

# Evaluation of beneficial and inhibitory effects of nitrate on nodulation and nitrogen fixation in common bean (*Phaseolus vulgaris*)

Yunfei Jiang<sup>1,4</sup>  | Dustin E. MacLean<sup>1</sup>  | Gregory E. Perry<sup>1</sup> |  
Frédéric Marsolais<sup>2</sup>  | Brett Hill<sup>3</sup> | K. Peter Pauls<sup>1</sup> 

<sup>1</sup>Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada

<sup>2</sup>London Research and Development Centre, Agriculture and Agri-Food Canada, London, Ontario, Canada

<sup>3</sup>Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada

<sup>4</sup>Aquatic and Crop Resource Development, National Research Council Canada, Saskatoon, Saskatchewan, Canada

## Correspondence

K. Peter Pauls, Crop Science Building, University of Guelph, 50 Stone Road E., Guelph, ON N1G 2W1, Canada.  
Email: ppauls@uoguelph.ca

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## Abstract

The effects of applied nitrate on symbiotic nitrogen fixation in legumes are complex. Both inhibition and promotion of nodulation by nitrate have been observed in a dose-dependent manner. The objectives of this study were to determine the effects of nitrate at different concentrations on root nodulation in different genotypes in common bean (*Phaseolus vulgaris*). Six genotypes were inoculated with the same rhizobial strain and grown hydroponically in growth pouches in a growth chamber and exposed to six nitrate concentrations, including 0, 2.5, 5, 10, 15, and 20 mM for 4 weeks. The tested genotypes included three recombinant inbred lines (RILs, 25, 46, and 70) that differed in their responses to nitrogen (based on observations of one field growing season), their parents (Mist and Sanilac—registered varieties), which are different in N-fixing abilities, and one nonnodulating mutant (R99). Our results showed that small amounts of nitrate (2.5 and 5 mM) promoted nodule formation and increased nodule biomass, compared with plants in the 0 nitrate control treatment. In contrast, nitrate concentrations over 10 mM inhibited nodulation, resulting in reductions in nodule number and nodule biomass. Nodulation was completely inhibited by 15-mM nitrate in all the genotypes. Regression analyses indicated that 5-mM nitrate is the optimum concentration for promoting nodulation as measured by the total number of nodules formed, the number of effective nodules formed, and the nodule biomass formed. In contrast, nitrogen fixation was inhibited by all levels of nitrate. No genotypic differences were observed in nodulation among the three RILs and their parental cultivars, but all were significantly different than R99, a non-nodulating mutant.

## KEYWORDS

nitrate, nodulation, *Phaseolus vulgaris*, root nodules, symbiotic nitrogen fixation

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## 1 | INTRODUCTION

Symbiosis between legume plants and rhizobia is of ecological and economic importance as this process produces a large amount of nitrogen (N) that enters both natural and agricultural systems. Symbiotic nitrogen fixation (SNF) is the process of converting atmospheric  $N_2$  into alternative nitrogenous compounds used by the host plant (Loomis & Connor, 1992). SNF in legumes occurs in nodules that are specialized plant organs attached to roots (for review, see Ferguson et al., 2019). Vegetative cells inside nodules house bacteroids that differentiate from free-living rhizobia and synthesize nitrogenase (Meakin et al., 2007). Nitrogenase is the enzyme that reduces dinitrogen to ammonia, a process that requires large quantities of ATP and low partial oxygen pressures (Meakin et al., 2007). Roots release flavonoid molecular signals into the rhizosphere that attract rhizobia to root hair surfaces (Ferguson et al., 2019). The rhizobia invade roots, travel to root cortex cells, and cause them to divide and form nodules. Bacteria receive nutrients and energy from plants. Small nodules are visible with the naked eye about 10 days after infection in soybean (Ohyama et al., 2011). Under field conditions, small nodules are visible within 2–3 weeks of planting. SNF is initiated when nodules become larger and turn pink or reddish in color. The pink or red color is caused by leghemoglobin (Lb), a nodule-specific high-affinity carrier protein that controls oxygen flow to rhizobia (Meakin et al., 2007). At the pod-filling stage, nodules of annual legumes generally lose their ability to fix  $N_2$ . Factors affecting nodulation performance include weather, legume species, degree of nodulation by effective strains, the supply of mineral N in the soil, and plant density (Loomis & Connor, 1992).

Bacteria do not usually fix  $N_2$  in the presence of mineral N. A high level of combined N usually inhibits SNF, whereas a small amount sometimes promotes nodule development (Ferguson et al., 2019; Streeter, 1988). A small application of combined N (1–2 mM) has been claimed to be needed for maximum growth and nodule formation in legumes (Streeter, 1988). For example, a long-term supply of 1-mM nitrate promotes nodulation in soybean root nodules (Yashima et al., 2005). An N application of 20–30 kg ha<sup>-1</sup>, applied as starter application, improved the growth and productivity of field pea (Erman, Ari, Togay, & Cig, 2009) and groundnuts (Sulfab, Mukhtar, Hamad, & Adam, 2011). The above observations likely indicate that the low levels of N that were used in the studies promoted plant health but did not exceed levels that would inhibit SNF (Ferguson et al., 2019).

Nitrate effects on nodule growth are complex and variable; the effects are either beneficial or inhibitory, depending on nitrate concentration, exposure period, and growth medium (Cabeza et al., 2014; Saito et al., 2014). Nitrate in soils limits root infection, nodule development, and nitrogenase activity (Dwivedi et al., 2015). High concentrations of nitrate reduce the binding of rhizobia to root hairs, decrease the number of infection threads, increase the number of aborted infection events, and inhibit Lb synthesis (Bonilla & Bolaños, 2010; Streeter, 1988). Sixteen bean cultivars experienced a reduction of nodule weight and visual nodulation scores when combined N increased from 0 to 3.5 mM, with continuing reductions being observed as N levels increased to 10 mM (Park & Buttery, 1989).

Exposure to 5-mM nitrate for 1 day almost completely depresses the increase of soybean nodule size, due to the cessation of cell expansion in nodules (Fujikake et al., 2003). However, nodule growth is able to recover quickly after nitrate is removed (Fujikake et al., 2003). Nitrate not only inhibits nodule initiation and formation but also depresses functions of existing nodules (Vessey & Waterer, 1992). After exposure to nitrate for several days, soybean nodules lost their activity (Schullerf, Minchinp, & Gresshoff, 1988). Nodule-specific nitrogenase activity,  $CO_2$  evolution, the proportion of [<sup>14</sup>C]-labeled photosynthate translocated to nodules, respiration in nodules, and the concentration of nodule starch are significantly decreased in soybean plants when exposed to 10-mM nitrate for 48 h (Vessey, Walsh, & Layzell, 1988). Dry weight per nodule and the rate of acetylene reduction decreased when white clover (*Trifolium repens*) root nodules were exposed to more than 7-mM nitrate for 2–3 days and no new nodules developed at high concentrations of nitrate (Davidson & Robson, 1986).

