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Sharon A. Clay
South Dakota State University, sharon.clay@sdstate.edu

David E. Clay
South Dakota State University, david.clay@sdstate.edu

D. P. Horvath
USDA, Agricultural Research Service

J. Pullis
Michigan State University

C. Gregg Carlson
South Dakota State University, Gregg.Carlson@sdstate.edu

See next page for additional authors

Authors
Sharon A. Clay, David E. Clay, D. P. Horvath, J. Pullis, C. Gregg Carlson, Stephanie A. Hansen, and Graig Reicks

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S. A. Clay,* D. E. Clay, D. P. Horvath, J. Pullis, C. G. Carlson, S. Hansen, and G. Reicks

ABSTRACT

Competition mechanisms among adjacent plants are not well understood. This study compared corn growth and yield responses to water, N, and shade at 74,500 plants ha^{-1} (1×) with responses to water and N when planted at 149,000 plant ha^{-1}. Plant biomass, leaf area, chlorophyll content, reflectance, and enzyme expression (transcriptome analysis) were measured at V-12. Grain and stover yields were measured with grain analyzed for ^{13}C isotopic discrimination (Δ) and N concentration. At V-12, 60% shade plants had increased chlorophyll and reduced leaf area and height compared to full sun plants. In the 2× treatment, plants had 11% less chlorophyll than 1× plants with leaf area and height similar to 60% shade plants. At harvest, plants in the 2× treatment were smaller, had increased water and N use efficiency, and an 11% per hectare yield increase compared with the 1× unstressed treatment. Per-plant yields from 60% shade and 2× treatments were 50% less than 1× unstressed treatment. Yield reduction in shaded plants was attributed to light stress. Lower yield in the 2× treatment was attributed to a population-density induced 20% decrease in the red/near-infrared (NIR) ratio, which resulted in downregulation of C_4 carbon metabolism enzymes (phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxylase, and pyruvate orthophosphate dikinase). Although the net impact of high plant density and shade stress on per-plant yield were similar, the stress compensation mechanisms differed.

Crowding-induced yield reductions can result from direct competition for available resources and/or growth alterations that influence plant resource requirements (Park et al., 2003). If yield reductions are the result of direct competition for resources (Jones et al., 1997), then it should be possible to measure competition-induced resource availability differences in the soil, plant, or both. However, if the second hypothesis is true then it should be possible to measure changes in growth physiology, with the net result being changes in resource use efficiency (Aphalo et al., 1999; Rajcan et al., 2004; Horvath et al., 2006, 2007).

Typically, competition among adjacent plants has been assessed by conducting experiments where plant densities of the same or different species, or the amount of resources (light, water, and/or nutrients) are varied (Park et al., 2003). Data collected from these studies can be used to derive many parameters. These parameters include (i) calculating competitive indices, (ii) determining the competitive balance among adjacent plants, and (iii) assessing relative performance. In addition, yield data from these studies can be fit to reciprocal yield loss or hyperbolic models to estimate (i) the yield of an isolated plant, (ii) crowding influence on yield, (iii) yield loss at a given plant density, and (iv) maximum and minimum yield loss within a range of densities (Cousens, 1985; Rejmanek et al., 1989; Blackshaw et al., 2002). A major problem with many competition studies is that compensation mechanisms are difficult to identify (Lindquist et al., 1996, 1999; Cousens et al., 2003; Park et al., 2003; Deen et al., 2003; Clay et al., 2005b). After evaluating common experiments conducted at many Midwestern U.S. locations and finding high variability in estimates of yield loss, Lindquist et al. (1999) suggested that only when the mechanistic responses of crops to stress and competition are understood can more accurate predictions of loss and better-informed management decisions be implemented.

Plants have the ability to sense proximity of their neighbors and alter growth through responses to changes in light quality using phytochrome receptors that are sensitive to red/NIR wavelengths (Smith et al., 1990; Weinig, 2000b; Liu et al., 2009). Phytochrome exists in inactive, red absorbing (P_r) and active, NIR absorbing (P_{fr}) forms. Light in open canopies has a high red (600–690 nm) to NIR (690–800 nm) ratio and this ratio favors the P_{fr} form of phytochrome. When phytochome is in the P_{fr} form, stem elongation and flowering is suppressed (Whitelam and Devlin, 1997; Weinig, 2000a).

Under crowded conditions, even before changes in energy availability can be detected, the red/NIR ratio of ambient light is altered due to chlorophyll disproportionately absorbing red light (Smith et al., 1990). The reflected light from vegetation, therefore, has a low red/NIR ratio that favors the development of the P_r form of phytochrome (Kasperbauer, 1971). Smith et al. (1990) reported greater than 80% reduction in red light

S.A Clay, D.E. Clay, C.G. Carlson, S. Hansen, G. Reicks, South Dakota State Univ., Plant Science Dep., Brookings, SD 57007; D.P. Horvath, USDA-ARS, Biosciences Research Lab., Fargo, ND, 58105; J. Pullis, Michigan State Univ. Ext., Rogers City, MI. 49779. Received 2 Dec. 2008. *Corresponding author (sharon.clay@sdstate.edu).

