Effects of Nitrogen Fertilizer and Defoliation on Growth, Foliar Nitrogen, and Foliar Coumestrol Concentrations of Soybean

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ABSTRACT

Growth of plants and resistance to pathogens and herbivores can be altered by a variety of environmental factors, including nutrient availability and defoliation. Using *Glycine max* cv. G3197, a cultivar tolerant of phytophthora rot, we tested the predictions that (1) nitrogen fertilizer would enhance and defoliation would reduce vegetative growth and reproduction, and (2) nitrogen fertilizer would decrease and defoliation would increase concentrations of coumestrol, an isoflavonoid produced in response to a variety of stimuli and associated with decreased performance of insect herbivores. Individual plants were fertilized with a nitrogen-free basal fertilizer and either 2.5 mM NH_4NO_3 or water throughout the six weeks of growth. Half of the soybeans were partially defoliated 4 weeks after germination by cutting off the distal half of all fully expanded leaves. Two weeks later plants were harvested, dried, and weighed. Nitrogen fertilizer increased shoot mass and defoliation decreased root mass but effects were not strong and varied among the four groups of replicate plants. Foliar coumestrol concentrations of uncut leaves declined with nitrogen fertilizer but were not consistently linked to defoliation. Nitrogen concentrations of leaves were not consistently associated with nitrogen fertilizer or defoliation. Nutrient availability and defoliation significantly altered plant growth and chemistry, but in this experiment the strength of such factors was in turn influenced by unidentified environmental variability.

Key words: clipping, resistance, isoflavonoids, environmental variability

INTRODUCTION

Environmental conditions affect growth and chemical content of plants, which in turn determine resistance to pathogens and herbivores. Factors such as soil moisture (McQuate and Connor, 1990a,b; Lambert and Heatherly, 1991; Jenkins et al., 1997), soil fertility (Chang et al., 1985; Manuwoto and Scriber, 1985), mycorrhizal infection (Davis

and Menge, 1981; Rabin and Pacovsky, 1985; Gange and West, 1994; Gange et al., 1994; Matsubara et al., 1995), light (Kennedy et al., 1981; Elden and Kenworthy, 1995), temperature (van de Klashorst and Tingey, 1979; Ward and Lazarovits, 1982; Ratanatham and Gallun, 1986), previous damage (Reynolds and Smith, 1985; Lin and Kogan, 1990; Bi et al. 1994), and agrochemicals (Rose et al., 1988) can enhance or negate resistance to insects and pathogens. Because these environmental factors are varied and often unpredictable, development of lines resistant to insect pests and pathogens can be difficult. Breeding soybean for resistance to defoliating insects such as Mexican bean beetle (*Epilachna varivestis* L.) is a case in point.   Although numerous cultivars have been bred for resistance, concern that resistance may break down with plant maturity and environmental conditions such as temperature has resulted in limited release to growers (Hammond et al., 1995; Jenkins et al., 1997).

Not only do crops experience a variety of environmental conditions that influence expression of resistance, they may also suffer attack by more than the pest or pathogen for which they were selectively bred. Consequently, it is useful to take a less focused approach and consider responses of cultivars to environmental factors beyond those for which breeding was targeted. Studies that examine effects of interacting environmental factors on underlying mechanisms of resistance can test predictions of hypotheses proposed to explain patterns of plant-herbivore or plant-pathogen relations and may provide insight for future crop breeding strategies.

In this study we examined growth and foliar concentrations of nitrogen and coumestrol of soybeans, *Glycine max* (L.) Merr., grown under two nitrogen and defoliation regimes. The cultivar, G3197, exhibits tolerance to the fungal root pathogen *Phytophthora sojae* (M. Miles, pers. comm.). Tolerance refers to the relative ability of plants to sustain pathogenic infection without exhibiting severe disease symptoms (Walker and Schmitthenner, 1984), or more generally, the ability to withstand damage without incurring loss of productivity (Deverall, 1982). Tolerant cultivars can be a viable alternative to cultivars with race-specific resistance, which have been selected for new races of pathogens (Smith, 1985; Schmitthenner, 1985; Ryley et al., 1989).

