



RESEARCH ARTICLE

Genotype and cytokinin effects on soybean yield and biological nitrogen fixation across soil temperatures

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Abstract

High nitrogen (N) supply is required for high-yielding soybean, but low soil temperatures in either early production systems or cool environments delay nodulation and limit biological nitrogen fixation (BNF). Because cytokinins are key signalling hormones in mediating nodule formation and our initial controlled environment experiment indicated that seed cytokinin treatment increased early BNF and total nodule area, it was used in field trials. Cytokinin was applied (seed or foliar) to two commercial soybean genotypes (DM50I17 and DM40R16) in field trials with early (September and early November) and conventional (late November) sowing dates in Argentina. In the field, DMR50I7 achieved consistent yields across sowing dates because increased BNF compensated for limited soil N uptake in early sowing dates, also leading to 25% higher nitrogen use efficiency (NUE). Surprisingly, soil N uptake was more cold-sensitive than BNF with greater and prolonged N fixation in early sowing, perhaps through delayed nodulation, leading to improved N harvest index. Cytokinin seed treatment increased BNF (26%) in DM40R16 especially in early sowing dates. Although cytokinin improved cold tolerance of BNF, this was not explained by altered nodulation and did not increase yield. Here we show genetic differences in N supply in commercial soybean genotypes and the importance of BNF to maintain yield in early sown soybean.

KEYWORDS

canopy N uptake, *Glycine max*, hormone application, nodulation, root zone temperature, seed priming, yield

1 | INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is one of the most important vegetable protein sources globally, contributing to the agricultural economies of many countries (Hungria & Mendes, 2015). Soybean has the highest nitrogen (N) requirement of all major crops (Sinclair & De Wit, 1975) with 80 kg canopy N required per metric tonne of seed,

and yield strongly correlated to N accumulation (Rotundo, Borrás, De Bruin, & Pedersen, 2014; Salvagiotti et al., 2008). As a legume, soybean uses two N sources, mineral soil N uptake and atmospheric or biological nitrogen fixation (BNF). Soybean can derive up to 70% of its N demand from BNF (Salvagiotti et al., 2008; Santachiara, Borrás, Salvagiotti, Gerde, & Rotundo, 2017) and high soil N concentrations limit BNF (Santachiara, Salvagiotti, & Rotundo, 2019).

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Temperature also affects the contribution of the two N sources to plant N status, with BNF generally considered more cold-sensitive than soil N uptake (Legros & Smith, 1994; Matthews & Hayes, 1982; Thomas & Sprent, 1984). In soybean, root zone temperatures (RZT) less than 25°C delay the onset of BNF, with nodule initiation limited at 10°C RZT and activity at 15°C (Legros & Smith, 1994; Mishra, Mishra, Selvakumar, Kundu, & Shankar Gupta, 2009; Poustini, Mabood, & Smith, 2005; Zhang, Lynch, & Smith, 1995). However, low soil temperatures may also limit mineral N uptake by restricting root growth and/or nitrate uptake as seen in controlled environments (Rufty, Raper, & Jackson, 1981; Tolley & Raper, 1985) but not in field trials.

Despite these limitations, which may limit early growth and subsequent yields, many regions recommend early sowing of soybean in cold soils (Di Mauro, Borrás, Rugeroni, & Rotundo, 2019; Rattalino Edreira et al., 2020) to take advantage of early rainfall, to avoid summer drought, reduce disease and insect damage and extend the growing season. Local soybean production has the potential to improve protein self-sufficiency (de Visser, Schreuder, & Stoddard, 2014), even though many European countries have suboptimal environments for soybean (Kurasch et al., 2017).

BNF depends on successful nodulation and rhizobial efficiency to fix atmospheric N₂ to ammonia. Previous work to mitigate the effects of low RZT on BNF have focused on identifying cold-tolerant rhizobia (Kühling, Hüsing, Bome, & Trautz, 2018; Yuan et al., 2020; Zhang, Charles, Driscoll, Prithiviraj, & Smith, 2002; Zimmer et al., 2016). However, the success of rhizobial inoculants can depend on their persistence in the soil and competition with native rhizobia, with local strains better adapted to adverse conditions (Thilakarathna & Raizada, 2017). Early nodule establishment in low RZT may therefore improve the effectiveness of cold-optimised inoculants. The photosynthetic cost of BNF, 16 mol ATP per mole N (Kahn, McDermott, & Udvardi, 1998), requires that plants balance this with their N requirements; however, N is more limiting to growth than carbon (C) uptake under low (~15°C) temperatures (Thomas & Sprent, 1984; Walsh & Layzell, 1986). Thus, promoting nodulation in cold environments is likely to be beneficial.

In optimal temperatures, certain nodule traits are associated with increased BNF. Nodule size positively correlates with increased N fixation (de Araujo, de Almeida Lopes, Mier y Teran, Palkovic, & Gepts, 2017; Tajima, Lee, Abe, Lux, & Morita, 2007; Voisin, Salon, Jeudy, & Warembourg, 2003a) and certain nodule sizes are considered optimal (King & Purcell, 2001; Purcell, De Silva, King, & Kim, 1997), with greater relative export of N products and import of C. Increased nodule weight following low RZT temperatures (15°C) may compensate for lower nodule activity (Zhang & Smith, 1994), suggesting that increased nodulation is beneficial for cold tolerance. The effects of early nodule establishment on BNF have been studied previously (Cerezini et al., 2016; Chibeba et al., 2015) but not in early sown soybean experiencing low RZT.

Different soybean genotypes vary in their ability to fix N in low temperature (Lynch & Smith, 1993; Zhang & Smith, 1994). As new soybean varieties show reduced BNF under optimal conditions (Nicolás, Arrabal Arias, & Hungria, 2002; van Kessel & Hartley, 2000), similar effects could occur under cold temperatures but with greater impacts on yield. Maintaining N uptake during seed filling is important

for high yield (Kumudini, Hume, & Chu, 2002; Zimmer et al., 2016) especially in early sown soybean. Although genotypes differed in BNF when grown in cool conditions, there was no effect on nodulation (Zimmer et al., 2016) and nodule traits were not associated with genotypic differences in cold tolerance.

An alternative approach to enhance nodulation and reduce the effects of cold is to manipulate endogenous hormone concentrations *in planta*, such as cytokinins (Ali, Hayat, Hasan, & Ahmad, 2008; Fatima, Bano, & Aslam, 2008; Heckmann et al., 2011; Lorteau, Ferguson, & Guinel, 2001). Cytokinin application may enhance nodulation by maintaining plant rhizobial communication in low RZT. Host plants initiate nodulation by secreting flavonoids that activate rhizobial genes, including *nod* genes which code nod factors (NF; Redmond et al., 1986; Caetano-Anollés & Gresshoff, 1993; Dénarié, Debelle, & Promé, 1996; Spaink, 2000). Root perception of NF leads to root hair deformation and rhizobial invasion of root cortical cells, via the infection thread, to elicit nodule formation. Suboptimal soil temperatures (less than 25°C) limit these stages of nodule establishment (Lindemann & Ham, 1979; Lynch & Smith, 1993; Matthews & Hayes, 1982), especially infection and early nodule development, because of limited *nod* gene expression and NF synthesis (Shiro, Kuranaga, Yamamoto, Sameshima-Saito, & Saeki, 2016; Zhang & Smith, 1994). Cytokinin induces early nodulin genes in plants acting in a similar way to NF signalling, inducing cortical cell division genes (Bauer, Ratet, Crespi, Schultze, & Kondorosi, 1996; Dehio & de Bruijn, 1992; Heckmann et al., 2011; Mathesius, Charon, Rolfe, Kondorosi, & Crespi, 2000). Therefore, early cytokinin application during nodule formation may compensate for delayed bacterial signalling and stimulate higher rates of nodule development and BNF.