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Abbreviations: NIR, near-infrared; PEPCK, phosphoenolpyruvate carboxykinase; PEPC, phosphoenolpyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase; PCR, real-time polymerase chain reaction, real-time; 2×, 149,000 plants ha^{-1}; 1×, 74,500 plants ha^{-1}.

within a canopy and greater than 20% reduction in red light measured 30 cm from a vegetative mustard (*Sinapis alba* L.) stand. The perception of the change in red/NIR signal in leaves and internodes triggers the shade-avoidance response that is displayed by plants developing longer and narrower leaves and longer stems (Child and Smith, 1987; Kasperbauer and Karlen, 1994). While the ability of the plant to respond to light signaling and alter growth generally decreases with increasing growth stage and plant density (Weinig, 2000b; Nafziger, 2006; Sarlangue et al., 2007), plants that begin growth under crowded conditions generally develop fewer roots, smaller storage organs, and have less reproductive development (Weinig, 2000b) than plants grown alone. Thus, in densely grown crops, shade avoidance can result in poor plant morphology, delayed or incomplete grain fill, and ultimately, low plant yields. Corn hybirds, because of breeding and selection, however, produce high yields at relatively high populations (Weinig, 2000b; Nafziger, 2006), but the mechanisms of competition with itself and other species are unclear.

New techniques based on ^{13}C isotopic discrimination and transcriptome analysis might provide insights into corn competition mechanisms (Clay et al., 2001, 2003, 2005a; Horvath et al., 2006, 2007). ^{13}C isotopic discrimination (Δ) in C_4 plants has been described by the equation,

$$\Delta = a + (b_4 + b_3 \times \alpha - a)\,(c_i/c_a) \qquad [1]$$

where a is ^{13}C fractionation that occurs in air (4.4‰), c_i and c_a are the intercellular and ambient partial pressures of CO_2, b_3 is discrimination by RuBb carboxylase (29‰), b_4 is the discrimination of PEP carboxylase against bicarbonate (–5‰), and α is the leakage rate of CO_2 out of the bundle sheath cells (Farquhar and Lloyd, 1993). This equation suggests that ^{13}C discrimination is a function of light, photosynthetic capacity, and resource availability. In the C_4 corn plant, water stress increases Δ, whereas N stress decreases Δ (Clay et al., 2005a; Kim et al., 2008).

Transcriptome analysis may also provide information about competition by comparing gene expression from plants grown under various conditions. This approach has been used to determine the regulation of corn and velvetleaf (*Abutilon theophrasti* Medik.) genes in response to growing in a monoculture versus in competition with another species (Horvath et al., 2006, 2007). The objective of this study was to compare the ability of two alternative hypotheses (resource competition and growth modification) to explain competition among adjacent corn plants.

MATERIALS AND METHODS

Experiments were conducted at Aurora, SD (96°40′ W and 44°18′ N) in 2005 and 2006. The soil parent materials were loess over glacial outwash, and the soil series was a Brandt silty clay loam (fine-silty, mixed, superactive, frigid Calcic Hapludolls). The surface horizon contained 110 g sand, 580 g silt, and 310 g clay kg^{-1}. Total nitrogen in the 0- to 15- and 15- to 60-cm depths were 5.1 and 10.2 Mg N ha^{-1}, respectively. Total C in the 0- to 15- and 15- to 60-cm depths were 44.6 and 78.5 Mg C ha^{-1}, respectively. The slope at the site was between 0 and 2%. Additional information about soil at the site is available in Clay et al. (1994, 1995).

Each year, a split-plot design was used with two water rates (natural rainfall and natural plus supplemental) in four blocks of a randomized complete block design. Water was applied when plant available water was less than 50% (checkbook method, Werner, 1993) through an overhead sprinkler irrigation system, with water randomized by plugging appropriate nozzles. Four treatments [1× corn density (74,500 plant ha^{-1}) full sun, 1× density 40% shade, 1× density 60% shade, and a 2× corn density (149,000 plants ha^{-1}) full sun] at either 0 or 228 kg N ha^{-1} (eight total treatments) were then randomly assigned within each water rate. Shade tents, 3 by 3 m in size and placed over 4 rows in the center of designated plots, were constructed of commercially available shade cloth and mounted on extending poles that kept the cloth about 30 cm above the crop. To minimize edge effects with water or inadvertent shading, all subplots were 7 m wide and 8 m long with 3-m alleyways cropped to 1× corn population to separate water treatments.

Soil samples were collected in the spring and fall of 2005 and 2006 from the 0- to 75-cm depth at 15-cm increments. Soil samples were analyzed for gravimetric soil moisture and inorganic N. For inorganic N, soil samples were air-dried (35°C), ground (2 mm), extracted with 1.0 M KCl, and analyzed for ammonia and nitrate N using the phenate and Cd reduction methods, respectively (Maynard and Kalra, 1993). In 2005, preseason inorganic N was 105 kg (NH_4 + NO_3–N) ha^{-1}, of which 60% was in the nitrate form. In 2006, preseason inorganic N was 153 kg (NH_4 + NO_3–N) ha^{-1}, of which 32% was in the nitrate form.

A commercially available corn hybrid with a 97 d maturity rating was planted in 76 cm rows for the 1× density or 38 cm rows for the 2× density during the first week of May in 2005 and 2006. Shade was applied at the V-5 growth stage and remained until harvest.