We restricted our investigation to the effects of treatments on early growth of plants and foliar concentrations of nitrogen and the isoflavonoid coumestrol. Coumestrol is an estrogen-based phytoalexin (Smith, 1982), which is a metabolite that undergoes increased or de novo synthesis and accumulates in plants following exposure to a variety of stimuli (Paxton, 1980; Deverall, 1982). Coumestrol can deter feeding (Sutherland et al., 1980; Burden and Norris, 1992) and reduce performance (Rose et al., 1988) of herbivorous insects and it has some antimicrobial properties (Weinstein and Albersheim, 1983). Production of coumestrol has been induced by light, wounding, and by a wall glucan elicitor preparation from *P. sojae* (Graham and Graham, 1996). Induction of coumestrol in *Phaseolus vulgaris* L. leaves by ultraviolet radiation has been shown to increase with plant age and water or mineral stress (Beggs et al., 1985).

An increase in carbon-based defense compounds in mineral-stressed plants can occur if photosynthesis is sink-limited and plants accumulate carbohydrate reserves (Chapin et al., 1990). Surplus carbohydrates may be shunted to secondary metabolic pathways that produce non-nitrogenous defense compounds (Bryant et al., 1983). Because well-nourished

plants have the resources to use fixed carbon in primary metabolic pathways, they are predicted to have lower levels of carbon-based defense compounds when undamaged. Instead, they may be defended by nitrogen-based compounds (Bryant et al., 1983). This inverse relationship between carbon-based defenses and nitrogen availability has been demonstrated for phenolics in fir trees (Muzika, 1993). However terpenes, which are also carbon-based compounds, did not respond to nitrogen fertilization, suggesting that production of carbon-based resistance compounds are not invariably linked to nitrogen availability (Muzika, 1993).

Coumestrol production is induced by damage (Graham and Graham, 1996). We predicted that damage caused by clipping leaves would increase concentrations of coumestrol in uncut leaves of both nitrogen-stressed and nitrogen-fertilized plants, but in the absence of clipping, nitrogen-stressed plants would have higher levels of coumestrol than would nitrogen-fertilized plants. We predicted that addition of nitrogen fertilizer would increase growth and reproduction.

MATERIALS AND METHODS

Plants

Due to time constraints the experiment was conducted in four parts (= "groups") that were started at three or four day intervals over the course of 11 days. Each group consisted of two plants from each treatment combination. Seeds for each group were surface sterilized in 20% bleach for one minute and germinated on wet paper towel at the same time. Germinated seeds were planted in 3.75 L pots containing autoclaved 3:1 prairie topsoil:perlite and randomly positioned (using random number table) in a greenhouse under artificial light which extended the daylength to 14 h. Pot positions within a group and the entire groups were rotated daily.

Each plant was assigned one of two nitrogen treatments after being placed in the pots: no added nitrogen or nitrogen added as fertilizer. All plants were fertilized daily with 200 ml of nitrogen-free basal fertilizer (Pacovsky and Fuller, 1988). Half of the plants were fertilized to excess with 2.5 mM NH_4NO_3 and the remaining were watered to excess with water. Four weeks after planting, half of the plants in each fertilizer treatment were partially defoliated by clipping off one half of each fully expanded leaflet. The clipped portions were lyophilized and weighed.

Two weeks after the clipping treatment all plants were harvested and divided into seven major components:
    1 reproductive tissue
    2 nodules
    3 nodule-free roots
    4 clipped leaves on clipped plants, leaves of similar age on unclipped plants
    5 expanded, uncut leaves
    6 unexpanded leaves
    7 remaining aboveground tissue (stems, petioles)

Leaves (components 4 - 6) were lyophilized, weighed, and stored at -35ºC. All other tissue was oven-dried and weighed. Components 5 and 6 were pooled within plants and analyzed for % nitrogen and coumestrol concentration (μg coumestrol mg^{-1} dry leaf mass).

