Increased nitrogen fixation and remobilization may contribute to higher seed protein without a yield penalty in a soybean introgression line

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Abstract

The development of soybean varieties with higher seed protein concentration has been hindered by a negative correlation between seed protein concentration and yield. Benning HP, a genotype that breaks this tradeoff, contains a high protein allele introgressed into the cultivar Benning. Because seed protein is thought to be limited by N, field and growth chamber experiments were performed to identify the N flux(es) that enable Benning HP's increased seed protein without a yield penalty. When the N source was completely controlled in growth chambers, Benning HP was able to fix more N than its recurrent parent, but this response depended on the strain of the rhizobia symbiont and was not observed at all developmental stages. In the field, Benning HP remobilized N from its leaves at a higher rate during seed fill, but this response was only observed in one of the years studied. These results demonstrate that Benning HP has higher potential for N fixation and N remobilization from vegetative tissue compared to its lower protein recurrent parent, but those traits are not consistently expressed and may depend on environmental and sink control.

Keywords: soybean; seed protein; nitrogen fixation; nitrogen remobilization

Abbreviations: DAT, days after transplanting; DAP, days after planting; LAI, leaf area index

1. Introduction

A substantial portion of the value in soybean [*Glycine max* (L.) Merr.] seed is derived from its meal protein, which is an important source of nutrition for poultry and livestock worldwide. Soybean seed protein concentration ranges from 35% to over 50% among genotypes (Hwang et al. 2014, Zhang et al 2018). However, the negative correlation between seed protein concentration and yield is a major hurdle in exploiting this phenotypic diversity to breed agronomically elite, high protein soybean varieties (Brzostowski et al., 2017). While yield has steadily increased, seed protein concentration has decreased with year of cultivar release (Mahmoud et al., 2006; Morrison et al., 2000). It has been hypothesized that the persistence of greenness, which prolongs CO_2 assimilation during seed fill—increasing yield—has reduced the N available for remobilization from vegetative tissue (Kumudini et al., 2002).

N is primarily transported into the seed coat in the form of ureides and from seed coat to developing seed as glutamine and asparagine (Rainbird et al., 1984b), and energy must be expended in the seed for the synthesis of storage proteins. *In vitro* studies of soybean seed development have found that seed protein accumulation is determined by the supply of N into the seed (Pipolo et al., 2004; Saravitz and Raper, 1995). Similarly, in *Brassica napus* and *Brassica carinata*, final seed protein concentration correlated positively with total amino acid content in leaf phloem sap (Lohaus and Moellers, 2000). When the soybean supply/sink ratio was altered by removing 50% of the pods at each node, seeds accumulated significantly more protein (as % of seed weight) when the assimilate supply per seed was increased (Rotundo et al., 2011).

Three N fluxes can potentially contribute to developing seeds: uptake from the soil, biological N fixation, and remobilization from vegetative tissue. The relative contribution of

these components to seed N is variable among genotypes and environmental conditions (Leffel et al., 1992b; Mastrodomenico et al., 2013; Zeiher et al., 1982). It has been hypothesized that the large N demand in developing soybean seeds requires substantial N remobilization from vegetative tissue, triggering leaf senescence and limiting the seed fill period (Sinclair and de Wit, 1976, 1975). Later work found that the proportion of seed N from remobilization to be anywhere from 30% to 100%, depending on genotype (Zeiher et al., 1982). Other studies found that up to 90% of seed N may be supplied by N fixation, and that N fixation continues through most of the seed fill period (Leffel et al., 1992a; Mastrodomenico et al., 2013). A study with three high protein and three average protein soybean genotypes found no correlation between redistributed N from vegetative tissue and total N in mature seeds (Egli and Bruening, 2007). These discrepancies in the estimated contribution of remobilization to seed N may be partly linked to genetic improvement of soybean during the 20th century: when varieties released in the 1930's were compared with varieties released in the 80's and 90's, the earlier genotypes remobilized the same amount of N from vegetative tissue, but the newer genotypes were able to accumulate significantly more N during the seed fill period than the older genotypes (Kumudini et al., 2002).

A genome-wide association study identified 17 loci on 10 chromosomes that are significantly associated with seed protein concentration (Hwang et al., 2014). One of the loci identified in that study, on chromosome 20, resulted in mean protein increase from 41.46% to 44.32%. In a genome-wide analysis including 934 accessions from maturity groups (MG) IV-VI, this high protein locus was refined to a 1 Mbp region (Vaughn et al., 2014), but the specific gene(s) that determine this trait have yet to be resolved. A protein-determining locus in this region of chromosome 20 (hereafter called Gm20) has also been identified in genotypes of *G. max* and *Glycine soja* (Diers et al., 1992; Warrington et al., 2015). One of these analyses was

75 performed in a recombinant inbred population derived from a cross between Benning, a high-
76 yielding MG VII cultivar with moderate seed protein (Boerma et al., 1997), and Danbaekkong
77 (Kim et al., 1996), a lower-yielding MG V cultivar with very high seed protein. This locus
78 explained 55% of the variation in seed protein within the bi-parental population (Warrington et
79 al., 2015). From this population, the Danbaekkong Gm20 allele was backcrossed into Benning to
80 create a near-isogenic line, Benning HP, with ca. 3% increase in seed protein over Benning,
81 similar agronomic traits, and no yield penalty (Prenger et al., 2019).

82 Benning HP's seed protein concentration improvement over Benning without yield loss is
83 unique; while the Gm20 allele is linked to seed protein, it does not break through the protein-
84 yield tradeoff in most genetic backgrounds (Brzostowski et al., 2017). An understanding of the
85 physiological process(es) by which Benning HP achieves this increase could lead to new
86 strategies for improving seed protein concentration in other genetic backgrounds. This study
87 aimed to identify differences in N fluxes that contribute to higher seed protein without a yield
88 loss in Benning HP, which possesses the Danbaekkong Gm20 allele, compared to its recurrent
89 parent Benning. N uptake and fixation were evaluated in growth chamber experiments, where N
90 sources and rhizobia could be completely controlled, while N remobilization from aboveground
91 vegetative tissue was evaluated in field experiments.
92
93

2. Materials and Methods

2.1. Field experiment

Seeds of soybean cultivars Benning (Boerma et al., 1997) and Benning HP (Prenger et al., 2019) were sown on 26 May 2016 and 30 June 2017 at Central Crops Research Station in Clayton, NC. Soybeans at this research station were planted in a three-year rotation with corn and cotton. In 2016 the experiment was planted in a Norfolk loamy sand, and in 2017 the experiment was planted in a field with variable Appling sandy loam and eroded Cecil sandy clay loam. Plots were arranged in a randomized complete block design with four replicates of each genotype and 96.5 cm row spacing. Within-row planting density was 39.4 seeds per m. In 2016, plots were 3 rows wide and 3.7 m long; in 2017, plot size was increased to 6 rows wide and 5.5 m long to facilitate destructive aboveground biomass and leaf area index (LAI) measurements.

