Canadian Journal of Plant Science
Revue canadienne de phytotechnie

**Evaluation of growth and nitrogen fixation of pea
nodulation mutants in western Canada**

Journal:	<i>Canadian Journal of Plant Science</i>
Manuscript ID	CJPS-2016-0383.R2
Manuscript Type:	Article
Date Submitted by the Author:	24-Apr-2017
Complete List of Authors:	Yang, Chao (Tony); University of Saskatchewan, Plant Sciences Bueckert, R.; University of Saskatchewan, Plant Sciences Schoenau, Jeff; Univ of Saskatchewan, Soil Science Diederichsen, Axel; Plant Gene Resources of Canada, Zakeri, Hossein; College of Agriculture, California State University, Chico, USA, Plant and Soil Sciences Warkentin, Tom; University of Saskatchewan, Crop Development Centre/Plant Sciences
Keywords:	nitrogen fixation, Pisum sativum, Pea, mutation, plant breeding

SCHOLARONE™
Manuscripts

Evaluation of growth and nitrogen fixation of pea nodulation mutants in western Canada

Chao Yang¹, Rosalind Bueckert¹, Jeff Schoenau², Axel Diederichsen³,

Hossein Zakeri⁴, and Thomas D. Warkentin^{1*}

1. Department of Plant Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, S7N 5A8, Canada (tom.warkentin@usask.ca)

2. Department of Soil Science, University of Saskatchewan, Saskatoon, Canada

3. Plant Gene Resources of Canada, Agriculture and Agri-Food Canada, Saskatoon, Canada

4. College of Agriculture, California State University, Chico, USA

Abstract

Optimized biological nitrogen fixation (BNF) in pea (*Pisum sativum* L.) could increase crop productivity and reduce nitrogen fertilizer use in Western Canada. We tested the BNF capabilities and growth of three pea nodulation mutants (Frisson P64 *Sym29*, Frisson P88 *Sym28* and Rondo-*nod3* (fix+)) compared to check cultivars (CDC Dakota, CDC Meadow, Frisson, Rondo and non-fixing negative control Frisson P56 (nod-)) under field conditions in Saskatchewan, Canada, in three environments. CDC Meadow and CDC Dakota produced greater dry biomass and seed yield, but less fixed nitrogen compared to the mutants. On average, Frisson P88 *Sym29* fixed 19% and 31% more nitrogen per plot compared to CDC Dakota and CDC Meadow, respectively. Rondo-*nod3* (fix+) fixed 12% and 23% more nitrogen per plot compared to CDC Dakota and CDC Meadow, respectively. All lines grown at Saskatoon in 2015 had longer time to flowering, greater biomass, greater grain yield, but less amount of nitrogen

fixation compared to these lines grown at Saskatoon in 2014 or Floral in 2015. Compared to the commercial checks, Frisson P88 *Sym29* and Rondo-*nod3* (fix+) had a high % nitrogen derived from atmosphere (*Ndfa*) and good nodulation under relatively high soil available nitrogen content, while requiring at least one week shorter growing period to reach maturity, indicating these mutants have potential as parents in breeding for improved BNF in pea.

Key words: nitrogen fixation, *Pisum sativum* L., western Canada, mutation, plant breeding

Introduction

Most of the biologically fixed nitrogen in agri-ecosystems arises from the symbiosis of nitrogen fixing bacteria with legume crops. In 1990s, the amount of nitrogen arising from cultivation of legumes was estimated to be up to 40 million tonnes annually worldwide, providing about 20% of the available nitrogen in agricultural systems globally (Crews and Peoples, 2004). Biological nitrogen fixation (BNF) is more desirable than use of nitrogen fertilizers due to economic and ecological reasons (Bohloul *et al.*, 1992). The improvement of BNF in agriculture can make a major contribution to sustainable agriculture (Wani *et al.*, 1995; Brewin and Legocki, 1996).

Among legume crops used in agriculture, field pea (*Pisum sativum* L.) is one of the most popular grain legumes. Peas provide 20-28% protein in the dry grains, and accounts for over 16% of pulse production worldwide (Drew *et al.*, 2012). BNF can contribute over 80% of nitrogen in pea plants, and provide 25 kg ha⁻¹ of nitrogen on average to the soil system for the succeeding crop (Ruisi *et al.*, 2012). Therefore, improving the BNF capacity of field pea in cultivar development programs has important economic and environmental implications. Considering the

large area of pea cultivation in Western Canada (Government of Saskatchewan, 2014, <http://www.agriculture.gov.sk.ca/pea>), even a small increase in BNF in pea through breeding will result in substantial benefits to farmers, and reduce reliance on nitrogen fertilization in the northern Great Plains region.

Previous research has identified some mutants that are highly efficient in BNF due to their deficiency in the autoregulation of nodulation (AON), such as the supernodulating mutant that is characterized by producing numerous small nodules, and the hypernodulating mutant that is characterized by producing a large nodule mass (Sidorova and Shumnyi, 2014). These pea mutants have been widely used in studies of the genetic control of BNF. For example, the supernodulating mutant carrying *Sym29* gene expresses a putative transmembrane, leucine-rich repeat receptor-like kinase that is a key regulator of the AON signalling pathway (Krusell *et al.*, 2002). Sagan and Duc (1996) reported another supernodulating mutant gene *Sym28* in pea that also displayed shoot-controlled super-nodulation and may be altered in a homologue of the *LjKLAVIER* gene that can regulate nodule numbers. Nitrate tolerant supernodulating mutants carrying the *nod3* gene drew the attention of researchers because of their nitrogen fixation capabilities in the presence of high nitrate concentration (Jacobsen and Feenstra, 1984).