Exogenous cytokinin applications induced positive effects in a number of legumes depending on the application method, timing and concentration (Cho, Suh, Park, & Wood, 2002; Koprna, De Diego, Dundáková, & Spíchal, 2016; Liu, Jensen, & Andersen, 2004); with high concentrations limiting nodule number (Lorteau et al., 2001; Mens, Li, Haaima, Gresshoff, & Ferguson, 2018). Cytokinin applications during early reproductive development (stages R1–R3) increased pod set (dos Passos, de Rezende, de Carvalho, & Savelli, 2008; Ibrahim, Bekheta, El-Moursi, & Gaafar, 2007; Nonokawa, Kokubun, Nakajima, Nakamura, & Yoshida, 2007; Yashima, Kaihatsu, Nakajima, & Kokubun, 2005). Cytokinin seed priming or application to recently emerged seedlings also increased yield of other legumes but effects are unknown in soybean (Dhruve & Vakharia, 2013; Fatima et al., 2008; Naeem, Bhatti, Ahmad, & Ashraf, 2004; Schroeder, 1984). Seed treatment with nonthermal plasmas increases soybean nodule nitrogenase activity, in part by increasing endogenous cytokinin concentrations (Pérez-Pizá et al., 2020). While cytokinin application can enhance BNF in chickpea (*Cicer arietinum*; Fatima et al., 2008), to our knowledge no studies have considered cytokinin application to improve BNF of early sown soybean.

Because N supply is the most limiting factor to soybean yield (Rotundo et al., 2014) and cold temperature (<25°C) limits its uptake (Rufty et al., 1981; Tolley & Raper, 1985; Zhang et al., 1995), we tested whether N uptake varied between different genotypes and with cytokinin application. A controlled environment experiment

assessed the effectiveness of cytokinin in enhancing BNF, then a field experiment with early and conventional sowing dates aimed to (a) examine low-temperature responses of different commercial soybean genotypes and (b) test whether cytokinin application could enhance BNF in cold temperature. Because nodule formation and BNF are sensitive to cold temperature, we hypothesised that early sowing would limit BNF and any genotypic differences in cold tolerance will reflect differences in N uptake. Moreover, we hypothesised that cytokinin treatment would enhance nodulation, helping to maintain BNF during exposure to low soil temperature.

2 | MATERIALS AND METHODS

2.1 | Site conditions, treatments and experimental design

A controlled environment experiment was conducted with soybean (*Glycine max* cv. Viola) to determine if cytokinin treatment could increase BNF by altering nodulation. Seeds were sown into 1 L pots in a randomised block design with 12 biological replicates (one plant per pot) per treatment. After autoclaving, fine grade (1–3 mm) vermiculite (Sinclair professional, Ellesmere Port, UK) was used as the substrate. Before sowing, seeds were surface-sterilised with 1% sodium hypochlorite and then repeatedly washed. Seeds were inoculated with 10^8 cells/ml of *Bradyrhizobium japonicum* USDA110 that was previously cultured on YEM agar (Somasegaran & Hoben, 1994) at 29°C. Two seeds were sown per pot, later thinned to one plant per pot just after emergence (VE). Pots were irrigated with modified N-limited Hoagland's nutrient solution that lacked NO_3^- , to prevent the inhibition of nodulation. Average greenhouse temperature was 29.8°C day/21.3°C night. Light was supplemented by high-pressure sodium lamps (600 W Greenpower, Osram, St Helens, UK) when photosynthetic photon flux density (PPFD) was less than $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ for a 12 hr photoperiod (7:00 a.m. to 7:00 p.m.).

The synthetic cytokinin kinetin (Sigma Aldrich) was applied via three application methods: seed priming, root (applied to substrate) and foliar spray. Seeds that were not primed in kinetin (root, foliar and control) were primed in water and plants not sprayed with kinetin (root, seed primed and control) were sprayed with water. For the seed priming treatment, 25 g of seed were submerged in 25 ml of 10^{-7} (high) and 10^{-9} mol/L (low) kinetin solution for 4 hr. Seeds were air-dried in the greenhouse before inoculation and sowing later that day. Foliar and root application took place at early growth stages, VC and V1, respectively. Foliar spray was applied with a handheld pump pressure sprayer and root application by pouring 20 ml of kinetin solution onto substrate. Again, concentrations of 10^{-7} (high) and 10^{-9} mol/L (low) kinetin solution were used for both foliar and root applications.

A field trial was conducted to determine genotypic differences in response to early sowing and assess the effectiveness of cytokinin treatments to improve BNF in low RZT under field conditions. Trials were sown during the 2018/2019 growing season, with three sowing dates of 25 September, 8 November (early November) and

25 November (late November), at Campo Experimental Villarino, located in Zavalla, Santa Fe, Argentina (33°1'S, 60°53'W; elevation 24.6 m). Soil and air temperature and potential evapotranspiration (Hargreaves & Samani, 1985) varied across sowing dates but precipitation did not (Figure 1 and Table S1, Supporting Information). The USDA soil series was a silty clay loam Vertic Argiudoll, Roldan series, and soybean was the previous crop. Soil (0–20 cm depth) had 2.86% organic matter, 13.9 mg/kg P, 5.8 pH and N-NO_3^- were 12.5 mg/kg in September, 22.9 mg/kg in early November, and 7.1 mg/kg in late November. This rainfed experiment was sown in a field having a double crop of wheat (*Triticum aestivum*) and soybean during the previous season.

