SaskCanola Saskatchewan Canola Development Commission

WGRF

Advancing Agriculture through Research

Final Report

1. Advanced N management for canola and soybean: Evaluation of a new biological for N-fixation in non-legumes (WGRF AGR 1921 / CARP ADF 2018.195)

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4. Abstract/Summary: *Maximum of 300 word in lay language*

We evaluated a microbial inoculant containing a free-living nitrogen-fixing bacteria (*Gluconoacetobacter diazotrophicus*) for its ability to biologically fix N for wheat, soybean and canola. Three inoculation methods were tested: direct application to the seed, foliar inoculation; and soaking the seeds in inoculant as they germinate. Plants were place in a sealed chamber and $^{15}N_2$ released into the chamber. Because the $^{15}N_2$ gas is the only source of ^{15}N for the plants, any ^{15}N that we measured in the plant tissues had to be fixed by the bacteria. We also had plants analyzed for DNA segment copies unique to the bacteria. If the bacterial DNA was detected in the plant it provided evidence that the bacteria had successfully colonized the plant tissue. Many of the tissues tested positive for colonization, but most of the ^{15}N analyses did not show N fixation. In many cases the %N in the plants that were colonized was lower than the control (not inoculated) plants. The lower % N was not due to the N being diluted through enhance growth (biomass) as there were no differences in biomass production among any of the inoculation treatments. Unfortunately we only measured good ^{15}N enrichment (indicating biological nitrogen fixation) in 3 soybean plants, 2 of which were inoculated foliarly. Of the different inoculation methods tested, soaking the seed during germination was most successful in delivering bacteria that colonized.

Foliar application also tended to be a successful delivery method, but directly applying the inoculant to seed was the least effective. While soaking the seed during germination was the most successful delivery method, it is also the least practical, as it is unlikely to be implemented on a field scale. More work on improving the method of delivery of the inoculant and stimulating the inoculant bacteria to fix N is needed.

5.0 Introduction:

Consumers are increasingly concerned about the environmental impact of agricultural products and practices. As a result, the environmental sustainability of crop production practices has become a critical aspect of market access (e.g., European Union's Renewable Energy Directive; Wall Mart's Project Gigaton" initiative). Carbon footprinting is an important component of any sustainability initiative, and crops produced with a low carbon footprint have a competitive advantage in the global marketplace. For field crops, N_2O emissions constitute a key sustainability indicator, and N_2O is an important component of the overall carbon footprint of any cropping system. Thus, cost effective practices and products that reduce the amounts of N fertilizer needed to produce a crop will go a long way to reducing negative environmental impacts. Capitalizing on microorganisms that fix atmospheric N_2 is one potential avenue of reducing the need for fertilizer N.

Many species of soil microorganisms exist in nature that are capable of N_2 fixation. Collectively these organisms are called diazotrophs, and include *Rhizobium* and *Frankia* spp. that symbiotically fix N in legumes and in some trees and shrubs and other free-living diazotrophs that fix atmospheric N_2 free from the influence of plant roots. A group of diazotrophs referred to as endophytes invade plant roots and either colonize the spaces between the cells (intercellular) or the cell matrix itself (intracellular). Unlike rhizobia, these microorganisms do not undergo major physiological changes within the root and generally exist as integrated unicellular units capable of thriving in the root environment. A wide variety of endophytic non-rhizobial diazotrophs have been isolated, including *Gluconacetobacter diazotrophicus*. *Gluconacetobacter diazotrophicus* was first isolated from sugarcane in Brazil (Cavalcante & Dobereiner, 1988) and has been demonstrated to definitively fix atmospheric N_2 and provide significant amounts of N to sugarcane plants (Boddey et al., 2001). Subsequently, *G. diazotrophicus* has been isolated from a diverse range of other plant species (Eskin et al., 2014) and there is mounting evidence that this bacterium can indeed fix atmospheric N to supply N to the plants with which it associates to improve yield. The bacterium has been used to inoculate a variety of agricultural/horticultural plants—including maize, rice, wheat, oilseed rape, tomato, and white clover—and, in all cases, low numbers of *G. diazotrophicus* resulted in extensive intracellular colonization of roots and root meristems (Cocking et al. 2006). As well, yield increases of between 5 and 15% have been reported in tomato (Luna et al. 2012), sugar beet (Abbudureheman, 2012), and maize (Riggs et al., 2001).

In 2012, Azotic Technologies Ltd. (a UK based biotech company) was founded to commercialize a seed applied inoculant (N-fix®) employing a food-grade (non-GMO) strain of *G. diazotrophicus* to promote N fixation in non-legume crops. The N-fix technology was initially developed by Professor Edward Cocking (Director of the Centre for Crop Nitrogen Fixation at the University of Nottingham) and was licensed from the University of Nottingham. The company expanded into North America (Azotic North America; www.azotic-na.com) where the product is marketed under the trade name Envita™. The company reports preliminary findings that show the product can produce a 5 to 15% yield increase when fertilizer N is applied at soil test recommendations, or can replace 25% of fertilizer N without reducing yields. More extensive field trials with corn are underway in the United States, and the product has been

registered for corn production in Canada—with most research centered in Ontario. In addition, Azotic-NA is looking to expand into western Canada.

