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

Robert Kempster[a], Mercedes Barat[c], Laura Bishop[b], Mariana Rufino[a], Lucas Borras[c], and Ian C. Dodd[a].

[a] Lancaster Environment Centre, Lancaster University LA1 4YQ, United Kingdom.

[b] Plant Impact, West Common, Hertfordshire, Harpenden, AL5 2JQ.

[c] IICAR, UNR-CONICET, Universidad Nacional de Rosario, Consejo Nacional de Investigaciones Cientificas y Tecnicas, Campo Experimental Villarino S/N, Provincia de Santa Fe, Zavalla, S2125ZAA, Argentina.

10   **Summary**

11   High nitrogen (N) supply is required for high yielding soybean, but low soil temperatures in

12 either early production systems or cool environments delay nodulation and limit biological nitrogen

13 fixation (BNF). Since cytokinins are key signalling hormones in mediating nodule formation and our

14 initial controlled environment experiment indicated that seed cytokinin treatment increased early

15 BNF and total nodule area, it was used in field trials. Cytokinin was applied (seed or foliar) to two

16 commercial soybean genotypes (DM50I17 and DM40R16) in field trials with early (September and

17 early November) and conventional (late November) sowing dates in Argentina. In the field, DMR50I7

18 achieved consistent yields across sowing dates since increased BNF compensated for limited soil N

19 uptake in early sowing dates, also leading to 25% higher nitrogen use efficiency (NUE). Surprisingly,

20 soil N uptake was more cold sensitive than BNF with greater and prolonged N fixation in early

21 sowing, perhaps through delayed nodulation, leading to improved N harvest index. Cytokinin seed

22 treatment increased BNF (26%) in DM40R16 especially in early sowing dates. Although cytokinin

23 improved cold tolerance of BNF, this was not explained by altered nodulation and did not increase

24 yield. Here we show genetic differences in N supply in commercial soybean genotypes and the

25 importance of BNF to maintain yield in early sown soybean.

26

27   **Key words**: *Glycine max*; Nodulation; Canopy N uptake; Hormone application; Seed priming;

28 Root zone temperature; Yield.

29

30 **1 | Introduction**

31   Soybean (*Glycine max* (L) Merr) is one of the most important vegetable protein sources globally,

32 contributing to the agricultural economies of many countries (Hungria & Mendes, 2015). Soybean

33 has the highest nitrogen (N) requirement of all major crops (Sinclair & De Wit, 1975) with 80 kg

34 canopy N required per metric tonne of seed, and yield strongly correlated to N accumulation

35 (Rotundo et al., 2014; Salvagiotti et al., 2008). As a legume, soybean uses two N sources, mineral soil

36 N uptake and atmospheric or biological nitrogen fixation (BNF). Soybean can derive up to 70% of its

37 N demand from BNF (Salvagiotti et al., 2008; Santachiara et al., 2017) and high soil N concentrations

38 limit BNF (Santachiara et al., 2019).

39   Temperature also affects the contribution of the two N sources to plant N status, with BNF

40 generally considered more cold sensitive than soil N uptake (Legros & Smith, 1994; Matthews &

41 Hayes, 1982; Thomas & Sprent, 1984). In soybean, root zone temperatures (RZT) less than 25°C

42   delay the onset of BNF, with nodule initiation limited at 10°C RZT and activity at 15°C (Legros &

43   Smith 1994; Mishra et al., 2009; Poustiniet al., 2005; Zhang et al., 1995). However, low soil

44   temperatures may also limit mineral N uptake by restricting root growth and/or nitrate uptake as

45   seen in controlled environments (Rufty et al., 1981; Tolley & Raper 1985) but not in field trials.

46   Despite these limitations, which may limit early growth and subsequent yields, many regions

47   recommend early sowing of soybean in cold soils (Di Mauro et al., 2019; Purcell et al., 2014;

48   Rattalino Edreira et al., 2020) to take advantage of early rainfall, to avoid summer drought, reduce

49   disease and insect damage and extend the growing season. Local soybean production has the

50   potential to improve protein self-sufficiency (De Visser et al.,2014), even though many European

51   countries have suboptimal environments for soybean (Kurasch et al., 2017).

52   BNF depends on successful nodulation and rhizobial efficiency to fix atmospheric N$_2$ to ammonia.

53   Previous work to mitigate the effects of low RZT on BNF have focused on identifying cold tolerant

54   rhizobia (Kühling et al., 2018; Yuan et al., 2020; Zhang et al., 2002; Zimmer et al., 2016). However,

55   the success of rhizobial inoculants can depend on their persistence in the soil and competition with

56   native rhizobia, with local strains better adapted to adverse conditions (Thilakarathna & Raizada,

57   2017). Early nodule establishment in low RZT may therefore improve the effectiveness of cold

58   optimised inoculants. The photosynthetic cost of BNF, 16 moles ATP per mole N (Kahn et al., 1998),

59   requires that plants balance this with their N requirements, however N is more limiting to growth

60   than carbon (C) uptake under low (~ 15°C) temperatures (Thomas & Sprent, 1984; Walsh & Layzell,

61   1986). Thus promoting nodulation in cold environments is likely to be beneficial.