Cytokinin treatments (kinetin; Sigma Aldrich) consisted of either seed priming (10^{-9} mol/L), foliar spray (10^{-7} mol/L) or water control. All seeds were submerged either in water (foliar and control) or cytokinin solution (seed) for 4 hr, air-dried and stored at 4°C until sowing the following day. Cytokinin treatment did not significantly affect emergence, measured 22 days after sowing. Foliar cytokinin treatment was applied at VC and V1 (rate of 50 L/ha), with control and seed-treated plants sprayed with water. We used two commercial soybean genotypes developed by Grupo Don Mario DM40R16 and DM50I17, maturity groups IV and V, respectively. For the late November sowing date, days from emergence to R7 (physiological maturity) for genotypes DM50I17 and DM40R16 differed by 12 days. Figure 1 shows the phenology of genotypes from each sowing date. After drying, seeds were coated with inoculant and osmoprotector at recommended rates with Rizoliq LLI (Rhizobacter, Argentina) and seed insecticide and fungicide, Cruiser Advanced (Syngenta, Argentina) at recommended rates. A complete block design was used with genotypes and cytokinin treatments randomised within blocks, resulting in three plot replicates for each cytokinin/genotype combination per sowing date. Plots were over-seeded and hand-thinned to a target plant population of 20 plants/m². Manual sowing was necessary because of enlarged seed following seed priming, where seeds were evenly distributed into furrows approximately 3 cm deep. Each plot was 6 m long with four rows 0.52 m apart (plot size was 12.5 m²), with all measurements comprising the two central rows. Weeds and pests were chemically controlled with commercially available products as needed.

2.2 | Biomass and N concentration

In the controlled environment experiment, plants were harvested at flowering stage (R1, ~30 DAS), shoots were removed from the roots at the cotyledons and leaf area was measured using a leaf area meter (Model Li-3100C; Li-Cor, Lincoln, NE, USA). Shoots were then dried at 60°C for 72 hr to obtain shoot dry weight. After drying, entire stems were milled for relative ureide analysis (Peoples, Faizah, Rerkasem, & Herridge, 1989; Santachiara, Salvagiotti, Gerde, & Rotundo, 2018).

In the field trial, above ground biomass was sampled at the R1, R3, R5 and R7 phenological stages (Fehr & Caviness, 1977) (Figure 1) from a 0.5 m² area, leaving the first and last plant of the rows to prevent border effects. From each harvest, leaf area was measured with a leaf area meter (Model Li-3100C; Li-Cor), and plants were separated

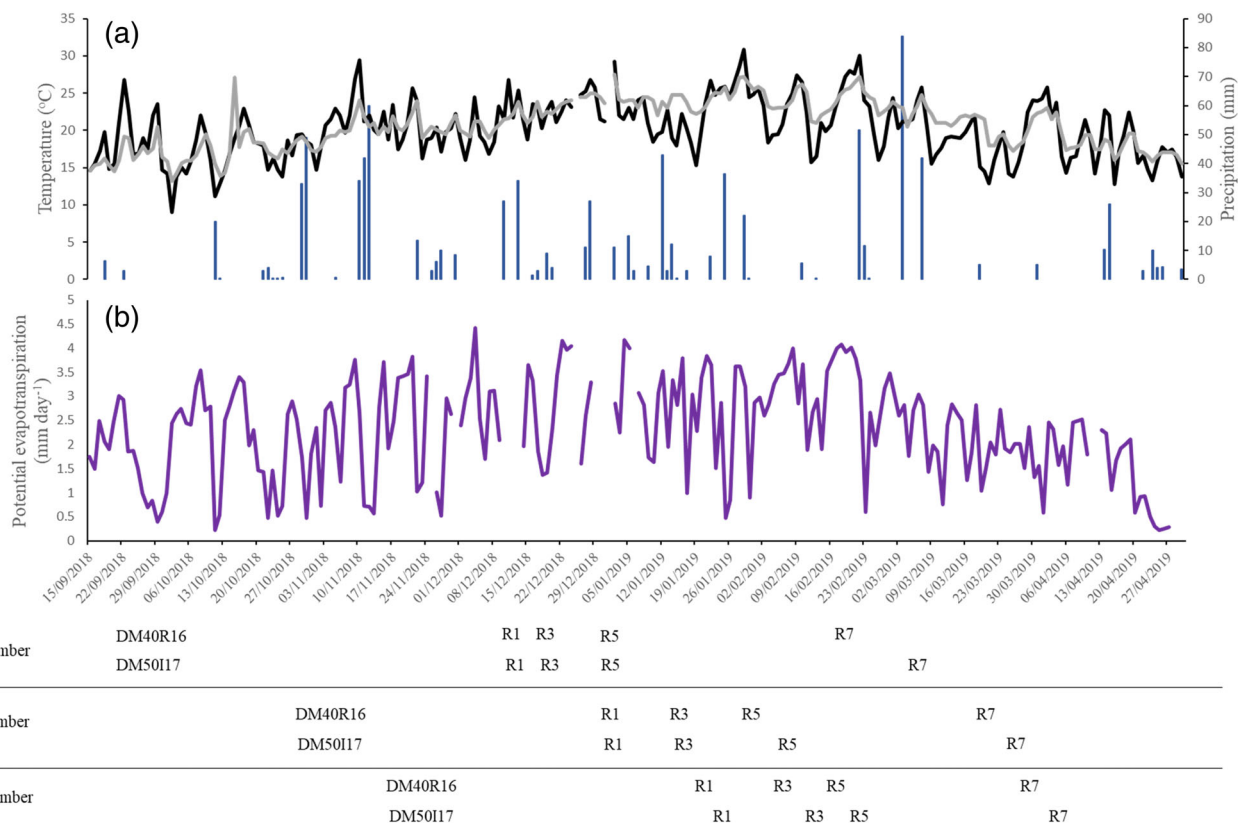


FIGURE 1 Daily air and soil (10 cm depth) temperature (black and grey line, respectively), precipitation (blue bars; a) and potential evapotranspiration (purple line; b). Phenological growth stages for each genotype (DM40R16 and DM50I17) in each sowing date, with genotype name denoting sowing date, are shown below

into leaves and stems and dried at 60°C in an air-forced oven. After drying, all plant parts were weighed to determine dry matter. Seed yield was determined at physiological maturity from the remainder of the plot (2.1 m²) using an experimental static harvester. After weighing, all plant biomass samples were milled to 1 mm. N concentration in leaves and stems was determined using Kjeldahl procedure (McKenzie & Wallace, 1954). N use efficiency was calculated by dividing total above ground biomass by total N uptake (Xu, Fan, & Miller, 2012). N harvest index was calculated by dividing total seed N content by total canopy N uptake at R7.

2.3 | Biological nitrogen fixation

Stem samples were used to determine BNF by calculating relative abundance of ureides in both controlled environment and field trials (Hungria & Araujo, 1994). Ureide products from fixation (allantoin and allantoic acid), nitrates and amino acids (asparagine and glutamine) were determined and the ratio of each was calculated. Ground stem samples (0.4 g) were used to extract ureide, nitrate and amino acid in 0.1 mol/L phosphate buffer and ethanol heated to 80°C. After cooling, extracts were filtered and centrifuged at 10,000g then stored at -20°C until analysis. The Young-Conway's method (Young & Conway, 1942), Cataldo method (Cataldo, Maroon, Schrader, &

Youngs, 1975) and ninhydrin method (Yemm, Cocking, & Ricketts, 1955) were used to colorimetrically measure ureide, nitrate and amino acid N, respectively. Relative ureide was calculated as

$$\text{Relative ureide} - \text{N}\% = \left(\frac{4U}{(4U + N + AA)} \right) \times 100,$$

where U, AA and N are molar concentrations of ureide, amino acids and nitrate, respectively (Herridge & Peoples, 1990). The amount of N fixed biologically (kg/ha), for each harvest, was calculated by multiplying relative ureide N (%) by aboveground total N (kg/ha; Herridge & Peoples, 1990). By adding the amount of biologically fixed N at each harvest date plus the amount accumulated between each harvest date, total N coming from BNF at physiological maturity (kg/ha) was determined. The ratio between biologically fixed N (kg/ha) and total N uptake at maturity provides the final percentage of N derived from fixation (Ndfa%) for the growth period. The difference between aboveground total N (kg/ha) and biologically fixed N (kg/ha) indicates soil mineral N absorption.