Autoregulation of nodulation (AON) is the mechanism that regulates the number of nodules formed in leguminous plants; AON-impaired mutants are partially tolerant to nitrate, and possess a hyper-nodulating phenotype (Ferguson et al., 2019; Reid, Ferguson, & Gresshoff, 2011). In a study conducted on *Medicago truncatula*, it was reported that nodule number per plant was reduced, and nodule initiation was inhibited with nitrate concentrations greater than 2.5 mM; however, root hair curling remained unaffected (van Noorden et al., 2016). The inhibitory effects of nitrate on nodulation involve modifications of flavonoid and defense metabolism, as well as changes to redox (van Noorden et al., 2016). Nitrate treatment (2 mM) induced *GmN1C1*, a candidate CLE peptide-encoding gene, which regulated nodule numbers; nodulation was inhibited in the roots of transgenic soybean plants through ectopic overexpression of the CLE peptide-encoding gene (Reid et al., 2011). *ACC SYNTHASE 10 (ACS10)*, an ethylene biosynthesis gene, is responsible for nitrate inhibition of nodulation in the leguminous plant, *M. truncatula* (van Zeijl et al., 2018).

Common bean (*Phaseolus vulgaris*) is the most important grain legume for human consumption. Its center of origin is believed to be Mesoamerica (Bitocchi et al., 2012). Common bean has generally been considered to be a weak  $N_2$  fixer compared with other legumes (Heilig, Beaver, Wright, Song, & Kelly, 2017). Previous research showed that approximately 75% of total N in faba bean, 62–94% of N in soybean, groundnut, pea, and lentil, and 54–58% of N in cowpea, chickpea, and pigeon pea was derived from SNF, whereas only 39% of N in common bean was derived from SNF (Dwivedi et al., 2015). However, there is significant genetic variability for SNF among common bean genotypes (Farid & Navabi, 2015; Kamfwa, Cichy, & Kelly, 2015), indicating that it is possible to improve SNF through breeding efforts. Early flowering bean genotypes were generally inferior in their SNF ability (Chaverra & Graham, 1992). Either promoting earlier nodulation or delaying nodule senescence can improve overall SNF (Chaverra & Graham, 1992). Common bean varieties with a longer vegetative duration generally have greater SNF ability (Farid, 2015). Climbing-type beans are superior in SNF compared with bush-type beans (Rennie & Kemp, 1983). Nodule number is positively correlated with N fixed in common bean (Pereira, Miranda, Attewell,

Kmiecik, & Bliss, 1993). Park and Buttery (1989) identified genotypes with superior nodulation and N<sub>2</sub> fixation characteristics that they believed would be useful for improving the nitrate-tolerant nodulating characteristics of beans.

Farid, Earl, and Navabi (2016) and Farid, Earl, Pauls, and Navabi (2017) compared yields in SNF-dependent versus N-fertilizer-dependent environments for 140 F<sub>4</sub>-derived F<sub>5</sub> recombinant inbred lines (RILs), developed from a cross between a low SNF bean genotype “Sanilac” and a high SNF bean genotype “Mist” (Farid & Navabi, 2015). The Nitrogen Management Yield Differential Indices (YDIs) that they calculated from the yields in the SNF-dependent versus N-fertilizer-dependent environments identified lines that were as productive in environments where their nitrogen requirements were met by SNF as in the N-fertilizer-dependent environments, including Mist (Farid et al., 2017). However, other lines in the population were less productive in SNF-dependent versus N-fertilizer-dependent environments, including Sanilac.

The current study utilized lines with contrasting YDIs from the previous study to determine the effects of nitrate on SNF, including the determination of critical thresholds of the beneficial and inhibitory effects of nitrate on SNF-related traits. In addition, the study examined relationships among nodule development and plant function traits.

## 2 | MATERIALS AND METHODS

Six bean genotypes were tested in a growth chamber, including three RILs (RIL25, RIL46, and RIL70) and their parents (Mist and Sanilac), that differed in their YDIs (Farid et al., 2017), and N-fixing abilities (Farid & Navabi, 2015), and one nonnodulating mutant, R99, used as a reference plant to estimate SNF potential (Buttery & Park, 1993).

A growth chamber experiment was conducted using a split-plot and a completely randomized design (CRD) arrangement with three replications, with nitrate concentration as the main-plot factor and genotype as the subplot factor. Seeds of the six genotypes were surface sterilized using 1% sodium hypochlorite for 3 min, rinsed five times with sterile distilled water, and germinated on wetted filter paper in petri dishes at room temperature, in the dark. After 2 days, germinated seeds were treated with a commercial peat-based inoculant containing *Rhizobium leguminosarum* biovar *viceae*, *R. leguminosarum* biovar *phaseoli*, and *Bradyrhizobium* sp. (McKenzie Seeds, Brandon, MB, Canada). The seeds were thoroughly coated with approximately 4 × 10<sup>5</sup> *Rhizobium* cells per seed. The inoculated seeds were transferred to growth pouches (16.5 × 30 cm, Mega International, St. Paul, MN, United States) with modified Hoagland's solutions with six different nitrate concentrations (0, 2.5, 5, 10, 15, and 20 mM with pH of 6.2) in six containers (main-plot factors). The plants were grown in individual growth pouches using an experimental arrangement similar to that described in a pea SNF study by Bourion et al. (2010). Each growth pouch was considered to be one replication. The plants within each whole-plot factor (nitrate concentration) of 18 plants (six genotypes × three replications) were randomly assigned.

A second inoculation (4 × 10<sup>5</sup> *Rhizobium* cells per seedling) was made by applying 2.5 mL of the above inoculant broth to the root region of each plant using a syringe 1 week after the first inoculation to ensure a sufficient number of rhizobia for symbiosis. The moisture levels in growth pouches were monitored daily and nutrient solutions were added when necessary to avoid drought stress. The plants were grown in a growth chamber at 25°C/18°C in light/dark conditions, respectively, with a 16/8 h photoperiod, at a light intensity of 400 mmol m<sup>-2</sup> s<sup>-1</sup> flux of photosynthetically active radiation, and a relative humidity of 70%.