On field sampling dates, four 2-cm diameter disks were cut from uppermost, fully expanded leaves of two plants per plot on each sampling day, and three pods were removed from main stem nodes in the middle (2016) or upper (2017) third of the canopy from two plants per plot on each sampling day. Samples were dried at 60°C for a minimum of 3 days for C and N analysis.

When Benning and Benning HP were in developmental stage R5 in 2017, all plants in 1 m of a center row in each plot were cut down at the soil level. These rows had not been used for tissue sampling. Plots were harvested at 84 – 88 days after planting (DAP). Leaflets and pods were separated from stems and petioles, and total leaf area was measured with a leaf area meter (LI-3100, LI-COR, Lincoln, NE). All tissues were dried at 60°C for a minimum of 3 days, and leaf, seed, pod, and stem + petiole dry weight was measured for each plant.

116 At maturity, center rows were harvested with a single-row plot combine (Almaco,
117 Nevada, IA). After seeds were weighed, seed protein concentration was measured using near-
118 infrared spectroscopy (DA-7250, Perten Instruments North America, Springfield, IL). Seed
119 protein concentration is reported on a 13% moisture basis.
120
121 *2.2. Chamber experiment 1*
122 Seeds of Benning and Benning HP were surface sterilized with 95% ethanol and 1% NaOCl
123 (Somasegaran and Hoben, 1985) and incubated in rolled, moistened germination paper at 29°C
124 for two days to germinate. Germinated seeds were transplanted into 0.5 L pots filled with
125 vermiculite. The vermiculite was saturated with water before planting. Three germinated seeds
126 were transplanted per pot and thinned to one plant per pot a week after transplanting. Plants were
127 grown at 26°C day/22°C night with a 9 h photoperiod, with the addition of a 3 h incandescent
128 dark interruption for the first 35 days to suppress flowering.
129 Five plants per genotype were assigned to each treatment. The four treatments were: (1)
130 NH_4NO_3 in nutrient solution, (2) inoculation with *Bradyrhizobium diazoefficiens* strain USDA
131 110 [formerly classified as *Bradyrhizobium japonicum* (Delamuta et al., 2013)], (3) inoculation
132 with *Bradyrhizobium elkanii* strain USDA 31, or (4) no N fertilizer or inoculation. The rhizobia
133 strains contrast in N fixation efficiency, with *B. diazoefficiens* USDA 110 fixing N more
134 efficiently than *B. japonicum* USDA 31 (Schubert et al., 1978). The N-free plants were included
135 to monitor for potential rhizobia contamination in the substrate and to measure N derived from
136 cotyledons prior to excision. Cotyledons were excised 8 days after transplanting (DAT) to hasten
137 reliance on N treatments. All plants were fed 50% Long-Ashton nutrient solution (Hewitt, 1966),
138 modified by omitting NH_4NO_3 for the inoculated plants and the N-free plants and with 7 mM

139 NH_4NO_3 for the remaining treatment. Inoculations were conducted immediately after
140 transplanting, by adding 1 mL of the respective rhizobia culture grown in yeast extract-mannitol
141 medium (Somasegaran and Hoben, 1985) and containing approximately 10^8 CFU ml^{-1} of *B.*
142 *diazoefficiens* USDA 110 or *B. elkanii* USDA 31 to the transplanted seed. Genotypes and
143 treatments were fully randomized within the growth chamber.
144 For the first 4 DAT, each pot received 20 – 30 ml of deionized water daily. Beginning at
145 5 DAT, each pot received 50 ml of water daily, and this was followed by 50 ml of nutrient
146 solution every other day. At 26 DAT, water and nutrient solution were both increased to 80 ml
147 daily. All plants were harvested 45 DAT. Plants were separated into shoots, roots and nodules
148 and dried at 60°C for 3 days for biomass determination, followed by C and N analysis.
149
150 *2.3. Chamber experiment 2*
151 Benning and Benning HP seeds were germinated as described above. Germinated seeds were
152 transplanted into 6 L pots filled with vermiculite and approximately 80 g of crushed oyster shells
153 to control acidification of the rhizosphere in later developmental stages (Israel and Jackson,
154 1982). The vermiculite was saturated with water before planting. Three germinated seeds were
155 transplanted per pot and thinned to one plant per pot a week after transplanting.
156 Germinated seeds were inoculated with *B. diazoefficiens* USDA 110 immediately after
157 transplanting as described above and grown in a controlled environment growth chamber under
158 the same conditions as described above. For each genotype, 20 pots were inoculated, and seven
159 pots remained uninoculated. Genotypes and treatments were fully randomized within the growth
160 chamber. For the first four DAT, 30 ml of deionized water were added daily; then, vermiculite
161 was flushed daily with 600 ml of deionized water followed by the addition of 400 ml of N-free

nutrient solution. Nutrient solution was modified 50% Long-Ashton solution as described above. After developmental stage V5, water and nutrient solution were increased to 1 L and 400 mL, respectively, twice per day. Cotyledons were excised at 8 DAT.

Five inoculated plants per genotype were harvested at 33, 65, 82, and 132 DAT, when the plants were in developmental stages V5, R5, R6, and R8 (maturity). The non-inoculated plants, which received no N, were also harvested at the first sampling date (33 DAT) to measure N derived from cotyledons prior to excision. At harvest dates, whole plants were separated into nodules, roots, vegetative shoot tissue, and reproductive tissue, and dried at 60°C for a minimum of 3 days. Biomass, C, and N content were measured for dry tissue. Seed protein concentration in mature seed was measured with near-infrared spectroscopy.