As a first step in evaluating the usefulness of the inoculant on economically important crops grown in western Canada, we tested Envita™ for its ability to fix and supply N to canola, wheat and soybean in controlled environment studies. Although soybean is a legume and, hence, fixes N through rhizobial symbioses, the short-season varieties developed for western Canada have lower seed protein contents than their long-season counterparts in the US and eastern Canada. Thus, it may be possible to increase protein contents with the Envita™ product. The primary goal of this project was to inoculate the select field crops with Envita™ and evaluate if the inoculated crops were acquiring N fixed by the *G. diazotrophicus.* ^{15}N-N_2 gas was supplied to the atmosphere surrounding the plants and the plants evaluated for ^{15}N uptake. Different methods of introducing the *G. diazotrophicus* to the plant were evaluated. We also collaborated with Dr. Sean Hemmingsway at NRC-Saskatoon to quantify numbers of the bacteria colonizing the tissues using a ddPCR technique that identifies DNA segments unique to *G. diazotrophicus.*

6.0 Methodology:

6.1 Overview of the experiments:

Our initial approach to evaluating if the Envita™ inoculant was fixing atmospheric N_2 and transferring it to the target plant tissues was to investigate the process in a soil-less, semi-sterile, N-free environment (Leonard Jars – section 6.6.1). An initial set of experiments was completed with wheat and soybean; using Leonard jars with Envita™ applied directly to the seed (section 6.5.1). We were unable to grown canola successfully in the Leonard jars. The wheat and soybean plants were pulse-labeled with $^{15}N_2$ (section 6.7), the plants harvested and analysed for ^{15}N content. Any ^{15}N measured in the plant tissues had to have been supplied by ^{15}N transfer of N fixed by the *G. diazotrophicus* organism in the inoculant to the target plant. We saw very limited evidence of N fixation, so conducted additional experiments in Leonard jars with wheat and soybean, where the inoculant was applied foliarly (section 6.5.3). After conversations with the parent company we changed to growing plants in soil-based systems (section 6.6.2). Different methods of applying the inoculant were also evaluated including direct seed inoculation (6.5.1), inoculation at germination (6.5.2) and foliar application (6.5.3). At the time we were starting these soil-based experiments, we were put in contact with Dr. Sean Hemmingsway, a molecular biologist at NRC-Saskatoon. He has developed a protocol for identifying colonization of *G. diazotrophicus* in plant tissues using droplet digital polymerase chain reaction (ddPCR) (section 6.7.1). We sampled several on-going experiments and had his lab analyse the tissues for *G. diazotrophius* colonization. A summary of the different experiments is included in Table 6.1.

Table 6.1 Summary of the different experiments conducted. Plants were grown in Leonard Jars (section 6.6.1) or in soil in pots (section 6.6.2), Plant tissues [shoot (Sht) and root (rt)] are the tissues analyed for ^{15}N and Gd . Details of the inoculation methods are outlined in section 6.5. Samples were evaluated for mass spectrometry and in later experiments for *G. diazotrophicus* copy number (section 6.7.1).

Plant	Plant tissue	Growth System	Inoculation method	Evaluated for ^{15}N	Evaluated for Gd
Wheat	Sht	Leonard jar	Direct seed	yes	no
Wheat	Sht	Leonard jar	Foliar	yes	no
Wheat	Sht	Soil	Direct seed	yes	no
Wheat	Sht	Soil	Foliar	yes	no
Wheat	Sht/Rt	Soil	Direct seed	yes	yes
Wheat	Sht/Rt	Soil	Germination	yes	yes
Wheat	Sht/Rt	Soil	Foliar	yes	yes
Soybean	Sht	Leonard jar	Direct seed	yes	no
Soybean	Sht	Leonard jar	Foliar	yes	no
Soybean	Sht/Pods	Soil	Direct seed	yes	no
Soybean	Sht/Pods	Soil	foliar	yes	no
Soybean	Sht/Rt	Soil	Direct seed	yes	yes
Soybean	Sht/Rt	Soil	Germination	yes	yes
Soybean	Sht/Rt	Soil	Foliar	yes	yes
Canola	Sht	Soil	Direct seed	yes	no
Canola	Sht	Soil	Foliar	yes	no
Canola	Sht/rt	Soil	Direct seed	yes	yes
Canola	Sht/rt	Soil	Germination	yes	yes
Canola	Sht/rt	Soil	Foliar	yes	yes

6.2 Treatments:

Three plant species, canola, wheat and soybean were evaluated. Treatments included: 1) no inoculant; 2) Envita™; 3) Heat-killed Envita™. The heat-killed treatment provides a control to evaluate if the Envita™ formulation (i.e., matrix that the G. diazotrophicus is supported in) affects the ^{15}N signal independently of the inoculant organism. Heat-killed treatments were treated with the Envita™ inoculant that had been autoclaved and cooled to room temperature. No-inoculant controls were treated with sterile non-chlorinated diH_2O. For these experiments soybean was not inoculated with a *Rhizobium* inoculant.