In this study, a hypernodulating pea mutant (Rondo-*nod3*(fix+)) developed from cultivar Rondo was selected for the test. This mutant carries the *nod3* gene that controls the production of substantially greater nodule numbers and accumulated 3-5 times more nitrogen in roots than other tested pea cultivars (Postma *et al.*, 1988). Two supernodulating mutants (Frisson P64 *Sym28* and Frisson P88 *Sym29*) from cultivar Frisson that carry *sym 28* and *sym29* genes, respectively (Sagan and Duc, 1996), which can produce larger nodule mass than the progenitor cultivar, were also selected for this study. All pea lines were tested for their BNF capabilities,

biomass production, and seed yield in symbiosis with *R. leguminosarum* bv. *viciae* under field conditions. The objectives of this study were 1) test differences of BNF capabilities among pea mutants and commercial pea cultivars under Western Canadian soil-climatic conditions; 2) evaluate the relationships among soil nutrients, plant growth and nitrogen fixation; 3) provide information that will assist in breeding pea cultivars with higher nitrogen fixing capability.

Materials and Methods

Pea germplasm

Two supernodulating mutants Frisson P64 *Sym29* and Frisson P88 *Sym28*, which produce many small nodules along with their progenitor Frisson, one hypernodulating mutant Rondo-*nod3* (fix+), which produces many large nodules along with its progenitor Rondo, and two pea cultivars, CDC Dakota and CDC Meadow (Warkentin *et al.*, 2007), grown in western Canada were tested for their nitrogen fixation capabilities under field conditions in Saskatchewan. A non-nodulating pea mutant Frisson P56 (nod-) (Sagan *et al.*, 1993a) was used as a non-fixing negative control. Seeds of CDC Dakota and CDC Meadow were provided by Crop Development Centre, University of Saskatchewan. Seeds of Frisson, Rondo, negative control and three pea mutants were obtained from the John Innes Institute (Norwich, UK).

Field experiments and ^{15}N application

Pea lines were planted at the Saskatoon University experimental field in 2014 (Dark Brown soil zone, 52°10'07''N, 106°30'50''W) and 2015 (Dark Brown soil zone, 52°10'20''N,

106°30'24''W), and at the Floral experimental field in 2015 (Dark Brown soil zone, 52°03'45''N, 106°26'20''W). Soil samples from the top 30 cm layer were collected in April from these three experimental field sites and sent to ALS Laboratory Group Agricultural Services Saskatoon (<http://www.alsglobal.com>) for soil nutrient analysis. Details on soil available nutrients at the three sites are summarized in Table 1.

A randomized complete block design (RCBD) with 5 replicates was utilized. Plot size was 1 m² including the 15 cm space between plots (microplot), with each microplot consisting of three rows with 30 cm row spacing. Seeding rate was 60 seeds per m². The commercial pea granular inoculant TagTeam MultiAction® (Monsanto BioAg) was applied at the recommended rate of 3 kg ha⁻¹. Two weeks after planting when pea plants were in the seedling stage, ¹⁵N treatment (¹⁵NH₄¹⁵NO₃, 10 atom%, ICON ISOTOPES, www.iconisotopes.com) was applied between the second and third row of each plot, followed by application of 500ml of distilled water to allow the ¹⁵N solution to penetrate the top soil layer and spread evenly. The same amount of ¹⁵N solution was applied again two weeks after the first application. Overall, the total ¹⁵NH₄¹⁵NO₃ application in each plot was 0.55 g. Weeds were hand-removed from the experiment during the entire growing season.

Evaluation of biomass and yield traits

During the two growing seasons, germination rate (GR), days to flowering (DTF), days to podding (DTP), days to maturity (DTM) and plant height were recorded. Plots were harvested at physiological maturity by hand, when total accumulated BNF in above ground plant tissue was maximized. Since plants in row one were without ¹⁵N, while plants in rows two and three were

^{15}N labeled, the above ground biomass was harvested from individual rows, i.e., plants with and without ^{15}N application from the same plot were collected separately. Samples were dried at 37°C for 24 hours, then dry biomass of each microplot and seed yield were recorded.

Evaluation of BNF

After drying, whole plant samples were finely ground using a Cyclone sample mill (UDY Corporation, Colorado, USA). Plant ^{15}N -to- ^{14}N ratio was measured by mass spectrometry (V.G. Isotech, Aston Way, Middlewich, Cheshire, CW10 OHT, United Kingdom). The amount and percentage of nitrogen derived from air were calculated as follows (Voisin *et al.*, 2002; Fried and Middelboe 1977):

$$\text{N}_{2\text{fixed}} = \%Ndfa \times \text{total N}_{\text{legume}} / 100$$

$$\%Ndfa = 100 \times (\delta^{15}\text{N}_{\text{legume}} - \delta^{15}\text{N}_{\text{reference plant}}) / (\epsilon_{\text{fix}} \times \delta^{15}\text{N}_{\text{reference plant}})$$

Where $\delta^{15}\text{N}_{\text{legume}}$ is ^{15}N isotope measured in tested pea tissues, $\delta^{15}\text{N}_{\text{reference plant}}$ is ^{15}N isotope measured in tissues of negative control (non-fixing mutant). $\%Ndfa$ is percentage of nitrogen derived from atmosphere, and $\text{total N}_{\text{legume}}$ is total nitrogen measured in tested pea tissues. ϵ_{fix} (-1 for pea) is the isotopic fractionation factor associated with N_2 fixation processes.