At V12/13, plant height, from the soil to tip of the plant, was measured on four plants per plot and averaged. Chlorophyll on the most recently expanded leaf was measured using a SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc., Osaka, Japan) on 19 July 2005 and 12 July 2006 when corn was at the V-12/V-13 stage of growth. In 2005, the SPAD value for a plot was the average of 5 plants located in the center of each plot and in 2006 the value was the average of 10 plants per plot. Relative chlorophyll content was calculated by dividing each plot reading by the highest plot reading within a year. Four plants per plot were destructively sampled and leaf area measured using a leaf area meter (Li-Cor 3100 C, Li-Cor Biosciences) and averaged. These plants were dried at 90°C to constant weight, dry weights were measured, and average plant dry weight calculated. In 2006, soil samples from the 0- to 15-cm depth also were collected at V12/13 to determine soil water.

Crop reflectance was collected with a Cropscan (Cropscan, Inc., Rochester, MN) on 12 July 2006 (Chang et al., 2005). Band width measured were the blue (440–530 nm), green (520–600 nm), red (630–690 nm), and NIR (760–900 nm). The blue/NIR, green/NIR, and red/NIR ratio values were determined. Photosynthetically active radiation (PAR) (400–700 nm) was measured above the crop and at soil level at

Table 1. Analysis of variance model showing main effects, interactions, and error terms used in Proc Mixed procedure for SAS (SAS Institute, 2000). Treatment is the shade/population main effect. Soil water data for July 2006 used a similar model except year was removed as a source of variation.

Source of variation		df
Year		1
Error (1)	Block (year)	6
Water		1
Year × water		1
Error (2)	Water × block (year)	6
Nrate		1
Year × Nrate		1
Water × Nrate		1
Year × water × Nrate		1
Treatment (shade/population)		3
Year × treatment		3
Water × treatment		3
Year × water × treatment		3
Nrate × treatment		3
Year × Nrate × treatment		3
Water × Nrate × treatment		3
Error (3)		84

the V-12 stage of corn growth using a line quantum sensor (Li-Cor Biosciences, Lincoln, NE) that measured photosynthetic photon flux density. The readings were taken at the spectral zenith on cloud-free days.

Corn grain and stover were harvested from center rows of plots for a specific row length at physiological maturity (black layer). The center two rows of the four rows covered with shade cloth were harvested. The number of ears harvested and plants per area were recorded so that yield and stover could be calculated on both per-plant and per-area bases. Grain and stover samples were analyzed for total N, $\delta^{15}N$, total C, and ^{13}C discrimination (Δ) (Farquar and Lloyd. 1993; Clay et al., 2003). The Δ values were used to calculate yield losses due to N and water stress using the method outlined by Clay et al. (2003).

Data for plant parameters including grain yield on an area and per-plant basis, stover yield, Δ, N concentration of grain and relative chlorophyll, and soil parameters of inorganic N and water at harvest were subjected to analysis of variance appropriate for a split-plot arrangement of treatments. Class values were year, block, water, N rate, and treatment. The model statement was *parameter* = year|water|Nrate|treatment; using block(year)water × block(year) in the random statement (Table 1). Data between years were combined because there was homogeneity of variance. Means for interactions and main effects are presented and when $P < 0.05$, separated using Fisher's Protected LSD Test (Milliken and Johnson, 1992).

Transcriptome Analysis (2006)

Leaf samples from three (60% shade and 1× and 2× populations) treatments, receiving N and irrigation water, were collected at the V-12 stage on 12 July 2006. Each sample consisted of 8 cm of the topmost fully expanded leaf from four plants that were combined into a single sample. The samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction.

The frozen plant material was finely ground to a powder using a mortar and pestle and RNA from about 1 g of this powdered plant material was extracted using the pine tree extraction method (Chang et al., 1993). Labeled cDNA was prepared from about 30 μg of total RNA with the use of Alexa Fluor cDNA labeling kit (Invitrogen Life Technologies, Inc.) according to manufacturer's protocols. Labeled cDNA was hybridized to the 15,680-element SAM 1.2 maize microarray chip developed by Iowa State University utilizing previously published protocols (Horvath et al., 2006).

A rolling circle dye swap hybridization model (Churchill, 2002) was used to compare gene expression between three replicates of 60% shade, 1× population, and 2× population treatments. A total of nine two-dye hybridizations were performed. Three hybridizations compared the 60% shade and 1× population treatment, three compared 2× and 1× population treatments, and three hybridizations compared 2× population and 60% shade treatments. Microarray chips were hybridized and washed according to the manufacturer's protocols. Intensities based on fluorescence for each probe were visualized and quantified with a GenePix scanner (MDS Analytical Technologies) and GenePix Pro software. GeneMaths XT software (Applied Maths Inc.) was used to log transform (log 2) the intensity readings and normalize the arrays against each other. Probes that had hybridization intensity less than 2 times the standard deviation plus the average of the negative controls were deleted (Horvath et al., 2007) and technical replicates for each probe were averaged. GeneMaths XT software was then used to identify *P* values based on ANOVA and individual *t* tests between treatments. Probes were considered differentially expressed if *P* values for any test were ≤0.05. All microarray data and collection conditions have been archived in the Gene Expression Omnibus (Series accession GSE13768) according to MIAME (Minimum Information About a Microarray Experiment) standards (Brazma et al., 2001).