2.4. Carbon/nitrogen measurements

Samples from the field and from chambers were dried at 60°C for a minimum of 3 days before analysis. Nodules and leaf disks were ground in 2 ml tubes containing two 3-mm stainless steel grinding balls, shaken at 1400 strokes min^{-1} for 45 sec in a homogenizer (Geno/Grinder 2000, Spex CertiPrep, NJ). Whole leaves, stems, seeds, pod shells, and roots were ground using a Wiley mill (Model 4, Thomas Scientific, NJ). Small amounts of seeds, such as the three pod samples collected on field sampling dates, were ground with a centrifugal mill (ZM100, Retsch, Germany). Tissue C and N content was measured with an elemental analyzer (FlashEA 1112, Thermo Scientific, Walham, MA), and the percentage of C and N per sample was calculated with the instrument's software (Eager Smart, Thermo Scientific, Walham, MA). Biomass and percent N values were multiplied to estimate total N in plant tissues.

185 *2.5. Leaf chlorophyll measurements*

186 In the field, two 2-cm diameter disks were cut from uppermost, fully expanded leaves of two
187 plants per plot on each sampling day and flash frozen in liquid N. These disks were sampled
188 from the same leaves on the same days as the disks used for leaf C and N measurements. Leaf
189 disks were stored at -80°C until chlorophyll extraction. Chlorophyll was measured according to
190 Porra et al. (1989). Briefly, frozen leaf disks were ground in chilled methanol. Methanol was
191 decanted and centrifuged at 2500 rpm for 10 min. The supernatant was transferred to a cuvette,
192 and absorbance was measured at 652.0 nm and 665.2 nm. Chlorophyll concentrations in the
193 supernatant (µg/mL) were calculated using the equations:

194 $$Chlorophyll\ a = 16.29(A^{665.2}) - 8.54(A^{652.0}) \quad (1)$$

195 $$Chlorophyll\ b = 30.66(A^{652.0}) - 13.58(A^{665.2}) \quad (2)$$

196 $$Chlorophyll\ a + b = 22.12(A^{652.0}) + 2.71(A^{665.2}) \quad (3)$$

197

198 *2.6. Statistical analysis*

199 Chamber experiments 1 and 2 were completely randomized designs. Two-way analysis of
200 variance (ANOVA) was performed in PROC MIXED (SAS Inc., Cary, NC), and genotype and N
201 treatment (experiment 1) or developmental stage (experiment 2) were treated as fixed effects.
202 Pairwise differences between genotypes within N treatment or developmental stage were
203 calculated with *F*-tests where appropriate using the slice option. *P*-values were adjusted to
204 control the false discovery rate where indicated in tables and figures (Benjamini and Hochberg,
205 1995).

206 Each year of the field experiment was a randomized complete block design. The blocking
207 factor was included in all statistical models as a random effect and was dropped from a model

when its covariance parameter estimate was equal to or less than 0. Final harvest parameters, LAI, and biomass harvest parameters were analyzed using type 3 ANOVA in PROC MIXED, with genotype treated as a fixed effect and block as a random effect.

To evaluate genotypic differences in leaf and seed N concentration and C/N, seed weight, and leaf chlorophyll concentration throughout each growing season, a linear model with repeated measures was fitted in PROC MIXED with restricted maximum likelihood estimation. Genotype and DAP were fixed effects, block was a random effect, and DAP was the repeated effect with genotype*rep as its subject. Differences between genotypes within DAP were calculated with *F*-tests using the slice option, and the resulting *p*-values were adjusted to control the false discovery rate. To evaluate the rates of change for leaf N concentration, leaf C/N, and leaf chlorophyll during the seed fill period, this model was changed so that DAP was a continuous variable rather than a repeated effect.

In chamber experiment 2 and in the field experiment, biomass parameters and N content were measured at successive developmental stages. These values were used to calculate N lost from vegetative tissue or gained in reproductive tissue between developmental stages. Because total N per organ was destructively measured for different groups of plants at each developmental stage, replicated values for N lost or N gained between stages could not be obtained and tested statistically. However, standard errors (SE) of the means were propagated using the formula:

$$SE_{difference} = \sqrt{{SE_a}^2 + {SE_b}^2} \qquad (4)$$

3. Results

3.1. Benning HP has greater N assimilation potential when inoculated with B. diazoefficiens USDA 110

In chamber experiment 1, plants were supplied with one of three N sources to determine if Benning HP is able to assimilate more N than Benning, and to determine if differences in N assimilation depend on the source of N. The three N sources tested were NH_4NO_3 fertilizer, inoculation with *B. diazoefficiens* USDA 110, or inoculation with *B. elkanii* USDA 31. All plants were harvested 45 DAT. Differences in nitrogen supply influenced the rate of development: at 45 DAT, plants inoculated with the more efficient rhizobia strain, USDA 110, were at approximately developmental stage V6, plants inoculated with less efficient rhizobia strain USDA 31 were around V4, and plants fed NH_4NO_3 were around V7. Non-inoculated plants did not form nodules, and vermiculite contains no plant-available N, so the N in the no-N plants was derived from stored N prior to cotyledon excision. No-N plants were extremely stunted and chlorotic, as would be expected for severe N deficiency. The average cotyledon N value for each genotype was subtracted from total plant N in the NH_4NO_3 fertilizer and rhizobia inoculation treatments to calculate total N assimilated.

N treatment and genotype had a significant effect on total N assimilation, while the genotype × treatment effect was not statistically significant (Table 1). Plants that received NH_4NO_3 assimilated the most N per plant (Fig. 1), reflecting the 2-3 weeks after transplanting and inoculation required to develop functional nodules. Plants inoculated with *B. diazoefficiens* USDA 110 fixed more N than plants inoculated with *B. elkanii* USDA 31. Given the low practical risk of accepting a higher type I error rate in the interaction effect to consider the more biologically interesting within-group tests, we conducted *F*-tests for differences between the

genotypes within each N treatment. Total N assimilated was not significantly different between genotypes in the plants that were fed fertilizer containing NH_4NO_3 ($p = 0.0828$) or inoculated with *B. elkanii* USDA 31 ($p = 0.5829$). When inoculated with *B. diazoefficiens* USDA 110, Benning HP assimilated significantly more N than Benning ($p = 0.0120$) (Fig. 1).

Total N assimilated per plant was divided by total nodule weight per plant to calculate N fixed per nodule weight. In chamber experiment 1, Benning HP fixed significantly more N per nodule weight than Benning when inoculated with *B. diazoefficiens* USDA 110 but not when inoculated with *B. elkanii* USDA 31 (Table S1).