62   In optimal temperatures, certain nodule traits are associated with increased BNF. Nodule size

63   positively correlates with increased N fixation (de Araujo et al., 2017; Tajima et al., 2007; Voisin et

64   al., 2003) and certain nodule sizes are considered optimal (King & Purcell, 2001; Purcell et al., 1997),

65   with greater relative export of N products and import of C. Increased nodule weight following low

66   RZT temperatures (15°C), may compensate for lower nodule activity (Zhang & Smith, 1994),

67   suggesting increased nodulation is beneficial for cold tolerance. The effects of early nodule

68   establishment on BNF have been studied previously (Cerezini et al. 2016; Chibeba et al. 2015) but

69   not in early sown soybean experiencing low RZT.

70   Different soybean genotypes vary in their ability to fix N in low temperature (Lynch & Smith,

71   1993; Zhang & Smith, 1994). As new soybean varieties show reduced BNF under optimal conditions

72   (van Kessel & Hartley, 2000; Nicolás et al.,2002), similar effects could occur under cold temperatures

73   but with greater impacts on yield. Maintaining N uptake during seed filling is important for high yield

74   (Kumudini et al.,2002; Zimmer et al., 2016) especially in early sown soybean. Although genotypes

75 differed in BNF when grown in cool conditions, there was no effect on nodulation (Zimmer et al.,

76 2016) and nodule traits were not associated with genotypic differences in cold tolerance.

77   An alternative approach to enhance nodulation and reduce the effects of cold is to manipulate

78 endogenous hormone concentrations *in planta*, such as cytokinins (Ali et al., 2008; Fatima et al.,

79 2008; Heckmann et al., 2011; Lorteau et al.,2001). Cytokinin application may enhance nodulation by

80 maintaining plant rhizobial communication in low RZT. Host plants initiate nodulation by secreting

81 flavonoids that activate rhizobial genes, including *nod* genes which code nod factors (NF) (Redmond

82 et al., 1986; Caetano-Anollés & Gresshoff, 1993; Denarie & Debelle, 1996; Spaink, 2000). Root

83 perception of NF leads to root hair deformation and rhizobial invasion of root cortical cells, via the

84 infection thread, to elicit nodule formation. Suboptimal soil temperatures (less than 25°C) limit these

85 stages of nodule establishment (Lindemann & Ham, 1979; Lynch & Smith, 1993; Matthews & Hayes,

86 1982), especially infection and early nodule development, due to limited *nod* gene expression and

87 NF synthesis (Shiro et al., 2016; Zhang & Smith 1994). Cytokinin induces early nodulin genes in plants

88 acting in a similar way to NF signalling, inducing cortical cell division genes (Bauer et al., 1996; Dehio

89 & Bruijn 1992; Heckmann et al., 2011; Mathesius et al., 2000). Therefore, early cytokinin application

90 during nodule formation may compensate for delayed bacterial signalling and stimulate higher rates

91 of nodule development and BNF.

92   Exogenous cytokinin applications induced positive effects in a number of legumes depending on

93 the application method, timing and concentration (Cho et al., 2002; Koprna et al., 2016; Liu et al,

94 2004); with high concentrations limiting nodule number (Lorteau et al., 2001; Mens et al. 2018).

95 Cytokinin applications during early reproductive development (stages R1-R3) increased pod set

96 (Ibrahim et al., 2007; Nonokawa et al., 2007; Passos et al., 2008; Yashima et al., 2005). Cytokinin

97 seed priming or application to recently emerged seedlings also increased yield of other legumes but

98 effects are unknown in soybean (Dhruve & Vakharia 2013; Fatimaet al., 2008; Naeem et al., 2004;

99 Schroeder, 1984). Seed treatment with non-thermal plasmas increase soybean nodule nitrogenase

100 activity, in part by increasing endogenous cytokinin concentrations (Pérez-Pizá et al., 2020). While

101 cytokinin application can enhance BNF in chickpea (*Cicer arietinum*; Fatima et al., 2008), to our

102 knowledge no studies have considered cytokinin application to improve BNF of early sown soybean.

