RESEARCH ARTICLE

Quantification of symbiotically fixed N_2 by faba bean and allocation of ^{15}N among above- and below-ground components of faba bean, canola, and barley on a Gray Luvisol

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Abstract: This study was carried out to estimate N-15 allocation into different components of the above- and below-ground plant parts, and to quantify biological N-fixation in faba bean using N-15 dilution method, and both canola and barley as reference crops. Four samplings were conducted during the crop growing season, both above- and below-ground plant parts, and soil were sampled. The above-ground plant was fractionated into leaves, stems, shell, and seeds for faba bean and canola; and dead-leaves, live-leaves, stems, sheaths, husks, and seeds for barley. Roots were extracted from soil by root-washing technique and further separated from dead debris by water floatation and hand-picking. N-15 excess of faba bean above-ground parts was lower than its roots, but the reverse was true for the non-legumes (canola and barley). N-15 fractionation was apparently taking place in various plant parts. Reproductive organs of faba bean had lower N-15 excess than the vegetative ones, but reproductive organs of non-legumes (canola and barley) had higher N-15 excess than the vegetative ones. N-fixation in faba bean was quantified by using N-15 dilution method with either canola or barley as a reference crop, the quantity of N derived from atmospheric fixation was 183-199 kg N/ha/yr in the above-ground parts of faba bean and 18-22 kg N/ha/yr in faba bean roots by September 1, when faba beans were not fully matured. Either canola or barley can be a valid reference crop for N-fixation estimation in N-15 dilution method. Total difference method agreed with N-15 dilution method with less than 10% variation in this study. A peak of N-fixation was observed after faba bean flowering and the rate of N-fixation during this period was 4.0-4.7 kg N/ha/day. This study provides the key inofmration for the quantity of N-fixation from atmosphere in faba bean growing on this soil of Canada.
Keywords:dinitrogen fixation; N-15 dilution method; Gray Luvisol; soil improvement; rotation

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1 Introduction

Biological dinitrogen fixation has commanded the attention of scientists concerned with plant mineral nutrition for more than 100 years (Date, 1973; Dixon and Wheeler, 1986; Faust, 1982; Havelka et al., 1982). It is a process to obtain N from the atmosphere through symbiotic association between Rhizobium and legume and, therefore, plays a very important role in the sustaining agriculture to maintain adequate nitrogen level of the soils (Ladd et al., 1981; 1983; Cleveland et al., 1999; Pereira e Silva et al., 2001; Gu and McGill, 2017). Substantial amount of nitrogen fixed in legume residues can be turned into following crops through mineralization of fixed N, this portion of nitrogen is potentially available in the nutrient cycling (Ladd et al., 1986; McGill and Christie, 1983; Müller, 1988; Müller and Sundman, 1988; Koponen et al., 2003). As public concern on environmental quality increases, organic farming, which is a production system highly relied on rotation, tilth, legume, green-manure, etc., has been practised in some extend (Patriquin et al., 1981; Sprent, 1986). The increasing price on fuel has been proven to be one primary impact on the cost of industrial N production and also a consuming burden for farmers in the future. Pollution from over utilization of chemical fertilizers is also a major issue to the ecological quality of coastal and ocean ecosystems (Li et al., 2013; Wang and Gu, 2013a, b; Wang et al., 2013; 2014; Chan et al., 2016; Li and Gu, 2016; Wu et al., 2017). Manures and legumes have shown their perspective future in nowaday agriculture (McGill, 1982; Patriquin et al., 1981; Yan et al., 2016; Xiong et al., 2017).

Nitrogen-15 isotope technique has been applied to the N related research, symbiotic N-fixation (McAuliffe et al., 1958; Rennie and Dubetz, 1986; Witty, 1983; Gu and McGill, 2017), mineralization and immobilization of nitrogen through microorganisms (Paul and Juma, 1981), and N turnover in reclaimation mined sites (Fyles and McGill, 1986) since the initial work had been done by McAuliffe and co-workers (1958). The basic principle involved in N-15 dilution method was elucidated by Fried and Dean (1952) and has been used in quantification of dinitrogen fixation in legumes thereafter (Fried and Broeshart, 1975; Goh et al., 1978; Rennie, 1982; Witty, 1983). Because a non-legume crop is usually introduced into the N-15 dilution technique in field quantification as a reference plant, the difference in labeled nitrogen between reference plant and legume is taken into account for the dinitrogen fixed by legume plant only (Vallis et al., 1967). This also leads the accuracy of the results are questionable in a study due to the morphological and physiological differences between root systems of the reference plants and legumes (McAuliffe et al., 1958; Vallis et al., 1977; Bergersen and Turner, 1983). The primary assumptions by McAuliffe et al. (1958) were that reference plant and legume absorb N-15 and non-labeled mineral N at the same ratio and there was no transferring of N from legume to associated plant. However, method was developed to estimate the proportion of soil indigenous N to the assimilated N (Ledgard et al., 1985a, b, c; Gu and McGill, 2017). Meanwhile, Witty (1983), Witty and Ritz (1984), and Chalk et al. (1983) recommended that the incorporation of organic form N-15 or accompanying inorganic N with carbon source to stabilize the mineralization process of N during a growing season. Until recently, it becomes possible to accurately quantify the dinitrogen fixation in annual legumes with N-15 isotope; even though difficulties still exist in the assay of dinitrogen fixation by legumes. The advantage from N-15 dilution is also of significant importance in the N cycle study and research (Goh et al., 1978; Boddey et al., 1983; Butler and ladd, 1985; Chalk, 1985; Ledgard et al., 1985a, b, c; Danso, 1986; Gu and McGill, 2017). In a recently review, Chalk (1985) had examined the N-15 dilution technique in a great details with condensed information from the work had been done so far. Problems, such as the discriminative uptake between nitrate and ammonium, application times, amount to apply, and the forms of nitrogen used, were discussed in the review (Chalk, 1985).