3.2. Benning HP's greater N fixation is not observed at every developmental stage

Subsequently, a second growth chamber experiment was conducted to determine if the observed N fixation advantage in Benning HP is consistent throughout development. In chamber experiment 2, Benning and Benning HP were inoculated only with *B. diazoefficiens* USDA 110 and harvested at developmental stages V5, R5, R6, and R8 (maturity). As expected, seed protein concentration at maturity was significantly higher in Benning HP than in its recurrent parent, and seed N concentration and C/N at maturity were also significantly different between the two genotypes (Table 2). Although seed production in a growth chamber does not scale to yield, seed weight per plant at maturity was not significantly different between the two genotypes.

Total N fixed per plant was measured at V5, R5, R6, and R8. Although the developmental stage effect was significant for every organ, the genotype and genotype*stage effects were only significant for whole plants and for reproductive tissue (pods + seeds) (Table 3). Based on pairwise tests within developmental stages, Benning HP had significantly more

total N in reproductive tissue only at maturity, which drove the difference in total N fixed at this developmental stage (Fig. 2).

As in the first chamber experiment, total N assimilated was divided by nodule weight to calculate N fixed per nodule weight. There were not significant differences between genotypes in N fixed per nodule weight at V5, R5, or R6 (Table S1). Insufficient nodule tissue remained for N measurement at R8, so N fixed per nodule weight at maturity could not be calculated.

3.3. In growth chambers, Benning HP's greater reproductive N at maturity must be attributed to N fixation

For chamber experiment 2, the maximum amount of N that could have been remobilized from vegetative tissue into reproductive tissue between developmental stages was estimated from differences in N content between developmental stages. Vegetative N lost was calculated as the difference in vegetative N (leaf, stem, root, and nodule) between developmental stages (Table 4). Because the N remaining in abscised leaves was not measured, this value represents the maximum potential N remobilization from vegetative into reproductive tissue during seed development. Reproductive N gained was calculated as the difference in reproductive N (seed and pod) between developmental stages. In this chamber experiment, the amount of reproductive N gained was always greater than vegetative N lost. Thus, N remobilization cannot have contributed all of the N in reproductive tissue, and the rest of reproductive N must come from N fixation occurring during seed fill. The two genotypes lost similar amounts of N from vegetative tissue and gained similar amounts of N in reproductive tissue from R5 to R6 (Table 4); thus, the estimate for N fixed from R5 to R6 was also similar between the two genotypes. From R6 to R8, the two genotypes again lost similar amounts of N from vegetative tissue. During the same

296 period, however, Benning HP gained substantially more N in its reproductive tissue. Thus,
297 Benning HP may have fixed twice as much N as Benning from R6 to R8, and the propagated
298 standard errors for these estimates do not overlap (Table 4).
299
300 *3.4. Higher N remobilization is not consistent in the field*
301 Benning and Benning HP were grown in the field in 2016 and 2017 to examine nitrogen
302 remobilization during seed fill. Seed protein concentration at harvest was significantly higher in
303 Benning HP than in Benning for both years, while yield was not significantly different between
304 the genotypes (Table 5). Seed N concentration was significantly higher in Benning HP only in
305 2017, and seed size was significantly smaller in Benning HP in both years.
306 LAI and aboveground biomass were measured in 2017 for 1 m rows harvested at DAP 84
307 – 88. Both genotypes were in developmental stage R5 at this time. LAI and aboveground
308 biomass were not significantly different between genotypes (Table S2). Percent N was measured
309 for aboveground organs to calculate total aboveground N as well as vegetative and reproductive
310 N. There were no differences between the genotypes in total aboveground N, aboveground
311 vegetative N (leaf N + stem N), or reproductive N (seed + pod shell N) at R5 (Table S2). At the
312 time of these measurements, seeds had gained one-third or less of their final dry weight (Fig.
313 3B).
314 The seed N gained from R5 to R8 was calculated as it was for growth chamber
315 experiment 2, from differences in N at each developmental stage (Table S3). For Benning and
316 Benning HP in the field, the amount of seed N gained from R5 to R8 was smaller than the
317 amount of aboveground N in vegetative tissue at R5 which could have been remobilized (Table
318 S2, S3). The standard error estimates for seed N gained overlap greatly. Thus, it was not possible

to estimate from these data how much N assimilation occurred during this time period or if N assimilation may have differed between genotypes.

Seed growth in Benning and Benning HP was examined in both years. Seed fill began fewer DAP in 2017, because the planting date was much later that year. In 2016, the overall effect of genotype on individual seed dry weight was significant (Fig. 3A). Individual seed weight tended to be higher in Benning late in seed fill in 2016, but pairwise comparisons were not significant on any specific sampling dates during seed fill, and genotype did not have a significant effect on individual seed weight during seed fill 2017 (Fig. 3B). Genotype had a significant effect on seed N concentration (Fig. 3C – D) and on seed C/N (Fig. 3E – F) throughout seed fill in both years, and the pairwise difference between genotypes was significantly different on almost every measurement day through both years. In 2017, seeds were too small on the first sampling date to measure C and N content.

In leaves, genotype had a significant effect on N concentration and on C/N across the growing season in 2016, but not in 2017 (Fig. S1A, S1B). Differences between the two genotypes were not significant on individual measurement days during vegetative development or during flowering, but leaf N concentration became significantly lower in Benning HP by the end of the seed fill period in 2016 (Fig. S1A), and leaf C/N diverged a few sampling dates earlier (Fig. S1C), suggesting more N depletion from leaves during the seed fill period. The same leaf N concentration and C/N data from the seed fill period were then analyzed with DAP treated as a continuous variable instead of a repeated effect to test if the slope of leaf N concentration and leaf C/N over time was different between the two genotypes. In 2016, genotype and genotype*DAP effects were significant across the seed fill period for leaf C/N and leaf N concentration (Fig. 4A, 4C). The slope of predicted leaf N concentration over time was

342 significantly lower for Benning HP than for Benning, while the slope of predicted leaf C/N over
343 time was significantly higher in Benning HP. However, genotype and genotype*DAP effects
344 were not significantly different in 2017 (Fig. 4B, 4D).