103   Since nitrogen supply is the most limiting factor to soybean yield (Rotundo et al., 2014) and cold

104 temperature (<25°C) limit its uptake (Rufty et al., 1981; Tolley & Raper, 1985; Zhang et al., 1995), we

105 tested whether N uptake varied between different genotypes and with cytokinin application. A

106 controlled environment experiment assessed the effectiveness of cytokinin in enhancing BNF, then a

107 field experiment with early and conventional sowing dates aimed to: (i) examine low temperature

108   responses of different commercial soybean genotypes and (ii) test whether cytokinin application

109   could enhance BNF in cold temperature. Since nodule formation and BNF are sensitive to cold

110   temperature, we hypothesised that early sowing would limit BNF and any genotypic differences in

111   cold tolerance will reflect differences in N uptake. Moreover, we hypothesised that cytokinin

112   treatment would enhance nodulation, helping to maintain BNF during exposure to low soil

113   temperature.

114   **2 | Materials and methods**

115   *Site conditions, treatments and experimental design*

116   A controlled environment experiment was conducted with soybean (*Glycine max* cv. Viola) to

117   determine if cytokinin treatment could increase BNF by altering nodulation. Seeds were sown into 1

118   L pots in a randomised block design with 12 biological replicates (one plant per pot) per treatment.

119   After autoclaving, fine grade (1-3 mm) vermiculite (Sinclair professional, Ellesmere Port, UK) was

120   used as the substrate. Before sowing, seeds were surface sterilised with 1% sodium hypochlorite and

121   then repeatedly washed. Seeds were inoculated with 10^8 cells ml^{-1} of *Bradyrhizobium japonicum*

122   USDA110 that was previously cultured on YEM agar (Somasegaran & Hoben, 1994) at 29°C. Two

123   seeds were sown per pot, later thinned to one plant per pot just after emergence (VE). Pots were

124   irrigated with modified N-limited Hoagland's nutrient solution that lacked NO_3^-, to prevent the

125   inhibition of nodulation. Average greenhouse temperature was 29.8°C day/21.3°C night. Light was

126   supplemented by high-pressure sodium lamps (600 W Greenpower, Osram, St Helens, UK) when

127   photosynthetic Photon Flux Density (PPFD) was less than 400 µmol m^{-2} s^{-1} for a 12 h photoperiod

128   (7.00 hrs to 19.00 hrs).

129   The synthetic cytokinin kinetin (Sigma Aldrich) was applied via three application methods: seed

130   priming, root (applied to substrate), and foliar spray. Seeds that were not primed in kinetin (root,

131   foliar, and control) were primed in water and plants not sprayed with kinetin (root, seed primed, and

132   control) were sprayed with water. For the seed priming treatment, 25 g of seed were submerged in

133   25 mL of 10^{-7} (high) and 10^{-9} mol L^{-1} (low) kinetin solution for 4 h. Seeds were air dried in the

134   greenhouse before inoculation and sowing later that day. Foliar and root application took place at

135   early growth stages, VC and V1, respectively. Foliar spray was applied with a handheld pump

136   pressure sprayer and root application by pouring 20 mL of kinetin solution onto substrate. Again,

137   concentrations of 10^{-7} (high) and 10^{-9} mol L^{-1} (low) kinetin solution were used for both foliar and root

138   applications.

139   A field trial was conducted to determine genotypic differences in response to early sowing and

140   assess the effectiveness of cytokinin treatments to improve BNF in low RZT under field conditions.

141   Trials were sown during the 2018/2019 growing season, with three sowing dates of 25th September,

142   8th November (early November) and 25th November (late November), at Campo Experimental

143   Villarino, located in Zavalla, Santa Fe, Argentina (33°1' S, 60°53 W; elevation 24.6 m). Soil and air

144   temperature and potential evapotranspiration (Hargreaves & Samani, 1985) varied across sowing

145   dates but precipitation did not (Figure 1; Supplementary Table 1). The USDA soil series (USDA) was a

146   silty clay loam Vertic Argiudoll, Roldan series, and soybean was the previous crop. Soil (0 to 20 cm

147   depth) had 2.86% organic matter, 13.9 mg kg^{-1} P, 5.8 pH, and N-NO3^{-} were 12.5 mg kg^{-1} in

148   September, 22.9 mg kg^{-1} in early November , and 7.1 mg kg^{-1} in late November. This rainfed

149   experiment was sown in a field having a double crop of wheat (*Triticum aestivum*) and soybean

150   during the previous season.