Faba bean (*Vicia faba* (L) minor.) is one of the most important grain and forage legumes widely distributed over the world. In Asia, China has the largest production of Faba bean (Tao, 1981). Faba bean (*V. faba* (L) minor.) becomes one of the symbiotic plant that has drawn widely attention in the Western Canada due to its agronomic performance and high protein content (Evans et al., 1972). In 1978, about 5000 hectares, of which 1500 hectares were irrigated, were grown in Alberta (Krogman et al., 1980). Faba bean is an important source of directory protein especially in the rural communities in the developing and less developed countries for human consumption (Saxena and Stewart, 1983). The amount of nitrogen fixed by faba bean ranges from 54 to 146 kg/ha have been reported in Western Canada (Dean and Clark, 1977; Richards and Soper, 1982). Faba bean production was initially introduced into Western Canada in 1972 (Evans et al., 1972; Gu and McGill, 2017) and not much knowledge on North American faba bean is available (Rennie and Dubetz, 1986). Nitrogen fixed in faba beans was increased significantly in the intercropped system with barley, but there was no evidence to verify the N transfer from faba bean to barley in that system (Danso et al., 1987). Faba bean (*Vicia faba* (L.) minor.) had reported to response to inoculant (Candlish and Clark, 1975), nitrogen fixation can be increased on these farm with selected rhizobium strain. N-fixation was studied in different conditions by field (Rennie, 1986), greenhouse, culture medium (Ismaili and weaver, 1986).

Since the first application of N-15 technique in agricultural research (McAuliffe et al., 1958), methods to quantify N-fixation have been challenged by the reliabilities of methodology. Acetylene reduction (AR) is still a useful method for its high sensitivity in the biological nitrogen fixation study. Varied results were obtained at the same site with different approachs in methodology (Smith and Hume, 1987). Among methods, total N balance method, N-15 dilution and acetylene reduction (AR), they may differ in a large extend. Several methods are available for the quantification of the biological nitrogen fixation, each has its own problems (LaRue and Patterson, 1981). Reasonable results are not adequate on which plant is the most suitable one for N-fixation quantification as a reference plant when using N-15 dilution methods. To assess N-fixation, an appropriate non-fixing reference crop is needed. For the experiment to be valid, there must be no N transfer from N-fixing system to non-fixing system (Ledgard et al., 1985d; McAuliffe et al., 1958; Gu and McGill, 2017). In pots low in N, 87% of the plant N was derived from fixation (A-value method), but fertilizer addition decreased fixation without increasing total N (LaRue and Patterson, 1981). Ledgard et al. (1985c) had observed that the natural abundance of N-15 in clover (*Trifolium subterraneum* L., cv. Woogenellup) roots was significantly higher than in the shoots, but there was no significant difference between shoots and roots of ryegrass (*Lolium rigidum* Gaudin cv. Wimmera). In N-15 enriched plot, N-15 abundance in the shoots of ryegrass was significantly higher than in the roots, but there was no significant difference between the shoots and roots of clover. Although these differences existed, there was not significant difference in the percentages of N-fixation calculated from data for whole plants or shoots alone, using N-15 enrichment or N-15 natural abundance.

The objectives of this study were to estimate allocation of N transfer among above and below ground components of crops with ^{15}N labeling technique and to justify and/or further estimate N-fixation in faba bean using canola and/or barley as reference crop(s). Results presented in this paper are part of our ^{15}N internal cycling study in agroecosystem. Dry matter production and N accumulation in above and below ground components of faba bean, canola and barley has reported (Gu and McGill, 2017).

2 Materials and Methods

2.1 Description of Study Site

This study was carried out in the Soil Science plots at Breton as described previously (Gu and McGill, 2017). Dark Gray Luvisols and Gray Luvisols are predominant soils in the study plots (Lindsay et al., 1968). The soil physical and chemical properties are summarized in Table S1.