Differential expression data resulting from microarray analysis were validated for select genes of interest using real-time polymerase chain reaction (PCR) (Table 2). The ubiquitin conjugating enzyme and a gene of unknown origin were included as the controls (housekeeping genes) for each analysis. Complementary DNA was generated from selected RNA samples using Stratagene's AffinityScript QPCR cDNA Synthesis Kit (Applied Biosystems, Cat. no. 600559). Manufacturer's protocols were followed, using supplied Oligo (dT) primers and 1800 ng total sample RNA for each 20 μL reaction. Reactions were incubated 30 min at 42°C. Primers were designed for select genes using IDT's Oligo Analyzer and PrimerQuest software. Real-time PCR using the Stratagene Brilliant II SYBR Green QPCR Master Mix Kit (Cat. no. 600828) was performed on a Stratagene Mx3000P real-time PCR system (Applied Biosystems, Foster City, CA) using the following parameters: 10 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 50°C and 30 s at 72°C, followed by a 1-min soak at 95°C. Dissociation curves wherein data were collected continuously were then performed using a 30 s soak at 50°C and ramped up to 95°C at a rate of 0.2°C s^{-1}. Threshold values were determined with MxPro v3.00 software (Stratagene Corp.) and read at a threshold set point of 0.559. Samples were run in triplicate and averaged for data analysis. The resulting Ct values were normalized to the average of the two control genes for each sample and relative expression levels were identified using the delta delta Ct method (Livak and Schmittgen, 2001).

Table 2. Selected enzymes with accession number, sequence of amino acids used for forward (F) and reverse (*R*) transcription, and melting temperature (Tm) used for real-time PCR analysis. The ubiquitin enzymes and unknown function enzymes were used as internal controls for each run.

Gene/ enzyme abbrev.	Enzyme	Accessionno.	F/R	Sequence	Tm
PEPCK	phosphoenolpyruvate carboxykinase	DV490568	F	ATG CTC CAC CCG ACC AA	56.8
			R	AAC ACC TCG GTC TTC TGG TA	55.4
PEPC	phosphoenolpyruavte carboxylase	CB886182	F	ACT TGG TGA TGT CAG GGA TAA	53.4
			R	CCT CAG ACC ATT CAG CAT AG	52.6
PPDK1	pyruvate orthophosphate dikinase	DV943173	F	GAC AAG CAC CTG AGC TG	53.4
			R	TTT GCT TCA GAC GAG AGG AT	53.2
PPDK2	orthophosphate dikinase	DV493144	F	GCT AGG TGC AGT AGG TG	52.2
			R	TGT GCT CAA GCT TCT CT	50.6
Ubiq	ubiquitin-conjugating enzyme E2	DV495589	F	CAC CAA GAC AGC ACT GC	54.3
			R	GAC AAG CTT GGT AGG ATA TGC	53.3
Unkn	unknown	DV549369	F	GGT ATT GTT GAT CCA CGA CAT A	52.4
			R	AAT CGG TCT ATT GTT GTG GCT C	55.1

RESULTS AND DISCUSSION

Climatic Conditions

Growing degree days (GDD) (base 10°C) totaled 1204 in both 2005 and 2006 growing seasons from planting to harvest. Growing degree days were about 10% above the 30-yr (1971–2000) GDD average of 1100. Growing season precipitation (March–October) in 2005 was 57.2 cm, which was slightly higher than the 30-yr normal of 52.8 cm. In 2005, total water in the rainfall + supplemental irrigation treatment was 66.7 cm, with most of the irrigation applied between July 15 and August 15. In 2006, total growing season water was 31.4 cm, which was almost 50% below the 30-yr normal. Total water in the rainfall + supplemental water treatment was 52.9 cm with irrigation starting in mid-June and supplied weekly as needed through mid-August.

Shade and Population Impacts on Growth and Yield Parameters

Even though the 2× and shade treatments had similar impacts on the amount of available photosynthetically active available light (PAR measurement, data not shown) in the canopy, it is likely that these treatments had different impacts on light quality. Testing red/NIR ratio under the shade cloth indicated that the cloth had no effect on the incoming ratio compared with full sun, however, reflectance values of the plants under shade were not measured. At V-12, the red/NIR ratio of the reflected light was 20% lower ($P = 0.03$) above the 2× (0.04) than the 1× (0.05) canopy. Differences in the red/NIR ratio have been linked to the shade-avoidance response with plants being taller with less leaf area (Kasperbauer and Karlen, 1994).

If the shade and 2× treatments had similar mechanistic impacts on growth, then similar phenotypic responses should be observed. At V-12, leaf areas were less in the 2× (8, 470 cm^2 $plant^{-1}$) and 60% shade (8600 cm^2 $plant^{-1}$) treatments than the 1× (10,000 cm^2 $plant^{-1}$) treatment. Reduced leaf area was associated with plants that were 10% shorter in both the 2× and 60% shade relative to the 1× treatment. However, the shaded plants accumulated more chlorophyll than the 1× plants whereas the 2× plants accumulated less chlorophyll than the 1× plants (Table 3).

