Utilizing Branched-chain Amino Acids for Increasing Shoot Density and Establishment Rate in Creeping Bentgrass

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SUMMARY. The branched-chain amino acids (BCAA) leucine (L), isoleucine (IL), and valine (V) are synthesized in plants and are essential to growth in most organisms. These compounds can be absorbed by the plant when foliarly applied, but plant catabolism of BCAA is not completely understood. A recent study observed that BCAA applied in a 2:1:1 or 4:1:1 ratio (L:IL:V) increased creeping bentgrass (*Agrostis stolonifera*) shoot density compared with applications of equal urea nitrogen (N) at 3.03 lb/acre N. The present study investigated whether those increases could translate to a quicker establishment rate of creeping bentgrass grown from seed in standard greenhouse pots. The BCAA applications were compared with equal N applications using urea and a commercially available amino acid product. All N treatments were applied at 3.03 lb/acre N, per application and applied a total of four times on a 14-day interval starting 14 days after seeding. Measurements included final shoot density counts and root and shoot weights, as well as digital image analysis of percent green cover for each greenhouse pot every 7 days. No differences were observed after 70 days in shoot weight, or percent green cover between BCAA treatments and urea; however, BCAA 2:1:1 and 4:1:1 increased shoot density 21% and 30%, respectively, compared with urea, and were equal to the commercially available amino acid product. Applications of BCAA 4:1:1 also increased creeping bentgrass rooting weight by a factor of 7 compared with urea N.

Commercial turfgrass growth products may include organic materials, as well as inorganic nitrogen (N), phosphorus (P), and potassium (K) (Aylward and Chief, 2005). Products containing these organic materials are referred to as specialty turfgrass fertilization products because of their ability to promote or improve growth over mineral nutrition only (Ervin and Zhang, 2008; Schmidt et al., 2003). A common organic additive in specialty turfgrass fertilization products is amino acids [AA (Schmidt, 1999; Schmidt and Zhang, 1997)]. Amino acids are simple organic compounds that contain an amino group [NH_2 (Taiz and Zeiger, 2006)], which makes them a possible source of plant N.

When foliarly applied, some of these organic materials can enter the plant through leaf tissue, where they become a possible N source for plant growth (Joy and Antcliff, 1966; Mäkelä et al., 1996). Compared with mineral nutrition, AA-containing products have been reported to improve root growth, increase the rate of establishment, aid recovery, and enable grasses to better withstand environmental stresses (Aamlid et al., 2017; Jones and Christians, 2011; Mertz, 2015; Mertz et al., 2017; Zhang et al., 2013). Furthermore, the industry standard urea N must be converted to ammonium (NH_4^+) before entering plant leaves and being assimilated into AA inside the plant (Hull et al., 2014); therefore, supplying AA directly

may be a more efficient method of providing plants with N.

Foliarly applied AA are capable of being plant absorbed due to openings in the leaf cuticle tissue that are negatively charged (Stiegler et al., 2013). When the pH of an AA solution is less than 9, the N-functional group of AA is positively charged (Lide, 1991), and this promotes AA plant absorption (Stiegler et al., 2013). AA that also have a hydrophobic side-chain can easily enter the plant through leaf tissue using diffusion forces, resulting in N uptake by the plant (Schönherr, 1976). The use of diffusion forces may result in a quicker rate of uptake relative to the ionic pathway (Schönherr, 1976).

As a result, some AA may be more effective than others as additives in fertilizers. Three AA that can be plant absorbed through both ion uptake and diffusion forces are the BCAA L, IL, and V (Platell et al., 2000). The BCAA could be effective as additives in foliar fertilization products because they have aliphatic, nonpolar side chains and are relatively hydrophobic (Harper et al., 1984). It is hypothesized that the hydrophobicity of the BCAA makes them more mobile through the epicuticular wax and cutin of the leaf cuticle, compared with other AA and standard N sources (e.g., urea). Once inside the leaf, the hydrophobicity of BCAA could also promote diffusion through the cell membranes of leaf tissues (Schönherr, 1976). These characteristics would favor plant absorption of BCAA (Marschner, 1995, 2011); however, research on plant and BCAA catabolism and their effect on plant growth when exogenously applied is limited.