Two, 3, and 4 weeks after the first inoculation with rhizobia, chlorophyll content of the first fully expanded leaf on the main stem was measured using a Soil-Plant-Analysis-Development (SPAD) chlorophyll meter (Spectrum Technologies, 3,600 Thayer Court, Aurora, IL, United States; Uddling, Gelang-Alfredsson, Piikki, & Pleijel, 2007).

Four weeks after the first inoculation with rhizobia, when plants were at the early flowering stage, the total number of nodules per plant and the number of effective (red) and ineffective (pale) nodules were counted. The nodules were detached from roots, dried in a forced-air oven at 60°C for 4 days and weighed to estimate nodule biomass per plant. The aboveground shoots were placed in envelopes and dried at 60°C for 7 days, and the dry weights were recorded.

Dried shoot samples were ground using a coffee grinder and homogenized using a Bean Ruptor 12 Homogenizer (OMNI International, Kennesaw, GA, United States). Ground shoot sample was weighed (5 mg) and put into a tin capsule (8 × 5 mm, Isomass Scientific Inc. Calgary, AB, Canada). The capsule was folded and compressed and placed into 96-well microplates. The <sup>15</sup>N natural abundance—δ<sup>15</sup>N (the per ml <sup>15</sup>N excess [‰<sup>15</sup>N]) and carbon isotope discrimination (CID, δ<sup>13</sup>C; an indicator of water use efficiency, Farquhar, Ehleringer, & Hubick, 1989) were analyzed using gas chromatography–mass spectrometry at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre (Lethbridge, AB), following the protocol described by Shearer and Kohl (1993). R99 was used as a nonnodulating reference plant to estimate percent of nitrogen derived from the atmosphere (%Ndfa) through the natural <sup>15</sup>N abundance method (Buttery & Park, 1993). More specifically, R99 grown under six different concentrations of nitrate were separated to serve as the reference plants for the corresponding nitrate treatments when estimating %Ndfa for other N-fixing bean genotypes. %Ndfa was calculated using the equation below (Shearer & Kohl, 1986).

$$\%Ndfa = \frac{(\delta^{15}N_{ref\ plant} - \delta^{15}N_{N\text{-fixing\ plant}})}{\delta^{15}N_{ref\ plant} - B}$$

where δ<sup>15</sup>N<sub>ref plant</sub> is the <sup>15</sup>N abundance of the reference plant (R99), δ<sup>15</sup>N<sub>N-fixing plant</sub> is the <sup>15</sup>N abundance of the N-fixing bean genotypes, and *B* is the <sup>15</sup>N abundance of legumes that are grown under N-free conditions and thus obtain their N from SNF entirely. A *B* value of −1.98 was used in the current study based on the results from Farid (2015). The amount of shoot N derived from SNF was calculated by multiplying shoot N content by %Ndfa.



## 2.1 | Data analyses

Analysis of variance was conducted with the mixed model of SAS 9.4 statistical software (SAS Institute Inc., Cary, NC), for a split-plot design with a CRD arrangement using nitrate concentration as the main-plot factor and genotype as the subplot factor. Multiple mean comparisons were declared statistically different at  $P < 0.05$ . The effects of nitrate concentration and genotype were considered as fixed effects, and replication was considered as a random effect. Regression analysis was performed to investigate the effects of nitrate concentrations on the total number of nodules, the number of red nodules, nodule dry weight, %Ndfa, and the amount of shoot N from SNF. The fits of linear or quadratic regression models were determined if regression coefficients were significantly different from zero in both models. For the quadratic regression models, the optimum nitrate concentration for nodulation was calculated by setting the quadratic function after the first derivative to zero. Principal component analysis (PCA) was performed to dissect the relationships among traits under investigation, using Minitab 17 statistical software (Minitab Inc. PA, United State).

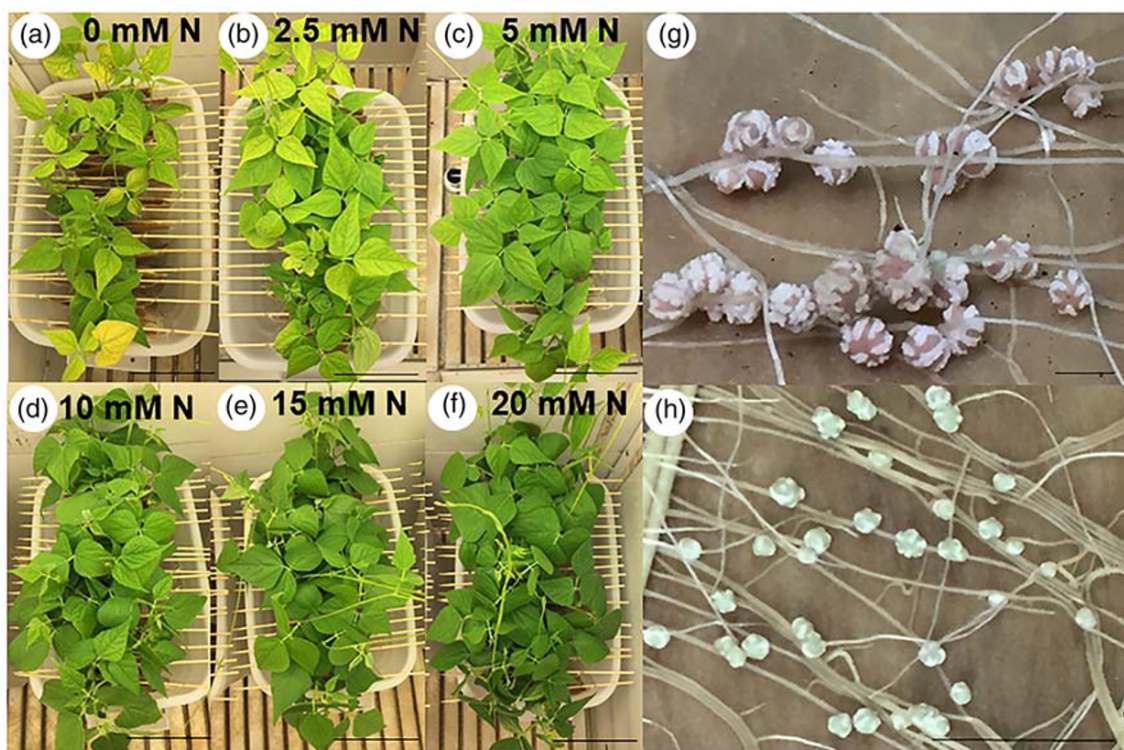
## 3 | RESULTS

Plants became more robust with greener and larger leaves when nitrate concentrations increased from 0 to 20 mM after 2–3 weeks of planting (Figure 1a–f). At zero or low nitrate (2.5 mM) levels,

genotypes differed in leaf greenness (chlorophyll content). In particular, R99 had abnormal yellow leaves, whereas the other five genotypes (Mist, Sanilac, RIL25, RIL46, and RIL70) that were able to fix nitrogen had greener leaves. Plants of these genotypes developing in relatively low nitrate (2.5 and 5 mM) solutions produced larger and more pink root nodules compared with those in nitrate free or at high nitrate (more than or equal to 10 mM) concentrations (Figure 1g versus Figure 1h; Table 1).