Statistical analysis

Cultivar and environmental effects on GR, DTF, DTP, DTM, plant height, plant dry biomass, seed yield, $\%Ndfa$, amount of fixed N, percentage nitrogen ($\%\text{N}$), and total nitrogen in plant above ground tissue were tested by ANOVA using SYSTAT 12 (Systat Software, Inc., Chicago,

IL, USA. <http://www.systat.com/>). The Shapiro-Wilk test was used to verify the normality of the data, and the Wilks' Lambda test was used to detect significant effects at the 5% level. Duncan's Multiple Range Test was used to evaluate significant pairwise difference among treatments. Redundancy analysis (RDA) was used to evaluate the relationship between soil nutrients and fixed N in this study in R Studio (version 0.99.887, R Studio Inc., <https://www.rstudio.com/>).

Results

Cultivar effects on growth and nitrogen fixation in pea

Monthly average temperatures during the growing season (from May to August) were quite similar in the 2014 and 2015 growing seasons, but monthly average precipitation in the two growing seasons were very different (Fig. 1). In particular, very high precipitation was received during the seedling and early growing stages in 2014. Compared to average precipitation over the last 30 years (<http://www.farmzone.com/statistics/precipitation/cl4057202/sk023/metric>), precipitation in May and June of 2014 were 17% and 58% higher than the average. In 2015, extremely low precipitation was received during the seeding and early growing stages in May and June, which were 98% and 77% lower than the last 30 years' average, respectively, but during the flowering and podding stages in July, precipitation was 34% higher than the last 30 years' average.

Small but significant differences in GR were detected among tested pea lines (Table 2). In particular, Frisson showed higher GR than others. CDC Meadow and CDC Dakota required a longer vegetative period to reach podding stage (DTP), maturity (DTM) and had taller above ground canopy than either supernodulating (Rondo-*nod3* (fix+)) or hypernodulating (Frisson P88

Sym29 and Frisson P64 *Sym 28*) mutants (Table 2). Longer growing period and larger above ground biomass of these two cultivars also led to greater dry biomass and seed yield. In particular, biomass of both cultivars was two times as large as in Rondo-*nod3* (fix+), and seed yield of CDC Dakota was nearly double compared to seed yield of Frisson P88 *Sym29* (Table 2). However, the shorter growing period of the mutants could be advantageous in the shorter season regions of pea production. No significant differences in % nitrogen in the dry tissue were detected among pea lines, but total nitrogen differed among lines (Table 2) due to differences in above ground biomass.

For fixed N, Frisson P88 *Sym29* and Rondo-*nod3* (fix+) showed higher amount of fixed N than other tested pea lines especially at Saskatoon-2014 and Floral-2015 (Table 3). Although CDC Meadow and CDC Dakota showed higher dry biomass than the mutants, higher %*Ndfa* of the mutants lead to relatively higher amount of fixed N in their above ground tissue (Table 4). Among the three pea mutants, hypernodulating line Frisson P88 *Sym29* and supernodulating line Rondo-*nod3* (fix+) had greater amount of fixed N compared to Frisson P64 *Sym 28*. As well, Frisson P88 *Sym29* and Rondo-*nod3* (fix+) showed higher nodulation (Fig. 2) and %*Ndfa* (Table 4) than their original parents Frisson and Rondo, respectively, under most tested environmental conditions. Also, these two mutants showed higher %*Ndfa* than CDC Dakota and CDC Meadow (Table 4), indicating the potential of using these two mutants for breeding in western Canada.

Environmental effects on pea growth and nitrogen fixation

Environmental factors significantly affected pea growth (Table 2). In particular, Saskatoon-2014 showed higher GR compared with other two environments, while the experiment conducted at

Saskatoon-2015 had on average longer DTF, DTP, greater biomass, seed yield, % nitrogen and total nitrogen compared with the experiments conducted at Floral-2015 and Saskatoon-2014. Environmental and soil factors also significantly affected nitrogen fixation in the tested pea lines. In general, total fixed nitrogen in all tested lines was lowest at Saskatoon-2015 (Table 3). In Saskatoon-2015, soil pH was slightly alkaline compared to the other two environments which were slightly acidic. In addition, Fe concentration were much lower in Saskatoon-2015 than the other two environments (Table 1). Potentially soil pH and micronutrient conditions may restrict nitrogen fixation and plant-rhizobia interactions (Chemining'wa and Vessey, 2006). Total fixed nitrogen in all tested lines was highest at Floral-2015 (Table 3), which had somewhat lower soil available nitrogen compared to the other two test environments (Table 1). Lower available soil nitrogen may promote the nitrogen fixation capability of pulse plants. The relationships between fixed nitrogen and soil nutrients were tested by RDA analysis (Fig. 3, $P = 0.001$). Positive relationships were detected between fixed nitrogen and soil available Fe, meanwhile negative relationships were detected between fixed nitrogen and soil available nitrogen. No significant interactions were detected between environments and lines (Table 2).