6.3 Preparation of Envita™ Inoculant Solution: The Envita™ product was received as a dry powder. 0.2 g of dry inoculant was reconstituted by mixing it with 2L non-chlorinated, deionized water (di-H_2O) with a pH between 4 and 7.5. The solution was mixed using a magnetic stirrer for 30 min at room temperature. The solution was refrigerated and used within 72 h. Unused dry inoculant was separated into 0.2 g aliquots into 2-mL microcentrifuge tubes which were individually placed into a 50mL Falcon tube containing a small amount of silica gel desiccant (Sigma-Aldrich). Falcon tubes were stored refrigerated (*ca*. 4°C) until needed.

6.4 Seed Sterilization Protocol: Seed sterilization and seeding of the Leonard jars were performed in a laminar flow biocontrol cabinet. All seed were initially rinsed in sterile di-H_2O. Each seed type required a slightly different sterilization protocol to achieve sterility without damaging the seed and affecting germination. Canola seed was surface sterilized by soaking in 30% H_2O_2 for 90 s followed by three rinses in sterile di-H_2O. Wheat seed was surface sterilized by soaking in 95% ethanol for 60 s, followed by a 10 min soak in a solution containing 5% NaOCl (commercial bleach) and 0.01% Tween-20. The bleach solution was decanted and discarded and the seeds rinsed 5 times for 30 s per rinse in sterile di-H_2O. Soybean seed was sterilized using the same protocol as wheat except that the initial soak in ethanol was for 30 s and the subsequent soak in bleach solution was for 5 min.

6.5 Seed Inoculation Protocols:

6.5.1 Direct Seed Application: Surface sterilized seed were placed into sterile Whirl-Paks containing sufficient reconstituted Envita™ to ensure that all seeds were in contact with the inoculant. The amount varied with seed size. The seeds were soaked for 10 min, the excess inoculant solution decanted and discarded and the seed transferred to disposable sterile petri-dishes (100 mm x 5 mm) fitted with a Whatman # 2 filter paper that was moistened with an additional 10-mL of inoculant. The seeds were incubated at room temperature in a dark cabinet overnight.

6.5.2 During Germination: For seed inoculation during germination sterile Whatman #2 filter paper was fitted into disposable sterile petri-dish (100 mm x 5 mm). Ten mL of the reconstituted Envita™ was pipetted onto the filter paper and 10-20 surface sterilized seeds placed onto the wetted filter paper. The petri dishes were lidded and stored in a dark cabinet at room temperature for 5 to 7 d. Five germinated seedlings were transplanted into the vermiculite chamber in the Leonard jar or into a pot with soil.

6.5.3 Foliar Application: Reconstituted Envita™ solution was prepared as described above, except that 0.1 g of the powder was mixed with 1.0L sterile, non-chlorinated di-H_20 and 1.0 mL of non-ionic surfactant (Silwet L-77) and transferred to a sterile spray bottle. The solution was sprayed onto the leaves at the 2 to 4 leaf stage – approximately 15 days after planting.

6.6 Plant Growth Systems:

6.6.1 Leonard Jars: Leonard jars are a semi-sterile, soil-less system (Fig. 6-1) for growing plants. It is essentially a small hydroponic unit where the plant roots anchor into a solid, inert medium that is connected to a reservoir containing N-free nutrient solution through a cotton wick. Approximately 500 mL of pre-wetted vermiculite was used to fill the top, inverted bottle. The neck of the bottle was plugged with a cotton ball to prevent the vermiculite from falling into the nutrient solution. The entire assembly was wrapped in foil and autoclaved. After cooling the apparatus to room temperature, the bottom reservoir was filled with 400 mL of autoclaved Hoagland's No. 2 Basal Salt Mix nutrient solution without nitrogen (Sigma-Aldrich Canada). Filling the reservoir before autoclaving and autoclaving the entire apparatus resulted in unequal and unpredictable loss of the nutrient solution during autoclaving, hence the reason we filled the reservoir after autoclaving. Seeds or seedlings with an appropriate treatment were seeded/transplanted into the vermiculite. The Leonard jars were placed in a Conviron controlled environment chamber with a 16h/8h day/night cycle with 28/22 °C day/night temperatures. Jars were completely randomized and were re-randomized weekly.