151   Cytokinin treatments (kinetin; Sigma Aldrich) consisted of either seed priming (10^{-9} mol L^{-1}),

152   foliar spray (10^{-7} mol L^{-1}) or water control. All seeds were submerged either in water (Foliar and

153   control) or cytokinin solution (seed) for four hours, air dried and stored at 4°C until sowing the

154   following day. Cytokinin treatment did not significantly affect emergence, measured 22 days after

155   sowing. Foliar cytokinin treatment was applied at VC and V1 (rate of 50 L ha^{-1}), with control and seed

156   treated plants sprayed with water. We used two commercial soybean genotypes developed by

157   Grupo Don Mario DM40R16 and DM50I17, maturity groups IV and V, respectively. For the late

158   November sowing date, days from emergence to R7 (physiological maturity) for genotypes DM50I17

159   and DM40R16 differed by 12 days. Figure 1 shows the phenology of genotypes from each sowing

160   date. After drying, seeds were coated with inoculant and osmoprotector at recommended rates with

161   RizoLiq LLI ® (Rhizobacter, Argentina) and seed insecticide and fungicide, Cruiser Advanced ®

162   (Syngenta, Argentina) at recommended rates. A complete block design was used with genotypes and

163   cytokinin treatments randomised within blocks, resulting in three plot replicates for each

164   cytokinin/genotype combination per sowing date. Plots were over-seeded and hand thinned to a

165   target plant population of 20 plants per m^2. Manual sowing was necessary due to enlarged seed

166   following seed priming, where seeds were evenly distributed into furrows approximately 3 cm deep.

167   Each plot was 6 m long with 4 rows 0.52 m apart (plot size was 12.5 m^2), with all measurements

168   comprising the two central rows. Weeds and pests were chemically controlled with commercially

169   available products as needed.

170   *Biomass and nitrogen concentration*

171   In the controlled environment experiment, plants were harvested at flowering stage (R1, ~30

172   DAS), shoots were removed from the roots at the cotyledons and leaf area was measured using a

173   leaf area meter (Model Li-3100C Li-Cor, NE, USA). Shoots were then dried at 60°C for 72 h to obtain

174   shoot dry weight. After drying, entire stems were milled for relative ureide analysis (Peoples et al.,

175   1989; Santachiara et al., 2018).

176   In the field trial, above ground biomass was sampled at the R1, R3, R5, and R7 phenological

177   stages (Fehr & Caviness, 1977; Figure 1) from a 0.5 m^2 area, leaving the first and last plant of the

178   rows to prevent border effects. From each harvest, leaf area was measured with a leaf area meter

179   (Model Li-3100C Li-Cor), and plants were separated into leaves and stems and dried at 60°C in an air

180   forced oven. After drying, all plant parts were weighed to determine dry matter. Seed yield was

181   determined at physiological maturity from the remainder of the plot (2.1 m^2) using an experimental

182   static harvester. After weighing, all plant biomass samples were milled to 1 mm. Nitrogen

183   concentration in leaves and stems was determined using Kjeldahl procedure (McKenzie & Wallace

184   1954). Nitrogen use efficiency was calculated by dividing total above ground biomass by total N

185   uptake (Xu et al., 2012). Nitrogen harvest index was calculated by dividing total seed N content by

186   total canopy N uptake at R7.

187   *Biological nitrogen fixation*

188   Stem samples were used to determine BNF by calculating relative abundance of ureides in both

189   controlled environment and field trials (Hungria & Araujo, 1994). Ureide products from fixation

190   (allantoin and allantoic acid), nitrates and amino acids (asparagine and glutamine) are determined

191   and the ratio of each was calculated. Ground stem samples (0.4 g) were used to extract ureide,

192   nitrate and amino acid in 0.1 mol L^{-1} phosphate buffer and ethanol heated to 80°C. After cooling,

193   extracts were filtered and centrifuged at 10,000 g then stored at -20°C until analysis. The Young-

194   Conway's method (Young & Conway 1942), Cataldo method (Cataldo et al., 1975) and ninhydrin

195   method (Yemm & Cocking, 1955) were used to colorimetrically measure ureide, nitrate and amino

196   acid N, respectively. Relative ureide was calculated as:

197      $Relative\ ureide - N\% = (\frac{4U}{(4U+N+AA)}) \times 100$

198   where U, AA and N are molar concentrations of ureide, amino acids and nitrate, respectively

199   (Herridge & Peoples 1990). The amount of N fixed biologically (kg ha^{-1}), for each harvest, was

200   calculated by multiplying relative ureide N (%) by aboveground total N (kg ha^{-1}; Herridge & Peoples,

201   1990). By adding the amount of biologically fixed N at each harvest date plus the amount

202   accumulated between each harvest date, total N coming from BNF at physiological maturity (kg ha^{-1})

203   was determined. The ratio between biologically fixed N (kg ha^{-1}) and total N uptake at maturity

204   provides the final percentage of N derived from fixation (Ndfa%) for the growth period. The

205   difference between aboveground total N (kg ha^{-1}) and biologically fixed N (kg ha^{-1}) indicates soil

206   mineral N absorption.