2.2 Plot Establishment and N-15 Application

As described in a previous paper (Gu and McGill, 2017), open-ended steel cylinders (20 cm Dia, 30 cm H) were used in this study. Faba bean (*V. faba* L., minor. cv. Ackerperle), canola (*Brassica napus* L., cv. Westar) and barley (*Hordeum vulgare* L., cv. Empress) were chosen. Treatments were faba bean, canola, barley and summer fallow and replicated four times. Crop seeding and plot establishment were also available else where (Gu and McGill, 2017). Cylinders were pushed into the soils on June 15, 1987 and followed by application of enriched N-15 on June 19. N-15 solutions were made from 99.5% N-15 urea (MSD Isotopes, Division of Merck Frosst Canada Inc., Montreal) of analytical grade to make two solutions with N-15 22.017 and 22.049% (53.763 mg N/ml and 32.264 mg N/ml). Application solution was made by dilute the original one and applied, the applied N at each time was corresponding to 0.5 kg N/ha. Therefore, cylinders which had remained till the last sampling had receiving N corresponding to 2 kg N/ha. During calculation of N-15 enriched urea needed, mineralization rate of 20 kg N/ha/yr was assumed from indigenous soil organic matter (Rennie, 1986).

The above stock solution was used in first, third and last applications, while another one with N-15 22.017% (32.264mg N/ml) was used for the second application only.

2.3 Sampling Schedules and Methods

Samplings were conducted four times in the whole growing season on July 8, July 24, August 19 and September 1, 1987 corresponding to 63, 77, 103 and 115 days after seeding barley and faba bean, but 38, 52, 78 and 90 days for canola. Plant tops were cut at the soil surface and put in oven at 65°C for one week, then weighed. Fractionation was carried out on those above ground materials and weights of each fraction were recorded. Soils inside the steel cylinders were subject to subsampling for the soils to root-washing, those for microbial biomass study and for total N and N-15 abundance determination at 0-10, 10-20 and 20-27 cm depths.

2.4 Samples Preparation and Analytical Methods

All samples of plant material components, dead crop debris and root washed of three-depth were grounded either in a Wiley mill, such as for faba bean root in 0-10 cm and stems of faba bean and canola, or coffee ground, such as for leaves. Then all samples, both plant materials and soils, were further grounded in a vibrating mill. Samples were analyzed for total N and N-15 abundance on the ANA-SIRA mass spectrometer (England).

2.5 Calculations

N-15 excess was used explicitly in this paper and it was defined by using results from Junk and Svec (1958).

N-15 excess % = N-15 abundance in sample% − 0.3663%

N-15 excess % in crop = Sum (N-15% × N in one fraction) × 100/ Sum(N in all fractions)

2.6 Statistical Analysis

Data collected from this study were subject to analysis of variance for treatment, sampling date, depths effects of the three crops in this research with BMDP statistical software (Dixon, 1983). Student-Newman-Keuls (Sokal and Rohlf, 1981) multiple comparison was conducted when F-test of ANOVA was significant.

3 Results

3.1 Above Ground Parts

Crop species, sampling dates, and interactions of crop and sampling effects on N-15 excess were all significant ($p < 0.01$) (Table S2). N-15 excess in faba bean tops differed significantly from canola and barley on any of the sampling in the crop growing season ($p < 0.01$). The range of N-15 excess change was between 0.291-0.772%, 1.994-4.236% and 1.854-4.082% for faba bean, canola and barley tops (Figure 1), respectively. N-15 excess in faba bean tops did not change significantly over time, but that in canola tops had significant increase continuously except for the period between the second and the third sampling; and that in barley tops increase till the third sampling then decrease on the last sampling. Compared with natural N-15 abundance (Junk and Svec, 1958), N-15 excess in crop tops was enriched by 79, 544 and 506% by the first sampling for faba bean, canola and barley, respectively. Percentage of N-15 excess change between sampling intervals was 165, -32 and 22% on the second, third and last samplings in faba bean tops; similarly, it was 49, 2 and 42% in canola tops; and 54, 43 and -18% in barley tops. Deviation of N-15 excess in canola and barley tops in the latter growing season will cause the quantity of N fixed in faba bean calculated from N-15 dilution vary possibly. However, the increase of N-15 excess in faba bean, canola and barley tops was 1.8, 12 and 9 times that of the natural N-15 abundance (Junk and Svec, 1958; Gu and McGill, 2017) on the last sampling. A peak of N-fixation is evident around the third sampling, when faba bean was in blooming, and the 32% depletion of N-15 excess in faba bean had supported when compared with the second sampling (Figure 2). On the third sampling, barley had 27% higher N-15 excess than canola, but canola N-15 excess was 21% higher than in barley on the last sampling. The compensation effect will results in less degree of variation when using either canola or barley as a reference crop. Therefore, it is reasonable to postulate N fixed in legume can be estimated with either canola or barley as reference crop.

Faba bean leaves and stems had almost identical N-15 excess on the first 2 samplings, and the percentage increase

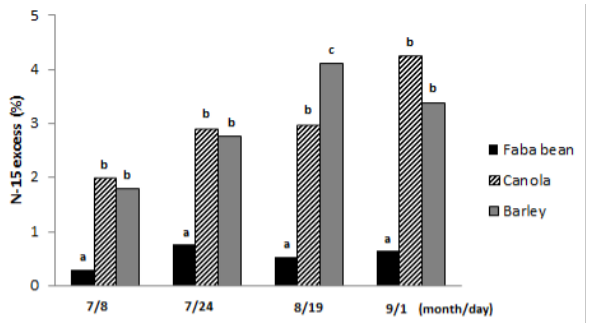

Figure 1. Variations of percentage N-15 excess in the above-ground parts of different crops over the growing season.