Nitrogen assay

Nitrogen was analyzed following a modification of the procedure of Robyt and White (1987). For each plant a 100 mg sample of ground, lyophilized leaves was digested in 5 ml of 7.5% (wt/v) sodium sulfate in 18 N sulfuric acid by heating to boiling, cooling, and adding 30% hydrogen peroxide dropwise over low heat until the mixture stopped bubbling and cleared. After cooling, the volume was brought up to 35 ml. Then 0.5 ml of sample was mixed with 0.1 ml Rochelle salt, 3.0 ml Nessler's reagent, and 6.4 ml distilled water and absorbance was measured at 506 nm. Digests were assayed in duplicate and the two values were averaged. Ammonium sulfate was used as a standard with a range of 0 to 1.29 μg N ml^{-1}. Assays were done by group and a standard curve was established for each group using the linear or quadratic function that provided the best fit. Data are reported as percent nitrogen per dry weight of leaf.

Coumestrol assay

Coumestrol was analyzed using a modification of the method of Burden and Norris (1992). For each plant a 250 mg sample was homogenized in 20 ml methanol and filtered through #1 Whatman filter paper. This filtrate was discarded after TLC analysis showed absence of coumestrol in this fraction. Fifty ml of methanol were added to the residue and the sample was shaken for 16 hours and vacuum filtered through #1 Whatman filter paper. The shaking and filtering was repeated and the two filtrates for each plant were pooled. The pooled sample was reduced to 2-4 ml by rotoevaporation, filtered through a 2.0 micron BS filter (Millipore, Milford, MA), and the volume was adjusted to 2 ml. Extracts were filtered using Sep-pak plus® C18 cartridges (Millipore, Milford, MA).

To confirm that there was no coumestrol loss due to the Sep-pak plus® C18 cartridge, a sep pak cartridge was prewet with 1 ml of water. Extract (400 μl) was eluted through the cartridge and the fraction was collected. The cartridge was washed (2X) with 400 μl of methanol and each of the three fractions was analyzed by HPLC to determine the coumestrol peak. For each fraction a 20 μl injection was analyzed by reversed phase HPLC using an ODS column and a 3 solvent elution system modified from Burden and Norris (1992). The pump flow rate was 1ml min^{-1} and the elution step gradient was solvent A (90:10 (v/v) 2% acetic acid:acetonitrile) for 5 min, solvent B (50% solvent A + 50% solvent C) for 10 min, and solvent C (100% acetonitrile) for 15 min. The elute was evaluated at 254 nm. The area under the peak which eluted at 19 min, the elution time for authentic coumestrol, was calculated for determining the apparent concentration of coumestrol. To confirm the identity of coumestrol by elution time, the fraction at the expected elution time was collected, dried under N_2 then chromatographed on an Alufolien plate using 80:20 (v:v) water/methanol as solvent. The presence of coumestrol was determined by presence under short-wave UV light of fluorescent spots which co-migrate with authentic coumestrol. A standard curve using authentic coumestrol (Spectrum Lab, Gardena, CA) was constructed ranging from 0.08 to 0.72 μg coumestrol. Data are reported as μg coumestrol mg^{-1} dry leaf mass.

<u>Statistical analysis</u>

Vegetative shoot mass (components 4 - 7), reproductive tissue, and dry root mass (components 2 + 3) were treated as univariates in a multivariate analysis of variance (MANOVA, SAS Institute Inc. 1989) using a three-way mixed effects model. Group (1-4) was treated as a random effect and nitrogen fertilizer (no, yes) and clipping (no, yes) as the fixed effects nested in group. When main effects were significant multiple pairwise comparisons of least-squares means were performed using the sequential Bonferroni method (Rice, 1989) at experimentwise $\alpha = 0.05$. This resulted in 4 comparisons if only one fixed effect was significant, 8 comparisons if both nitrogen and clipping treatments were significant, and 16 comparisons if there was a significant interaction. To meet the assumptions of normality and homoscedasticity the reproductive tissue data were square-root transformed and the root data were rank-transformed.

Percent nitrogen and coumestrol concentration were univariates in a multivariate analysis of variance (MANOVA) with group as a random effect and nitrogen fertilizer and clipping as fixed effects nested in group. Sequential Bonferroni comparisons were performed as needed. Coumestrol data were squared to meet assumptions of analysis of variance.