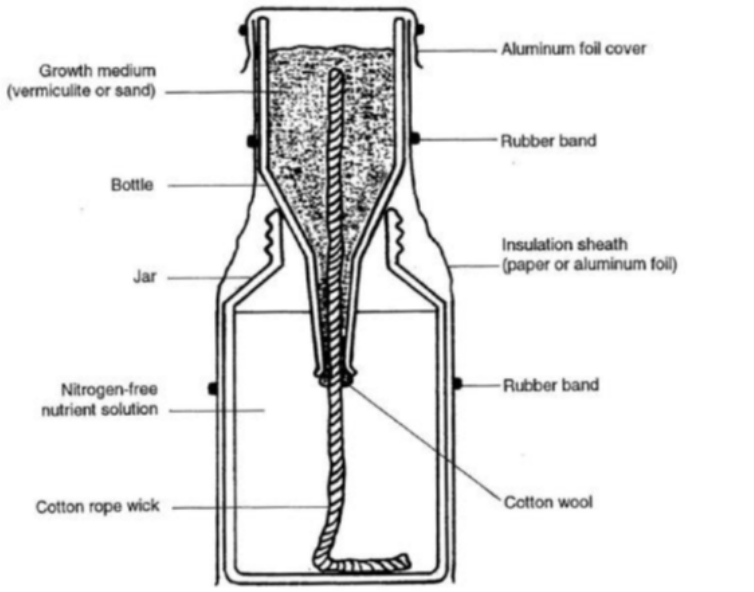


Figure 6-1. Leonard jar assembly (from Somasegaran and Hoben, 1985).

6.6.2 Soil-based: Growing the plants in soil in pots was done after the Leonard Jar trials. Plants grown in soil tend to be more robust and healthier than those grown in Leonard Jars. Soil used was a low N soil (4.2 mg available N kg^{-1} soil) with a loam texture that was mixed with sand (1:1 sand:soil) to further lower the N concentration (final available N concentration was 2.2 mg N kg^{-1}) and to facilitate recovery of roots in some experiments. 450 g of soil was weighed into a 10-cm diameter pot. Soil was maintained near 70% water holding capacity with regular watering. A dilute N-free nutrient solution was applied weekly at the same time as a regular watering event. Plants were grown in a Conviron controlled environment chamber with a 16h/8h day/night cycle with 28/22 °C day/night temperatures. Treatment pots were completely randomized in the growth chamber and re-randomized weekly.

6.7 Pulse-Labelling with $^{15}N_2$

Pulse labeling of the plants began 21 days after germination (DAG). Plants grown in Leonard jars were pluse labeled once; plants grown in soil were pulse labeled 3 times. Pulse labeling occurred in an air-tight labeling chamber fitted with a port for injecting ^{15}N-N_2.(Figure 6-2). Th atmospheric concentration of CO_2 was maintained between 450 and 600 ppmV. On a labeling day the Leonard jars or pots with the growing plant were placed in the chamber, the door sealed, and 3-L 99 atom% $^{15}N_2$ injected into the chamber. A fan circulated the $^{15}N_2$ through the chamber atmosphere. The chamber was fitted with an air conditioning unit to cool the air temperature to 28 °C during the labeling event. The plants were kept in the labeling chamber for 48 h. After 48 h the plants were removed, and returned to their Conviron controlled environment chamber. Pulse labeling was repeated 28DAG and 35DAG. Forty-eight hours after the third labeling event the plants were destructively harvested.