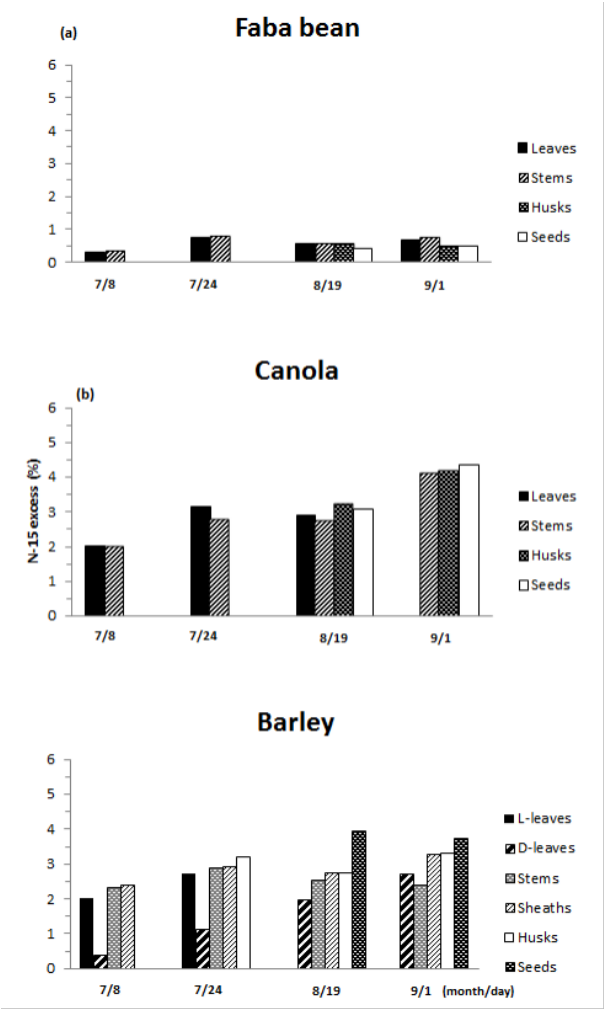

Figure 2. N-15 excess in various components of crop tops over thr growing season.

of N-15 excess was 168% for leaves and 164% for stems from the first to the second samplings (Figure 3). N was transported to these fractions simutanously during this period and there is an equal translocation of $^{15}N/^{14}N$ to both vegetative components as shown by the increase of N-15 in leaves and stems in responding to the second application of N-15 solution. However, N-15 excess in leaves, stems, shells and seeds had lowered by 30% since the second sampling. Significantly dry matter increase was observed between second and third sampling (Gu and McGill, 2017), so the decrease of N-15 excess during this period is symbolized by symbiotic N-fixation process. During this period, ^{14}N of atmospheric origin has diluted the N-15 excess in the legume, faba bean, and the more N fixed the lower N-15 will be in the faba beans. It should be noticed that N-15 excess of seeds on the third sampling was 25% lower than in leaves, stems and shells, which are pre-seed formed organs. The interpretation is that N, which is mostly of atmospheric origin, was preferentially transported into seeds than any other components. Alternative explanation is that fixed N of low N-15 enrichment was exported from the nodules and roots development depends on soil N, which is highly enriched. Fixed N transported into the seeds was higher than into other organ by 25% on the third sampling. With further developing, N-15 excess in leaves, stems and seeds had enriched by 25, 39 and 15% while decreased by 6% in shells. Apparently, the assimilation of highly enriched N was transported more into the vegetative organs, leaves and stems; less amount has been to reproductive organs, shells and seeds; on the contrary, more symbiotic fixed N, which is of lower N-15 enrichment during this period may be transported into seeds mostly and less amount was exported to leaves and stems. The fractionation of labeled N-15 has been taken place during the crop development among above and below ground components, and the plant parts. There is a peak for symbiotic N-fixation around the third sampling (August 19) after faba bean was in blooming. During this period, N-15 in plant components had dropped, particularly in seeds.

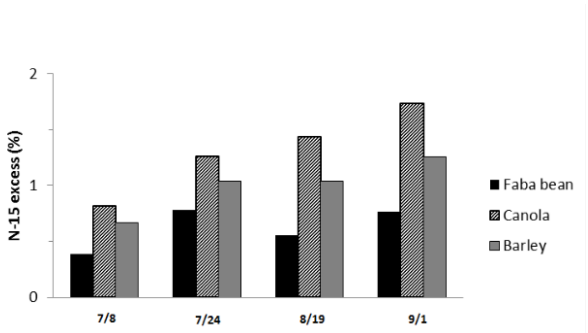

Figure 3. Percentage N-15 excess in the crop roots (0-27cm) of this study.