345 Leaf chlorophyll was measured for the same DAP to assess whether differences in N
346 remobilization rates could be detected through changes in leaf chlorophyll content. Despite the
347 differences found for leaf N concentration and leaf C/N, the genotype and genotype*DAP effects
348 for leaf chlorophyll in 2016 were not significantly different between Benning and Benning HP
349 (Fig. S2).

4. Discussion

The primary goal of this study was to identify the N flux(es) that enable higher seed protein without a yield penalty in Benning HP compared to its recurrent parent, Benning. In these experiments, Benning HP was capable of greater N fixation when N sources were controlled in growth chambers and of a higher rate of N depletion from vegetative tissue during seed fill in the field, but neither of these traits was observed consistently. Strikingly, Benning HP plants inoculated with *B. diazoefficiens* USDA 110 in growth chambers fixed over twice as much N on average from R6 to maturity. Differences in seed protein concentration between two unrelated genotypes of similar yield potential have previously been linked to higher N fixation during seed fill period (Leffel et al., 1992a). In a greenhouse study, N fixation across reproductive development, measured by integrating acetylene reduction measurements over time, was found to be significantly and positively correlated with seed protein concentration, while integrated N fixation was not significantly correlated with yield (Fabre and Planchon, 2000).

B. diazoefficiens USDA 110 is more efficient at fixing N than *B. elkanii* USDA 31, and this has been linked to the presence of a hydrogenase in the nodule which recycles H_2 evolved from the nitrogenase-catalyzed reaction (Caldwell and Vest, 1970; Schubert et al., 1978). As expected, plants inoculated with *B. diazoefficiens* USDA 110 in this study fixed more N and had greater dry weight across genotypes than those inoculated with *B. elkanii* USDA 31. The lower N fixation efficiency of *B. elkanii* USDA 31 restricted the higher N fixation potential of Benning HP that was observed with *B. diazoefficiens* USDA 110. The native rhizobia population was not characterized for the field experiment, but a prior analysis in a different soil type at the same research station found the majority of nodules to be infected with rhizobia belonging to serotypes 31/94, 46/76, and 76 (Ramirez et al., 1997). In that study, only about 15% of the samples isolated

373 from the research station had high N fixation capacity, as does *B. diazoefficiens* USDA 110,
374 while ca. 20% had low N fixation capacity like *B. elkanii* USDA 31, and the rest had
375 intermediate N fixation capacity. Thus, it is possible that in our field experiment, Benning HP's
376 potential to supply more N to developing seeds from N fixation was restricted by the efficiency
377 of rhizobia strain(s) that were present in soil.

378 The rate of N remobilization, measured as the slope of leaf C/N over time, from Benning
379 HP leaves was only higher in one of the two years of the field experiment, despite Benning HP
380 maintaining higher seed protein concentration and similar yield to Benning in both years. This
381 variability in N remobilization between years, together with the growth chamber results, do not
382 support the self-destructive hypothesis, which suggested that high seed N requirements in
383 soybean trigger leaf senescence and thus limit the seed fill period (Sinclair and de Wit, 1975).
384 Under the artificial, but optimal, environmental conditions of the growth chambers, the high N
385 requirement in developing seeds was met by sustained N fixation though seed fill in both
386 Benning and Benning HP, as remobilized N accounted for less than half of the N in each
387 genotype at maturity. In the field, Benning HP produced the same seed yield as Benning despite
388 remobilizing N from leaves at a higher rate in 2016. This suggests that under favorable
389 environmental conditions, leaves can continue to assimilate carbon and thus fuel N fixation well
390 into the seed fill period, and that environmental cues may play a larger role than seed N demand
391 in triggering leaf senescence. Because the carbon demand for N fixation is high (Rainbird et al.,
392 1984a), this implies that leaf senescence may actually have been delayed relative to seed
393 development if photosynthesis continued at rates necessary to sustain nodule function.

394 The data from these experiments cannot conclusively determine if seed composition
395 differences in Benning HP are source- or sink-driven. However, differences in seed N

concentration and seed C/N between Benning and Benning HP were established early in seed development, when seeds had gained less than a third of their final weight. Leaf C/N had not yet diverged between the two genotypes this early in seed development. These observations, together with the intermittent differences in N fixation and N remobilization rate, suggest that the Benning HP's higher seed N may be more dependent on N sink strength than on uniformly higher N fixation or N remobilization.

In growth chamber experiment 2, the higher total N assimilation in Benning HP at maturity was accompanied by substantially higher mature seed weight per plant (ca. 20%), congruent with a previous finding that total N at R7 is positively correlated with yield across genotypes (Rotundo et al., 2014). The seed protein gain for chamber-grown Benning HP was similar to that in the field, ca. 3-4%. As Benning HP's maximum protein gain over Benning seems to be in this 3-4% range, the potential for greater N fixation during seed fill in Benning HP could contribute to yield maintenance despite higher seed protein.

In the field, individual seed weight was lower in Benning HP when considered across the seed fill period in 2016, and Benning HP's 100 seed weight at harvest was significantly lower in both years. This is consistent with the findings of Prenger et al. (2019). Based on these findings, it is possible that Benning HP's higher seed protein concentration is related to reduced weight accumulation per seed relative to N, but yield is maintained because the of Benning HP's ability to remobilize or fix sufficient N to support a similar total weight of seeds. Genotypic differences in seed sink strength for N were also observed in an *in vitro* study comparing cotyledon N uptake by normal and high protein genotypes at a range of N concentrations (Hayati et al., 1996). In that study, cotyledon N concentration in both genotypes responded positively to N availability in solution, but the high protein genotype's seeds were able to take up more N at every