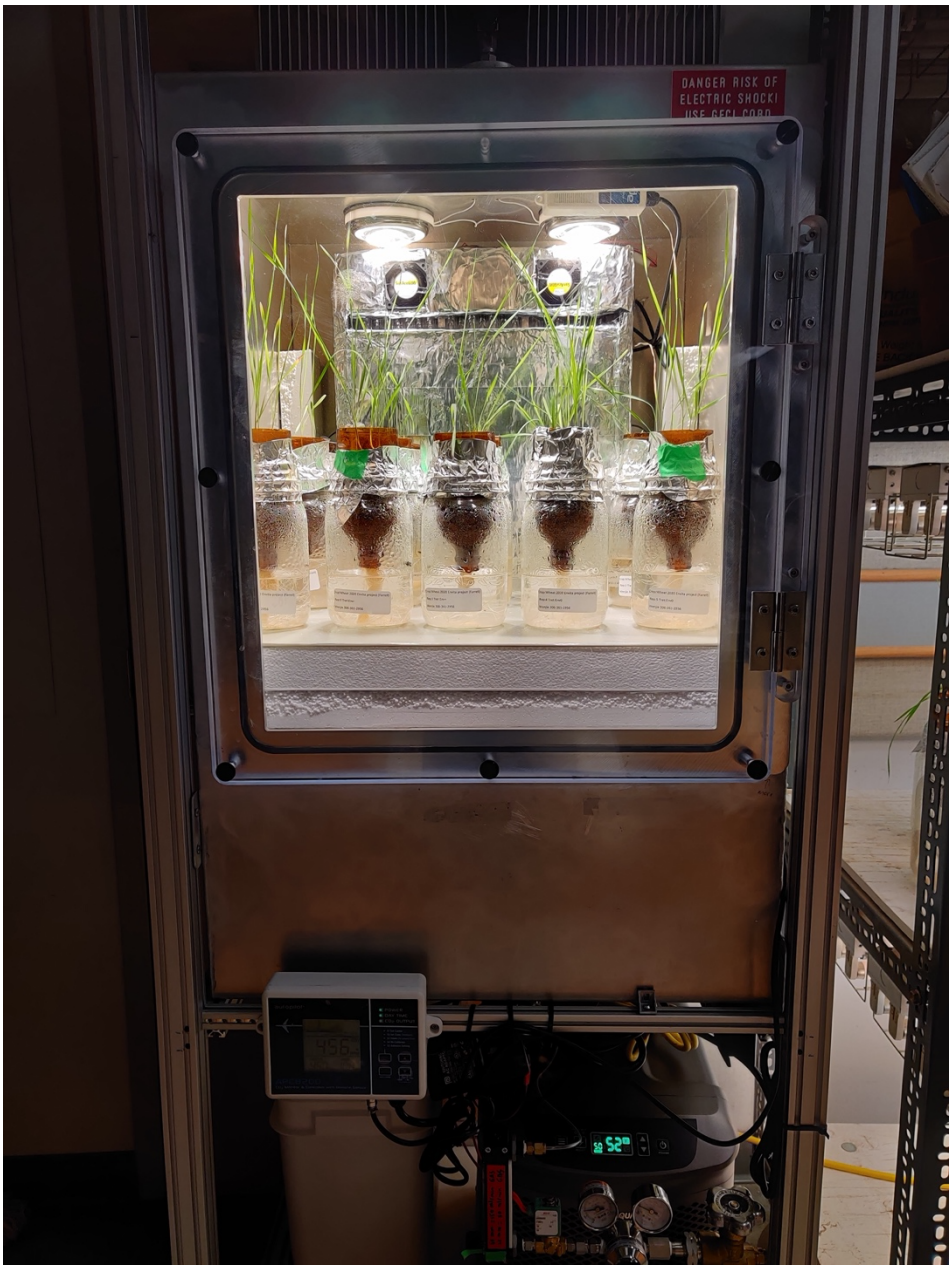


Figure 6-2. Photograph of the $^{15}N_2$ labeling chamber, containing wheat plants growing in Leonard jars undergoing pulse labeling.

6.8 Plant harvest and processing

Plants were separated into shoots and roots and the roots washed free of the rooting media. In early experiments only shoots were analysed; in later experiments shoots and roots were analysed separately. In one of the soybean experiments pods with developing seed were separated from shoots and analysed for ^{15}N content separately. The tissues were oven-dried at 60 °C in a forced-air oven and dry biomass recorded. These dried tissues were ground using a Wiley Mill and then further ground in a ball mill. Subsamples of the dried, ground shoot and root samples were encapsulated in tin capsules for on an Isotope Ratio Mass Spectrometer for atom % ^{15}N content and %N.

In later experiments where tissues were analysed for ddPCR (section 6.7.1, Table 6.1) a subsample of fresh tissue was collected, transported on ice to the Hemmingsway lab where the materials were freeze dried in preparation for the ddPCR analysis. Liquid N_2 was not used in the freeze drying process to avoid any contamination/alteration of the ^{15}N signals in the test tissues

6. 8.1 Droplet digital polymerase chain reaction (ddPCR analysis)

Dr. Sean Hemmingsway, a researcher at NTC-Saskatoon, developed a protocol for identifying colonization of *G. diazotrophicus* in plant tissues using droplet digital polymerase chain reaction (ddPCR). The procedure is a sensitive, precise and accurate method for amplifying sequences of different nucleic acids. Nucleic acid segments that are unique to *G. diazotrophicus* are amplified and quantified. The method quantifies number of copies of the target sequence. Data interpretation is as follows: positive (>8 copies); borderline (5-8 copies); negative (<5 copies). Because of the requirement for fresh tissues, only later experiments were sampled and analysed for ddPCR – would could not reanalyse early experiments because the tissues were dried.

6.9 Data analysis and presentation

Because we had limited success with identifying ^{15}N in the plant tissues associated with *G. diazotrophicus* inoculation, the standard reporting of means or medians does not make sense. Instead, individual data (i.e., individual replicates) are presented for the Envita™ inoculated treatments relative to the mean of the control plants (which did not exhibit much variability) so as to capture the extreme variability of the Envita™ inoculation data. Similarly because of the extreme variability statistical analyses performed only on the biomass data for the final soil experiment. Biomass data was subjected to ANOVA using a one-way analysis for treatment (α=0.05). Atom % ^{15N} values for plants inoculated with the heat-killed Envita were never different from the controls and as such are not presented.