Discussion

Environmental effects on nitrogen fixation

Percent nitrogen and total nitrogen in above ground plant tissue differed among the three tested environmental conditions. In general, the amount of fixed nitrogen in pea in this study was relatively lower than previous reports (McCauley *et al.*, 2012; Ashworth *et al.*, 2015). McCauley reported amount of fixed nitrogen in pea varied between 74 to 84 kg per ha, while Ashworth

reported approximately 84 kg per ha. The key events of nodulation occur early in the growing season and weather conditions were not ideal for maximizing nitrogen fixation in either season. In 2014 spring conditions were much wetter than average (Fig. 1), while in 2015 spring conditions were much drier than average. These key moisture effects could have reduced total nitrogen fixation in these experiments. Soil moisture effects on fixed nitrogen have been reported in many legume crops, including common bean (Devi *et al.*, 2013; Farid and Navabi, 2015), soybean (Djekoun and Planchon, 1991; Sprent, 2006), faba bean (Sangakkara *et al.*, 1996), lupin and peanut (Sinclair and Serraj, 1995). In these studies, authors reported a negative impact of water shortage on fixed nitrogen in legume crops, with nodule senescence, and restricted nitrogenase activity (Becana *et al.*, 1986). For example in 2014, although spring conditions were wet, summer conditions were relatively dry and this could have restricted growth and nitrogen fixation of pea plants.

Effect of soil nutrients on fixed nitrogen

Previous studies showed that fixed nitrogen and %*Ndfa* are characteristics not only determined by legume genotypes and rhizobia strains, but also impacted by soil nutrients such as soil available N (Peoples *et al.*, 1995; Peoples *et al.*, 2009). High concentration of soil nitrogen has shown inhibition effects both experimentally and practically on BNF and %*Ndfa* in several legume crops including soybean, chickpea and faba bean in Australia (Peoples *et al.*, 2001). In Nepal and Vietnam, reduced fixed nitrogen in soybean and groundnut was also related to high soil nitrogen due to high fertilizer nitrogen application by local farmers (Maskey *et al.*, 2001; Hoa *et al.*, 2002). In this study, available soil nitrogen was between 55 to 71 kg/ha under the three tested environmental conditions. Based on report from the Saskatchewan Ministry of

Agriculture (<http://www.saskatchewan.ca/business/agriculture-natural-resources-and-industry/agribusiness-farmers-and-ranchers/crops-and-irrigation/pulse-crop-bean-chickpea-faba-bean-lentils/dry-pea>), soil available nitrogen higher than 40 kg/ha can delay nodulation and BNF in pea, and nitrogen level higher than 50 kg/ha would restrict nodulation and BNF in pea in Saskatchewan. In general, in this study overall %*Ndfa* of all tested pea lines was between 37% to 81% which was similar to previous reports of BNF in pea that ranged between 33% to 75% (Peoples *et al.*, 2009; Sagan and Duc, 1996). However, compared to the mutants, the cultivars showed relatively higher total nitrogen but lower %*Ndfa*, suggesting soil nitrate in this study may have restricted BNF of the pea cultivars more than the mutants. Relatively high soil available nitrogen did not significantly restrict nodulation of the mutants, as many nodules were detected. Previous research showed that pea mutants with good nitrate-tolerant symbiosis were recommended for use in pea breeding to produce progeny with improved BNF capacity and grain yield (Novák *et al.*, 2009; Sagan and Duc, 1996). For example, Sagan *et al.*, (1993b) reported that *nod++* pea mutants such as Frisson P64 *Sym28* showed higher nodulation potential than their original parent Frisson, but did not necessarily have greater %*Ndfa* and yield especially under nitrogen deficient field conditions. However in our study, Frisson P64 *Sym28* and Frisson P88 *Sym29* had greater %*Ndfa* and fixed nitrogen compared to their original parent Frisson, suggesting their nitrate-tolerant potential in nitrogen fixation, as nitrogen levels in the tested experimental nurseries was relatively high. Therefore, results shown here indicate that the pea mutants appear to have good nitrate tolerance and could be utilized as parents in breeding.

Cultivar effects on fixed nitrogen

Previous studies have documented cultivar effects on BNF in different legume crops. In a study of soybean, the cultivar effects on variability in fixed nitrogen was as high as 70% (Bello *et al.*, 1980). In lentil, differences in fixed nitrogen among tested cultivars was as high as 81% (Hafeez *et al.*, 2000). In dry bean, differences in fixed nitrogen among different small-seeded Central American cultivars were up to 4-fold (Farid and Navabi, 2015). In this study, as expected, the highest yielding pea lines were the two commercial pea cultivars, however their BNF capacities were not outstanding especially when soil nutrient conditions were not favored for nitrogen fixation. In recent decades, pea improvement has focused on improving grain yield, grain quality, and disease resistance (Micke, 1993; Warkentin *et al.*, 2015). Therefore, these traits must be maintained while new traits including high nitrogen fixation capability should also be included in order to improve economic and environmental benefits (Herridge and Rose, 2000).