419 concentration. Seed N concentration was significantly different between Benning and Benning
420 HP through most of seed development. The lack of significance for the seed N concentration in
421 2016 harvest data is probably the result of variability through the canopy (Huber et al., 2016).
422 Harvest samples were taken from the combine, which homogenized all the seed from a row. In
423 contrast, samples during seed development were selected from the same canopy position.
424 Directly measuring leaf N requires specialized, expensive instrumentation, and requires
425 destructive sampling. Chlorophyll content also declines as leaves senesce, and chlorophyll can be
426 extracted in relatively simple assay (Porra et al., 1989) or estimated non-destructively in the field
427 (e.g., Lichtenthaler et al., 1996; Cassol et al., 2008; Steele et al., 2008). The N in chlorophyll is
428 not directly exported from senescing leaves (Hörtensteiner and Feller, 2002), but N from the
429 concurrent breakdown of chloroplast proteins is an important source of N for remobilization to
430 vegetative tissue (Fischer, 2007; Liu et al., 2008). Thus, the decline in leaf chlorophyll content
431 could be a convenient proxy for estimating N remobilization in future studies. We measured leaf
432 chlorophyll in 2016 to determine if it could be used as a surrogate for N remobilization rates.
433 However, the decline of leaf chlorophyll content during seed fill did not reveal the difference in
434 N depletion that was measured in the field in 2016. These results indicate that chlorophyll would
435 not be reliable for detecting subtle but significant differences in leaf N metabolism.
436 These experiments revealed that the introgressed region of chromosome 20 from
437 Danbaekkong in the Benning background can increase N fixation as well as the rate of N
438 remobilization from senescing soybean leaves, but that the phenotypic expression of these traits
439 may depend on environment and biotic interactions. Whether the changes in these N fluxes are
440 source or sink driven remains unresolved, and the molecular mechanisms behind these changes
441 have yet to be elucidated. Although the Gm20 protein locus has been narrowed to a <1Mb region

(Vaughn et al., 2014), the protein gain combined with maintaining the high yield potential of Benning evidently involves multiple genes, as evidenced by large region of Danbaekkong's chromosome 20 that is present in Benning HP (Prenger et al., 2019). If the gene(s) involved can be identified, a new path to improving soybean seed composition through breeding or biotechnology could be illuminated.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Table S1. N fixed per nodule weight means (mg/g; top) and *p*-values from ANOVA (bottom) in chamber experiments 1 and 2. Letters indicate significant differences between genotypes within a treatment ($p < 0.05$).

Table S2. Leaf area index, aboveground biomass, and aboveground N measured at 84 – 88 DAP in 2017, when Benning and Benning HP were in developmental stage R5; $n = 4$.

Table S3. Seed N gained from R5 to R8 in the field in 2017. Seed N gained was calculated as the difference in mean seed N between R5 and R8. Values are mean ± standard error; errors were propagated as described in the Methods; $n = 4$.

Figure S1. Leaf N concentration and leaf C/N across the growing season for Benning and Benning HP grown in the field in 2016 and 2017. Leaf tissue was sampled from the uppermost, fully expanded leaf in the canopy. Significant pairwise differences (FDR-corrected $p < 0.05$) between genotypes on a sampling day are indicated by asterisks. Points and error bars are means ± standard error.

Figure S2. Leaf chlorophyll content during the 2016 seed fill period, R5 and R6. Chlorophyll was isolated from tissue sampled from the uppermost, fully expanded leaf in the canopy. Slopes were not significantly different between the two genotypes.

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598

599

600 **Figures and Tables**

601 Table 1. ANOVA results for chamber experiment 1, comparing N assimilation at 45 DAT in

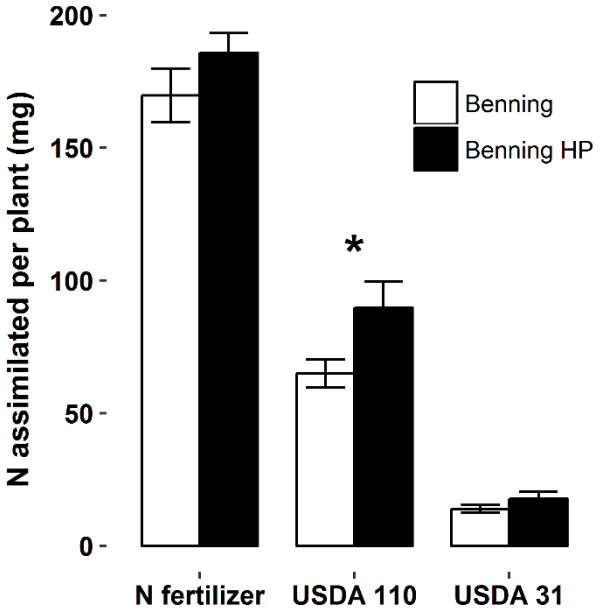
602 Benning and Benning HP from NH_4NO_3 fertilizer, inoculation with *B. diazoefficiens* USDA 110,

603 and *B. elkanii* USDA 31.

Source	DF	Sum of Squares	Mean Square	Error DF	F Value	*p*-value
genotype	1	2012.6	2012.6	30	6.87	**0.0136**
N treatment	2	160308	80154	30	273.51	**< 0.0001**
genotype × N treatment	2	663.6	331.8	30	1.13	0.3357
residual	30	8791.8	293.1	.	.	.

604

605 Figure 1. Total nitrogen assimilated per plant (mg) for plants supplied with NH_4NO_3 fertilizer,
606 inoculated with *B. diazoefficiens* USDA 110, or inoculated with *B. elkanii* USDA 31 in chamber
607 experiment 1. Whole plants were harvested 45 days after transplanting. Values are mean ±
608 standard error; $n = 4$. Asterisks indicate significant differences ($p < 0.05$) between genotypes
609 within N treatment.



610

611 Table 2. Seed protein concentration, seed N concentration, seed C/N, and total seed weight per
612 plant at maturity from plants relying on nitrogen fixation with *B. diazoefficiens* USDA 110 in
613 chamber experiment 2. Seed protein concentration is expressed on a 13% moisture basis. *P*-value
614 for genotype effect on each variable shown in bottom row. Values are means for each genotype;
615 $n = 5$.

Genotype	Seed protein concentration (%)	Seed N concentration (%)	seed C/N	Seed weight per plant (g)
Benning	37.5	6.5	8.2	69.0
Benning HP	41.3	7.7	6.8	82.8
p-value	**<0.0001**	**0.0179**	**0.0022**	0.1554

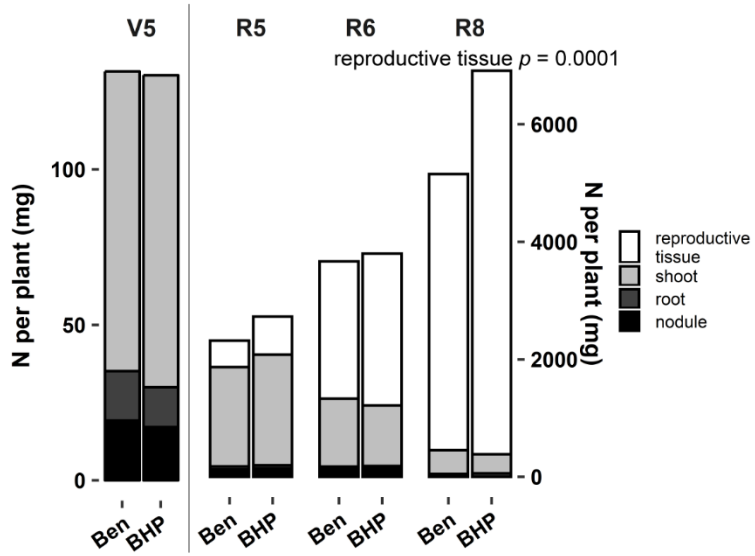
616

617 Table 3. ANOVA results for chamber experiment 2, total nitrogen fixed per plant and nitrogen
618 per organ measured in Benning and Benning HP at four developmental stages. Analysis
619 corresponds with data presented in Figure 2. Reproductive tissue includes seeds and pods.