207   *Nodulation*

208   In controlled environment experiments, root samples were frozen at -20°C until analysed, since

209   these labour intensive measurements took 30 minutes per sample. Roots were scanned (Epson

210   expression 11000XL Pro with transparent unit), then nodules were then removed from roots and

211   again scanned (Figure 2). Roots and nodules were then dried at 60°C for 72 h to get nodule and root

212   dry weight. ImageJ (1.51K; Schneider et al., 2012) was used to analyse root and nodule scans. Nodule

213   position was estimated by digitally measuring the distance from the root crown to each nodule,

214   using the plant label as a size reference. Nodule scans were used to both count and estimate the

215   area of each nodule per plant using the "Analyse particle" function in ImageJ. Nodules were

216   categories into size classes with the number of nodules between 3.5 mm and 4.4 mm diameter

217   referred to herein as "4 mm nodules".

218   In the field trial, roots were sampled when each plot reached at R1, R3 and R5. Three plant

219   samples were taken and frozen at -20°C until analysis. Root samples were thawed and washed

220   before nodules were detached and photographed on a white surface with a size reference label.

221   ImageJ (1.51K; Schneider et al., 2012) was used to count and measure nodule area (mm^2). Once

222   imaged, nodules were dried at 60°C and weighed.

223   *Data analysis*

224   A one-way analysis of variance (ANOVA) was run with the data from controlled environment

225   experiment with cytokinin treatment as the main effect. For field trial data, ANOVA included sowing

226   date, genotype and cytokinin treatment as main effects, with Protected Fisher's least significant

227   difference calculated for significant (p = <0.05) effects. Models were validated by checking the

228   normality of the residuals and by plotting residuals against fitted values. All data analysis was

229   performed in R software (RStudio Team, 2020).

230   **3 | Results**

231   *Controlled environment experiment*

232   Cytokinin seed priming treatment (10^{-9} mol L^{-1}) approximately doubled BNF (Table 1; p = 0.05)

233   and increased total nodule area (63%; p < 0.05; Figure 2) compared with the control. Root cytokinin

234 application (10^{-7} mol L^{-1}) also increased total nodule area (64%; $p < 0.05$). Cytokinin treatments had

235 no significant effect on total nodule weight, therefore in the subsequent field trial only nodule area

236 was reported. Cytokinin seed treatments roughly halved ($p < 0.05$; Figure 2) the distance of nodules

237 from the root crown (mm), meaning they were less spread across the root system. Cytokinin

238 treatments did not alter shoot weight ($p = 0.146$), root weight ($p = 0.129$; Table 1) or leaf area per

239 plant ($p = 0.126$). Therefore, cytokinin seed priming (10^{-9} mol L^{-1}) was the most promising treatment,

240 able to increase BNF and nodulation.

241 *Field trial yield and growth*

242   Sowing date did not significantly affect seed yield ($p = 0.252$) but genotype did ($p = 0.011$), with

243 12% higher yield in DM50I17 than DM40R16. There was no significant genotype x sowing date

244 interaction ($p = 0.513$), suggesting no difference in cold tolerance between genotypes

245 (Supplementary Figure 1). Cytokinin seed priming did not significantly alter yield, but foliar

246 treatment reduced yield ($p < 0.05$) by 18.6% from control (Table 2). However, a treatment x sowing

247 date interaction ($p = 0.03$) occurred, with cytokinin foliar treatment only significantly ($p = < 0.05$)

248 decreasing yield of early November sown crops. Thus, cytokinin treatments do not seem to benefit

249 yield and may be detrimental in conventional sowing. Grain quality, indicated by seed N content,

250 was not significantly affected by sowing date, genotype, or cytokinin. However, cytokinin seed

251 treatment more than doubled grain N of DM40R16 in early November sowing compared to control

252 ($p < 0.05$), leading to a marginal cytokinin x genotype x sowing date interaction ($p = 0.057$; Table 2).

253   September sown crops had significantly ($p < 0.05$) lower specific leaf area, 30% and 45% less

254 than the early November and late November crops, respectively. Specific leaf area of DM50I17 was

255 14% higher than DM40R16 ($p = 0.01$). Both genotypes significantly decreased their specific leaf area

256 from September to late November, DM40R16 by 69% and DM50I17 by 28% (Supplementary Figure

257 1), without a significant genotype x sowing date interaction ($p = 0.128$) indicating no difference in

258 cold tolerance.

259 *Nitrogen source, use efficiency and harvest index*

260   Late November sowing accumulated more canopy N (12% and 21%) than the September and

261 early November sowing (Table 2), but there was no significant genotype or cytokinin effects.