2.4 | Nodulation

In controlled environment experiments, root samples were frozen at -20°C until analysed, because these labour-intensive measurements

took 30 min per sample. Roots were scanned (Epson Expression 11000XL Pro with transparent unit), then nodules were removed from roots and again scanned (Figure 2). Roots and nodules were then dried at 60°C for 72 hr to get nodule and root dry weight. ImageJ (version 1.51k; Schneider, Rasband, & Eliceiri, 2012) was used to analyse root and nodule scans. Nodule position was estimated by digitally measuring the distance from the root crown to each nodule, using the plant label as a size reference. Nodule scans were used to both count and estimate the area of each nodule per plant using the “Analyse particle” function in ImageJ. Nodules were categories into size classes with the number of nodules between 3.5 and 4.4 mm diameter referred to herein as “4 mm nodules.”

In the field trial, roots were sampled when each plot reached at R1, R3 and R5. Three plant samples were taken and frozen at -20°C until analysis. Root samples were thawed and washed before nodules were detached and photographed on a white surface with a size reference label. ImageJ (version 1.51k; Schneider et al., 2012) was used to count and measure nodule area (mm²). Once imaged, nodules were dried at 60°C and weighed.

2.5 | Data analysis

A one-way analysis of variance (ANOVA) was run with the data from controlled environment experiment with cytokinin treatment as the

main effect. For field trial data, ANOVA included sowing date, genotype and cytokinin treatment as main effects, with protected Fisher's least significant difference calculated for significant ($p \leq .05$) effects. Models were validated by checking the normality of the residuals and by plotting residuals against fitted values. All data analysis was performed in R software (RStudio Team, 2020).

3 | RESULTS

3.1 | Controlled environment experiment

Cytokinin seed priming treatment (10^{-9} mol/L) approximately doubled BNF ($p = .05$; Table 1) and increased total nodule area (63%, $p < .05$; Figure 2) compared with the control. Root cytokinin application (10^{-7} mol/L) also increased total nodule area (64%, $p < .05$). Cytokinin treatments had no significant effect on total nodule weight; therefore, in the subsequent field trial only nodule area was reported. Cytokinin seed treatments roughly halved ($p < .05$; Figure 2) the distance of nodules from the root crown (mm), meaning they were less spread across the root system. Cytokinin treatments did not alter shoot weight ($p = .146$), root weight ($p = .129$; Table 1) or leaf area per plant ($p = .126$). Therefore, cytokinin seed priming (10^{-9} mol/L) was the most promising treatment, able to increase BNF and nodulation.

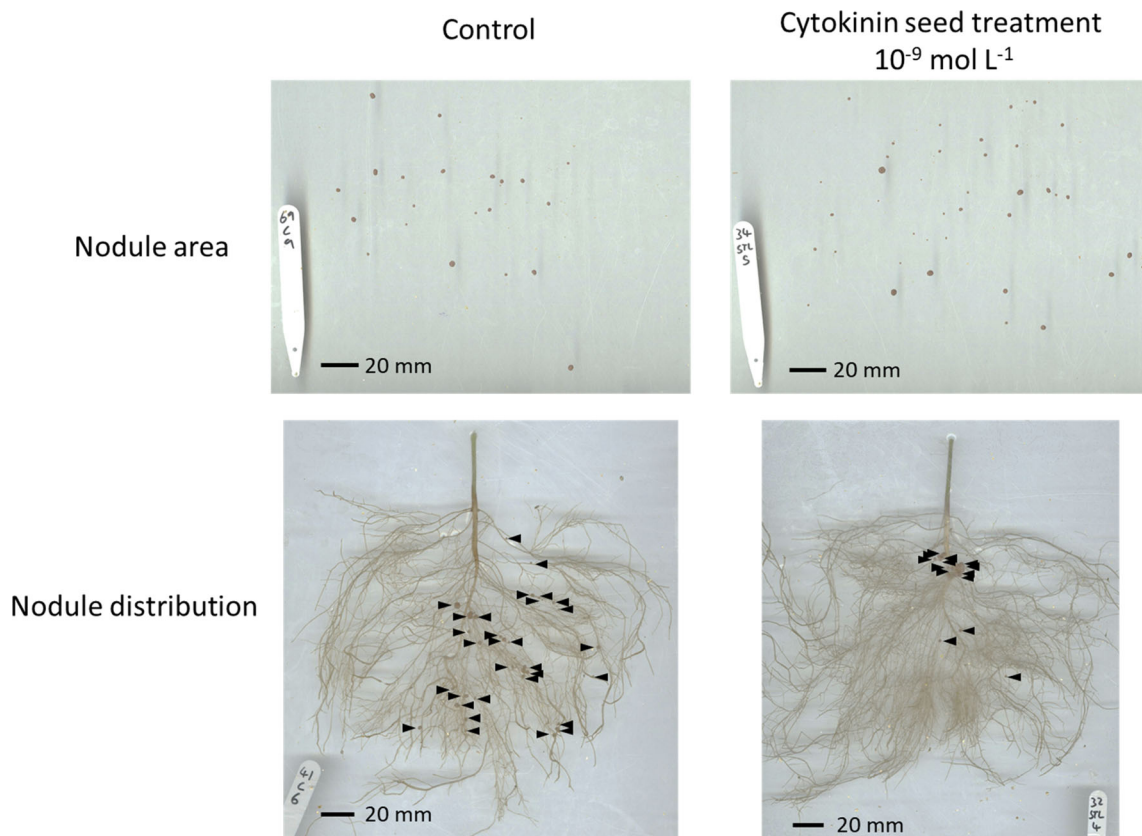


FIGURE 2 Nodule and root scans of plants with close to average nodule area and distribution for respective treatment. Size guide calculated from plant label with line representing 20 mm. Nodule locations indicated with arrows

3.2 | Field trial yield and growth

Sowing date did not significantly affect seed yield ($p = .252$) but genotype did ($p = .011$), with 12% higher yield in DM50I17 than DM40R16. There was no significant genotype \times sowing date interaction ($p = .513$), suggesting no difference in cold tolerance between genotypes (Figure S1). Cytokinin seed priming did not significantly alter yield, but foliar treatment reduced yield ($p < .05$) by 18.6% from control (Table 2). However, a treatment \times sowing date interaction ($p = .03$) occurred, with cytokinin foliar treatment only significantly ($p \leq .05$) decreasing yield of early November sown crops. Thus, cytokinin treatments do not seem to benefit yield and may be detrimental in conventional sowing. Grain quality, indicated by seed N content, was not significantly affected by sowing date, genotype or cytokinin. However, cytokinin seed treatment more than doubled grain N of DM40R16 in early November sowing compared to control ($p < .05$), leading to a marginal cytokinin \times genotype \times sowing date interaction ($p = .057$; Table 2).