RESULTS

<u>Biomass</u>

Nitrogen fertilizer, clipping, and group significantly affected the pattern of soybean growth (MANOVA -Table 1). The magnitude of standardized canonical coefficients indicate that the multivariate effect was primarily due to effects on vegetative shoot mass (Table 1). Shoots constituted the largest portion of the biomass and so factors that improved growth tended to have the greatest absolute effect on shoots. However, most of the variation due to clipping was explained by root mass, indicating that clipping had a greater effect on roots than on shoot mass (Table 1).

The pattern of allocation to the three biomass components was constant across nitrogen and clipping treatments, as indicated by positive standardized canonical coefficients (Table 1). Addition of nitrogen tended to increase growth and clipping tended to decrease growth. However, the effects of nitrogen were significant only for the two shoot components (significant F tests, Table 1) and then only in some pairwise comparisons of nitrogen treatments within groups (Fig. 1a,b). Clipping significantly affected vegetative shoot mass and root mass (Table 1), but the reduction in growth was significant only in some pairwise comparisons of clipping treatment within groups (Fig. 1d,f). Nitrogen and clipping treatments did not interact (Table 1), indicating that the response to clipping did not depend upon nitrogen availability.

Plant group had a strong effect on all biomass components but the nature of the effect was not consistent across components (Table 1). Mean vegetative shoot mass was significantly smaller in group 4 than in group 1 (P=0.0005) and 3 (P=0.0004), mean mass of reproductive tissue was greater in group 4 then in group 2 (P=0.0005), and mean root mass was smaller in group 2 than in group 1 (P= 0.0012) or group 3 (P=0.0076). Thus,

fertilizer and clipping treatments had qualitatively similar effects across groups, but the magnitude of the effects varied among plant parts within groups.

Plant chemistry

Nitrogen fertilizer, clipping, and group significantly affected foliar concentrations of nitrogen and coumestrol. The magnitude of standardized canonical coefficients indicates that effects on coumestrol concentration explained most of the variation due to the fixed effects (Table 2). The relationship between nitrogen and coumestrol concentrations tended to remain constant among treatments (standardized canonical coefficients all positive - Table 2).

Nitrogen fertilizer significantly affected foliar nitrogen concentration (Table 2) and the trend was towards greater nitrogen concentrations in the foliage of nitrogen-fertilized plants (Fig. 2). However, the size of the effect was significant in only two groups and those two groups exhibited opposite trends (Fig. 2). Nitrogen-fertilized plants had significantly higher foliar nitrogen concentration than unfertilized plants in group 2, but had significantly lower concentrations than unfertilized plants in group 4 (Fig. 2). Clipping did not significantly affect foliar nitrogen concentration (Table 2).

Both nitrogen fertilizer and clipping treatments affected coumestrol concentrations (Table 2). When significant, the effect of nitrogen fertilizer on coumestrol concentration was consistent regardless of clipping. Nitrogen fertilizer decreased coumestrol concentration in unclipped plants in groups 1 and 2, and also decreased coumestrol concentration in clipped plants in group 3, and marginally in groups 1 and 2 (Fig. 3). Effects of clipping were less clear. Clipping significantly affected coumestrol concentration but only among unfertilized plants and the direction of the effect differed between groups (Fig. 3).

A fungal pathogen was observed on one or more plants in each group by the time of harvest and seven of the eight plants in group 4 had a significant number of infected leaves. Although leaves used in chemical assays were fungus-free, systemic induction of coumestrol could have elevated levels in the leaves we analyzed. We examined the correlation between coumestrol concentration and the proportion of leaf mass attacked by the fungus to determine whether coumestrol concentration was associated with the pathogenic infection. Coumestrol concentration was not correlated with severity of fungus infection for either clipped (Pearson correlation, $r = 0.19980$, $P = 0.4581$), or unclipped leaves ($r = 0.096545$, $P = 0.7223$).

DISCUSSION

The effects of nitrogen and clipping on growth and chemistry of soybeans were in general agreement with predictions based on published accounts and hypothesized models. Nitrogen fertilizer increased growth and decreased foliar coumestrol concentrations. Clipping decreased growth but had variable effects on coumestrol concentrations. The effects of these treatments were not pronounced and varied considerably among groups.