N-15 excess in two non-legumes increased over sampling dates in the growing season (Figure 3). N-15 excess in leaves and stems of canola was the same as that for faba bean on the first sampling, and there was an equal allocation of inorganic N from soil to the two components. On the second sampling,

leaves and stems had enriched by 55 and 40% compared with the first sampling. Since the N accumulated in leaves was higher than in stems (Gu and McGill, 2017), inorganic N was transported to leaves more than to stems. The 5% variation of N-15 enrichment may be due to allocation assimilated N was preferentially transported for leaves development first and then for the stems. N-15 excess had decreased by 6 and 1% on the third sampling for leaves and stems, there may be an internal N allocation from vegetative organs to the reproductive ones when reproductive stage reached. During this period, N-15 excess in leaves, shells and seeds was respectively 7, 20 and 13% higher than that in stems. N-15 excess did not vary much among leaves, stems, shells and seeds although N accumulated in the four components differed. A preferential transport of N to shells and seeds could be possible since the N-15 excess was lower in leaves and stems than in the reproductive organs on the third sampling. On the last sampling when canola and barley was fully mature, N-15 excess followed decreasing sequence in seeds, shells and stems. Leaves had disappeared by the time of sampling, it was possible that N had allocated from leaves, stems and shells to seeds. Since total N accumulated in seeds was much higher than in other components (Gu and McGill, 2017), a little increase of N-15 excess could be a large amount of N translocated. Nutrient allocation within plant is governed by developing stage of a crop, the shift of growth from vegetative to reproductive is accompanied by nutrients reallocation from one part to another.

During barley growth, N-15 excess in dead-lives was much lower, by 6-7 folds due to N can be translocated to new developing components. N distribution over various organs follows a decreasing order as sheaths, stems, live-leaves and dead-lives in the growing season (Figure 3). During the first 2 sampling, N-15 excess had increased by 30% in various organs and also followed a decreasing order as sheaths, stems, live-leaves and dead-leaves. Increase of N-15 excess was 33, 38, 206 and 44% in barley sheaths, stems, dead-leaves and live-leaves, respectively. However, N-15 excess of dead-leaves was 81-83% lower than that in live-leaves, stems and sheaths on the first sampling; 59-62% on the second sampling. During late development, N-15 excess increased by 96% in seeds on the third sampling and decreased but still higher than in other components on the last sampling. The high enrichment of N-15 in seeds of the third sampling was corresponding to the grain-filling stage, therefore N was translocated into the seeds from various components of crop and soil. N-15 excess had decreased by 27 and 6% in seeds and stems, but increased respectively by 36, 17 and 20% in dead-leaves, sheaths and husks during the third and the fourth samplings. As seed-filling proceeds, either less enriched N or ^{15}N from other components were transported into seeds.

3.2 Below-ground Parts

N-15 excess in 0-27cm depth of non-legumes (canola and barley) roots was increasing consistently with N-15 application over the four sampling conducted (Figure 4). N-15 excess of faba bean roots differed from non-legumes and did not respond to N-15 application continuously due to symbiotic N-fixation. Compared with natural N-15 abundance, N-15 excess of crop roots had increased by 106, 225 and 182% in faba bean, canola and barley on the first sampling, respectively. Increase of N-15 excess between sampling intervals was 54, 13 and 20% in canola over the four samplings[Why do four samplings only have three sets of data?]; it was 56, 0 and 40% in barley; and 100, -28 and 38% in faba bean. N-15 excess of the legume (faba bean) differed from non-legumes (canola and barley) due to the symbiotic N-fixation by legume. Between the second and the third sampling, N-15 excess had decreased by 28% due to translocation of symbiotically fixed N, which is the nitrogen from the atmosphere and lower in N-15 enrichment exported from root nodules. Decrease of N-15 enrichment on the third sampling in faba bean corresponded to depletion of N-15 in faba bean tops and rapidly increase of crop tops at the third sampling. Root dry matter of canola and barley did not change significantly over the four samplings (Gu and McGill, 2017); however, N-15 excess in the two non-legume roots increased continuously (Figure 4). Increase of N-15 excess between intervals was 54, 13 and 20% in canola; and it was 56, 0 and 40% in barley.

3.3 Quantity of N fixed in Faba Bean

N-15 excess in faba bean roots was higher than in its tops on any of the samplings, but reverse was observed on canola and barley (Figures 3 and 4). The differences in N-15 enrichment are thought caused by biological fractionation due to mass differences between N-15 and N-14. Percentage enrichment was higher in faba bean roots by 34, 1, 5 and 16% than tops on the four samplings. Increase of enrichment in tops was 142, 134, 108 and 146% higher than in canola roots; and 178, 174, 292 and 131% in barley. Root nodules of faba bean were mostly distributed within 15 cm, a few were observed down to 15 cm. The large nodules were the ones near tap roots and within 10 cm depth.

Crop species, sampling dates, depth, and interactions of crop and depth effects on N-15 excess were significant ($p < 0.01$). Interactions of sampling and depth effects were significant on N-15 excess ($p < 0.05$). And interactions of crop and sampling, and that of crop, sampling, and depth effects were not significant on N-15 excess ($p < 0.1$). N-15 excess of 0-10, 10-20 and 20-27 cm roots had shown that highest N-15 excess was found in canola roots at 0-10 cm depth compared with barley and faba bean of the same depth. Generally, there was not significant difference in N-15 excess between 10-20 and 20-27 cm, and between species of crops at these two depths (Figure 4). This observation could be due to the physical properties of the Gray Luvisolic soil and the root morphology caused by the soil conditions. Roots for these three crops were concentrated in 0-15 cm, there was a denser, compacted layer beyond. In this case, crop roots distributed in upper layer and absorbed applied N-15 in soil more efficiently. Lower roots, which are of small amount, consequently only have little accessibility of N-15