The main effect of nitrate concentration was significant on all traits under investigation, except for leaf chlorophyll content after 4 weeks of rhizobia inoculation and the number of white nodules. After 2 and 3 weeks of rhizobia inoculation, leaf chlorophyll content increased with an increase in nitrate concentration (Table 1). After 4 weeks of rhizobia inoculation, however, leaf chlorophyll content did not differ among six concentrations of nitrate. The application of 2.5- and 5-mM nitrate promoted nodulation compared with plants without nitrate application, producing more total nodules, effective nodules, and nodule dry weight per plant. However, chlorophyll content after 2 and 4 weeks of rhizobia inoculation, the number of white nodules, and the amount of shoot N from SNF did not differ among the treatment of 0-, 2.5-, and 5-mM nitrate (Table 1). High nitrate concentrations, including 15 and 20 mM, completely inhibited nodulation, leading to almost zero effective nodules and nodule dry weight.

The two-way interaction between nitrate concentration and genotype was not significant for leaf chlorophyll content or shoot dry weight 2 weeks after rhizobia inoculation but had significant effects on leaf chlorophyll content after 3 and 4 weeks of rhizobia



**FIGURE 1** Effects of nitrate concentrations on plant canopy and nodules in common bean (*Phaseolus vulgaris*). (a–f) bean plants receiving 0-, 2.5-, 5-, 10-, 15-, and 20-mM nitrate after 18 days of planting; (g) effective/red nodules (RIL25 with 2.5-mM nitrate application); and (h) ineffective/pale nodules (RIL25 with 10-mM nitrate application). Scale bar = 10 cm in (a)–(f); scale bar = 1 cm in (g) and (h)

**TABLE 1** Effect of nitrate concentration and genotype on symbiotic nitrogen fixation (SNF)-related traits in common bean

Effect	SPAD (Week 2)	SPAD (Week 3)	SPAD (Week 4)	Total nodule number	Red nodule number	White nodule number	Nodule wt per plant (g)	Shoot dry w (g)	$\delta^{13}\text{C}$	%Ndfa	Shoot N from SNF (mg)
Nitrate (N)	7.61***	42.14***	4.45 ns	57.47***	11.24***	11.24 ns	83.63***	15.97**	10.86***	156.35***	57.77***
Genotype (G)	4.75***	7.01***	15.49***	30.76***	15.18***	15.18***	21.32***	3.97**	3.77**	58.04***	28.11***
N * G	1.34 ns	2.44**	2.16**	3.11***	4.33***	4.33*	4.77***	0.97 ns	1.97*	8.23***	4.02***
Nitrate (mM)											
0	37.5 bc	23.7 c	20.5 a	153 b	3 b	92 a	0.0421 b	0.2216 b	-29.27 a	65.74 a	0.1005 a
2.5	35.4 c	27.3 bc	35.1 a	248 a	124 a	110 a	0.0900 a	0.7915 ab	-30.27 b	60.99 ab	0.1018 a
5	38.5 a-c	31.1 b	32.2 a	262 a	148 a	141 a	0.1063 a	1.1716 a	-30.25 b	52.51 b	0.0865 a
10	41.3 ab	36.5 a	32.7 a	143 b	36 b	97 a	0.0294 b	1.3841 a	-30.89 b	13.76 c	0.0269 b
15	42.8 a	38.0 a	34.2 a	45 c	1 b	47 a	0 c	1.4338 a	-30.86 b	4.80 c	0.0125 b
20	41.9 a	39.9 a	35.7 a	13 c	0 b	13 a	0 c	1.5668 a	-30.44 b	6.35 c	0.0173 b
Tukey value	4.35	4.24	20.4	55.7	84.5	1141.9	0.02025	0.78608	0.752	42.211	0.18489
Genotype											
Mist	38.6 ab	33.8 a	33.8 a	160 a	75 a	80 b	0.0534 a	1.1267 a	-30.85 b	44.19 a	0.0825 a
Sanilac	41.1 a	33.4 a	34.3 a	195 a	56 a	133 a	0.0519 a	1.0942 ab	-30.30 ab	43.18 a	0.0671 a
RIL25	41.3 a	34.2 a	35.3 a	186 a	70 a	112 ab	0.0507 a	1.1135 ab	-30.56 ab	38.91 a	0.0659 a
RIL46	40.0 a	33.4 a	32.4 a	178 a	73 a	79 b	0.0606 a	1.2108 a	-30.30 ab	40.45 a	0.0669 a
RIL70	40.8 a	33.9 a	31.2 a	146 a	44 a	95 ab	0.0512 a	1.1256 a	-30.17 ab	37.42 a	0.0628 a
R99	35.5 b	27.3 b	23.3 b	0 b	0 b	0 c	0 b	0.8985 b	-29.81 a	0.00 b	0 b
Tukey value	4.35	4.24	4.7	55.7	33.1	50.8	0.02025	0.21888	0.751	11.765	0.02667

Note: Means with a common letter within each column and each main effect did not differ at  $P < 0.05$ .

Abbreviations:  $\delta^{13}\text{C}$ , carbon isotope discrimination; %Ndfa, percent of nitrogen derived from atmosphere; ns, non-significant; SPAD, Soil-Plant-Analysis-Development chlorophyll meter; wt, weight.

\* $0.01 \leq P < 0.05$ .