The BCAA are capable of increasing protein synthesis, with the degree of increase being related to the intake ratio of L to IL and V (Holeček, 2002; Kimball and Jefferson, 2001, 2006; Pasiakos et al., 2011). Preliminary research conducted on creeping bentgrass indicated that

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
0.0929	ft ²	m ²	10.7639
9.3540	gal/acre	L·ha ⁻¹	0.1069
2.54	inch(es)	cm	0.3937
6.4516	inch ²	cm ²	0.1550
1.1209	lb/acre	kg·ha ⁻¹	0.8922
28.3495	oz	g	0.0353
1	ppm	mg·L ⁻¹	1
(°F - 32) ÷ 1.8	°F	°C	(°C × 1.8) + 32

a three-way application of BCAA (L:IL:V) may also result in additional beneficial plant growth compared with mineral nutrition only (Mertz et al., 2019). In that study, BCAA increased creeping bentgrass shoot density by 30% and 40% compared with urea N (3.03 lb/acre N) when applied at a 2:1:1 and 4:1:1 ratio (L:IL:V) (Mertz et al., 2019). No such increases were observed when BCAA were applied as single AA or in two-way combinations, which was found to be similar to previous studies (Holeček, 2002; Kimball and Jefferson, 2001, 2006; Pasiakos et al., 2011).

The objectives of this study were to investigate the use of BCAA for increasing the shoot density of creeping bentgrass and determine whether these increases in shoot density previously observed could also translate to a quicker rate in establishment of creeping bentgrass.

Materials and methods

EXPERIMENTAL DESIGN AND APPLICATION OF TREATMENTS. The experiment was initiated in 2014 at the Iowa State University Charles V. Hall Greenhouses (Ames, IA) and conducted over two runs using a completely randomized design and four replications. Run 1 was initiated on 1 Dec. 2014, and run 2 began 30 Mar. 2015. In each run, initial N applications were applied 14 d after seeding (DAS) and continued at 14-d intervals. Nitrogen sources consisted of L, V, IL, L + V, L + IL, IL + V, L + IL + V (2:1:1), and L + IL + V (4:1:1), in addition to an untreated control [UTC (i.e., no N fertilizer)], equivalent urea N, and a commercially available AA fertilizer treatment were included for comparisons (Table 1). All N sources were evaluated at an N application rate of 3.03 lb/acre N (Table 1).

Run 1 treatment applications occurred on 15 and 29 Dec. 2014, as well as 12 and 26 Jan. 2015. Run 2 treatment applications occurred on 13 Apr., 27 Apr., 11 May, and 25 May 2015. Deionized water (pH 5.8) was used to dissolve N sources into solution before foliar application with a hand sprayer using a carrier volume of 87 gal/acre. Following application, treatments were allowed to dry on the leaf tissue before being watered in.

Table 1. Description of the nitrogen (N) fertility treatments applied to creeping bentgrass growing in sand under controlled-environment conditions at Ames, IA, in 2014 and 2015.

N source ^z	N-content	Product rate ^y	N rate (lb/acre) ^y
Untreated control	0	0	0
Urea	46	6.6 lb/acre	3.03
Leucine (L) ^x	10.7	28.37 lb/acre	3.03
Isoleucine (IL) ^x	10.7	28.37 lb/acre	3.03
Valine (V) ^x	12	25.24 lb/acre	3.03
L + IL	10.7	28.37 lb/acre	3.03
L + V	11.4	26.85 lb/acre	3.03
IL + V	11.4	26.85 lb/acre	3.03
L:IL:V (2:1:1)	11.1	27.57 lb/acre	3.03
L:IL:V (4:1:1)	11.1	27.85 lb/acre	3.03
Commercially available amino acid product ^w	6	18 L/acre	3.03

^zRun 1 treatment applications occurred on 15 and 29 Dec. 2014, as well as 12 and 26 Jan. 2015. Run 2 treatment applications occurred on 13 Apr., 27 Apr., 11 May, and 25 May 2015. During each run of the study, a total of four N applications were made using each N source.

^y1 lb/acre = 1.1209 kg·ha⁻¹, 1 L/acre = 2.4711 L·ha⁻¹ = 0.2642 gal/acre.

^xThe branched-chain amino acids L, IL, and V were purchased from Hard Eight Nutrition LLC (Henderson, NV).

^wThe commercially available amino acid product GreenNCrease was obtained from Ajinomoto North America, Inc. (Eddyville, IA) and is a combination of amino acids, sugars, organic acids, polysaccharides, and ammonium sulfate.