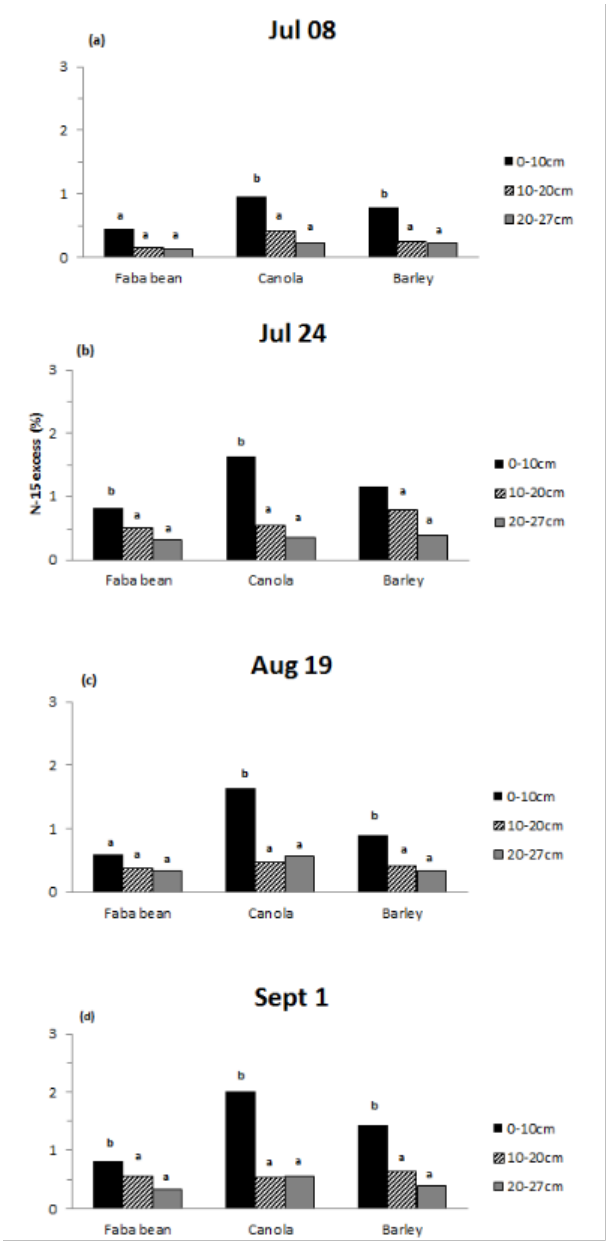

Figure 4. N-15 excess in crop roots over the growing season. a-b means followed by the same letters do not differ significantly on one date.

in the lower depth due to the physical condition for growth and development.

Compared with faba bean root at 0-10 cm, N-15 excess was higher by 123% in canola roots, and 51% in barley roots on the first sampling; it was 97% in canola and 18% in barley on the second sampling; 161% in canola and 35% in barley on the third sampling; and 140% in canola and 58% in barley on the last sampling. N-15 enrichment was higher in canola roots than in barley on all samplings. Discrepancy exists on N-15 excess patterns over the four samplings between crop tops and roots indicates biological fractionation is not negligible in N-15 applied research.

3.4 Quantity of N Fixed in Faba Bean

Significant differences in dry matter production and N accumulation between the legume (faba bean) and two non-legumes (canola and barley) were observed (Gu and McGill, 2017), but not between canola and barley. In addition, N-15 excess in legume (faba bean) tops differed significantly from that in either canola or barley tops. Significant difference between canola and barley was observed only on August 19 (Figure 1), and percentage of N-15 excess varied slightly between canola and barley. N-15 excess of canola were more enriched by 26% than that of barley on the last sampling. N-15 excess of the legume (faba bean) roots differed from the two non-legumes (canola and barley). Surprisingly, N-15 excess in the two non-legumes deviated by 18% on the last sampling. However, only crop tops were further considered to estimate N-fixation in faba bean. Further proceeding on using either canola or barley as a reference crop of the N-15 dilution technique had shown that agreeable results were obtained no matter canola or barley was chosen as a reference (Tables 1 and 2). The percentage of N fixed in faba bean calculated using canola or barley as a reference crop was between 70-76%, which corresponded to 183-199 kg N/ha/yr. The variation was due to the cascading of N-15 excess in canola and barley on the third and the last samplings.

Dry matter production and N accumulated were enhanced mostly between the second and the third sampling in faba bean due to symbiotic N-fixation. N fixed in faba bean tops was 104-122 kg N/ha/yr between the second and the third sampling. The rate of N-fixation in faba bean was calculated based on the quantitative data from N-15 dilution, it reached 4.0-4.7 kg N/ha/day using canola or barley as a reference crop; and it was 0.75 kg N/ha/day between the first and the second sampling, and less than 0 to 2.3 kg N/ha/day between the third and the last sampling (Table 2). A peak of N-fixation in faba bean after flowering (second sampling) was evident.