The harvest index (grain to above ground biomass ratio) of both 60% shade and 1× treatment was almost identical (55%) (data not shown). Grain yields per unit area decreased with increasing shade (11,900 kg ha^{-1} 1× vs. 6300 kg ha^{-1} 60% shade) (Table 3) and this response was attributed to light stress. Based on soil samples, the yield loss could not be attributed to less available water or N. In addition, the loss could not be alleviated if additional water was added (Table 3). Associated with lower yields with 60% shade were (i) higher mid-season chlorophyll and soil water amounts and (ii) higher end-of-the-season grain N concentrations and Δ values than those measured in the 1× population (Table 3). The higher Δ value in shaded treatments was attributed to lower assimilation of CO_2 into sugar due to lower photon flux density (Wong et al., 1985) or preferential leakage of $^{13}CO_2$ from the bundle sheath cells due to lower light energy availability slowing photosynthesis.

Grain per unit area and above ground biomass yields averaged over N and water treatment were about 11% higher in the 2× than the 1× treatment (Table 3). Plants in the 2× treatment also had lower chlorophyll content at V-12 and, at harvest, lower Δ values and N concentration in grain than 1× plants (Table 3). The yield increase per unit area in the 2× treatment was attributed to increased resource use efficiency. For example, Δ-based yield losses due to water (YLWS) and N stress (YLNS) calculations (Clay et al., 2005a) showed that increasing the population from 1× to 2× reduced YLWS from 1120 to 612 kg grain ha^{-1} ($P = 0.0026$). Associated with this reduction in YLWS was 8% less soil water at harvest (Table 3).

Unlike the yield per unit area, the yields per plant for the 60% shade and 2× treatments were reduced by 50% compared with the 1× treatment. However, grain N concentrations and Δ values were different among these treatments (Table 3). These data indicate that different mechanisms led to the per-plant grain reduction.

Transcriptome Analysis

At V-12, there were a total of 477 differentially expressed genes, out of ~14,000 unique probes on the microarray chip, in the 1×, 2×, and 60% shade treatments (see Supplemental Tables 1, 2, 3, and 4). Of these genes, 338 genes were differentially expressed when either the 60% shade or the 2× treatment was compared with 1×.

Thirty-three genes had similar responses in the 60% shade and 2× treatment when compared with 1× treatment. Of the similarly expressed genes, an unexpected result was the

Table 3. The influence of water, N rate, and treatment [1× (corn population = 74,500 plants ha^{-1}) 0% shade; 1× 40% shade; 1× 60% shade; and 2× (149,000 plants ha^{-1}) 0% shade] on yield, ^{13}C discrimination, and N concentration of grain, stover biomass amounts, relative chlorophyll and soil water content at V-12 stage of corn growth, and inorganic N and water content of soil at harvest. All data except water content at V-12 is averaged over years. Relative chlorophyll content was calculated by dividing values by the maximum value recorded at the sampling date in 2005 and 2006.

				Plant parameters						Soil parameters		
		Treatment		Grain						Harvest		19 July 2006
Water	N rate	Population	Shade	Yield	Yield	Δ	N conc.	Stove biomass	Relative chloro.	Inorganic N	Water	Water
	kg ha^{-1}		%	kg ha^{-1}	g plant^{-1}	‰	g kg^{-1}	kg ha^{-1}		kg ha^{-1}	cm	cm
Dryland	0	1×	0	10,100	135	3.33	13.3	19000	0.76b†	103f	21.8	10.9
Dryland	0	1×	40	8,700	117	3.36	14.0	15000	0.82a	104f	22.7	13.3
Dryland	0	1×	60	6,400	86	3.67	15.5	12300	0.82a	127def	23.1	15.0
Dryland	0	2×	0	12,700	85	3.20	12.9	23100	0.70c	100f	22.3	11.0
Dryland	228	1×	0	11,800	158	3.35	14.7	21400	0.83a	189bc	25.2	11.2
Dryland	228	1×	40	8,700	117	3.37	14.5	15400	0.84a	269a	22.1	13.2
Dryland	228	1×	60	6,000	81	3.69	15.6	11100	0.84a	169bcde	23.0	14.9
Dryland	228	2×	0	13,100	88	3.28	13.9	24400	0.75b	122ef	22.4	10.3
Irrigated	0	1×	0	12,200	164	3.27	13.6	22000	0.76b	102f	23.1	13.8
Irrigated	0	1×	40	9,500	128	3.31	13.9	16600	0.83a	112ef	22.6	16.9
Irrigated	0	1×	60	6,500	88	3.51	15.0	12300	0.81a	123def	22.9	16.9
Irrigated	0	2×	0	13,300	89	3.12	13.4	23800	0.65c	134cdef	21.1	14.5
Irrigated	228	1×	0	13,300	179	3.30	14.2	23400	0.81a	200b	26.4	13.9
Irrigated	228	1×	40	9,300	124	3.43	14.8	16100	0.85a	144bcdef	22.9	16.6
Irrigated	228	1×	60	6,200	83	3.58	15.4	11100	0.82a	155bcdef	23.0	17.5
Irrigated	228	2×	0	13,600	91	3.23	13.3	25100	0.74b	143bcdef	22.7	12.8
P				0.091	0.078	0.576	0.646	0.91	0.046	0.007	0.23	0.888
Dryland		1×	0	11,000b	147b	3.39	14.2	20200	0.79	146ab	23.5	11.0
Dryland		1×	40	8,700c	117c	3.41	14.4	15200	0.83	186a	22.4	13.2
Dryland		1×	60	6,200d	83d	3.70	15.6	11700	0.83	148a	23.0	14.9
Dryland		2×	0	12,900a	86d	3.31	13.8	23700	0.72	111b	22.3	10.6
Irrigated		1×	0	12,800a	171a	3.23	13.7	22700	0.79	151ab	24.8	13.9
Irrigated		1×	40	9,400c	126c	3.32	14.1	16300	0.84	128b	22.8	16.8
Irrigated		1×	60	6,400d	85d	3.52	15.2	11700	0.82	139b	22.9	17.2
Irrigated		2×	0	13,400a	90d	3.10	12.9	24500	0.70	139b	21.9	13.6
P				0.021	0.021	0.07	0.686	0.082	0.24	0.034	0.48	0.785
Dryland	0			9,500	102	3.45a	14.1	17400	0.77	108	22.5	12.6
Dryland	228			9,900	107	3.47a	15.0	18100	0.81	187	23.2	12.3
Irrigated	0			10,400	112	3.25b	13.8	18700	0.76	118	22.4	15.5
Irrigated	228			10,600	114	3.34a	14.1	18900	0.81	161	23.8	15.2
P				0.514	0.514	0.02	0.07	0.512	0.91	0.094	0.40	0.882
		1×	0	11,900b	159a	3.31c	14.0b	21500b	0.79b	149	24.1a	12.4b
		1×	40	9,100c	122b	3.37b	14.2b	15800c	0.84a	157	22.6b	15.0a
		1×	60	6,300d	84c	3.61a	15.4a	11700c	0.82a	144	23.0ab	16.1a
		2×	0	13,200a	88c	3.21d	13.4c	24100a	0.71c	125	22.1b	12.1b
P				0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.17	0.005	0.0001