The BCAA used in this study were obtained from Hard Eight Nutrition LLC (Henderson, NV). GreenNCrease, the commercially available AA product used, was obtained from Ajinomoto North America Inc. (Eddyville, IA) and is a combination of AA, sugars, organic acids, polysaccharides, betaine, and ammonium sulfate. GreenNCrease was previously reported to increase the shoot density of a creeping bentgrass fairway by Law et al. (2013).

The N content of each treatment was tested and verified independently by Waypoint Analytical (Memphis, TN) and was as follows: L and IL 10.7% N, V 12% N, urea 46% N, and GreenNCrease 6% N.

CREeping BENTGRASS ESTABLISHMENT. A completely randomized design with four replications (44 pots total) was used during each run of the study. Standard 4.5-inch-diameter plastic pots were used to establish '007' creeping bentgrass from seed (43.6 lb/acre). Each pot was filled with a calcareous sand root zone that had a pH of 8.2. The sand root zone particle size analysis was 8.2% very coarse, 35.3% coarse, 44.3% medium, 11.9% fine, 0.1% very fine, and 0.2% silt and clay by volume, which met United States Golf Association specifications for putting green use (Beard, 2002).

Throughout the study, greenhouse conditions consisted of average

day and night temperatures of 23.9 and 20.1 °C, respectively. Supplemental lighting was provided when daytime irradiance dropped below 200 μmol·m⁻²·s⁻¹ photosynthetic photon flux (PPF), so that plants consistently received 16 h of light per day and ranged from 350 to 385 μmol·m⁻²·s⁻¹ PPF. Relative humidity levels ranged from 25.1% to 44.9%. At the time of seeding, the application of a modified Hoagland's solution was made to promote uniform germination (Carrow et al., 1975). The modified Hoagland's solution was N free and applied at a rate of 43.56 lb/acre P (24 ppm P, 63 ppm K, 19 ppm magnesium, and 1.21 ppm iron).

During creeping bentgrass establishment, pots were kept moist using an automatic misting bench. Germination occurred at 10 DAS and pots were transferred from the automatic misting bench to a different bench for manual hand watering. Pots received 1 inch of irrigation per week throughout the duration of the study.

Creeping bentgrass plants were initially cut when the samples had reached a 1.3-inch height on average, and then maintained at a 1-inch height of cut (HOC) weekly with hand shears. This HOC was chosen based on the methods used by Mertz et al. (2019), and limitations of the hand shears. At the time of cutting, samples

were inverted, and clippings were removed to avoid influencing digital image analysis (DIA) measurements.

DATA COLLECTION. Digital images were captured weekly during each run of the study using a digital camera (CoolPix S8200; Nikon, Melville, NY) and a specialty light box (Ikemura, 2003). Initial images were captured 21 DAS and DIA (Soldat et al., 2012) was conducted on all images using the software ImageJ (National Institutes of Health, Bethesda, MD). ImageJ identifies the number of pixels in an image that contain a green color that is within a range determined by the investigators (Ferreira and Rasband, 2012). Images were analyzed using the following ranges: hue 40 to 110, saturation 74 to 255, and brightness 117 to 255 (Mertz and Christians, 2016).

The measurement feature within ImageJ was used to obtain the number of pixels per centimeter of each image, which allowed the number of green pixels present in each image to be converted to green-pixel area (square centimeters). This calculated value was then divided by the overhead surface area present in each plastic pot to quantify pot percentage cover of each sample, similar to methods used by Baker et al. (1996).

Additionally, plant biomass was determined at the study's conclusion. Plant biomass measurements included root weight determined through the loss on ignition method (Sluiter et al., 2008; Storer, 1984), as well as above-ground, oven-dry shoot weight (Scientific Engineering Response Analytical Services, 1994). The number of shoots present in each sample was also physically counted at the end of the study using modified methods described by Law et al. (2013).

STATISTICAL ANALYSES. Data were analyzed using the GLM procedure in SAS (version 9.3; SAS Institute, Cary, NC.). Due to a nonsignificant interaction between treatment and runs ($P = 0.386$), the results have been combined across runs and are presented as a single dataset. Fisher's protected least significant difference was used for mean separation and performed at a significance level of $P < 0.05$.

Results

Applications of BCAA 2:1:1 and 4:1:1 (L:IL:V) and the commercially available AA treatment increased

creeping bentgrass shoot density by 21%, 30%, and 26%, respectively, compared with urea N (Table 2). All BCAA treatments, other than IL, V, and IL + V, resulted in creeping bentgrass shoot density measurements that were greater than the UTC and equal to urea N. The BCAA treatments of IL, V, or IL + V resulted in creeping bentgrass shoot density that was greater than the UTC, but less than urea N.