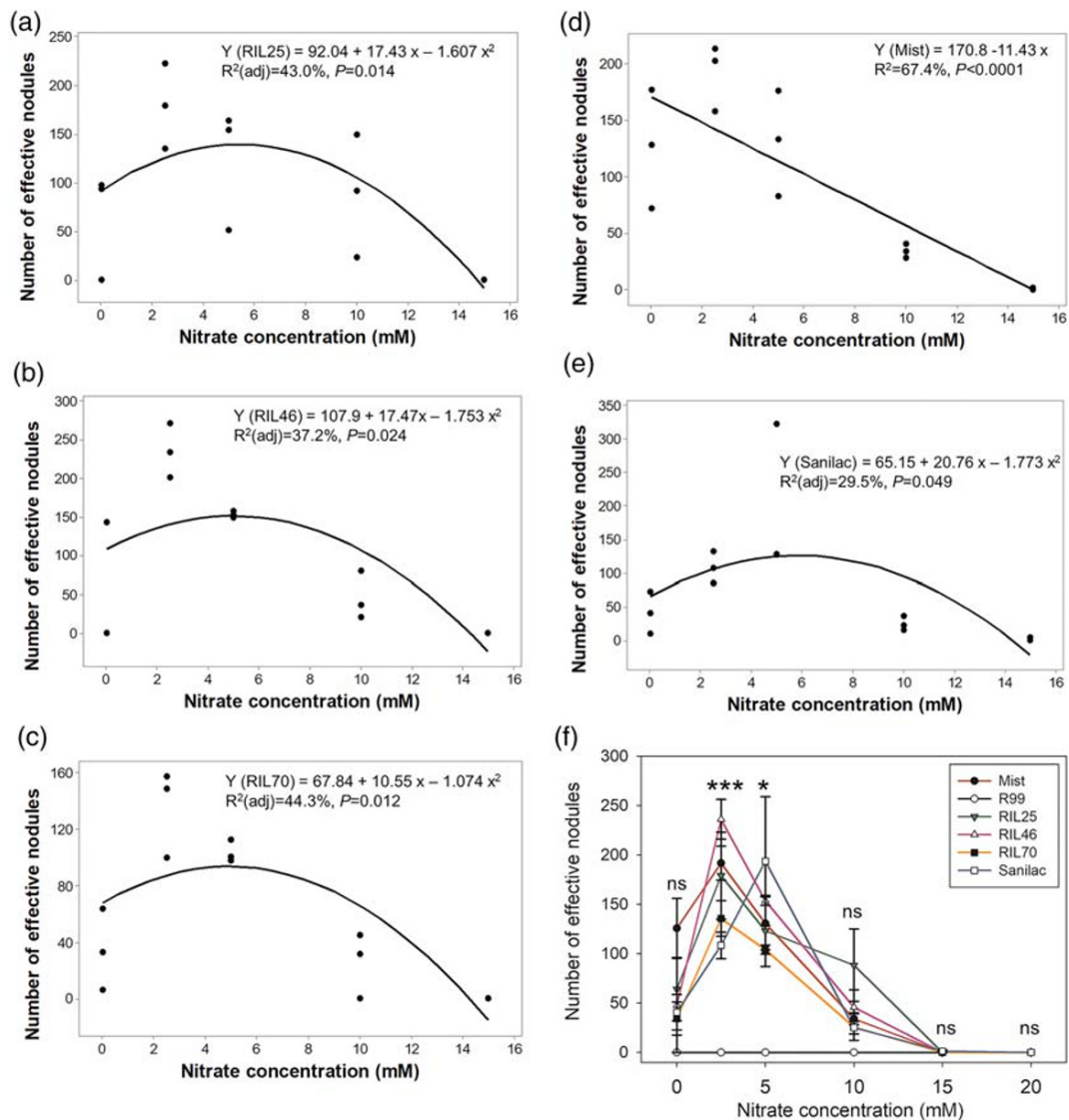
\*\* $0.001 \leq P < 0.01$ .

\*\*\* $P < 0.001$ .

inoculation, as well as on the numbers of total, effective (red), and ineffective (white) nodules per plant, nodule dry weight per plant,  $\delta^{13}\text{C}$ , %Ndfa, and the amount of shoot N derived from SNF (Table 1). The six genotypes differed for all the traits under investigation. The nonnodulating mutant, R99, had less leaf chlorophyll content compared with the other five genotypes after 3 and 4 weeks of rhizobia inoculation. The other five genotypes, capable of forming nodules and fixing nitrogen (including RIL25, RIL46, RIL70, Mist, and Sanilac) had

more total nodules, red nodules, nodule dry weight, %Ndfa, and the amount of shoot N from SNF when compared with R99, but they did not differ from each other for these traits.

Regression analyses were conducted to examine the effects of different nitrate concentrations on SNF-related traits, including the number of effective nodules (Figure 2a–e) and %Ndfa (Figure 3a–e) in five bean genotypes (RIL25, RIL46, RIL70, Mist, and Sanilac) with five nitrate concentrations—0, 2.5, 5, 10, and 15 mM. Regression analyses