† For each treatment effect, means within a column followed by the same letters are not significantly different at *P* = 0.05.

upregulation of 4 drought response genes in the 60% shade and 2× treatments (Supplemental Table 2). Drought response gene activation most likely would correlate with stomata closure. Stomata close if ABA content in the leaves increases from roots exposed to high (flooding) or low (drought) soil water conditions (Jackson and Hall, 1987; Rock, 2000). In addition, stomata will close if a stress stops the phloem export of sugar from the leaves (Else et al., 1996). In this study, the lower Δ value in grain would support that stomata closed in the 2× treatment (Table 3).

There were 171 genes that were expressed differently between the 60% shade and 2× treatments. Notable differences between the 60% shade and 2× treatment included a series of heat shock proteins and several key C-metabolism genes, most of which were downregulated in the 2× population treatment (Table 4). A similar response was observed for C-metabolism genes when corn was competing with velvetleaf (Horvath et al., 2006), suggesting that this response in corn is common to both intra- and inter-species competition. Amino acid metabolism genes were upregulated in the 60% shade treatment and either downregulated or not influenced by 2× treatment (Table 4). Amino acids are the building blocks of proteins, vitamins, enzymes, and chloroplasts. The higher chlorophyll content in the shade treatment may be related to the upregulation of amino acid metabolism genes (Table 3).

To confirm that C-metabolism genes were differentially expressed among treatments, real-time PCR analysis was conducted on four C-metabolism genes, DV490568 (PEPCK), CB886182 (PEPC), DV943173 (PPDK1), and DV493144 (PPDK2) (Fig. 1). The microarray and PCR analyses had similar responses and confirmed that these four genes were downregulated in the 2× compared to the 60% shade treatment (Fig. 1).

Table 4. The average relative expression of selected differentially-expressed probes (genes) in the 60% shade, and two population treatments (74,500 and 149,000 plants ha^{-1}). Gene identities were found by submitting the probe accession number into the Microarray Data Interface (MADI) database to get the gene sequence followed by a Basic Local Alignment Search Tool (BLAST)† query of the sequence. A complete listing of differentially expressed genes and *P* values can be found in supplemental Tables 1, 2, 3, and 4. The general functions were amino acid metabolism (AAM), carbon metabolism (CM), drought stress (DS), light response (LR), and protein stability (PS). Relative expression levels for the designated treatments are indicated in log base 2. Samples were collected at V-12 in 2006.