September sown crops had significantly ($p < .05$) lower specific leaf area, 30 and 45% less than the early November and late November crops, respectively. Specific leaf area of DM50I17 was 14% higher than DM40R16 ($p = .01$). Both genotypes significantly decreased their specific leaf area from September to late November, DM40R16 by 69% and DM50I17 by 28% (Figure S1), without a significant genotype \times sowing date interaction ($p = .128$) indicating no difference in cold tolerance.

3.3 | N source, use efficiency and harvest index

Late November sowing accumulated more canopy N (12% and 21%) than the September and early November sowing (Table 2), but there was no significant genotype or cytokinin effects. However, September sown plants derived significantly more ($p = .001$; Table 2) of their N from BNF (Ndfa%) than later sown plants: 20 and 11% greater than in early November and late November sowing. Genotype did not affect Ndfa%, but late November sown DM50I17 had lower (39%) BNF than September, while this effect was not seen in DM40R16 (Figure 3a), as indicated by a genotype \times sowing date interaction ($p < .001$). Percent BNF was also increased in DM50I17 compared with DM40R16 in early November sowing date ($p < .05$; Figure 2a). Therefore, early sowing increases plant reliance on BNF compared to those sown at more conventional times, with BNF of DM50I17 (but not DM40R16) significantly affected by sowing date.

The effect of sowing date on BNF changed across the growth period (Table 3). At early reproductive stages (R1 and R3), BNF was higher in late November than September (74 and 40%, respectively; $p < .05$) sowing. However, at R7, BNF in September was 26% greater than late November ($p < .05$). Genotype affected BNF only at R1, with DM50I17 deriving 46% more N from fixation than DM40R16 ($p < .05$). The effectiveness of cytokinin treatments in altering BNF also varied at different stages. At R1, foliar application of cytokinin reduced BNF by 30% ($p < .05$) but at R3 cytokinin seed priming increased BNF by 13% ($p < .05$). Thus, delayed BNF because of early sowing leads to increased BNF at maturity, with genotypic differences

TABLE 1 Effect of different cytokinin application methods (foliar, root and seed) at two different concentrations (high 10^{-7} mol/L and low 10^{-9} mol/L) on plant nodule phenotype

Treatment		BNF (Ndfa %)	Total nodule area (mm ²)	Total nodule dry weight (mg)	Nodule distribution (mm from crown ^a)	Nodule number	Average nodule size (mm ²)	Average nodule weight (mg)	Root dry weight (mg)
Control	Control	17.5b ±3.1	93.7b ±12.1	28.4 ±3.1	84.9a ±7.4	24.6b ±7.2	4.25 ±0.40	1.41 0.18	203 ±15
Foliar	High	21.9b ±3.2	121.2ab ±12.6	34.7 ±3.3	65.1ab ±8.6	33b ±7.6	4.47 ±0.42	1.47 0.20	0.254 ±16.7
	Low	24.8ab ±3.6	143a ±13.3	38.2 ±3.3	74.5a ±6.6	55.8a ±8.0	2.96 ±0.44	0.87 0.20	250 ±16.7
Root	High	22.3b ±3.6	122.9ab ±14.1	28.8 ±3.6	77.2ab ±6.4	38.1ab ±8.53	3.70 ±0.47	0.92 0.21	237 ±17.7
	Low	22.0b ±3.9	154.1a ±12.6	30.8 ±3.2	83.9a ±6.1	59.5a ±7.6	3.15 ±0.42	0.71 0.19	217 ±15.8
Seed	High	20.5b ±3.6	135a ±14.1	32.6 ±3.6	34.7c ±7.4	41.5ab ±8.5	3.69 ±0.47	1.03 0.21	237 ±17.7
	Low	34.0a ±3.6	153.3a ±13.3	34.5 ±3.4	46.7bc ±6.6	44.4ab ±8.0	3.70 ±0.44	0.98 0.20	260 ±16.7
Treatment		0.054	0.014	0.349	<0.001	0.024	0.147	0.065	0.129
SE		10.21	39.85	10.08	14.83	23.89	1.31	0.60	50.1
df		53	58	57	24	58	58	57	57

Note: Values are averages, ±SE with letters denoting significant difference at $p < .05$ as determined by least significant difference (LSD) test with results of one-way ANOVA (bold values denoting significance at $p \leq .05$) below with model residual SE and df.

^aDigitally calculated average distance of nodule from root crown.

only seen at early reproductive stages and effects of cytokinin treatment being stage-dependent.

Soil N uptake was 23% higher for the late November than the September sowing ($p < .05$; Table 2). Soil N uptake was higher (12%; $p = .027$) in DM50I17 than DM40R16. Again, there was a genotype \times sowing date interaction ($p < .001$), with increased soil N uptake (~32%) in the late November sowing of DM50I17 compared

with other sowing dates of both genotypes (Figure 2b). Therefore, soil N uptake is limited by early sowing date and only DM50I17 increased soil N uptake in response to later sowing.

Overall, cytokinin treatment did not increase BNF (Table 2). However, cytokinin seed treatment increased BNF of DM40R16 by 21% compared to control ($p < .05$) but not in DM50I17, giving a significant cytokinin \times genotype interaction ($p = .002$; Figure 4a). The effect of

TABLE 2 Seed yield at R8, specific leaf area at R1, grain nitrogen content, percent biological nitrogen fixation (BNF), soil N uptake, N use efficiency (NUE; biomass/N uptake) and N harvest index (NHI; grain N/N uptake) at R7

Source of variance		Seed yield (kg/ha)	Grain N (g)	Specific leaf area (cm ² /g)	BNF (Ndfa%)	Soil uptake (kg/ha)	Total canopy N (kg/ha)	NUE (kg/kg)	NHI (%)
Sowing date	Sep	3,922	5.18	226b	44.6a	191b	348b	31.1a	79.6b
	Early Nov	4,210	4.27	294a	35.8c	197b	310b	32.3a	86.8a
	Late Nov	4,254	4.7	327a	39.5b	235a	397a	24.2b	74.1c
Genotype	DM40R16	3,895b	4.78	264b	40.4	195b	337	28.1	81.3
	DM50I17	4,362a	4.66	301a	39.4	219a	366	30.3	79.0
Cytokinin	Control	4,329a	4.31	304	38.8	214	370	30.8	79.7
	Seed	4,408a	5.24	277	42.0	204	355	28.6	80.3
	Foliar	3,649b	4.61	266	39.0	199	329	28.1	80.5
Sow (S)		0.252	0.135	<0.001	<0.001	0.003	<0.001	<0.001	<0.001
Genotype (G)		0.011	0.737	0.025	0.401	0.027	0.095	0.127	0.086
Cytokinin (C)		0.002	0.114	0.154	0.102	0.216	0.141	0.266	0.979
G \times S		0.513	0.636	0.128	<0.001	<0.001	0.638	0.067	0.465
C \times S		0.030	0.099	0.069	<0.001	0.042	0.731	0.729	0.010
C \times G		0.854	0.571	0.408	0.002	0.009	0.255	0.491	0.561
C \times G \times S		0.984	0.057	0.466	<0.001	0.923	0.109	0.162	0.067
Residual SE		638.9	1.3	59.2	4.9	38.8	61.9	5.2	3.4
LSD 5%		431.9	0.89	40.0	3.4	26.2	41.8	3.5	2.3