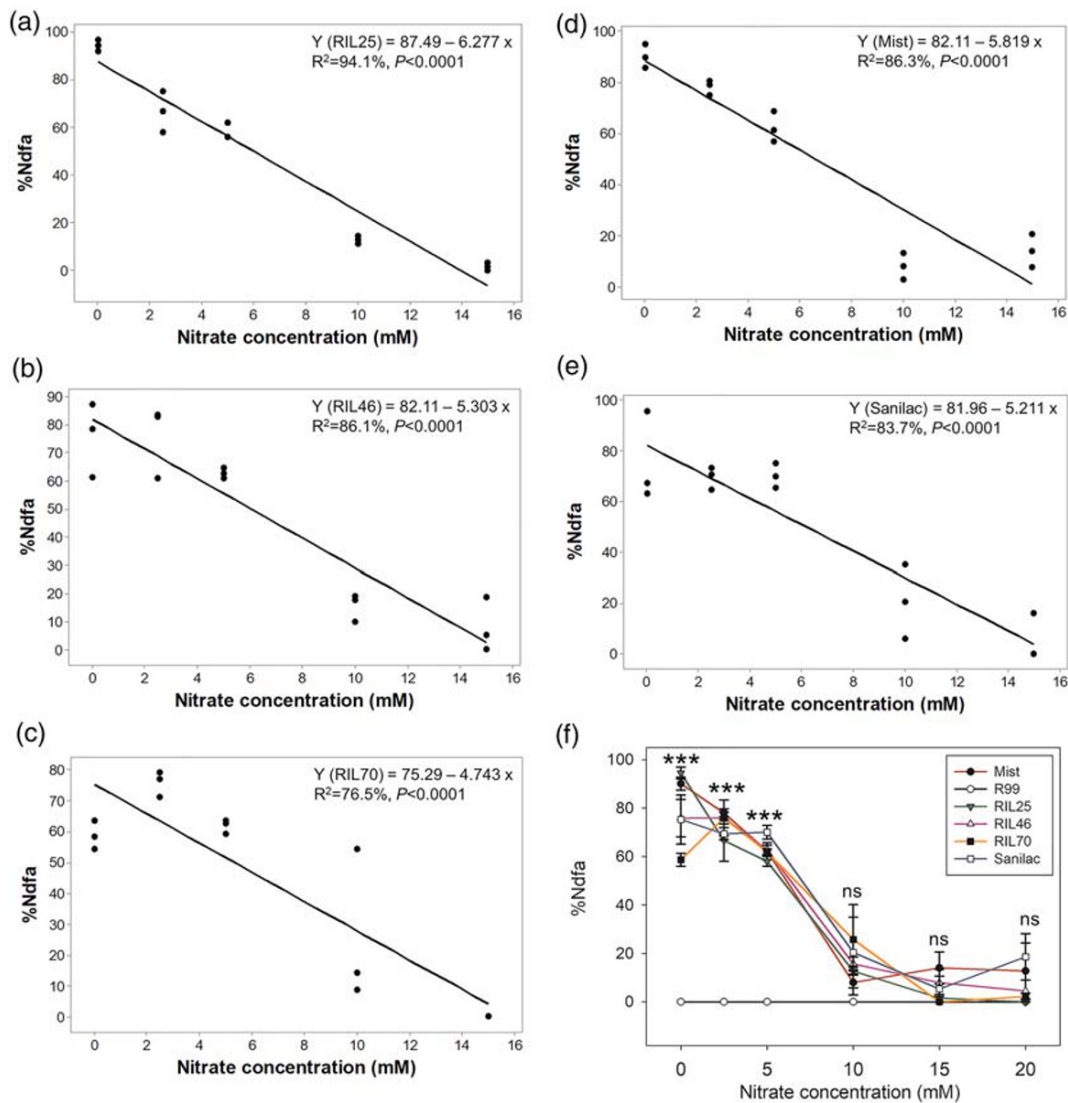


**FIGURE 2** Effect of nitrate concentration on the number of effective/red nodules per plant for different bean genotypes. (a–e) regression analyses of nitrate effect in (a) RIL25, (b) RIL46, (c) RIL70, (d) Mist, and (e) Sanilac and (f) line graph of nitrate effect; significance test was done at a specific nitrate concentration to compare the difference among six bean genotypes;  $0.01 \leq P < 0.05$ ;  $0.001 \leq P < 0.01$ ;  $P < 0.001$ ; ns, not significant

of other SNF-related traits are included in the Supporting Information—the total number of nodules (Figure S1A–E), nodule dry weight per plant (Figure S2A–E), and the amount of shoot N from SNF (Figure S3A–E). The number of effective nodules was reduced with increasing nitrate concentrations in four bean genotypes, and the optimum nitrate concentrations for the production of the most effective nodules were at 5.4, 5.0, 4.9, and 5.9 mM for RIL25, RIL46, RIL70, and Sanilac, respectively. The number of effective nodules was negatively associated with nitrate concentrations in a linear fashion in Mist (Figure 2a–e). %Ndfa was negatively associated with nitrate concentrations in a linear way in all genotypes (Figure 3a–e). The total number of nodules decreased with increasing nitrate concentrations in a quadratic fashion, and the optimum nitrate concentrations for total nodules occurred at 4.6, 4.9, 5.5, 4.9, and 5.6 mM for RIL25, RIL46, RIL70, Mist, and Sanilac, respectively (Figure S1A–E). Nodule

dry weight was negatively associated with nitrate concentrations in a quadratic way, and the optimum nitrate concentrations for nodule dry weight were 5.6, 4.5, 5.3, 3.9, and 5.4 mM for RIL25, RIL46, RIL70, Mist, and Sanilac, respectively (Figure S2A–E). The amount of shoot N derived from SNF was negatively associated with nitrate concentration in linear models in RIL25, RIL46, Mist, and Sanilac, and in a quadratic model in RIL70 (Figure S3A–E).

At a given specific nitrate concentration, no genotypic difference of the number of red nodules (Figure 2f) and %Ndfa (Figure 3f) was observed among the five nodulating genotypes, which was significantly different from the nonnodulating genotype, R99. Similar genotypic observations were found on other traits, including total number of nodules (Figure S1F), nodule dry weight per plant (Figure S2F), and the amount of shoot N from SNF (Figure S3F).



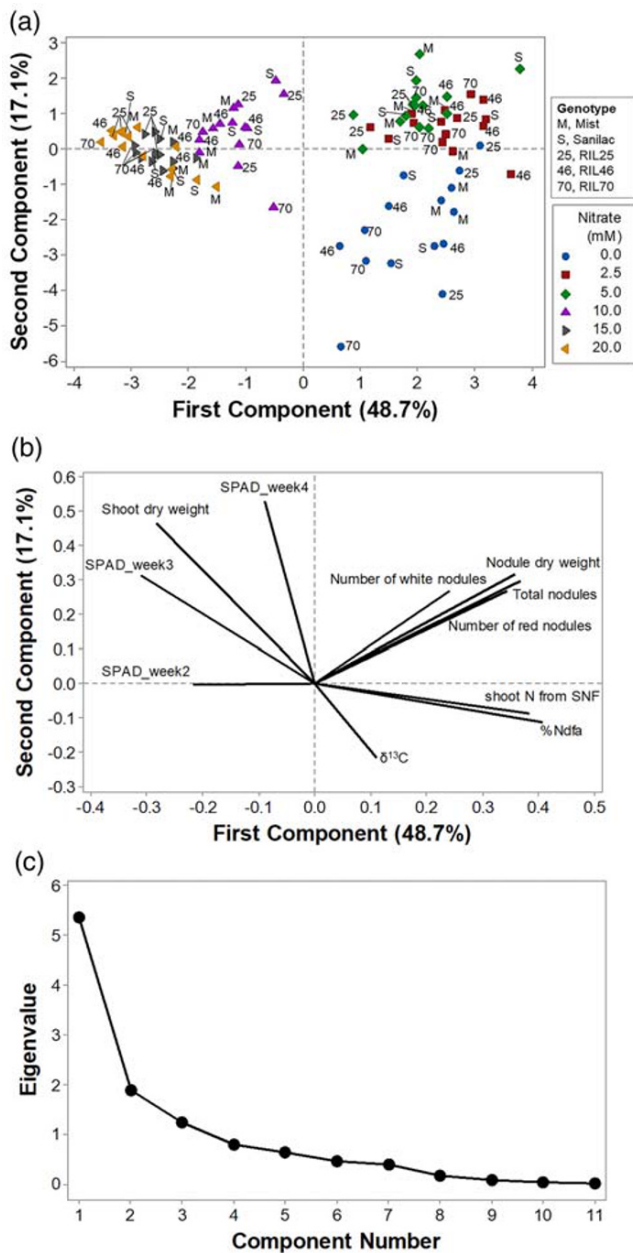
**FIGURE 3** Effect of nitrate concentration on percent of nitrogen derived from the atmosphere (%Ndfa) for different bean genotypes. (a–e) regression analyses of nitrate effect in (a) RIL25, (b) RIL46, (c) RIL70, (d) Mist, and (e) Sanilac and (f) line graph of nitrate effect; significance test was done at a specific nitrate concentration to compare the difference among six bean genotypes;  $*0.01 \leq P < 0.05$ ;  $**0.001 \leq P < 0.01$ ;  $*** < 0.001$ ; ns, not significant