The extent of nitrogen fixation reported in pea is inconsistent (McCauley *et al.*, 2012; Ruisi *et al.*, 2012; Schwenke *et al.*, 2015). McCauley found the amount of fixed nitrogen in above ground tissues of pea and lentil to vary at different growing stages due to different seeding times in the northern Great Plains region of North America. Ruisi *et al.* (2012) reported that pea fixed less nitrogen than chickpea and faba bean, but more nitrogen than lentil in Mediterranean regions, while Schwenke *et al.* (2015) found that the total plant biomass nitrogen attributable to nitrogen fixation in pea was about 14% higher than in chickpea, but 10% less than in faba bean in a subtropical area near Tamworth, Australia. In a greenhouse assay, differences of fixed nitrogen among five tested pea cultivars varied from 379 to 578 mg per plant (Abi-Ghanem *et al.*, 2011). Nitrogen fixation is an energy consuming process. The mutants that produced either numerous small nodules or fewer large nodules would potentially be devoting more of their photosynthate carbon to nodule formation, and this could limit the photosynthate dedicated to nitrogen fixation.

and impact AON (Voisin *et al.*, 2007). Voisin *et al.*, (2013) reported that root and shoot growth of hypernodulating mutants were reduced to 80% and 60%, respectively, when compared to their wild type progenitors due to the carbon limits caused by massive nodule production. Carbon limits could partially explain why genotype had a significant effect on total nitrogen in above ground tissue (Table 2), and why higher %*Ndfa* and fixed nitrogen of the mutants did not necessarily lead to increased biomass and grain yield.

Cultivar effects on fixed nitrogen in pea plants may also be due to their influence on the composition of *R. leguminosarum* bv. *viciae* in the rhizosphere. For example, the *nod3* gene carried by Rondo-*nod3* (fix+) could regulate systemic signals involved in nodule production in the root system (Postma *et al.*, 1988), whereas Frisson P88 *Sym29* carries gene *sym29*, which is the pea orthologue of the HAR1 gene involved in a putative receptor kinase for shoot-controlled regulation of nodulation and root development (Krusell *et al.*, 2002). As a consequence, root morphogenesis and exudates might differ between mutants carrying these different genes, and signal exchange between the plant and rhizobia may also differ (Yang and Crowley, 2000), which could further influence the nodulation and nitrogen fixation efficiency by impacting the recognition and symbiosis of rhizobia to their host plants. In this study, significant cultivar effects were detected among tested pea lines. We found all three tested mutants had shorter growing period, much higher nodulation and %*Ndfa* compared to the two commercial cultivars, which may give them potential for being used in further breeding for short growing season areas in western Canada and other continental regions. Fixed nitrogen in above ground tissue of hypernodulating mutant Frisson P88 *Sym29* was higher than Frisson P64 *Sym28* and Rondo-*nod3* (fix+), suggesting good potential for this mutant being used in breeding. The %*Ndfa* results in our study showed that these mutants have the ability to derive a higher proportion of nitrogen

from fixation than their original parents and two most commonly planted commercial pea cultivars CDC Dakota and CDC Meadow. Compared to these commercial pea cultivars, Frisson P88 *Sym29* and Rondo-*nod3* (fix+) showed much higher nodule production and %*Ndfa* under field conditions, indicating their potential in nitrogen fixation, if energy supply is not a limitation. Therefore, including these mutants in further pea breeding programs may produce hybrids with better nitrogen fixation and yield. A small %*Ndfa* was detected in the non-fixing mutant Frisson P56 (nod-) (Table 4). Ghachtouli et al. (1995) reported that Frisson P56 (nod-) showed strong symbiosis with arbuscular mycorrhizal (AM) fungi, thus, the fixed nitrogen detected may have been due to nutrient transfer from neighboring nitrogen fixing pea plots in this experiment via AM activity.

Conclusion

Considerable variation in yield and BNF was found among tested pea lines. Commercial pea cultivars showed greater biomass and seed yield compared to tested mutants since they were bred for specific adaptation to western Canadian environmental conditions. However, the mutants Frisson P88 *Sym29* and Rondo-*nod3* (fix+) showed greater BNF, higher nodulation and %*Ndfa* among tested pea lines, indicating the potential of these two pea lines as parents in breeding for improved nitrogen fixation. Environmental factors significantly influenced the growth and nitrogen fixing ability of the tested pea varieties at three sites. Environments with moderate soil moisture content, neutral pH, relatively low available nitrogen concentration should be the best for differentiating pea lines in terms of nitrogen fixation.

Acknowledgement

We are grateful to the Saskatchewan Ministry of Agriculture, Agriculture Development Fund for financial support of this research. We thank Dr. Mike Ambrose from John Innes Centre for kindly providing three pea mutants. We also grateful to Scott Ife, Brent Barlow and Myles Stocki for their technical support.