Note: Data from three sowing dates (September, early November and late November), in two genotypes (DM40R16 and DM50I17) with cytokinin application (water control, seed soak or foliar spray). Values are averages ($n = 9$), \pm SE with letters denoting significant difference at $p < .05$ as determined by least significant difference (LSD) test with ANOVA (bold values denoting significance at $p < .05$), residual SE and LSD results below with model residual SE. Residual df are 36.

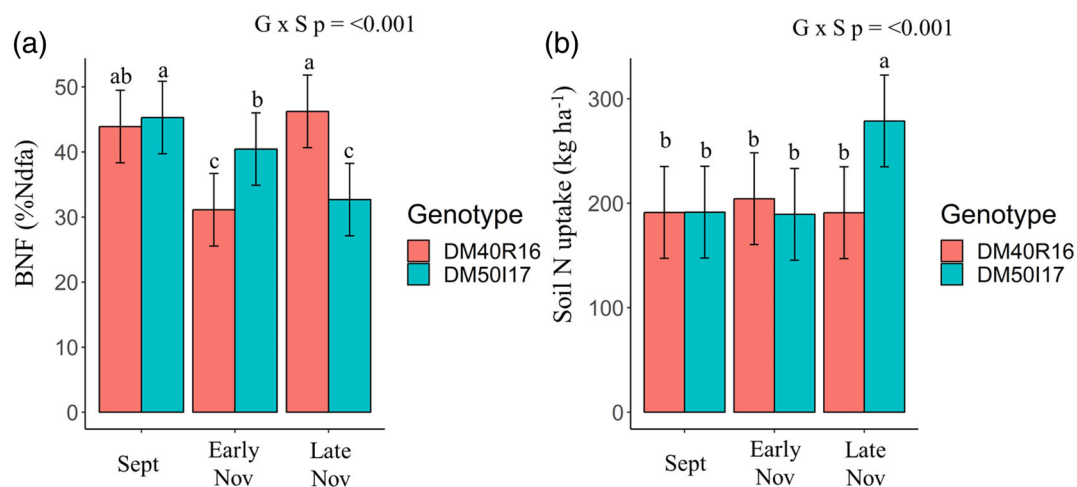


FIGURE 3 Percent BNF (a) and soil N uptake (b) in two genotypes, DM40R16 (red) and DM50I17 (teal), across three sowing dates. Data are mean \pm SE of nine plots, with different letters above bars indicating significant differences according to least significant difference (LSD) test

TABLE 3 Biological nitrogen fixation (BNF) at R1, R3, R5 and R7

Source of variance		BNF (%)			
		R1	R3	R5	R7
Sowing date	Sep	8.21c	43.4b	38.2b	47.4a
	Early Nov	23.8b	31.7c	53.9a	36.2b
	Late Nov	31.7a	72.5a	54.9a	35.1b
Genotype	DM40R16	17.3b	48.3	48.5	40.7
	DM50I17	25.2a	50.2	49.5	38.5
Cytokinin	Control	23.5a	47.2b	49.8	37.98
	Seed	24.0a	53.3a	47.5	41.6
	Foliar	16.3b	47.1b	49.7	39.2
Sow (S)		<0.001	<0.001	<0.001	<0.001
Genotype (G)		<0.001	0.262	0.615	0.215
Cytokinin (C)		<0.001	0.004	0.523	0.233
G × S		<0.001	0.009	0.006	<0.001
C × S		<0.001	0.072	0.185	<0.001
C × G		0.022	0.806	<0.001	0.002
C × G × S		0.034	<0.001	0.014	<0.001
Residual SE		5.0	6.1	6.7	6.4
LSD 5%		3.4	4.1	4.5	4.3

Note: Data from three sowing dates (September, early November and late November), in two genotypes (DM40R16 and DM50I17) with two cytokinin applications (water control, seed soak or foliar spray). Values are averages ($n = 9$), \pm SE with letters denoting significant difference at $p < .05$ as determined by least significant difference (LSD) test with ANOVA (bold values denoting significance at $p < .05$), residual SE and LSD results below with model residual SE. Residual df are 36.

cytokinin also depended on sowing date ($p < .001$), with foliar cytokinin treatment increasing BNF in early November but decreasing BNF in late November sowing ($p < .05$; Figure 4c). Thus, cytokinin seed treatment tends to increase BNF, but this is genotype and sowing-date-dependent.

Cytokinin seed priming decreased soil N uptake by 35% ($p < .05$) in DM40R16 but did not affect DM50I17, resulting in a cytokinin \times genotype interaction ($p = .009$; Figure 4b). Foliar cytokinin treatment decreased soil N uptake by 47% ($p < .05$) compared with the control in the early November but not other sowing dates, resulting in a significant cytokinin \times sowing date interaction ($p < .05$; Figure 4d). Thus, cytokinin seed priming reduces soil N uptake, but this is genotype and sowing-date-dependent.

Nitrogen use efficiency (NUE) was higher in September and early November than late November ($\sim 24\%$, $p < .05$; Table 2). For the September sowing date, NUE was 25% greater in DM50I17 than DM40R16, with a marginal effect (genotype \times sowing date interaction; $p = .067$; Figure S2). Nitrogen harvest index (NHI) was also higher in September and early November than late November (7 and 15%, $p < .05$). Therefore, assimilation of N into canopy and grain was more efficient in early sowing dates.