A PCA was conducted to dissect the relationships among lines treated with different amounts of nitrate and evaluated for a number of traits, including nodule number, nodule weight, %Ndfa, amount of shoot N from SNF, shoot weight,  $\delta^{13}\text{C}$  in shoot, and leaf chlorophyll content measured using a SPAD meter. The first two PCs accounted for 65.8% of the total variation in the measured traits (Figure 4a). Two groups of plants receiving zero or low (0-, 2.5-, and 5-mM nitrate) and high (10-, 15-, and 20-mM nitrate) concentrations of nitrate were separated by the first PC (48.7% of total variation), with zero and low nitrate concentrations located on the positive side of the x axis and high nitrate concentrations clustered on the negative side of the x axis. PC2 (17.1% of total variation) roughly separated plants with and without nitrate supply into two groups. Data points of plants receiving 2.5- and 5-mM nitrate overlapped each other, indicating these two nitrate concentrations exerted similar effects on traits of interest. Similarly, the positions of the plants treated with 15- and

20-mM nitrate overlapped each other in the PCA plots. SNF-related traits, including total number of nodules, the number of red nodules and white nodules, nodule dry weight, the amount of shoot N from SNF, and %Ndfa were positively correlated with the PC1, whereas canopy robustness-related traits, including shoot dry weight and leaf chlorophyll content, were negatively correlated with PC1 (Figure 4b). The first two PCs explained most of the total variation (Figure 4c).

Positive correlations were observed between %Ndfa and several traits, including values of 0.969 for the amount of shoot N from SNF, 0.733 for nodule dry weight, 0.675 for total nodule number, 0.672 for the number of red nodules, 0.372 for the number of white nodules, and 0.239 for  $\delta^{13}\text{C}$  (Table 2). %Ndfa was negatively correlated with shoot dry weight and leaf chlorophyll content. Correlation coefficients of  $-0.759$ ,  $-0.460$ ,  $-0.660$ , and  $-0.225$  were measured for shoot biomass and leaf chlorophyll content after 2, 3, and 4 weeks of rhizobia inoculation.





**FIGURE 4** Principal component analysis of the relationships among bean plants (RIL25, RIL46, RIL70, Mist, and Sanilac) treated with six levels of nitrate (0, 2.5, 5.0, 10.0, 15.0, and 20.0 mM) and assessed for nodule number, nodule weight, percent of nitrogen derived from the atmosphere (%Ndfa), amount of shoot N from symbiotic nitrogen fixation (SNF), shoot weight, carbon isotope discrimination ( $\delta^{13}\text{C}$ ) in shoot, leaf chlorophyll content measured using a Soil-Plant-Analysis-Development (SPAD) chlorophyll meter. (a) Score plot of first two principal components (PCs); (b) loading plot of first two PCs; and (c) scree plot of PC numbers on eigenvalues

## 4 | DISCUSSION

### 4.1 | Inhibitory or beneficial effects of nitrate application

Both inhibitory and beneficial effects of additional nitrate application were observed in the present study, depending on nitrate

concentration. Low levels of nitrate stimulated nodule growth, due to an increase in plant vigor (Ferguson et al., 2019; Streeter, 1988). Beneficial effects of nitrate were also observed in certain legumes between the pod-filling stage and maturity, as the demand for C and N is relatively high at that stage (Becana & Sprent, 1987). Our results showed that low levels of nitrate (2.5 and 5 mM) increased the number of effective nodules and nodule biomass, and nitrate concentrations greater than 10 mM and above inhibited nodulation. Interestingly, no beneficial effect of nitrate, even at low concentrations, was observed for the amount of nitrogen fixed by the 4-week-old plants. These results suggest that the elaboration of the nodules needed for nitrogen fixation and the biological and biochemical activity that carry out that function are separately and differently affected by nitrate. Previous research showed that nitrate (more than 2–3 mM) inhibited nitrate reductase activity, decreased the content of Lb and soluble nodule protein, and accelerated the senescence of nodules in soybean (Becana & Sprent, 1987). Using RNAseq, researchers found that all genes related to Lb were down-regulated in *M. truncatula* when exposed to nitrate continuously (Cabeza et al., 2014). Inhibitory effects of nitrate on nodulation were also associated with cellular iron allocation and mitochondrial ATP synthesis. Genes related to nodule senescence were differentially expressed between control and nitrate-treated nodules (Cabeza et al., 2014).

Only long-term exposure (4 weeks) to nitrate effects was evaluated in the present study. Previous studies showed that short-term exposure to nitrate had a reversible effect on nodule activity, whereas SNF ability was irreversibly lost when exposed to nitrate for a long period of time (Becana & Sprent, 1987). How the irreversible effects were related to carbohydrate deprivation or the accumulation of  $\text{NO}_2^-$  was not clear in the 1980s (Becana & Sprent, 1987). More energy is needed to fix  $\text{N}_2$  compared with the utilization of  $\text{NO}_3^-$  (Ferguson et al., 2019; Sprent & Raven, 1985). Therefore, in the presence of nitrate, plants are able to detect levels of nitrate and adjust SNF accordingly.

### 4.2 | Genotypic effect on SNF traits

Common bean has been noted in several studies to be a weak  $\text{N}_2$  fixer in comparison with other leguminous plants; however, genotypic effects (genetic variability) play a large role in common beans ability to fix nitrogen as several *P. vulgaris* genotypes have been reported to have higher SNF capability when compared with other genotypes. (Farid & Navabi, 2015; Kamfwa et al., 2015; Farid et al., 2016; Farid et al., 2017; Wilker et al., 2019). It is possible that, compared with other legumes, common bean can take better advantage of low N quantities present in the soil under field conditions. Therefore, we recommend that more work is needed to be done to explore the underlying reason why common bean is considered to be a weak  $\text{N}_2$  fixer in comparison with other legumes.