References

- Abi-Ghanem, R., Carpenter-Boggs, L., Smith, J.L. 2011. Cultivar effects on nitrogen fixation in peas and lentils. *Biol. Fertil. Soils* 47: 115-120.
- Ashworth, A.J., West, C.P., Allen, F.L., Keyser, P.D., Weiss, S.A., Tyler, D.D., Taylor, A.M., Warwick, K.L., Beamer, K.P. 2015. Biologically fixed nitrogen in legume intercropped systems: Comparison of nitrogen-difference and nitrogen-15 enrichment techniques. *Agronomy Journal* 107: 2419-2430.
- Becana, M., Aparicio-tejo, P., Peña, J., Aguirreolea, J., Sánchez-díaz, M. 1986. N₂ fixation (C₂H₂-reducing activity) and leghaemoglobin content during nitrate- and water-stress-induced senescence of *Medicago sativa* root nodules. *Journal of Experimental Botany* 37: 597-605.
- Bello, A.B., Ceron-Dias, W.A., Nickell, C.D., Elsheriff, E.O., Davis, L.C. 1980. Influence of cultivar, between-row spacing and plant population of fixation of soybeans. *Crop Sci* 20: 751-755.
- Bohlool, B.B., Ladha, J.K., Garrity, D.P., George, T. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. *Plant Soil* 141: 1-11.
- Brewin, N.J., Legocki, A.B. 1996. Biological nitrogen fixation for sustainable agriculture. *Trends Microbiol.* 4: 476-477.
- Chemining'wa, G.N., Vessey, J.K. 2006. The abundance and efficacy of *Rhizobium leguminosarum* bv. *viciae* in cultivated soils of the eastern Canadian prairie. *Soil Biology and Biochemistry* 38: 294-302.
- Crews, T.E., Peoples, M.B. 2004. Legume versus fertilizer sources of nitrogen: Ecological tradeoffs and human needs. *Agric. Ecosyst. Environ.* 102: 279-297.
- Devi, M.J., Sinclair, T.R., Beebe, S.E., Rao, I.M. 2013. Comparison of common bean (*Phaseolus vulgaris* L.) genotypes for nitrogen fixation tolerance to soil drying. *Plant Soil* 364: 29-37.
- Djekoun, A., Planchon, C. 1991. Water status effect on dinitrogen fixation and photosynthesis in soybean. *Agron. J.* 83: 316-322.
- Drew, E.A., Denton, M.D., Sadras, V.O., Ballard, R.A. 2012. Agronomic and environmental drivers of population size and symbiotic performance of *Rhizobium leguminosarum* bv. *viciae* in Mediterranean-type environments. *Crop Pasture Sci.* 63: 467-477.
- Farid, M., Navabi, A. 2015. N₂ fixation ability of different dry bean genotypes. *Can. J. Plant Sci.* 95: 1243-1257.

- Ghachtouli, N. El., Paynot, M., Morandi, D., Martin-Tanguy, J., Gianinazzi, S. 1995. The effect of polyamines on endomycorrhizal infection of wild-type *Pisum sativum*, cv. Frisson (nod+myc+) and two mutants (nod-myc+ and nod-myc-). *Mycorrhiza* 5: 189-192.
- Hafeez, F.Y., Shah, N.H., Malik, K.A. 2000. Field evaluation of lentil cultivars inoculated with *Rhizobium leguminosarum* bv. *viciae* strains for nitrogen fixation using nitrogen-15 isotope dilution. *Biol. Fertil. Soils* 31: 65-69.
- Herridge, D., Rose, I. 2000. Breeding for enhanced nitrogen fixation in crop legumes. *Field Crops Research* 65: 229-248.
- Hoa, N.T.L., Thao, T.Y., Lieu, P., Herridge, D.F., Herridge, D. 2002. N₂ fixation of groundnut in the eastern region of south Vietnam. *Inoculants and Nitrogen Fixation of Legumes in Vietnam*. ACIAR Proc. 109: 19-28.
- Jacobsen, E., Feenstra, W.J. 1984. A new pea mutant with efficient nodulation in the presence of nitrate. *Plant Science Letters* 33: 337-344.
- Krusell, L., Madsen, L.H., Sato, S., Aubert, G., Genua, A., Szczyglowski, K., Duc, G., Kaneko, T., Tabata, S., De Bruijn, F., Pajuelo, E., Sandal, N., Stougaard, J. 2002. Shoot control of root development and nodulation is mediated by a receptor-like kinase. *Nature* 420: 422-426.
- Maskey, S.L., Bhattarai, S., Peoples, M.B., Herridge, D.F. 2001. On-farm measurements of nitrogen fixation by winter and summer legumes in the hill and terai regions of nepal. *Field Crops Research* 70: 209-221.
- McCauley, A.M., Jones, C.A., Miller, P.R., Burgess, M.H., Zabinski, C.A. 2012. Nitrogen fixation by pea and lentil green manures in a semi-arid agroecoregion: Effect of planting and termination timing. *Nutr. Cycl. Agroecosyst.* 92: 305-314.
- Micke, A. 1993. Mutation breeding of grain legumes. *Plant Soil* 152: 81-85.
- Novák, K., Biedermannová, E., Vondrys, J. 2009. Symbiotic and growth performance of supernodulating forage pea lines. *Crop Sci.* 49: 1227-1234
- Peoples, M.B., Bowman, A.M., Gault, R.R., Herridge, D.F., McCallum, M.H., McCormick, K.M., Norton, R.M., Rochester, I.J., Scammell, G.J., Schwenke, G.D. 2001. Factors regulating the contributions of fixed nitrogen by pasture and crop legumes to different farming systems of eastern Australia. *Plant Soil* 228: 29-41.
- Peoples, M.B., Brockwell, J., Herridge, D.F., Rochester, I.J., Alves, B.J.R., Urquiaga, S., Boddey, R.M., Dakora, F.D., Bhattarai, S., Maskey, S.L., Sampet, C., Rerkasem, B., Khan, D.F., Hauggaard-Nielsen, H., Jensen, E.S. 2009. The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis* 48: 1-17.
- Peoples, M.B., Ladha, J.K., Herridge, D.F. 1995. Enhancing legume N₂ fixation through plant and soil management. *Plant Soil* 174: 83-101.
- Postma, J.G., Jacobsen, E., Feenstra, W.J. 1988. Three pea mutants with an altered nodulation studied by genetic analysis and grafting. *J. Plant Physiol.* 132: 424-430.
- Ruisi, P., Giambalvo, D., Di Miceli, G., Frenda, A.S., Saia, S., Amato, G. 2012. Tillage effects on yield and nitrogen fixation of legumes in mediterranean conditions. *Agronomy Journal* 104: 1459-1466.
- Sagan, M., Duc, G. 1996. *Sym28* and *Sym29*, two new genes involved in regulation of nodulation in pea (*Pisum sativum* L.). *Symbiosis* 20: 229-245.
- Sagan, M., Messenger, A., Duc, G. 1993a. Specificity of the *Rhizobium*-legume symbiosis obtained after mutagenesis in pea (*Pisum sativum* L.). *New Phytol.* 125: 757-761.
- Sagan M., Ney B., Duc G. 1993b. Plant symbiotic mutants as a tool to analyse nitrogen nutrition and yield relationship in field-grown peas (*Pisum sativum* L.). *Plant Soil* 153: 33-45.