Probe Accession no.	MADI search followed by blast† query	Abbrev. in text	General function	Treatment			Probability level		
				60% shade	1×‡	2×§	60% shade vs. 1×	1× vs. 2×	60% shade vs. 2×
CB833479	phosphoglycerate dehydrogenase		AAM	0.210	0.088	0.087	0.010		
DV622248	pollen phosphoglycerate dehydrogenase		AAM	–0.355	–0.562	–0.604			0.026
BM267424	serine carboxypeptidase		AAM	–1.700	–1.918	–2.248			0.039
DV491622	serine carboxypeptidase I		AAM	0.240	0.062	0.110	0.006		
DV492790	serine carboxypeptidase II		AAM	–0.335	–0.517	–0.479			0.062
DV490568	phosphoenolpyruvate carboxykinase	PEPCK	CM	–0.647	–1.045	–0.897	0.025		0.029
CV886182	phosphoenolpyruvate carboxylase	PEPC	CM	1.368	1.297	1.131		0.012	0.064
DV493144	pyruvate orthophosphate dikinase	PPPDK2	CM	1.537	1.202	0.841			0.001
DV943320	pyruvate orthophosphate dikinase		CM	–0.211	–0.496	–0.560	0.025		0.029
DV942348	pyruvate orthophosphate dikinase		CM	1.398	1.062	0.847	0.038		0.001
DV943173	pyruvate orthophosphate dikinase	PPDK1	CM	1.254	1.100	0.787			0.026
BG842364	pyruvate orthophosphate dikinase		CM	1.797	1.747	1.390			0.046
BI992020	ABA-responsive protein-like		DS	0.129	0.090	0.247		0.007	
CD485070	ABI3-interacting protein 2		DS	0.110	–0.031	0.037	0.016	0.017	
DV493006	ABI3-interacting protein 2; CnAIP2		DS	–0.439	–0.865	–0.674	0.010	0.047	
DV491206	abscisic acid-induced protein		DS	0.222	0.037	0.194	0.030	0.013	
DV490515	early-responsive to dehydration stress protein 3		DS	–0.536	–2.44	–0.427		0.041	
DV621501	early-responsive to dehydration stress protein 4		DS	–0.038	–0.177	–0.034	0.039	0.017	
DV942249	inducer of CBF expression 2		DS	0.331	0.003	0.228	0.030	0.013	
DV489545	constitutive photomorphogenic 11		LR	0.328	0.149	0.200		0.039	
CB816024	constitutive photomorphogenic 11		LR	–1.287	–0.908	–0.964	0.048		
DV490341	early light-inducible protein ELIP		LR	–0.21	–0.354	–0.385			0.026
BM382140	photosystem I reaction center subunit VI		LR	–0.475	–0.721	–0.644	0.005		
DN228731	phytochrome interacting factor-like 5		LR	–2.448	–2.211	–1.381			0.007
DV491432	protoporphyrinogen IX oxidase		LR	–2.684	–2.419	–2.188			0.012
BM073828	thylakoid lumen protein, chloroplast precursor-like		LR	0.749	0.590	0.714	0.013	0.055	
DV549676	heat shock protein 101		PS	–0.820	–0.747	–0.646			0.023
DV493376	heat shock protein 82		PS	0.549	0.514	0.518	0.047		
DV495288	heat shock protein 82		PS	–0.089	–0.222	–0.351			0.034
CB331204	heat shock protein 90		PS	0.605	0.318	0.338		0.045	0.018
DV493748	heat shock protein 90		PS	0.805	0.634	0.489			0.045
CB833764	heat shock protein 90		PS	0.319	–0.020	–0.046			0.150

† Microarray Data Interface (MADI) can be accessed through http://schnablelab.plantgenomics.iastate.edu:8080/madi/browse/spotQuery.do [verified 2 Sept. 2009]. Basic Local Alignment Search Tool (Blast) analysis data can be accessed through www.ncbi.nlm.nih.gov/blast/Blast.cgi [verfied 2 Sept. 2009].

‡ 74,500 plants ha^{-1}.

§ 149,000 plants ha^{-1}.

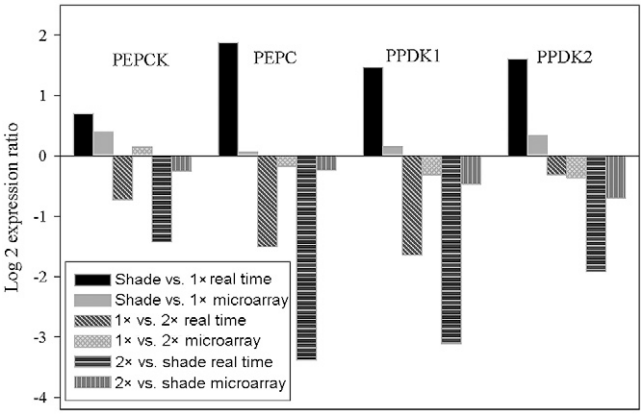

Fig. 1. A comparison of results from real-time PCR and microarray analysis for designated genes. Comparisons were made between the 1× vs. 60% shade, 1× vs. 2× populations, and 60% shade vs. 2× population treatments. The genes investigated were phosphoenolpyruvate carboxykinase (PEPCK, DV490568), phosphoenolpyruvate carboxylase (PEPC, CB886182), pyruvate orthophosphate dikinase (PPDK1, DV943173), and pyruvate orthophosphate dikinase (PPDK2, DV493144).

Using Δ and Transcriptome Differences to Understand the Mechanisms Controlling Corn Growth and Yield in Shade vs. Crowded (2×) Conditions

On a per-plant basis, the 2× and 60% shade treatments had grain yields that were 50–60% of the yield of the 1× plants (Table 2). The similarity of these yield values might lead to the conclusion that similar responses to shade and crowding (2×) stress occurred in both treatments. However, results from chlorophyll, ^{13}C isotope, and transcriptome analyses indicated that shade and crowding had different impacts on these parameters (Tables 3 and 4; Supplemental Tables 1–4).