Compared with the UTC, the BCAA V and commercially available AA treatment increased creeping bentgrass root weight at 70 DAS (Table 2). All other treatments, including urea, resulted in creeping bentgrass root weights that were equal to the UTC. The BCAA V and 4:1:1 ratio, as well as the commercially available AA treatment did increase root weight compared with urea N, but only the commercially available AA treatment was greater than the UTC.

Compared with the UTC and urea N, the BCAA V and commercially available AA treatment increased creeping bentgrass above-ground shoot weight at 70 DAS (Table 2). All other BCAA N sources resulted in above-ground shoot weights that were less

than commercially available AA treatment and V but were equal to urea N and the UTC.

Differences in percent cover were observed on four of eight measurement dates, and occurred at 28, 35, 42, and 49 DAS (Table 3). At 28 DAS, the BCAA treatments L, IL, V, IL + V, and 2:1:1 (L:IL:V) resulted in a lower percent cover compared with urea N. At 35 DAS, percent cover measurements were less than urea for the BCAA treatments IL and IL + V. From 35 DAS through the end of the study, there were no differences between urea N, commercially available AA treatment, and BCAA, regardless of combination. On average over all measurement dates, the BCAA treatments of L + V, 2:1:1, 4:1:1, and commercially available AA treatment were the only ones to have a mean percent cover greater than the UTC but were still equal to urea N (Table 3).

Discussion

Creeping bentgrass receiving applications of BCAA 2:1:1 and 4:1:1 (L:IL:V) and commercially available AA treatment resulted in a 21%, 30%, and 26% increase in shoot density, respectively, compared with urea N.

Table 2. Effects of nitrogen (N) fertility treatment on creeping bentgrass root weight, shoot weight, and shoot density after growing in sand for 70 d under controlled-environment conditions at Ames, IA, in 2014 and 2015.^z

N source	Shoot density (no./2.85 cm ²) ^y	Root wt (g) ^x	Shoot wt (g) ^w
Untreated control	26.9	0.04	0.05
Urea	37.9	0.01	0.06
Leucine (L) ^v	35.0	0.03	0.10
Isoleucine (IL) ^v	28.0	0.03	0.10
Valine (V) ^v	33.0	0.08	0.13
L + IL	37.5	0.03	0.06
L + V	38.8	0.02	0.07
IL + V	30.0	0.01	0.08
L:IL:V (2:1:1)	46.0	0.01	0.08
L:IL:V (4:1:1)	49.3	0.07	0.11
Commercially available amino acid product ^u	48.0	0.09	0.17
LSD (0.05) ^t	3.8	0.04	0.06

^zDue to a nonsignificant interaction between N source and runs, results have been combined across runs and presented as a single dataset.

^yAverage number of creeping bentgrass shoots counted visually in each 4.5-inch-diameter (11.43 cm) greenhouse pot. The data are reported in terms of 2.85 cm² (0.442 inch²) so that comparisons may easily be made to previous published work; 1 shoot/2.85 cm² = 2.2637 shoots/inch².

^xRoot weight as determined by loss on ignition method of each 4.5-inch-diameter greenhouse pot; 1 g = 0.0353 oz.

^wOven-dry weight of shoots measured in grams of each 4.5-inch-diameter greenhouse pot.

^vThe branched-chain amino acids L, IL, and V were purchased from Hard Eight Nutrition LLC (Henderson, NV).

^uThe commercially available amino acid product GreenNCrease was obtained from Ajinomoto North America, Inc. (Eddyville, IA) and is a combination of amino acids, sugars, organic acids, polysaccharides, and ammonium sulfate.

^tMean separation was done using Fisher's least significant difference (LSD) at $P < 0.05$.