3.4 | Nodulation

At R1, the late November sowing had 63 and 46% more nodules than September and early November sowing, respectively ($p < .05$;

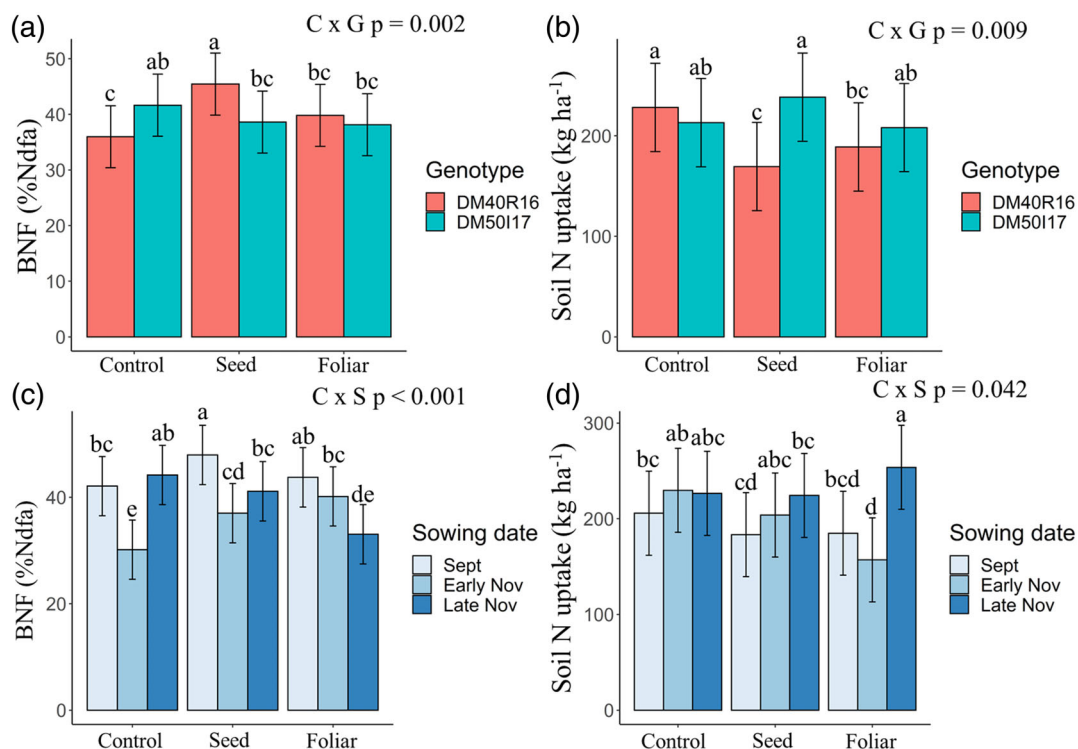


FIGURE 4 Effect of genotype (a, b) and sowing date (c, d) on the response of biological nitrogen fixation (BNF) (a, c) and soil N uptake (right) to cytokinin treatment in two genotypes, DM40R16 (red) and DM50I17 (teal) and three sowing dates. Data are mean \pm SE of nine (a, b) or six (c, d) plots, respectively. Different letters above bars indicate significant differences ($p < .05$) according to least significant difference (LSD) test

Table 4). At R5 the opposite was evident, with nodule number increased in the September than late November sowing (by 38%; $p < .05$). There was a marginal genotypic effect, with DM50I17 having more nodules than DM40R16 (32%, $p = .057$) at R1 but not at R3 or R5. Cytokinin application did not affect nodule number at any of the stages. Like BNF, early sowing date only affected nodulation at R1 and R5 and not R3, decreasing nodule number at R1 but increasing it at R5.

Average nodule size followed a similar pattern with increased (37%, $p < .05$; Table 4) nodule size at R1 in late November than September sowing. At R3 and R5, nodules were larger in September than late November sowing (19 and 33%, respectively; $p < .05$). Thus, early sowing delayed both nodule development and senescence.

Similar trends occurred in other nodule traits (Table S2). At R1 and R3, the number of 4 mm nodules were greater in late November than September sowing, but at R5 the September sowing date had more than double the number of 4 mm nodules than those sown in late November. Equally, at R1, total nodule area in late November sowing was close to four times that of September while at R5 total nodule area in late November was more than 50% that of September. This gives further evidence that early sowing delays nodulation.

4 | DISCUSSION

4.1 | Genotypic responses to early planting

Cold environments restrict plant N accumulation, with BNF thought to be more sensitive than soil N uptake. Although cold soil temperature limits total N accumulation (Table 2), surprisingly soil N uptake was more affected by low RZT than BNF, contrary to previous findings in controlled environments (Legros & Smith, 1994; Matthews & Hayes, 1982; Thomas & Sprent, 1984). Here, BNF was 11% higher in September than the late November sowing, but soil N uptake was 23% lower. As early sowing reduces soybean root growth (Turman, Wiebold, Wrather, & Tracy, 1995) thus limiting N uptake at low RZT (Alsajri et al., 2019; Ouertani et al., 2011; Rufty et al., 1981; Tolley & Raper, 1985), this may explain why soil N uptake is more limited in the field compared to pot grown plants in controlled environments. Differences in soil depth exploration affects the amount of N available to field-grown crops (Voisin et al., 2003a), whereas root exploration in pots is unlikely to be limiting. In cool growing conditions, increased BNF may compensate for limited soil N availability thus maintaining yield. BNF increases with evapotranspiration (Cleveland et al., 1999) and therefore increases in potential evapotranspiration across sowing dates (Figure 1 and Table S1) do not account for higher BNF in early

TABLE 4 Nodule number and average nodule size at three growth stages (R1, R3 and R5)

Source of variance	Nodule number			Average nodule size (mm ²)			
	R1	R3	R5	R1	R3	R5	
Sowing date	Sep	19.4c	68.5	60.8a	7.67b	9.5a	11.3a
	Early Nov	28.2b	60.6	40.4b	9.53a	8.65ab	11.6a
	Late Nov	51.9a	61.7	37.5b	10.47a	7.7b	7.54b
Genotype	DM40R16	30.6	60.3	39.9	9.75a	8.93	10.6
	DM50I17	35.8	66.9	52.5	8.70b	8.32	9.74
Cytokinin	Control	36.3	73.9	45.9	9.03ab	8.46	10.1
	Seed	31.8	59.1	47.5	8.49b	8.39	9.8
	Foliar	31.4	57.7	45.2	10.2a	9.02	10.6
Sow (S)	<0.001	0.618	0.01	<0.001	0.005	<0.001	
Genotype (G)	0.123	0.364	0.057	0.036	0.144	0.591	
Cytokinin (C)	0.421	0.132	0.959	0.024	0.400	0.192	
G × S	0.605	0.449	0.256	0.269	0.373	0.741	
C × S	0.013	0.641	0.574	0.863	0.071	0.527	
C × G	0.450	0.886	0.602	0.349	0.969	0.900	
C × G × S	0.582	0.038	0.448	0.659	0.415	0.410	
Residual SE	12.1	26.0	23.5	1.78	1.52	2.25	
LSD 5%	8.2	17.6	15.9	1.20	1.02	1.52	

Note: Data from three sowing dates (September, early November and late November), in two genotypes (DM40R16 and DM50I17) with cytokinin application (water control, seed soak or foliar spray). Values are averages ($n = 9$), \pm SE with letters denoting significant difference at $p < .05$ as determined by least significant difference (LSD) test with ANOVA (bold values denoting significance at $p < .05$), residual SE and LSD results below with model residual SE. Residual df are 36.

planting. Differences in the timing and severity of cold stress might also explain the disparity between controlled and field environments, even though to our knowledge, the effects of early sowing on soybean N source have not been shown previously.