262 However, September sown plants derived significantly more ($p = 0.001$; Table 2) of their N from BNF

263 (Ndfa%) than later sown plants: 20% and 11% greater than in early November and late November

264 sowing. Genotype did not affect Ndfa%, but late November sown DM50I17 had lower (39%) BNF

265 than September, while this effect was not seen in DM40R16 (Figure 3A), as indicated by a genotype x

266 sowing date interaction ($p < 0.001$). Percent BNF was also increased in DM50I17 compared with

267   DM40R16 in early November sowing date (p < 0.05; Figure 2A). Therefore, early sowing increases

268   plant reliance on BNF compared to those sown at more conventional times, with BNF of DM50I17

269   (but not DM40R16) significantly affected by sowing date.

270   The effect of sowing date on BNF changed across the growth period (Table 3). At early

271   reproductive stages (R1 and R3), BNF was higher in late November than September (74% and 40%,

272   respectively; p < 0.05) sowing. However, at R7, BNF in September was 26% greater than late

273   November (p < 0.05). Genotype affected BNF only at R1, with DM50I17 deriving 46% more N from

274   fixation than DM40R16 (p < 0.05). The effectiveness of cytokinin treatments in altering BNF also

275   varied at different stages. At R1, foliar application of cytokinin reduced BNF by 30% (p < 0.05) but at

276   R3 cytokinin seed priming increased BNF by 13% (p < 0.05). Thus, delayed BNF due to early sowing

277   leads to increased BNF at maturity, with genotypic differences only seen at early reproductive stages

278   and effects of cytokinin treatment being stage dependent.

279   Soil N uptake was 23% higher for the late November than the September sowing (p < 0.05; Table

280   2). Soil N uptake was higher (12%; p = 0.027) in DM50I17 than DM40R16. Again, there was a

281   genotype x sowing date interaction (p < 0.001), with increased soil N uptake (~32%) in the late

282   November sowing of DM50I17 compared with other sowing dates of both genotypes (Figure 2B).

283   Therefore, soil N uptake is limited by early sowing date and only DM50I17 increased soil N uptake in

284   response to later sowing.

285   Overall, cytokinin treatment did not increase BNF (Table 2). However, cytokinin seed treatment

286   increased BNF of DM40R16 by 21% compared to control (p < 0.05) but not in DM50I17, giving a

287   significant cytokinin x genotype interaction (p = 0.002; Figure 4A). The effect of cytokinin also

288   depended on sowing date (p < 0.001), with foliar cytokinin treatment increasing BNF in early

289   November but decreasing BNF in late November sowing (p < 0.05; Figure 4C). Thus cytokinin seed

290   treatment tends to increase BNF, but this is genotype and sowing date dependent.

291   Cytokinin seed priming decreased soil N uptake by 35% (p < 0.05) in DMR40R16 but did not

292   affect DM50I17, resulting in a cytokinin x genotype interaction (Figure 4B; p = 0.009). Foliar cytokinin

293   treatment decreased soil N uptake by 47% (p < 0.05) compared with the control in the early

294   November but not other sowing dates, resulting in a significant cytokinin x sowing date interaction

295   (Figure 4D; p < 0.05). Thus, cytokinin seed priming reduces soil N uptake but this is genotype and

296   sowing date dependent.

297   Nitrogen use efficiency (NUE) was higher in September and early November than late November

298   (~24%; p < 0.05; Table 2). For the September sowing date, NUE was 25% greater in DM50I17 than

299   DM40R16, with a marginal effect (genotype x sowing date interaction p = 0.067; Supplementary

300   Figure 2). Nitrogen harvest index (NHI) was also higher in September and early November than late

301   November (7% and 15%; p < 0.05). Therefore, assimilation of N into canopy and grain was more

302   efficient in early sowing dates.

303   *Nodulation*

304   At R1, the late November sowing had 63 and 46% more nodules than September and early

305   November sowing, respectively (p < 0.05; Table 4). At R5 the opposite was evident, with nodule

306   number increased in the September than late November sowing (by 38%; p < 0.05). There was a

307   marginal genotypic effect, with DM50I17 having more nodules than DM40R16 (32%; p = 0.057) at R1

308   but not at R3 or R5. Cytokinin application did not affect nodule number at any of the stages. Like

309   BNF, early sowing date only affected nodulation at R1 and R5 and not R3, decreasing nodule number

310   at R1 but increasing it at R5.

311   Average nodule size followed a similar pattern with increased (37%; p < 0.05; Table 4) nodule

312   size at R1 in late November than September sowing. At R3 and R5, nodules were larger in September

313   than late November sowing (19 and 33%, respectively; p < 0.05). Thus, early sowing delayed both

314   nodule development and senescence.

315   Similar trends occurred in other nodule traits (Supplementary Table 2). At R1 and R3, the number of

316   4 mm nodules were greater in late November than September sowing, but at R5 the September

317   sowing date had more than double the number of 4 mm nodules than those sown in late November.