Table 3. Effects of nitrogen (N) fertility treatment on percentage pot green-cover of creeping bentgrass growing in sand under controlled environment conditions for 70 d at Ames, IA, in 2014 and 2015.^z

N source	Pot green cover (%) ^y								Mean
	21 DAS ^x	28 DAS	35 DAS	42 DAS	49 DAS	56 DAS	63 DAS	70 DAS	
Untreated control	9.9	1.8	0.3	1.2	2.6	4.7	11.3	12.4	5.3
Urea	6.9	7.7	7.8	8.5	11.9	10.7	12.2	13.2	9.6
Leucine (L) ^w	8.0	3.0	3.0	7.1	10.0	11.4	14.9	12.2	8.5
Isoleucine (IL) ^w	9.2	1.7	0.6	2.9	6.1	9.9	14.4	16.1	7.2
Valine (V) ^w	8.5	3.4	3.9	7.2	9.0	12.5	16.1	17.6	9.2
L + IL	8.3	5.4	5.5	6.6	10.9	12.1	15.8	13.6	9.6
L + V	8.1	3.9	3.5	9.8	16.1	11.4	13.8	15.6	9.9
IL + V	8.4	1.6	1.3	4.8	10.1	14.1	12.8	14.4	8.4
L:IL:V (2:1:1)	8.0	2.7	3.1	7.4	11.5	14.0	17.6	17.3	10.1
L:IL:V (4:1:1)	8.0	6.6	7.5	12.1	19.5	18.4	18.5	20.9	13.2
Commercially available amino acid product ^v	8.7	7.2	7.5	11.3	15.9	17.1	18.4	19.0	12.6
LSD (0.05) ^u	NS	3.8	5.4	6.0	8.1	NS	NS	NS	4.5

^zDue to a nonsignificant interaction between N source and runs, results have been combined across runs and presented as a single dataset.

^yPercent green cover of each 4.5-inch-diameter (11.43-cm) greenhouse pot was determined using the digital image analysis software ImageJ.

^xDays after seeding.

^wThe branched-chain amino acids L, IL, and V were purchased from Hard Eight Nutrition LLC (Henderson, NV).

^vThe commercially available amino acid product GreenNCrease was obtained from Ajinomoto North America, Inc. (Eddyville, IA) and is a combination of amino acids, sugars, organic acids, polysaccharides, and ammonium sulfate.

^uMean separation was done using Fisher's least significant difference (LSD) at $P < 0.05$; NS = nonsignificant.

These results coincide with earlier findings and confirm the use of BCAA for increasing creeping bentgrass shoot density when applied in a three-way combination (Mertz et al., 2019). Compared with the commercially available AA product, increases in shoot density with the three-way BCAA combinations were similar, however, increases in creeping bentgrass shoot density observed with these treatments do not appear to translate to an increased rate of establishment compared with urea N, based on DIA results from this study. On the basis of the results of this study, in both cases where BCAA and the commercially available AA treatments led to an increased creeping bentgrass shoot density compared with urea N, there were no differences between those same treatments and urea for percent green cover on any dates, as determined using DIA.

The BCAA treatments also resulted in equivalent root and shoot weights compared with urea, regardless of combination; however, the BCAA did result in lower root and shoot weights for some combinations compared with the commercially available AA treatment. Only V and the 4:1:1 BCAA treatments resulted in equal root and shoot weights compared with the commercially available AA treatment. Therefore, it appears that BCAA are having little to no effect

on the degree of above- and below-ground plant biomass being produced compared with urea and also the commercially available AA product.

The BCAA treatments of 2:1:1 and 4:1:1 increasing creeping bentgrass shoot density, without increasing the accumulated above-ground biomass and also percent cover, indicates that this may be some type of plant growth regulator (PGR) response. Previously, AA have been reported to alter endogenous plant hormone levels following exogenous application (Carbonera et al., 1989; Merewitz et al., 2012; Zhang et al., 2013), and this demonstrates how AA applications can result in observable PGR activity. It appears that this PGR effect only occurs when BCAA are applied in a three-way combination and is not N related because applied N was equal across all treatments other than the UTC.

On the basis of these results, further testing of single AA and two-way BCAA combinations is unnecessary and increases to shoot density occur when BCAA are applied as a complete three-way combination only. Nonetheless, more research should be conducted on three-way BCAA applications to identify the optimal application ratio and ultimately provide the best increases to creeping bentgrass shoot density.

Although the 4:1:1 BCAA ratio showed the greatest increases in creeping bentgrass shoot density, future research should be conducted to determine whether those increases may possibly be further improved. It is hypothesized that this could be done by incorporating more L into the combination and extending the tested ratios beyond 4:1:1. This would complement existing BCAA research, where ratios greater than 4:1:1 have been reported to provide increased benefits (Dreyer et al., 2008; Glynn et al., 2010; Tipton et al., 1999).

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