available. Two identical growth chambers were used for the study, one maintained under ambient CO_2 and one supplying CO_2 from a cylinder controlled by an Autopilot APC8200 controller, with fluctuations of ± 50 µmol mol^{-1} from the set point. The first batch (control and *e*CO_2) was conducted from June 20 to July 26, 2023, and the second batch (*e*T and *e*CO_2 + *e*T) from September 12 to October 18, 2023.

Red clover and cowpea seeds used in this study were purchased from commercial seed companies. Prior to the initiation of each batch of treatments, seeds were sown in 3.8 L pots (16.0 cm diameter and 17.5 cm depth) in a Promix Bx potting media. In an initial pilot study, we observed that seed germination and seedling establishment were slower for red clover than cowpea. Therefore, before initiating each batch of treatments, red clover was sown two weeks earlier than cowpea to ensure both species would be at a similar developmental stage when stress treatments were applied. After seeding, pots were maintained in the growth chamber under 16 h light at 24 °C and 8 h dark at 18 °C with ambient CO_2 (~ 400 mmol mol^{-1}), 65% average relative humidity, and 350 mmol m^{-2} s^{-1} light intensity. Pots were watered twice a day, once with fresh water and once with water fertilized with a greenhouse nutrient solution containing 150 ppm of N as well as other micro and macronutrients (Supplementary Table S1). At each time, pots were watered until water was observed to drain from the pots' bottom holes. One week after germination, seedlings were thinned to eight (red clover) and four (cowpea) seedlings per pot, based on the pot size to support their growth. Pots were considered experimental units.

At the beginning of each batch, when red clover and cowpea were 26 days and 12 days old, respectively, four pots ($n = 4$) of each species were randomly assigned to each of the two growth chambers. In the first batch, the growing conditions of the two stress treatments were (1) ambient control (24/18 °C (day/night) and ~ 400 ppm CO_2) and (2) *e*CO_2 (24/18 °C (day/night) and ~ 750 ppm CO_2). In the second batch, the growing conditions were (3) *e*T (35/26 °C (day/night) and ~ 400 ppm CO_2) and (4) *e*CO_2 + *e*T (35/26 °C (day/night) and ~ 750 ppm CO_2). Across all treatments, the average relative humidity was maintained at 65% with a light intensity of 350 mmol m^{-2} s^{-1}. In each batch, plants were exposed to experimental growing conditions for a period of 10 days. Immediately after this time, plant physiological and physical measurements were made and tissue samples were collected for biochemical analysis (described below). Over the duration of the study, growth chamber temperature, CO_2 level, and moisture status of the growing media in each pot were monitored to ensure these parameters were maintained at the set levels.

Physiological parameters

Plant physiological variables, including sub-stomatal CO_2 concentration, net photosynthetic rate, stomatal conductance, transpiration rate, water use efficiency, and vapor pressure deficit, were assessed using a portable photosynthesis system (CIRAS-3; PP System, Amesbury, MS, United States). A leaf cuvette/chamber (7×25 mm) with a CO_2 reference of 390 µmol mol^{-1}, a cuvette flow rate of 250 cc min^{-1}, and an LED light source of 1200 µmol m^{-2} s^{-1} was used. The chlorophyll content was measured using a portable SPAD meter (SPAD 502, Konika Minolta Inc. Tokyo, Japan). All physiological measurements were taken on fully expanded leaves from three randomly selected plants in each experimental unit (pot).

Plant physical measurements and tissue sample collection

Plant height and number of leaves per plant were measured on two randomly selected individuals in each experimental unit. All leaves and stems from the same two individuals were collected and the leaves were separated to determine leaf area using ImageJ. After the leaf area measurements, the leaves and all remaining plant tissues from the two individuals were combined and oven-dried at 70 °C until they attained consistent weight and then weighed to determine aboveground dry biomass.

Plant tissue samples, including leaves, stems, and flowers, were collected from the remaining individuals in each experimental unit and this biomass was placed into a 50 ml conical centrifuge tube, immediately stored in a cooler box, flash frozen in liquid N, and stored at − 80 °C until total phenolic content and phytoestrogen analysis.

Total phenolics

Total phenolic compounds were quantified using a high-throughput modified Folin Ciocalteu reagent method[55]. Approximately 20 mg of freeze-dried powdered samples were placed into 15 ml centrifuge tubes, and 2 ml of ice-cold 95% (vol/vol) methanol was added. Subsequently, the samples were vortexed thoroughly and left at room temperature in the dark for 48 h, with occasional vortexing during this period. Next, samples were centrifuged at 4,500 rpm for 5 min at room temperature using a Sorvall ST16 centrifuge (Thermo Fisher Scientific, United States), with the supernatant collected into a new 2 ml microtube. Supernatant (100 µl), along with a standard (gallic acid) and 95% (vol/vol) methanol as a blank, was added into triplicate 2 ml microtubes and 200 µl of 10% (vol/vol) Folin-Ciocalteu reagent was added and thoroughly vortexed. Following this step, 800 µl of 700 m*M* Na_2CO_3 was added to each tube, and the mixture was incubated at room temperature for 2 h. A volume of 200 µl from the assay tube was transferred to a clear 96-well microplate, and the absorbance was read at 765 nm (Molecular Devices Spectra MAX 190, Sunnyvale, CA, United States). A standard curve was established using the blank-corrected A765 values of the gallic acid standards (ranging from 0 to 1.5 m*M*). The total phenolics were then calculated as gallic acid equivalents using the regression equation derived from the gallic acid calibration curve.