The large and varied effect of group (as defined earlier) was surprising, considering that this experiment was conducted in a small 4.5 m^2 greenhouse that was on the west side of the building and was partially covered by a tree thus reducing the effects of small varia-

tions in the weather. Although more controlled than in a field setting, factors such as light intensity and temperature fluctuate inside a greenhouse with daily changes in cloud cover and air temperature. Some fluctuations in environmental factors in a greenhouse can significantly affect plant growth (Summerfield, 1976; Potvin, 1993). Because small numbers of replicates were harvested at intervals, such fluctuations could have caused differences among groups without producing a discernible pattern.

Addition of nitrogen significantly increased shoot but not root growth. Changes in root:shoot investment are predicted if plants adjust resource allocation to minimize resource limitation (Mooney, 1972; Bloom et al., 1985), although see Coleman et al. (1993) for other outcomes. In our study addition of fertilizer created an imbalance between carbohydrates and nitrogen, and plants responded by investing less in roots for nutrient uptake and more in shoots for carbon fixation. Similarly, defoliation reduces photosynthesis and thus creates an imbalance in the ratio of carbon to nutrients (Bryant et al., 1983). As with addition of fertilizer, plants responded to the nutrient imbalance resulting from clipping by producing relatively less root and relatively more shoot mass.

We predicted that addition of fertilizer would not only increase growth but also increase the nitrogen concentration in leaves. This could occur if nitrogen fertilizer provides nitrogen in surplus and plant growth becomes limited by some other factor. A significantly greater nitrogen content of leaves in group 2 was associated with a marginally significant increase in vegetative shoot mass. However, foliar nitrogen concentration in group 3 showed no change with increased vegetative shoot mass, and foliar nitrogen concentration for group 4 decreased with increased production of reproductive tissue. Rather than concentrate minerals these groups responded to increased nitrogen availability by making more plant tissue, apparently diluting nitrogen concentrations. A similar result was observed in *Polygonum pensylvanicum*, where a pulse of fertilizer increased vegetative and fruit mass but did not affect leaf nitrogen (Mabry et al., 1997).

As predicted, nitrogen fertilization decreased foliar concentration of coumestrol. This is in agreement with observations of decreased coumestrol concentrations in roots of nitrogen fertilized "Amsoy 71" soybeans (Morandi and LeQuere, 1991) and with predictions of the carbon/nutrient balance model (Bryant et al., 1983). According to this model, nutrient limited plants shunt surplus carbon to secondary metabolic pathways and produce non-nitrogenous defense compounds. When not nutrient-limited they use carbon fixed in photosynthesis for growth, resulting in lower levels of carbon-based compounds such as coumestrol.

Coumestrol is distasteful to some herbivores (Kogan, 1977) and can decrease survival rate of larvae that consume foliage protected by coumestrol (Kogan and Paxton, 1983). However the effectiveness of coumestrol as a deterrent is dose dependent. Levels of foliar coumestrol in our experiment were similar to levels reported by Burden and Norris (1992) for 'Davis' soybean (0.063 μg mg^{-1}). Although "Davis" soybeans have constitutive antixenosis, Burden and Norris (1992) found that the coumestrol levels were considerably lower than 0.7 μg mg^{-1}, the minimum concentration required to deter feeding by Mexican bean beetles. They speculated that coumestrol alone is not responsible for constitutive antixenosis, but contributes to a complex array of plant stimuli affecting herbivore feeding.

Clipping did not consistently affect coumestrol concentration. It is possible that coumestrol levels increased shortly after clipping but dropped during the two weeks between clipping and harvest. Although isoflavonoid concentrations often exhibit temporal variation (Bohm, 1987; Burden and Norris, 1992) they do not necessarily respond to damage. Coumestrol levels of "Davis" soybean were not altered significantly by application of p-chloromercuriphenyl sulfonic acid, which nonetheless induced resistance to soybean looper, *Pseudoplusia includens*, and increased levels of the phytoalexin glyceollin (Liu et al., 1993). Glyceollin is a phytoalexin associated with resistance to phytophthora (e.g., Ward et al., 1979; Hahn et al., 1985) and, with coumestrol, is part of the suite of chemicals that can deter both insect herbivores and pathogens. We did not analyze glyceollin levels and plants may have responded to clipping by increasing concentrations of this phytoalexin.