Other methods are also available for quantification of N fixed in faba bean, among these total difference method (TDM) is a conventionally used based on crop dry matter production and Kjeldahl analysis of N. Usually, discrepancy is quite large between methods for quantification of N fixed in legume. Attempt had been made to evaluate the results from total difference and N-15 dilution methods. TDM is comparative to N-15 dilution method and the results from TDM was

Table 1. Dinitrogen fixation in the above-ground parts of faba bean by N-15 dilution method.

Ref. Crop	7/8	7/24	8/19	9/1
	(month/day)			
	(%)			
Canola	72	66	73	76
Barley	70	65	80	70
	(kg N/ha/year)			
Canola	53	65	169	199
Barley	52	64	186	183

Table 2. Dinitrogen fixation rate in the above-ground parts of faba bean N-15 dilution method.

Ref. Crop	7/8	7/24	8/19	9/1
	(month/day)			
	(kg N/ha/year)			
Canola	/	0.75	4	2.3
Barley	/	0.75	4.7	-0.2

generally higher than N-15 dilution over samplings. The variation by the N-15 dilution and TDM is within 10% ($<$ 20 kg N/ha/yr). The N fixed in faba bean tops was 215 kg N/ha/yr using TDM, and the percentage of N derived from N-fixation was 82-86% (Table 3). This agreement is much better than that between N-15 and acetylene reduction as reported (Smith and Hume, 1987). The advantage of more widely application of Kjeldahl method can make N-fixation be quantified with a routine tool although N-15 was not possible to be used.

Table 3. Dinitrogen fixation in the above-ground parts of faba bean by total difference method.

Ref. Crop	7/8	7/24	8/19	9/1
	(month/day)			
	(%)			
Canola	69	76	84	86
Barley	70	67	83	82
	(kg N/ha/year)			
Canola	51	75	195	225
Barley	52	66	192	215

Crop roots are always neglected in N-fixation study, but root were extracted from soil by root-washing technique in our study. N derived from atmosphere in the roots accounted for 19-49% of the N accumulated in roots, which was 19-22 kg N/ha/yr left in the roots using N-15 dilution method and canola and barley as reference crop (Table 4).

Table 4. Dinitrogen fixation in the above-ground parts of faba bean by total difference method.

Ref. Crop	7/8	7/24	8/19	9/1
	(month/day)			
	(%)			
Canola	36	30	49	46
Barley	27	19	34	38
	(kg N/ha/year)			
Canola	9	8	20	22
Barley	6.7	5.1	14	18

4 Discussion

4.1 Above-ground Parts

The reference crop constitutes the principle source of error in the N-15 technique for determining N fixed in field study. Our results have shown that N-15 excess of the legume differed from non-legumes (canola and barley) significantly ($p < 0.01$), but there was not significant difference in N-15 excess between canola and barley. Therefore, both canola and barley are valid reference candidate and can be used to quantify N-fixation in a legume. Theoretically, N-15 dilution technique is only valid under conditions (McAuliffe et al., 1958): 1) both legume and non-legume assimilate $^{15}N/^{14}N$ at the same ratio, but not necessarily the same quantity; and 2) no fixed N transfer from legume to non-legume in mixed cropping condition, particularly under pasture. The second condition was eliminated in our study by setting treatment plots for legume and non-legume separately, and also by applying highly enriched N-15 material to mask the background variation among plots between legume and non-legume. However, the first condition was evaluated indirectly by comparing N-15 assimilation between the legume and the two non-legumes. There is no general agreement on the best non-fixing reference plant though different alternatives are available. In a comparison of various non-fixing plant, Rennie (1982) reported N-fixation in soybean cultivars was overestimated when non-nodulating isolines were used, and the uninoculated and ineffectively inoculated treatments were the best controls. Of three non-legumes tested, barley was considered to be useful as a reference crop, but rapeseed and sudan grass were not. However, Wagner and Zapata (1982) suggested that non-nodulating soybean and sudan grass were as good as uninoculated soybaen as reference crop for soybaen. Barley was considered to be a very satisfactory reference crop for *V. faba* (Wagner and Zapata, 1982; Gu and McGill, 2017). However, Chalk et al. (1983) suggested that non-fixing reference crop could be replaced by N-15 mineralized from N-15 pre-enriched soil, which is the N-15 excess of inorganic N from soil organic matter N.

Allocation of N-15 was preferentially transported to reproductive organs after entering the stage of reproductive growth. Dilution of N-15 excess in faba bean indicated symbiotic N-fixation in faba bean, the decrease of N-15 excess in faba

bean tops was 85 and 81% compared with canola and barley, respectively. Variation caused by non-legumes is only 6% in term of percentage N fixed in faba beans on the last sampling. These results provided a basis for further quantification of N fixed in faba bean using either canola or barley as a reference crop. A decrease of N-15 excess was observed after faba bean flowering both for tops and roots, and similar results were reported by Richards and Soper (1979). N is translocated at different rates to different plant parts, depending on the state of maturity. However, N pool absorbed by plants at different period are not of the same ^{15}N enrichment. Since different amounts of N of dissimilar ^{15}N enrichments are translocated to different parts at the various physiological stages, it is not surprising to find that individual plants have different $^{15}N/^{14}N$ ratio as shown in Figure 2. The discrepancy was enhanced when plant began reproductive growth and this may be caused by growth centre shifted from vegetative to reproductive and reallocation of nitrogen among plant components. These differences in isotopic composition within different plant parts constitute an important potential source of error in N-fixation studies (Gu and McGill, 2017). A proper sampling for N-15 analysis is difficult when plant parts of different densities such as seeds, pods, stems, and leaves are in the same sample. This can be overcome by fractionation plants into recognizable plant parts, such as seeds, pods, leaves, and straw, each of which is much similar physically and homogenous in ^{15}N composition.