Test	Source	DF	Sum of Squares	Mean Square	Error DF	F Value	*p*-value
whole plant	genotype	1	3204975	3204975	31	8.23	**0.0074**
	stage	3	171682989	57227663	31	146.92	**< 0.0001**
	genotype × stage	3	4487722	1495907	31	3.84	**0.0190**
	residual	31	12075101	389519	.	.	.
reproductive tissue	genotype	1	4146291	4146291	23	14.17	**0.0010**
	stage	2	122288498	61144249	23	208.91	**< 0.0001**
	genotype × stage	2	3981910	1990955	23	6.8	**0.0048**
	residual	23	6731820	292688	.	.	.
shoot	genotype	1	100	100	31	0	0.9661
	stage	3	17146518	5715506	31	104.5	**<.0001**
	genotype × stage	3	146004	48668	31	0.89	0.4573
	residual	31	1695501	54694	.	.	.
root	genotype	1	61	61	31	0.36	0.5507
	stage	3	9912	3304	31	19.75	**< 0.0001**
	genotype × stage	3	305	102	31	0.61	0.6155
	residual	31	5186	167	.	.	.
nodules	genotype	1	709	709	24	3.5	0.0738
	stage	2	100095	50048	24	246.57	**< 0.0001**
	genotype × stage	2	668	334	24	1.64	0.2141
	residual	24	4871	203	.	.	.

620

Figure 2. N per plant (mg) in Benning and Benning HP relying on nitrogen fixation with *B. diazoefficiens* USDA 110 in chamber experiment 2. Values are means (n = 5). Annotations indicate FDR-corrected p-value for significant differences between genotypes in N content per organ; ANOVA results corresponding with these data are presented in Table 3. Scale on left applies to V5; scale on right applies to R5 – R8. Ben = Benning; BHP = Benning HP.



627 Table 4. Nitrogen lost per plant (mg) from vegetative tissues and nitrogen gained in reproductive
628 tissues between developmental stages in chamber experiment 2. Nitrogen fixed during each
629 period was estimated as the difference between reproductive N gained and vegetative N lost.
630 Values are mean ± standard error; errors were propagated using equation 4 as described in the
631 Materials and Methods.

Genotype	R5 – R6			R6 – R8		
	Vegetative N lost	Reproductive N gained	Estimated N fixed	Vegetative N lost	Reproductive N gained	Estimated N fixed
	---------- (mg plant^{-1}) ----------					
Benning	540 ± 213	1887 ± 153	1373 ± 262	875 ± 164	2363 ± 488	1488 ± 515
Benning HP	867 ± 169	1940 ± 121	1073 ± 208	833 ± 46	3945 ± 359	3112 ± 362

632

633 Table 5. Mean yield, seed protein concentration, seed N concentration, and seed size at

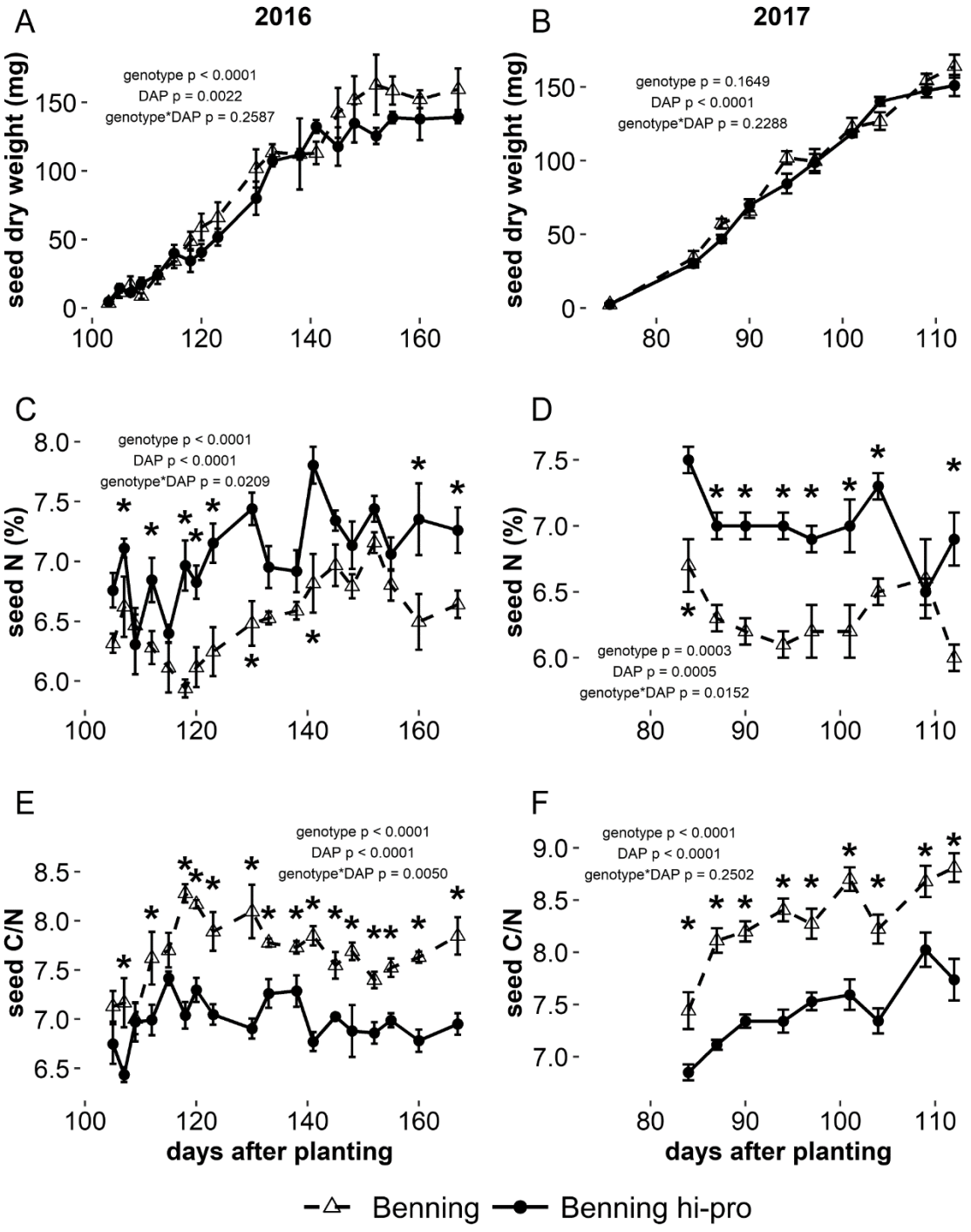
634 developmental maturity in field experiments. Seed protein is expressed on a 13% moisture basis;