CONCLUSION

In our experiment, the pattern of soybean growth and concentrations of foliar nitrogen and coumestrol were in general agreement with predictions. Plants responded to nitrogen deficiency and defoliation in a manner that was consistant with the predictions of the carbon/nutrient balance model. However, variability among groups was high. Although significant in the greenhouse setting, small differences in response to levels of nitrogen and defoliation may be swamped out under field conditions, where spatial and temporal variation in additional features such as soil quality, drainage, and herbivore load are likely to be significant.

Despite strong selection for specific attributes, soybeans still possess some inherent plasticity in growth and allocation. This plasticity may provide the basis for tolerance to a breadth of factors detrimental to growth and productivity, and may be a useful feature to exploit in some agricultural systems.

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Table 1. MANOVA and ANOVA results for three biomass components of clipped or unclipped plants given nitrogen-free or 2.5 mM NH_4NO_3 fertilizer. Analysis of root mass was performed on rank transformed data and analysis of pod mass was performed on square root-transformed data.

Source	df	F-Shoot mass (P)	F- Root mass (P)	F-Pod mass (P)	Pillai's trace (P)	Standardized canonical coefficients shoot mass	root mass	pod mass
N(group)	4	7.36 (0.0015)	2.11 (0.1264)	3.36 (0.0355)	1.0996 (0.0200)	1.1041	0.6001	0.6971
Clip(group)	4	4.85 (0.0094)	7.22 (0.0016)	0.94 (0.4649)	1.2256 (0.0062)	0.6714	1.3091	0.2975
N x Clip(group)	4	1.05 (0.4112)	0.94 (0.4676)	0.50 (0.7354)	0.5118 (0.6267)	1.8759	-0.7164	-0.2421
Group	3	9.24 (0.0009)	5.72 (0.0074)	6.34 (0.0049)	1.4454 (0.0001)	-1.3702	1.0048	1.3066

Table 2. MANOVA and ANOVA results for nitrogen concentration and coumestrol concentration for clipped or unclipped plants given nitrogen-free or 2.5 mM NH_4NO_3 fertilizer. Analysis of coumestrol concentration was performed on squared data.

Source	df	F-nitrogen (P)	F- coumestrol (P)	Pillai's trace (P)	Standardized canonical coefficients nitrogen	coumestrol
N(group)	4	6.22 (0.0032)	72.12 (0.0001)	1.3609 (0.0001)	0.4970	4.7822
Clip(group)	4	1.12 (0.3819)	29.05 (0.0001)	1.0950 (0.0006)	1.1270	4.8959
N x Clip(group)	4	0.54 (0.7119)	35.27 (0.0001)	0.9893 (0.0026)	1.0667	4.8911
Group	3	113.38 (0.0001)	31.25 (0.0001)	1.2740 (0.0001)	3.4914	3.3066