4.2 Below-ground Parts

Zapata et al. (1987) stated in their study that N contribution from roots was to be small and did not take into consideration. Actually, roots of faba beans had higher N-15 excess than their tops by 3-19% over the four samplings, reversely, canola and barley roots had lower N-15 excess than the tops (Figure 3). Kohl et al. (1982) observed that a connection between N fixing in soybaen and some process which leads to partitioning of N isotopes between nodules and the rest of the plant. The mechanism involves isotopic fractionation associated with synthesis of compounds rich in N which are then exported from the nodule leaving behind N-15 enriched metabolites for the synthesis of nodule tissue. Reschel and Sauito (1986) reported soybean roots did not contain N from fixation and an assumption was made that N fixed was not primarily distributed to tops of the nodulated plants. But, fixed N was found in roots when N-15 labeled nitrate was applied. The reason is not well understood; however, plant roots proliferation may depend on the assimilated N from soil while plant growth use synthesized N transported in forms of glutamine and asparagine from nodules of faba bean.

4.3 Quantity of N fixed in Faba Bean

Quantification of symbiotic N-fixation in faba beans have been reported (Danso et al., 1987; Richards and Soper, 1979; 1982; Wagner and Zapata, 1982; Zapata et al., 1987). With total difference method (TDM), Richards and Soper (1982) estimated N-fixation accounted for 63-71% of the N in faba bean shoots, which was 51-111 kg N/ha/yr in Manitoba. Wagner and Zapata (1982) used nitrogen A-value method to quantify the percentages of N derived from fixation by faba bean, and value between 71-84% corresponding to 125-144 kg N/ha/yr was obtained. In a further report, Zapata et al. (1987) concluded that 79% N in faba bean was derived from fixation, equals to 165 kg N/ha/yr using N-15 dilution technique. Larger amount of N is being fixed in faba bean through symbiotic N-fixation. By September 1, when faba beans were not fully mature, percentage of N derived from N-fixation was between 70-76% corresponding to 183-199 kg N/ha/yr with both canola and barley as reference crops in the Breton plots (Table 1). N fixed in faba bean roots amounted between 18-22 kg N/ha/yr; therefore, the total N in faba bean tops and roots totaled to 201-221 kg N/ha/yr in Breton plots in Alberta, Canada.

No matter canola or barley was used as a reference crop in N-15 dilution method, results obtained are reliable within 5%. Wagner and Zapata (1982) had obtained similar results that there was no significance among N-fixation quantified by applying barley, sudan grass or oil radish as a reference crop. Non-fixing crop will usually give a consistent estimation on N fixed in a legume. The variation between applying different reference crop was much smaller than between methods, acetylene reduction (AR) and N-15 dilution method (Smith and Hume, 1987). This suggested that total difference method is still a valid tool to approach N-fixing problem solving because N-15 method is limited by instrumental and N-15 material in application. A peak of N-fixation was observed after faba bean flowering (between the second and the third sampling), and the rate of N-fixation reached 4.0-4.7 kg N/ha/day using canola and barley as reference crops. This is in much better agreement with Zapata et al. (1987). Before or after this period, N-fixing was much lower.

4.4 Improvement of Soil Quality

Selective crops may significantly improve the soil physical and chemical properties because of the high biomass produced, soil texture improvement through penetration of the roots, high quantity of N input into the nutrients-limited soil (Gu and McGill, 2017). Due to plat growth, rhizosphere soil may promote the activity of soil microorganisms to enhence degradation and transformation for bioremediation and cleaning up of pollutants (Gu and McGill, 2017). Nitrogen dynamic is a major controlling factor to the ecological function and health of the ecosystem (Li et al., 2013; Wang and Gu, 2013a, b; Wang et al., 2013; 2014; Li and Gu, 2016; Wu et al., 2017) and proper management can maintain efficient use of N without over fertilization to cause pollution of the ecosystem.

5 Conclusions

We conclude that both canola and barley can be used validly as non-fixing plant in N-15 dilution method to quantify N-fixation in faba bean, particularly on this Gray Luvisolic soil

of Canada. Fractionation of N-15 was observed in legume and also non-legumes among above and below ground components and also various plant parts in this study. Faba bean roots had 18-22 kg N/ha/yr left from N-fixation and quantity of N fixed in faba bean tops was 183-199 kg N/ha/yr in Breton plots by the N-15 dilution method, corresponding to 70-76%. A peak of N-fixation was evident after faba bean in blooming, and a rate of 4.0-4.7 kg N/ha/day was obtained from N-15 dilution method. Both total difference and N-15 dilution methods provide valid quantification within 5% variation. Faba bean can be an effective crop for improving soil quality and bioremediation because of the large quantity of N assimilated.

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Electronic Supplementary Materials

This article contains supplementary materials in the following website:
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