7.0 Research accomplishments:

Objectives (*Please list the original objectives and/or revised objectives if approved revisions have been made to original objective. A justification is needed for any deviation from original objectives)*	***Progress (e.g. completed/in progress)***
Verify that biological N fixation (BNF) occurs in canola, wheat and soybean inoculated with a commercial strain of *Gluconobacter*	Completed (results described in Section 8)

Evaluate N characteristics and yield of canola, wheat and soybean treated with Envita™.	Completed (results described in Section 8)
Evaluate the effect of inoculation of canola, wheat and soybean with Envita™ on nitrous oxide (N_2O) emissions.	**Not done – because we were unable to consistently introduce *G. diazotrophicus* and verify that N_2 fixation was occurring there was no point in performing this objective. Effort was instead focused on evaluating different methods of introducing the organism and evaluating through the collaboration with Dr. Hemmingsway at NRC that the bacteria was colonizing the plant tissues.**

This project was severely affected by the operating restrictions imposed by the U of S in response to the COVID 19 pandemic. The pandemic affected our ability to acquire inoculant from the producer as well as conduct laboratory analyses due to restricted access to labs.

At the start of the project in 2019, inoculant was supplied from the parent plant in the UK. The original batch of inoculant we received was not viable. The replacement batch arrived just prior to the U of S closure in March 2020. The inoculant was checked for viability and was viable on receipt, but with the lab closures could not be used right away and was stored refrigerated. The shelf-life of the inoculant is 6 months. The student did not gain access to the lab until November 2020 at which time the inoculant had expired. A third batch was received in winter 2021. In Spring 2021 inoculant was being produced at a plant in Ontario so we had easier access to it.

8.0 Discussion:

We were unable to evaluate objective 3: "*Evaluate the effect of inoculation of canola, wheat and soybean with Envita™ on nitrous oxide (N_2O) emissions*" because we were unable to consistently achieve effective inoculation/colonization of the G*. diazotrophicus* organism. Canola did not grow well in the Leonard jars, hence no data are presented for canola. Early trials with wheat and soybean grown in Leonard Jars (Fig. 8-1) revealed no biological N fixation (BNF) occurring in the wheat when the inoculant was applied directly to the seed or as a foliar inoculant. Fairly high enrichment in two of five soybean plants in Leonard jars where the inoculant was applied foliarly indicated that BNF was occurring. In contrast there was only slight ^{15}N enrichment in soybean that was seed inoculated (Fig. 8-1). The Leonard jars are a semi-sterile system, in that at seeding the system is sterile, but after seeding the jars are placed in a non-sterile environmental chamber so are subject to contamination through air-borne contaminants. However, they are free of 'natural' soil organisms and hence do not undergo competition for resources with other soil organisms. Furthermore the Leonard jar systems are N free, except for being exposed to N_2 in the atmosphere, and

the $^{15}N_2$ atmosphere during pulse labeling. Any ^{15}N that is found in the plant tissues is from BNF of the $^{15}N_2$ enriched atmosphere. The data from the two ^{15}N-enriched soybean plants with foliar inoculation show clear evidence of the introduced *G. diazotrophicus* fixing N_2 and transferring it to the soybean tissues. The slight enrichment in the seed inoculated soybean is too small to conclusively indicate BNF and even if BNF was occurring the amount of N acquired through this pathway is too small to be practically significant.

Because the Leonard jars are essentially N free except for atmospheric N_2, their usefulness is limited to evaluation of small plants. Generally after a maximum of 4 weeks, plants show symptoms of stress. After these initial Leonard jar trials we shifted our approach to growing the plants in soil in pots. Plants grown in soil in the pots were more robust and visibly healthier than those grown in Leonard jars. Even though the soil had a low N content (2.2 mg/kg available N), in the absence of active BNF the plants still had access to N via root uptake. The first set of trials in the soil systems examined ^{15}N incorporation into shoots when the Envita™ inoculant was supplied either as a seed or a foliar inoculant (Figs. 8-2 and 8-3). Neither wheat, canola nor soybean showed any indication of BNF, in that there was no ^{15}N enrichment of the plants (Figs. 8-2 and 8-3). The Leonard jar experiments indicated that successful foliar inoculation is possible, but these initial soil experiments demonstrated that actually introducing the *G. diazotrophicus* organism to colonize the target plant tissues is challenging. Compared to the Leonard jar system, soils have the added challenge of microbial populations competing with the introduced organisms.

The final set of soil-based experiments expanded the inoculation methods evaluated to include soaking the germinating seed in the inoculant and then transplanting the small seedling to the soil (section 6.5.2). For these experiments we also had shoot and root tissues evaluated for *G. diazotrophicus* via the ddPCR method at NRC-Saskatoon (section 6.7.1). Both ^{15}N enrichment and %N in shoots and roots are reported.