Figure 1. Impact of nitrogen fertilizer (a-c) and clipping (d-f) on mass of lyophilized aboveground vegetative tissue, mass of oven-dried reproductive tissue, and rank of oven-dried root mass for the four groups ($\overline{X}$ ±1se). * significant pairwise comparison at overall $\alpha = 0.05$. (*) significant at overall $\alpha = 0.10$.
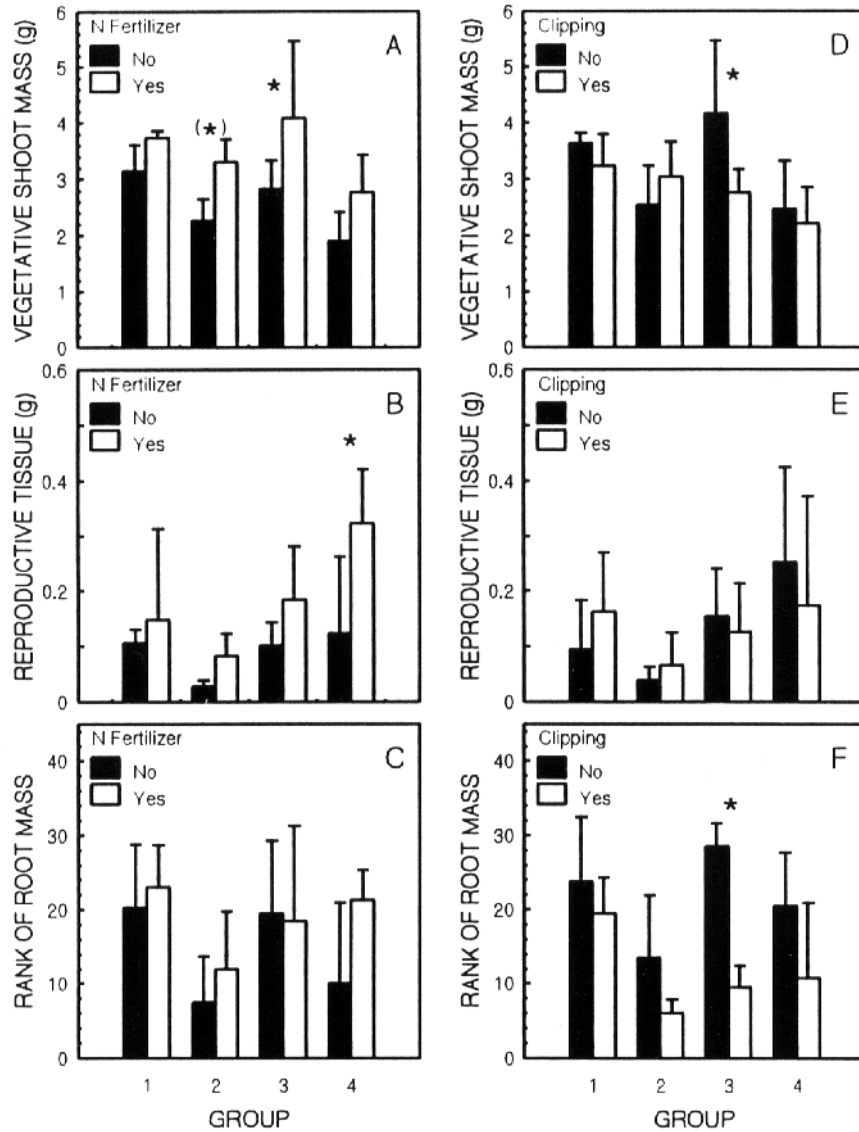
Figure 2. Impact of nitrogen fertilizer on nitrogen content of lyophilized leaves ($\overline{X}$ ±1se)
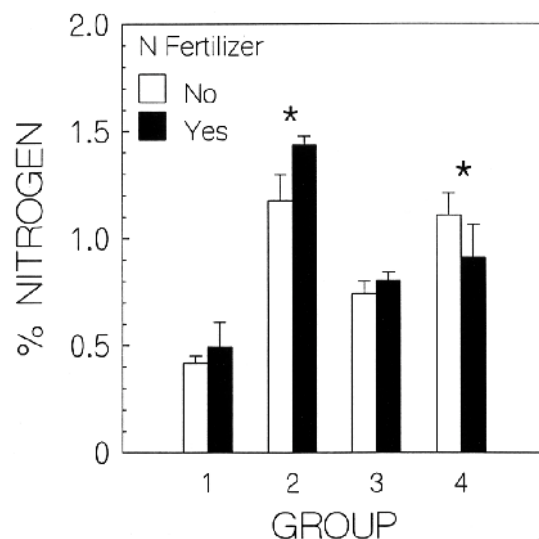* significant pairwise comparison at overall $\alpha = 0.05$.

Figure 3. Impact of nitrogen fertilizer and clipping treatment on coumestrol content of
lyophilized leaves ($\overline{X}$ ±1se). * significant pairwise comparison at overall $\alpha =$
0.05. (*) significant at overall $\alpha = 0.10$. Brackets below the graph indicate
pairs for which clipping was a significant effect.