- Sangakkara, U.R., Hartwig, U.A., Nösberger, J. 1996. Soil moisture and potassium affect the performance of symbiotic nitrogen fixation in faba bean and common bean. *Plant Soil* 184: 123-130.
- Schwenke, G.D., Herridge, D.F., Scheer, C., Rowlings, D.W., Haigh, B.M., McMullen, K.G. 2015. Soil N₂O emissions under N₂-fixing legumes and N-fertilised canola: A reappraisal of emissions factor calculations. *Agric. Ecosyst. Environ.* 202: 232-242.
- Sidorova, K.K., Shumnyi, V.K. 2014. Symbiotic mutants of pea (*Pisum sativum* L.), an important genetic source for selection for increased nitrogen fixation. *Dokl. Biol. Sci.* 454: 59-61.
- Sinclair, T.R., Serraj, R. 1995. Dinitrogen fixation sensitivity to drought among seed legume species. *Nature* 378: 344.
- Sprent, J.I. 2006. The effects of water stress on nitrogen-fixing root nodules. *New Phytol.* 71: 603-611.
- Voisin, A-S., Salon, C., Munier-Jolain, N. G., Ney, B. 2002. Quantitative effects of soil nitrate, growth potential and phenology on symbiotic nitrogen fixation of pea (*Pisum sativum* L.). *Plant Soil* 243: 31-42
- Voisin, A-S., Bourion, V., Due G., Salon C. 2007. Using an ecophysiological analysis to dissect genetic variability and to propose an ideotype for nitrogen nutrition in pea. *Annals of botany* 100: 1525-1536.
- Voisin, A-S., Cazenave, A-B., Due, G., Salon, C. 2013. Pea nodule gradients explain C nutrition and depressed growth phenotype of hypernodulating mutants. *Agron. Sustain. Dev.* 33: 829-838.
- Wani, S.P., Rupela, O.P., Lee, K.K. 1995. Sustainable agriculture in the semi-arid tropics through biological nitrogen fixation in grain legumes. *Plant Soil* 174: 29-49.
- Warkentin, D.T., Smýkal, P., Coyne, J.C., Weeden, N., Domoney, C., Bing, D.J., Leonforte, A., Xuxiao, Z., Dixit, P.G., Boros, L., McPhee, E.K., McGee, J.R., Burstin, J., Ellis, N.T.H. 2015. In Pea. Pages 37-83 in De Ron, M.A. eds. *Grain Legumes*. Springer New York, New York, NY.
- Warkentin, T., Vandenberg, A., Tar'an, B., Banniza, S., Barlow, B., Ife, S. 2007. CDC Meadow field pea. *Can. J. Plant Sci.* 87: 909-910.
- Yang, C.H., Crowley, D.E. 2000. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl. Environ. Microbiol.* 66: 345-351.

Table 1. Soil properties and available nutrients from the 0-30 cm soil layer at the three sites in Saskatchewan, Canada used to evaluate pea lines for biological nitrogen fixation.

	pH	EC	OM%	N	P	K	S	Cu kg/ha	Mn	Zn	B	Fe
Saskatoon-2014	5.9	0.1	3.6	71	138	1505	9	6	137	4	4	321
Saskatoon-2015	7.6	0.1	4.2	57	150	1505	9	3	20	7	4	81
Floral-2015	6.1	0.2	4.2	55	114	1505	41	3	101	7	6	287

Note: all soil nutrients data were measured in ALS Laboratories (Saskatoon, Saskatchewan. <http://www.alsglobal.com>). EC: electrical conductivity. OM%: percentage of organic matter.