Consistent with the prior studies, wheat shoots did not show any indication of ^{15}N enrichment (Fig. 8.4). Three of the wheat replicates that were inoculated with the Envita™ as a foliar application were positive for *G. diazotrophicus,* indicating that the bacteria had colonized the shoot tissue. But, the presence of the bacteria in the shoot tissue did not translate to BNF or increased %N in the shoots (Fig. 8.4). Biomass was similarly unaffected by inoculation (Table 8-1). In contrast, analysis of roots indicated some slight ^{15}N enrichment of roots, and many of the roots were colonized by *G. diazotrophicus* (Fig. 8-5). None-the-less all of the inoculated plants had lower %N than the controls (Fig. 8.5) and no differences in biomass production (Table 8-1), indicating that the slight enrichment and hence evidence of BNF was not contributing positively to the N balance of the wheat plants.

There was significant ^{15}N enrichment in three of the soybean plant shoots; two of which also showed positive evidence of *G. diazotrophicus* colonization (Fig. 8-6). These same three plants also showed ^{15}N enrichment in the roots (Fig. 8-7) and the same pattern of two roots positive for *G. diazotrophicus* and one negative for *G. diazotrophicus.* All of the root systems of soybean were examined for *Rhizobium* induced nodulation and were found to not have nodules, but it is likely that the one plant that was negative for *G. diazotrophicus* had a nodule(s) that was/were not detected. Interestingly the single

plant that was inoculated by the germination method that had the highest total ^{15}N enrichment had a lower percentage N than the controls (Fig. 8-7); indeed all of the soybean plants inoculatied at germination had lower percentages of N in the root tissues but no difference in root biomass (Table 8-2). The lower %N was not due to dilution by larger plants as might be expected. The reason for the lower %N values in the roots is not known, but it could be speculated that the introduced *G. diazotrophicus* may be scavenging some of the plant N or soil N for its own use.

Results for canola were the most inconsistent of the three plant species (Figs. 8-8, 8-9 and Table 8-3). All of the plants inoculated with the germination method showed some ^{15}N enrichment, albeit slight in the shoots (Fig 8-8) and roots (Fig. 8-9) but not all tested positive for *G. diazotrophicus* colonization. Furthermore, even though ^{15}N enrichment indicates the transfer of ^{15}N fixed by some means to the canola plants, there were not consistently higher percentages of N in the tissues nor did the plants produce more biomass (Table 8-3).

It is clear from the data that the mere presence of *G. diazotrophicus* colonizing the plant tissues (either shoots or roots) does not mean that the bacteria are fixing N and transferring it to the plant. Among the three target plants there were 25 positive tests for *G. diazotrophicus* in shoots (3 in wheat; 11 in soybean; 11 in canola) and 28 positive tests in roots (10 in wheat; 14 in soybean; 4 in canola) but except in foliar applications in three Soybean plants only very limited evidence of BNF. It is interesting that soybean, the only legume tested, seemed to be the most receptive to inoculation. Soybean is arguably the least important of the species tested because it can already fix atmospheric N through its symbiosis with *Rhizobium*. But it may be that the physiology of the plant better enables it to receive and utilize N fixed by *G. diazotrophicus*.

The slight ^{15}N enrichments in several of the plants that tested negative for *G. diazotroph*icus suggests that there may be some fixation and transfer of ^{15}N outside of the plant. *Gluconobacter diazotrophicus* is a free-living bacteria (does not require a plant to survive). There may be very small amounts of N fixed by *G. diazotrophicus* outside of the plant that is then taken up by the plant root systems. The magnitude of the enrichments could also simply be a function of the variability of the system – although the very consistent values obtained for the control plants (data not shown) suggests otherwise.

Ultimately, in terms of getting the *G. diazotrophicus* into the plant tissues, the germination method had the most positive tests followed by foliar application and then direct seed application. While interesting and informative, soaking seeds at germination is the least practical method of introducing the organism as an agricultural inoculant. On a field scale, it might be possible to introduce the inoculant as a liquid formulation shortly after germination and emergence but the probable timing restrictions and difficulty applying it are likely to be prohibitive. Certainly more work needs to address inoculation strategies that would easily translate to a field scale and how to translate successful colonization of the bacteria into transfer of biologically fixed N to the plant.