318   Equally, at R1, total nodule area in late November sowing was close to four times that of September

319   while at R5 total nodule area in late November was more than 50% that of September. This gives

320   further evidence that early sowing delays nodulation.

321   **4 | Discussion**

322   *Genotypic responses to early planting*

323   Cold environments restrict plant N accumulation, with BNF thought to be more sensitive than

324   soil N uptake. Although cold soil temperature limits total nitrogen accumulation (Table 2),

325   surprisingly soil nitrogen uptake was more affected by low RZT than BNF, contrary to previous

326   findings in controlled environments (Legros & Smith 1994; Matthews & Hayes 1982; Thomas &

327   Sprent 1984). Here, BNF was 11% higher in September than the late November sowing, but soil N

328   uptake was 23% lower. As early sowing reduces soybean root growth (Turman et al., 1995) thus

329   limiting N uptake at low RZT (Alsajri et al., 2019; Ouertani et al., 2011; Rufty et al., 1981; Tolley &

330   Raper 1985), this may explain why soil N uptake is more limited in the field compared to pot grown

331   plants in controlled environments. Differences in soil depth exploration affects the amount of N

332   available to field-grown crops (Voisin, 2003), whereas root exploration in pots is unlikely to be

333   limiting. In cool growing conditions, increased BNF may compensate for limited soil N availability

334   thus maintaining yield. BNF increases with evapotranspiration (Cleveland et al., 1999), therefore

335   increases in potential evapotranspiration across sowing dates (Figure 1; Supplementary table 1) do

336   not account for higher BNF in early planting. Differences in the timing and severity of cold stress

337   might also explain the disparity between controlled and field environments, even though to our

338   knowledge, the effects of early sowing on soybean N source have not been shown previously.

339   Despite different cycle lengths (Figure 1), early maturing DM40R16 (MG IV) was no more

340   sensitive to cold than DM50I17 (MGV), both with similar yield and specific leaf area in response to

341   early and conventional sowing dates (Supplementary Figure 1). Previously, early maturing soybean

342   genotypes appeared more sensitive to low temperatures, due to shorter vegetative growth (George

343   et al., 1988; Heatherly, 2005; Salmeron et al., 2014) but this was not seen here. However, soil N

344   uptake in DM50I17 was more cold sensitive than DM40R16, requiring increased BNF in early sowing

345   to allow maintained yield (Figure 3). As total canopy N at maturity was equal in genotypes in each

346   sowing date, the 25% increase in NUE in September sown DM50I17 likely maintained yield

347   (Supplementary Figure 2), possibly because an increased proportion of N was derived from fixation.

348   Therefore, increased BNF, enabling consistent N supply and enhanced NUE, overcame cold

349   sensitivity. Maximising BNF may require decreased fertiliser N applications, as these inhibit

350   nodulation (Santachiara et al., 2019), but this will depend on soil N levels at sowing as early canopy

351   growth is critical for crop establishment. Available mineral N accumulates during the growing

352   season, as soil temperature increases, due to organic matter mineralization (Haynes et al.,1993).

353   Early sowing delayed BNF (Table 3), as previously reported (Zimmer et al., 2016), but

354   additionally we show delayed decline of BNF resulting in higher rates of fixation in late reproductive

355   stages. Increased BNF enhanced nitrogen harvest index (Santachiara et al., 2018) and early soybean

356   sowing increased seed quality (Rahman et al., 2005) as here and marginally increased grain N

357   content in early sowing dates (Table 3). Biologically fixed N is more rapidly assimilated into pods and

358   seed whereas N from soil is first assimilated into vegetative tissue then remobilised into

359   reproductive parts (Ohyama, 1983). High N demand during grain filling promotes foliar senescence

360   due to remobilisation of N from vegetative tissue, with high yielding varieties maintaining N supply

361   during seed filling (Kumudini et al., 2002). Therefore early sowing increased BNF at late reproductive

362   stages, which likely helped maintain yield when soil N supply was limited as a consequence of early

363   sowing.

364   Nodule lifespan in many legumes is environment and genotype dependent (Vessey, 1992), but

365   the effect of early soybean sowing on nodule senescence has not been considered previously (Puppo

366   et al., 2004). Here, early sowing delayed nodule senescence (Table 4), perhaps due to more

367   favourable RZT in later growth. Limited canopy N accumulation in early growth may limit later pod

368   filling due to reduced N available for remobilisation, leading to increased N demand in reproductive

369   stages. Carbon competition between pods and nodules was previously thought to occur, thus

370   reproductive N supply from BNF would limit yield. However, male-sterile soybean show similar

371   declines in BNF in later growth, suggesting limited C competition between pods and nodules