Table 2. Genotype and environmental effects on plant GR (germination rate in percent), DTF (days to flowering), DTP (days to podding), DTM (days to maturity), height, above ground dry biomass, percentage nitrogen, and total nitrogen per m² in above ground dry biomass, as well as seed yield of pea lines grown in three environments in Saskatchewan

		GR	DTF	DTP	DTM	Height (cm)	Dry Biomass (g / m ²)	Dry Seed Yield (g / m ²)	Percent N (%)	Total N (g / m ²)
Pea lines (G)	CDC Dakota	77b	59a	61a	91a	96a	1518a	515a	2.6a	39.5a
	CDC Meadow	78b	54b	58ab	90a	87a	1231a	497a	2.2a	27.1b
	Frisson	83a	52b	56b	85b	60c	932b	354b	2.1a	19.6bc
	Frisson, P64 <i>Sym 28</i>	77b	52b	60a	81c	76ab	850b	329bc	2.8a	23.8b
	Frisson, P88 <i>Sym 29</i>	78b	52b	57b	81c	73b	829b	262c	2.9a	24.1b
	Rondo	80ab	53b	57b	85b	56c	1007ab	442ab	2.3a	23.2b
	Rondo- <i>nod3</i> (fix+)	77b	51b	56b	84b	58c	648c	291bc	2.4a	15.6c
	Frisson, P56 (nod-)	82a	50b	55b	83bc	47d	608c	219c	2.0a	12.2c
<i>P</i> value		0.0204	<0.0001	0.0005	<0.0001	<0.0001	<0.0001	<0.0001	NS	<0.0001
Environment (E)	Saskatoon-2014	83a	52.2b	54.7b	86a	68a	665c	373b	1.7c	11.3c
	Saskatoon-2015	77b	55.3a	60.8a	86a	70a	1251a	443a	3.3a	41.3a
	Floral-2015	75b	50.8b	54.7b	83b	69a	867b	275c	2.3b	19.9b
	<i>P</i> value	0.0001	0.006	0.005	0.0003	NS	0.038	0.0001	<0.0001	<0.0001
G*E	<i>P</i> value	NS	NS	NS	NS	NS	NS	NS	NS	NS

Note: differences between small letters in each column indicates significant difference of the tested variable either among pea lines or the environments based on Duncan’s Multiple Range Test.

Table 3. Environmental and genotype effects on fixed nitrogen (kg/ha) of pea lines grown in three environments in Saskatchewan

Environment	Pea lines								<i>P</i> value
	CDC Dakota	CDC Meadow	Frisson	Frisson, P64 <i>Sym 28</i>	Frisson, P88 <i>Sym 29</i>	Rondo	Rondo- <i>nod3</i> (fix+)	Frisson, P56 (nod-)	
Saskatoon-2014	26.1bB	31.9bB	29.4abB	41.6aA	45.9bA	25.3bB	43.0bA	7.1aC	<0.0001
Saskatoon-2015	25.4bAB	23.2cAB	21.1bB	24.9bAB	29.4cA	26.4bAB	26.6cAB	3.7bC	0.001
Floral-2015	42.0aB	39.1aB	34.5aC	40.5aB	53.4aA	49.7aA	52.1aA	5.6abD	<0.0001
<i>P</i> value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Note: difference between small letters in each column means significant difference of fixed N in each pea line among three environments; difference between capital letters in each row means significant difference of fixed N among eight tested pea lines in each environment, based on Duncan's Multiple Range Test.

Table 4. Environmental and genotype effects on %*Ndfa* of pea mutants and cultivars grown in three environments in Saskatchewan

Environment	Pea lines								<i>P</i> value
	CDC Dakota	CDC Meadow	Frisson	Frisson, P64 <i>Sym 28</i>	Frisson, P88 <i>Sym 29</i>	Rondo	Rondo- <i>nod3</i> (fix+)	Frisson, P56 (nod-)	
Saskatoon-2014	48.1bC	55.8aC	59.5aC	70.8aB	80.6aA	49.5aC	75.6aAB	10.5aD	<0.0001
Saskatoon-2015	58.2aA	47.5bAB	57.4aA	40.1bB	52.3bAB	44.2bB	63.2bA	7.2aC	0.012
Floral-2015	41.9cD	40.2cD	37.5bD	45.5bC	59.1bB	52.8aBC	69.1abA	8.5aE	<0.0001
<i>P</i> value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.034	0.04	ns	

Note: difference between small letters in each column means significant difference of %*Ndfa* in each pea line among three environments; difference between capital letters in each row means significant difference of %*Ndfa* among eight tested pea lines in each environment, based on Duncan’s Multiple Range Test.

Fig 1. Monthly average temperature (°C) and precipitation (mm) in 2014 and 2015 based on data from Saskatoon RCS station (Environment Canada). Note that this is the nearest station to the Saskatoon and Floral research sites used in this research.

Fig. 2 Representative photos of nodulation of tested pea lines under field conditions (a: CDC Meadow, b: CDC Dakota, c: Frisson, d: Rondo, e: Frisson, P64 *Sym28*, f: Frisson, P88 *Sym29*, g: Rondo-*nod3* (fix+), h: Frisson, P56 (nod-))

Fig. 3 Redundancy analysis (RDA) results of relationships between fixed nitrogen and soil nutrients ($P = 0.001$)

For Review Only

Fig. 1

Fig. 2

Fig. 3