In the 2× treatment, both chlorophyll and Δ were decreased relative to 1×, whereas in the 60% shade, Δ and chlorophyll increased. Differences in chlorophyll and grain N concentrations were not attributed to increased N stress for several reasons including: (i) inorganic N levels at harvest were similar in the 1× and 2× treatments (Table 3) and (ii) Δ-calculated YLNS was less ($P = 0.088$) in the 2× (1238 kg grain ha^{-1}) than 1× (1629 kg N ha^{-1}) treatment.

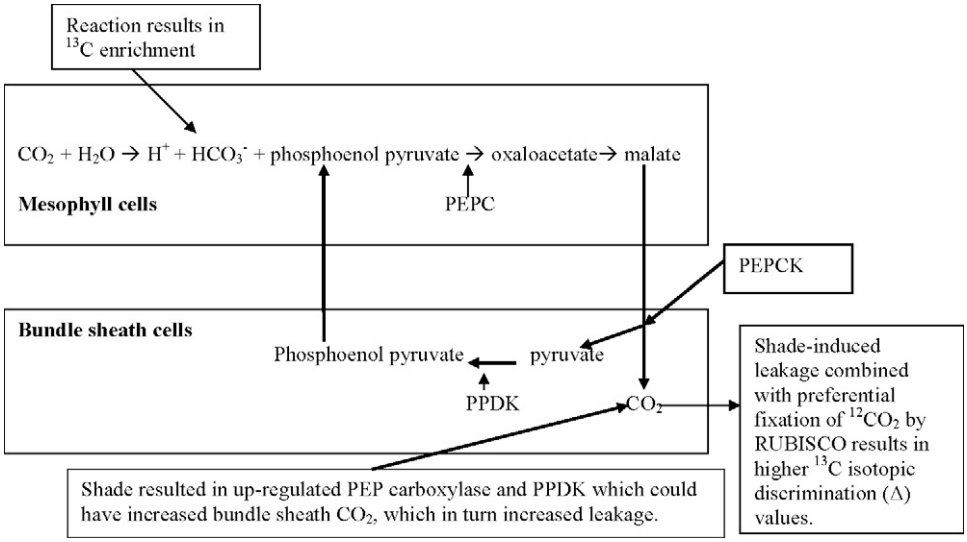

Fig. 2. Schematic of critical steps in C_4 carbon metabolism, enzymes involved in those steps, and steps where ^{13}C topic discrimination occurs. In the 60% shade treatment relative to 2× population treatment, PEPC, PEPCK, and PPDK were upregulated.

The CO_2 fixation pathway for a C_4 plant highlighting the C-metabolism genes that were differentially expressed between the 60% shade and 2× population treatments (Fig. 2) was prepared to better understand the relationship among the C-metabolism enzymes and how this may influence isotopic discrimination (Δ) and overall plant growth. Briefly, CO_2 fixation starts in the mesophyll cells with the enzyme PEPC utilizing bicarbonate (HCO_3^-) to catalyze the carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate. If the stomata are open, this carboxylation reaction results in oxaloacetate that is relatively enriched in ^{13}C whereas if the stomata are closed, this product will be depleted in ^{13}C. Oxaloacetate is then converted to malate that is transported to the bundle sheath cells, where PEPCK catalyzes the conversion of malate to pyruvate plus CO_2, with the CO_2 fixed in sugars by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Because Rubisco discriminates against $^{13}CO_2$, the preferential leakage of $^{13}CO_2$ may occur if PEPCK and Rubisco are not tightly linked, as predicted in Eq. [1] by the α value (Farquhar and Lloyd, 1993). The net result would be a higher Δ value in the fixed carbon, which was observed in the 60% shade treatment (Table 3). Continuing through the cycle, the PEP molecule is regenerated from pyruvate with PPDK enzyme and ATP and PEP recycled back to the mesophyll. The fixed carbohydrate products of photosynthesis are distributed throughout the plant (Fig. 2). Downregulation of the C-metabolism genes, PEPC, PEPCK, and PPDK, in the 2× treatment may indicate an adaptive compensation for a lower red/NIR ratio of light under crowded conditions expressed as less chlorophyll, lower yield per plant, but an unchanged Δ value compared to 1× treatment.

SUMMARY AND CONCLUSIONS

This study assessed two different mechanisms (direct competition for resources and growth alteration) responsible for competition-induced per-plant yield reductions. Even though the per-plant grain yields and leaf areas were similar in the 60% shade and 2× treatments, the treatments had different impacts on ^{13}C isotopic discrimination, chlorophyll content, and the expression of key enzymes (PEPCK, PEPC, and PPPDK) involved in C_4 carbon metabolism. Changes in gene expression between plants in 1× and 2× populations may have been affected by the 20% reduction in the red/NIR ratio that was measured at V-12. The net effect of crowding in the 2× population compared with the 1× population was a 10% increase in the grain yield per unit area. Higher yields per unit area in the 2× treatment were attributed to increased resource use efficiency.

Different plants may have different responses to crowding and competition. For example, in companion studies, Horvath et al. (2007) reported that velvetleaf upregulated its photosynthetic capacity in response to competition compared to the downregulation observed in corn in this study and when competing with velevetleaf (Horvath et al., 2006). These results suggest that competition models may be species-specific and could be improved by considering changes in: (i) enzyme regulation; and (ii) behavior modification, with concomitant changes in plant growth characteristics, resource requirements, and use efficiencies.

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