Despite different cycle lengths (Figure 1), early maturing DM40R16 (MG IV) was no more sensitive to cold than DM50I17 (MGV), both with similar yield and specific leaf area in response to early and conventional sowing dates (Figure S1). Previously, early maturing soybean genotypes appeared more sensitive to low temperatures, because of shorter vegetative growth (George, Singleton, & Bohlool, 1988; Heatherly, 2005; Salmeron et al., 2014) but this was not seen here. However, soil N uptake in DM50I17 was more cold-sensitive than DM40R16, requiring increased BNF in early sowing to allow maintained yield (Figure 3). As total canopy N at maturity was equal in genotypes in each sowing date, the 25% increase in NUE in September sown DM50I17 likely maintained yield (Figure S2), possibly because an increased proportion of N was derived from fixation. Therefore, increased BNF, enabling consistent N supply and enhanced NUE, overcame cold sensitivity. Maximising BNF may require decreased fertiliser N applications, as these inhibit nodulation (Santachiara et al., 2019), but this will depend on soil N levels at sowing as early canopy growth is critical for crop establishment. Available mineral N accumulates during the growing season, as soil temperature increases, because of organic matter mineralisation (Haynes, Martin, & Goh, 1993).

Early sowing delayed BNF (Table 3), as previously reported (Zimmer et al., 2016), but additionally we show delayed decline of BNF resulting in higher rates of fixation in late reproductive stages. Increased BNF enhanced NHI (Santachiara et al., 2018) and early soybean sowing increased seed quality (Rahman, Hampton, & Hill, 2005) as here and marginally increased grain N content in early sowing dates (Table 3). BFN is more rapidly assimilated into pods and seed, whereas N from soil is first assimilated into vegetative tissue then remobilised into reproductive parts (Ohya, 1983). High N demand during grain filling promotes foliar senescence because of remobilisation of N from vegetative tissue, with high-yielding varieties maintaining N supply during seed filling (Kumudini et al., 2002). Therefore, early sowing increased BNF at late reproductive stages, which likely helped maintain yield when soil N supply was limited as a consequence of early sowing.

Nodule lifespan in many legumes is environment-dependent and genotype-dependent (Vessey, 1992), but the effect of early soybean sowing on nodule senescence has not been considered previously (Puppo et al., 2004). Here, early sowing delayed nodule senescence (Table 4), perhaps because of more favourable RZT in later growth. Limited canopy N accumulation in early growth may limit later pod filling because of reduced N available for remobilisation, leading to increased N demand in reproductive stages. Carbon competition between pods and nodules was previously thought to occur; thus, reproductive N supply from BNF would limit yield. However, male-sterile soybean shows similar declines in BNF in later growth, suggesting limited C competition between pods and nodules (Imsande & Ralston, 1982; Riggle, Wiebold, & Kenworthy, 1984).

Therefore, delayed nodule senescence and prolonged BNF may benefit early soybean production.

Although nodule size has been suggested to influence BNF more than other nodule traits (de Araujo et al., 2017; Tajima et al., 2007; Voisin, Salon, Jeudy, & Warembourg, 2003b), contrarily greater fixation was seen in DM50I17 with smaller nodules than DM40R16. We confirm a genotypic effect on the timing of BNF (Hamawaki & Kantartzi, 2018) and additionally show this occurs for nodulation; however, nodulation and the timing of BNF were not correlated (Tables 3 and 4). Low RZT delays BNF and nodule formation (Zhang et al., 1995) in both genotypes (Tables 3 and 4) and therefore does not explain differences in N supply across sowing dates. To better understand N dynamics, nodulation should be monitored at different stages as significant genotypic differences were detected only at R1 and R5 not R3 (Table 4). Commercial genotypes with differential N accumulation patterns (Rotundo et al., 2014) may in part be because of improved nodulation missed previously.

4.2 | Effectiveness of cytokinin treatment

In controlled environment trials, cytokinin seed priming increased nodulation thereby enhancing BNF, with increased total nodule area (Table 1). Low concentration of cytokinin (10^{-9} mol/L) was more effective in promoting nodulation (Table 1), likely because high cytokinin concentrations stimulate ethylene production, which limits nodulation (Lorteau et al., 2001). Further development of cytokinin-based treatments to enhance nodulation should investigate cytokinin and ethylene levels in field-grown plants.

Reduced nodule distribution on roots following cytokinin seed priming (Table 1 and Figure 2) suggested enhanced nodule initiation during early growth. At certain distances from the root tip, susceptibility to nodulation is greatest because of root hair formation (Bhuvanewari, Mills, Crist, Evans, & Bauer, 1983; Calvert, Pence, Pierce, Malik, & Bauer, 1984); thus, less distributed nodules (closer to the root crown) resulted from earlier formation. Because low RZT typically delays nodulation, enhanced nodule formation caused by cytokinin treatment may be beneficial. Further investigations of how cytokinin application affects early nodule signalling are required, for example if cytokinin seed priming stimulates early nodulin gene expression.

In our field trial, cytokinin seed priming increased BNF, although this depended on genotype (in DM40R16), sowing date (Figure 4) and stage (Table 3). However, cytokinin seed priming also reduced soil N uptake perhaps by reducing root growth as cytokinin application can limit root elongation and lateral root formation by increasing ethylene levels (Bertell & Eliasson, 1992). Although root growth was not measured in field trials, cytokinin application did not decrease root growth in controlled environment (Table 1) and continuous cytokinin treatment was required to inhibit root growth (Bertell & Eliasson, 1992). Cytokinin treatment was marginally more effective in early sowing dates, with a cytokinin \times sowing date interaction (Figure 4c). Thus, cytokinin effects in enhancing BNF are more beneficial in low

temperature when plants depend more on N supply from BNF. Although cytokinin treatments show promise in enhancing BNF, the complexity of their response, seen here and previously (Koprna et al., 2016), requires further trials. These may include a greater variety of genotypes, particularly of varying maturity groups (Salmeron et al., 2014), different treatment concentration (10^{-6} mol/L) or cytokinins (6-benzylaminopurine or N^6 -(Δ^2 -isopentenyl)-adenine) used previously (Mens et al., 2018).

5 | CONCLUSIONS

Novel results herein are fourfold. First, we field-test cytokinin treatments for their effectiveness in altering nodulation and BNF. Although our controlled environment trial suggested cytokinin treatment can enhance BNF and early nodule establishment, our field trials do not fully support their agronomic benefit, as additional cytokinin treatments did not increase total N uptake or yield. Second, characterisation of soybean N uptake during cold stress shows maintenance of N supply is important for maintaining yield in low temperature, with soil N uptake more sensitive to cold than BNF, contrary to much of the relevant literature. We hypothesise this is because of limited root growth in early sowing. BNF was important in maintaining N supply in early sowing leading to consistent yields across sowing dates. This is of great consequence to soybean N management as it emphasises the importance of strategies to enhance BNF in cool environments. Third, we show that soil N supply was more sensitive in one genotype but was able to compensate with increased BNF to secure its N supply across soil temperatures, thus stabilising yields. This indicates the importance of appropriate selection for early sowing. Lastly, early sowing can delay nodulation and BNF, but this may be beneficial by prolonging BNF and improving N harvest index at the end of the season.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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