372   (Imsande & Ralston 1982; Riggle et al., 1984). Therefore delayed nodule senescence and prolonged

373   BNF may benefit early soybean production.

374   Although nodule size has been suggested to influence BNF more than other nodule traits (de

375   Araujo et al., 2017; Tajima et al., 2007; Voisin et al., 2003), contrarily greater fixation was seen in

376   DM50I17 with smaller nodules than DM40R16. We confirm a genotypic effect on the timing of BNF

377   (Hamawaki & Kantartzi 2018) and additionally show this occurs for nodulation; however nodulation

378   and the timing of BNF were not correlated (Table 3 and 4). Low RZT delays BNF and nodule

379   formation (Zhang et al., 1995) in both genotypes (Tables 3 and 4) and therefore does not explain

380   differences in N supply across sowing dates. To better understand N dynamics, nodulation should be

381   monitored at different stages as significant genotypic differences were detected only at R1 and R5

382   not R3 (Table 4). Commercial genotypes with differential N accumulation patterns (Rotundo et al.,

383   2014) may in part be due to improved nodulation missed previously.

384   *Effectiveness of cytokinin treatment*

385   In controlled environment trials, cytokinin seed priming increased nodulation thereby enhancing

386   BNF, with increased total nodule area (Table 1). Low concentration of cytokinin (10^{-9} mol L^{-1}) was

387   more effective in promoting nodulation (Table 1); likely because high cytokinin concentrations

388   stimulate ethylene production, which limits nodulation (Lorteau et al., 2001). Further development

389   of cytokinin-based treatments to enhance nodulation should investigate cytokinin and ethylene

390   levels in field-grown plants.

391   Reduced nodule distribution on roots following cytokinin seed priming (Table 1 and Figure 2),

392   suggested enhanced nodule initiation during early growth. At certain distances from the root tip,

393   susceptibility to nodulation is greatest due to root hair formation (Bhuvaneswari et al., 1983; Calvert

394   et al., 1984) thus less distributed nodules (closer to the root crown) resulted from earlier formation.

395   Since low RZT typically delays nodulation, enhanced nodule formation caused by cytokinin treatment

396   may be beneficial. Further investigations of how cytokinin application affects early nodule signalling

397   are required, for example if cytokinin seed priming stimulates early nodulin gene expression.

398   In our field trial, cytokinin seed priming increased BNF, although this depended on genotype (in

399   DM40R16), sowing date (Figure 4) and stage (Table 3). However, cytokinin seed priming also reduced

400   soil N uptake perhaps by reducing root growth as cytokinin application can limit root elongation and

401   lateral root formation by increasing ethylene levels (Bertell & Eliasson 1992). Although root growth

402   was not measured in field trials, cytokinin application did not decrease root growth in controlled

403   environment (Table 1) and continuous cytokinin treatment was required to inhibit root growth

404   (Bertell & Eliasson 1992). Cytokinin treatment was marginally more effective in early sowing dates,

405   with a cytokinin x sowing date interaction (Figure 4C). Thus, cytokinin effects in enhancing BNF are

406   more beneficial in low temperature when plants depend more on N supply from BNF. Although

407   cytokinin treatments show promise in enhancing BNF, the complexity of their response, seen here

408   and previously (Koprna et al., 2016), requires further trials. These may include a greater variety of

409   genotypes, particularly of varying maturity groups (Salmeron et al., 2014), different treatment

410   concentration (10^{-6} mol L^{-1}) or cytokinins (6-benzylaminopurine or N6-(Δ2-isopentenyl)- adenine)

411   used previously (Mens et al., 2018).

412   *Conclusions*

413   Novel results herein are four-fold. Firstly, we field-test cytokinin treatments for their

414   effectiveness in altering nodulation and BNF. Although our controlled environment trial suggested

415   cytokinin treatment can enhance BNF and early nodule establishment, our field trials do not fully

416   support their agronomic benefit, as additional cytokinin treatments did not increase total N uptake

417   or yield. Second, characterisation of soybean N uptake during cold stress shows maintenance of N

418   supply is important for maintaining yield in low temperature, with soil N uptake more sensitive to

419   cold than BNF, contrary to much of the relevant literature. We hypothesise this is due to limited root

420   growth in early sowing. BNF was important in maintaining N supply in early sowing leading to

421   consistent yields across sowing dates. This is of great consequence to soybean N management as it

422   emphasises the importance of strategies to enhance BNF in cool environments. Third, we show that

423   soil N supply was more sensitive in one genotype but was able to compensate with increased BNF to

424   secure its N supply across soil temperatures, thus stabilising yields. This indicates the importance of

425   appropriate selection for early sowing. Lastly, early sowing can delay nodulation and BNF, but this

426   may be beneficial by prolonging BNF and improving N harvest index at the end of the season.

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