Phytoestrogens

Targeted quantification of isoflavone phytoestrogens (including formononetin, biochanin A, genistein, daidzein, glycitein and prunetin) was done in above-ground plant tissue samples using high-performance liquid chromatography (HPLC) according to Payette et al.[56]. Before solvent extraction, samples underwent freeze-drying and were ground into a powder by hand using a mortar and pestle. Approximately 100 mg of plant powder was placed into 15 ml centrifuge tubes, followed by the addition of 4750 µl of 80% aqueous methanol and 250 µl of internal standard flavone (400 ng/µl). The mixture was quickly vortexed and then sonicated for 10 min at room temperature. Following sonication, samples were shaken at high speed in a multi-tube vortexer (VWR Scientific Products, West Chester, PA, United States) for 2 h, and soaked overnight before centrifugation to sediment biomass. The resulting supernatant (1.5 ml) was filtered through a 0.45 µm PVDF syringe filter into a 2 ml HPLC vial and stored at 4 °C until HPLC analysis (within 24 h). An HPLC system with a diode array detector (Agilent Technology 1100 Series, Palo Alto, CA) was employed to identify and quantify phytoestrogens in the samples, following the conditions outlined in Payette et al. (2021). An Atlantis T3 C_{18} column (150 × 2.1, 3 µm) from Waters Corp. (Milford, MA) was employed and maintained at 40 °C. The mobile phase consisted of solvent A (H_2O with 0.1% CH_2O_2) and solvent B (acetonitrile), with separation achieved using a gradient ranging from 15 to 50% acetonitrile over the initial 35 min, followed by a rapid increase to 85% acetonitrile in the final 5 min of the run. The flow rate was maintained at 0.3 ml/min with detector operated at 260 nm. Phytoestrogens were identified by comparing their retention times and UV-visible spectra with analytical standards of biochanin A, formononetin, genistein, daidzein, glycitein and prunetin. Calibration curves were constructed using these standard compounds spiked with the internal standard (flavone) for concentrations ranging from 1.2 to 32 µg/ml, which were then used to measure the concentration of these compounds in the experimental samples. Concentrations were calculated on a dry matter basis.

Statistical analysis

The effect of the stress treatments on plant response variables was assessed using analysis of variance (ANOVA) with partial (type II) sums of squares using R Studio version 2024.04.0. Species were analyzed separately due to high between-species levels of variation in their physiological and biochemical response data. Homogeneity of variances and normal distribution of residuals were analyzed using residual plots and the Levene ('car' R package) and Shapiro–Wilk tests, respectively. Number of leaves per plant, aboveground biomass, and WUE of red clover did not satisfy variance and distributional assumptions of ANOVA, even after transformation was applied. Therefore, these variables were analyzed using the nonparametric Kruskal-Wallis test. The Tukey HSD test ($\alpha = 0.05$) was used for all other variables to compare differences among means. Concentrations of phytoestrogens in cowpea were below detection level; therefore, only phytoestrogens in red clover were statistically analyzed.

We calculated Spearman correlations to examine relationships between the concentrations of individual phytoestrogen compounds in red clover and the physiological and biochemical responses. We used partial least squares regression (PLSR) to examine the relationships between the physiological and biochemical variables and total phytoestrogen concentration in red clover. PLSR calculates latent variables (factors) to determine the relationship between predictor and response variables and has advantages over traditional multiple regression approaches in that it performs well when sample sizes are low relative to the number of predictors or when predictors are highly correlated[57]. Predictor variables in the full model were sub-stomatal CO_2 concentration, stomatal conductance, net photosynthetic rate, transpiration rate, water use efficiency, vapor pressure deficit, SPAD, and total phenolics, with total phytoestrogen concentration as the response variable. Data were centered and scaled prior to analysis. The optimal number of latent factors was determined using the predictive residual sum of squares (PRESS) criterion. A K-fold cross-validation and the nonlinear iterative partial least squares (NIPALS) algorithm were utilized. The model was further refined by including only variables with variable importance for projection (VIP) scores greater than 0.8. Both the Spearman correlation analysis and PLSR analysis were conducted using JMP Pro Version 16.1.0.

Data availability

The datasets used and/or analyzed in this study are available upon reasonable request to the corresponding author.

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Author contributions

Palash Mandal: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Visualization. Marta R. M. Lima: Funding acquisition, Methodology, Writing – review & editing. Anna K. Wallingford: Writing – review & editing. Nicholas D. Warren: Methodology, Writing – review & editing. André F. Brito: Funding acquisition, Writing – review & editing. Richard G. Smith: Supervision, Funding acquisition, Conceptualization, Methodology, Data curation, Resources, Writing – review & editing.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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