635 $n = 4$.

	2016				2017			
Genotype	Yield (g m^{-2})	Seed protein concentration (%)	Seed N concentration (%)	Seed size (g 100 $seed^{-1}$)	Yield (g m^{-2})	Seed protein concentration (%)	Seed N concentration (%)	Seed size (g 100 $seed^{-1}$)
Benning	206.5	39.1	6.5	16.9	218.1	34.8	5.9	16.1
Benning HP	214.6	41.9	6.8	14.8	194.3	39.0	6.5	15.1
p-value	0.6005	**0.0011**	0.4093	**0.0406**	0.1843	**<0.0001**	**0.0267**	**0.0155**

636

637 Figure 3. Individual seed weight, seed N concentration, and seed C/N during seed fill for
638 Benning and Benning HP grown in the field. Significant pairwise differences (FDR-corrected $p <$
639 0.05) between the genotypes within days are indicated by asterisks. Points and error bars are
640 means ± standard error.



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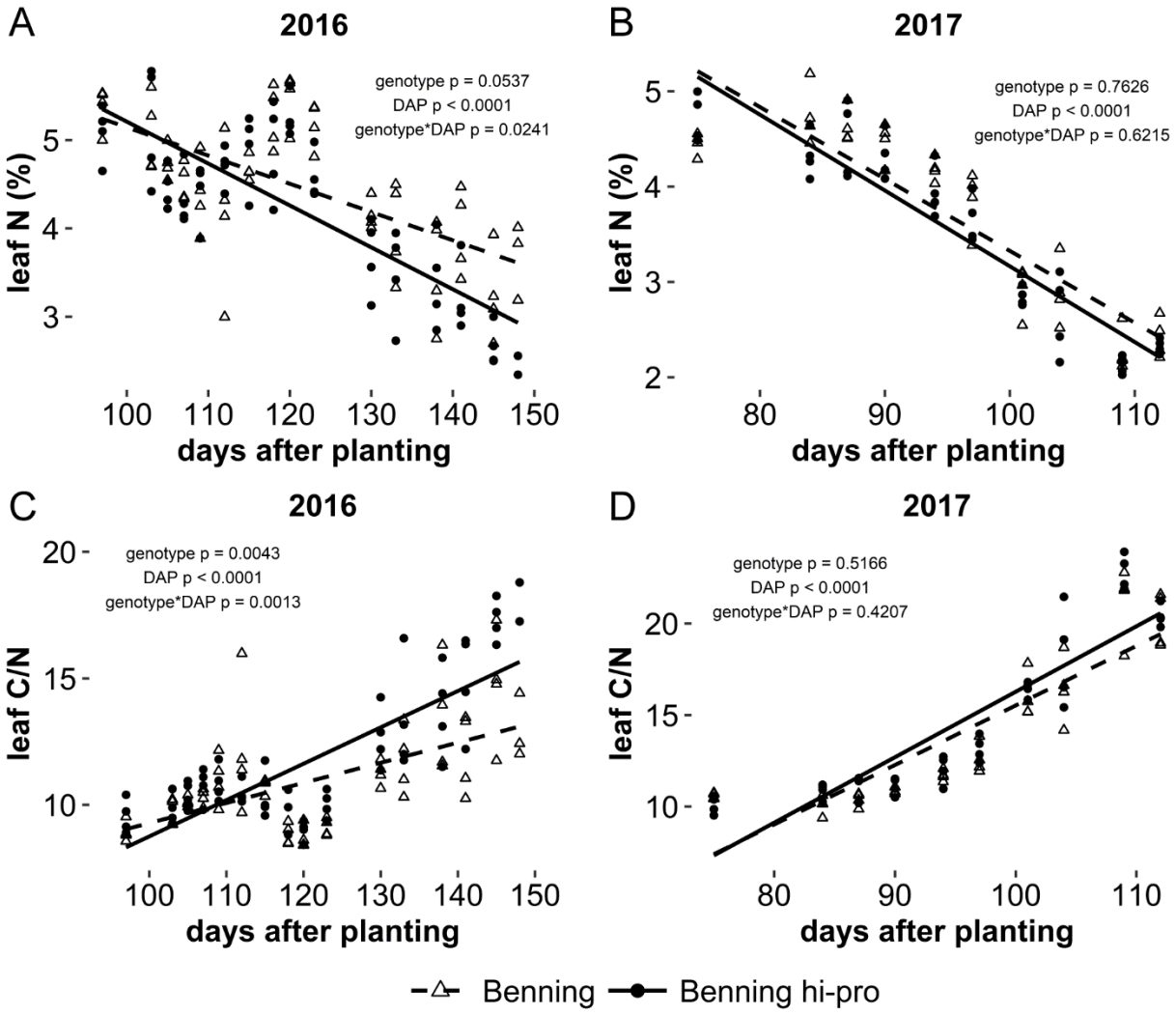
642

643 Figure 4. Leaf N concentration and C/N change during the seed fill period, R5 and R6. Dashed
644 and solid lines are predicted values for leaf N concentration and C/N in Benning and Benning HP
645 from the linear model. *P*-values indicate significance of fixed effects in the model.

646



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