Plant genotype is a key factor determining the efficiency of SNF in legumes, such as in field pea (Bourion et al., 2010) and common bean (Farid et al., 2016; Farid et al., 2017; Ramaekers, Galeano,



**TABLE 2** Correlation matrix between symbiotic nitrogen fixation (SNF)-related traits in common bean

	SPAD (Week 2)	SPAD (Week 3)	SPAD4 (Week 4)	Total nodules	Red nodules	White nodules	Nodule weight	Shoot weight	$\delta^{13}\text{C}$	%Ndfa
SPAD (Week 3)	0.347**									
SPAD (Week 4)	-0.020 ns	0.415***								
Total nodules	-0.305**	-0.473***	-0.009 ns							
Red nodules	-0.360***	-0.455***	0.105 ns	0.810***						
White nodules	-0.153 ns	-0.276**	-0.073 ns	0.805***	0.351**					
Nodule weight	-0.314**	-0.369***	0.089 ns	0.871***	0.845***	0.544***				
Shoot weight	0.314**	0.692***	0.456***	-0.274**	-0.276**	-0.097 ns	-0.219*			
$\delta^{13}\text{C}$	0.072 ns	-0.275**	-0.234*	0.161 ns	0.089 ns	0.143 ns	0.186 ns	0.247*		
%Ndfa	-0.460***	-0.660***	-0.225*	0.675***	0.672***	0.372***	0.733***	-0.759***	0.239*	
Shoot N from SNF	-0.487***	-0.578***	-0.164 ns	0.612***	0.637***	0.313**	0.689***	-0.723***	0.089 ns	0.969***

Abbreviations:  $\delta^{13}\text{C}$ , carbon isotope discrimination; %Ndfa, percent of nitrogen derived from atmosphere; ns, not significant; SPAD, Soil-Plant-Analysis-Development chlorophyll meter.

\* $0.01 \leq P < 0.05$  (significance level for the correlation coefficient [ $r$ ]).

\*\* $0.001 \leq P < 0.01$  (significance level for the correlation coefficient [ $r$ ]).

\*\*\* $P < 0.001$  (significance level for the correlation coefficient [ $r$ ]).

Garzón, Vanderleyden, & Blair, 2013). In the present study, no obvious differences were observed for the SNF traits among these five nodulating genotypes, namely, Mist, Sanilac, RIL25, RIL46, and RIL70, at specific nitrate concentrations. These results were surprising and did not meet our hypothesis, because we were expecting that these selected bean genotypes would have different SNF abilities and would perform differently under different nitrate concentrations. Therefore, we concluded that the nitrate effect on SNF measured in the laboratory does not discriminate among genotypes and cannot be directly compared with the effect measured in the field (Farid et al., 2016; Farid et al., 2017). Perhaps, there are some additional components in the field, that condition the genotype effect, such as native rhizobia that are missing in the laboratory experiment.

However, the laboratory method for studying the nitrate effect on nitrogen fixation within genotypes is reproducible and very convenient for studying different developmental times and nitrate concentration effects on nodule formation and nitrogen fixation processes within genotypes.

### 4.3 | Relationship among SNF-related traits

Our results showed that the amount of shoot N from SNF was most closely correlated with %Ndfa among the traits measured, followed by nodule dry weight, total number of nodules, and the number of red nodules. There is a high collinearity between shoot N from SNF and %Ndfa, because the calculation of shoot N from SNF was based on %Ndfa and shoot biomass. When direct

measurements of SNF, %Ndfa, is not available, indirect measurements such as nodule biomass and nodule number are recommended for estimating SNF ability. Measurement of leaf chlorophyll content is not recommended to estimate SNF when there are other sources of nitrogen available. In fact, results from both the PCA and the correlation matrix showed that %Ndfa was negatively correlated with leaf chlorophyll and shoot biomass, because additional N sources generally promote leaf chlorophyll content and shoot weight but negatively impact SNF-related traits.

### 4.4 | Carbon isotope discrimination ( $\delta^{13}\text{C}$ )

CID ( $\delta^{13}\text{C}$ ), an indicator of water use efficiency (WUE), was reported to be negatively associated with SNF (Kumarasinghe, Kirida, Mohamed, Zapata, & Danso, 1992). Plants with lower  $\delta^{13}\text{C}$  discrimination (less negative  $\delta^{13}\text{C}$  values) generally have higher WUE during photosynthesis and are thus more drought tolerant. Greater WUE and leaf N contribute to the "evolutionary success" and increased fitness and survival of N-fixing plants in arid and semiarid climates (Adams, Turnbull, Sprent, & Buchmann, 2016). Only a weak relationship between  $\delta^{13}\text{C}$  and %Ndfa, with the correlation coefficient of 0.239, was observed in our study, because plants in the current study were not exposed to drought stress. According to Knight, Verhees, Van Kessel, and Slinkard (1993), the negative correlation between  $\delta^{13}\text{C}$  and %Ndfa is only observed under drought stress conditions.

Plants with the 0-mM nitrate application had lower  $\delta^{13}\text{C}$  discrimination compared with plants receiving 2.5- to 20-mM nitrate,

indicating that plants without additional nitrate supply had greater WUE, most likely because these plants were generally less robust with smaller leaf area and thus experienced less water loss from evaporation. The nonnodulating genotype, R99, also had lower  $\delta^{13}\text{C}$  value (greater WUE) compared with other N-fixing genotypes that had N availability from both SNF and nitrate application and thus greater leaf area and more evapotranspiration than R99.

## 5 | CONCLUSION

A small amount of nitrate (2.5 and 5 mM) promoted nodulation in common bean, by increasing the number of total nodules and effective nodules and nodule biomass. On the contrary, a high amount of nitrate (greater than 10 mM) inhibited nodulation. No significant difference of nitrate tolerance was observed among three RILs and their parental lines. In the future, research that is directed to uncovering the intricate mechanisms underlying beneficial and inhibitory effects of nitrate on nodulation is recommended.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTIONS

Yunfei Jiang and K Peter Pauls conceptualized this research. Yunfei Jiang and Dustin MacLean conducted the experiments. Frédéric Marsolais and Brett Hill facilitated and conducted the nitrogen fixation measurements. K Peter Pauls supervised the work. Yunfei Jiang prepared the original manuscript draft and all authors contributed to the manuscript revision.

## ETHICAL STATEMENT

This article does not contain any studies with human or animal subjects.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

Yunfei Jiang  <https://orcid.org/0000-0003-4260-6513>

Dustin E. MacLean  <https://orcid.org/0000-0003-1169-9737>

Frédéric Marsolais  <https://orcid.org/0000-0003-3914-141X>

K. Peter Pauls  <https://orcid.org/0000-0003-0636-9151>

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