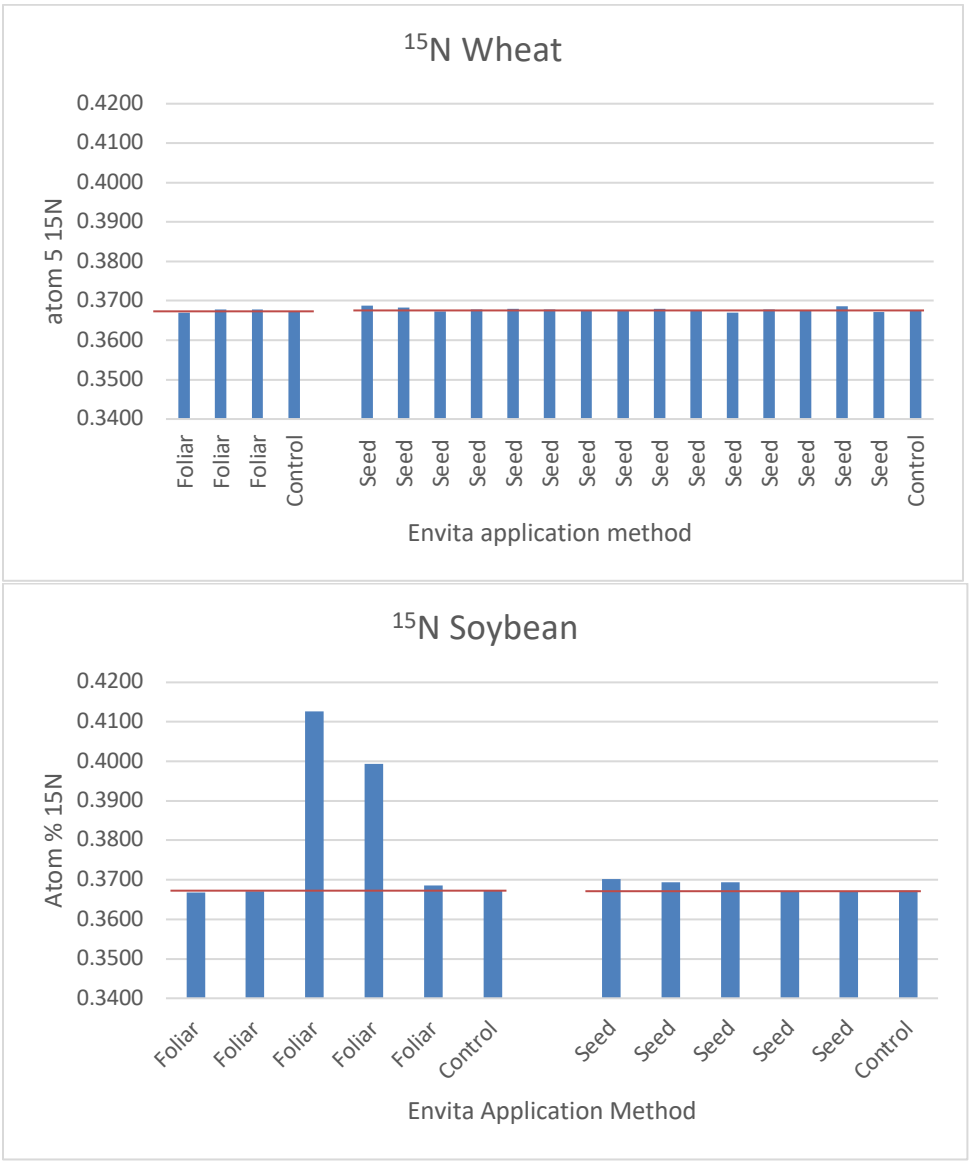
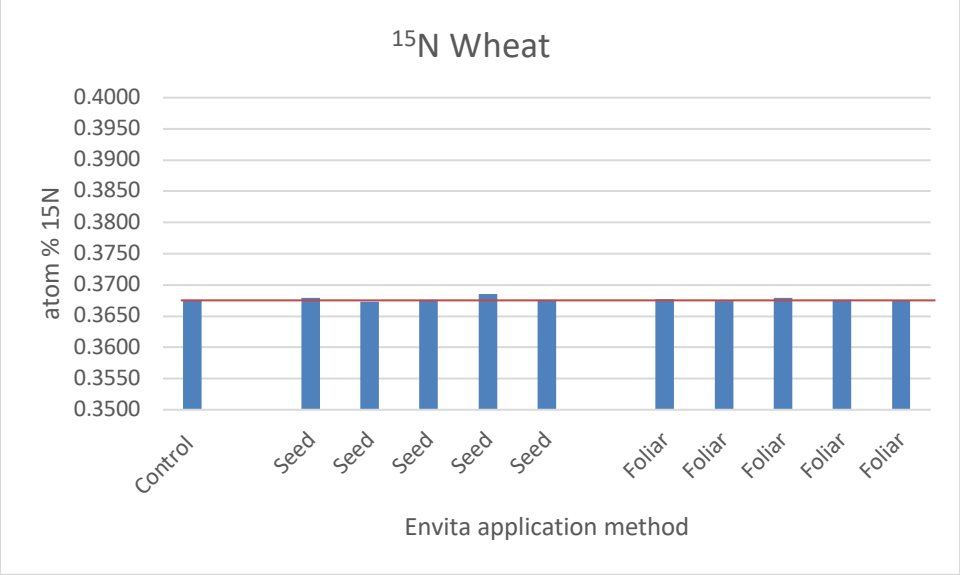


Fig. 8-1. Atom % ^{15}N values for **wheat** and **soybean** grown in **Leonard Jars** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1). The red line indicates the control plants (average of n=4) grown without Envita™ inoculation.



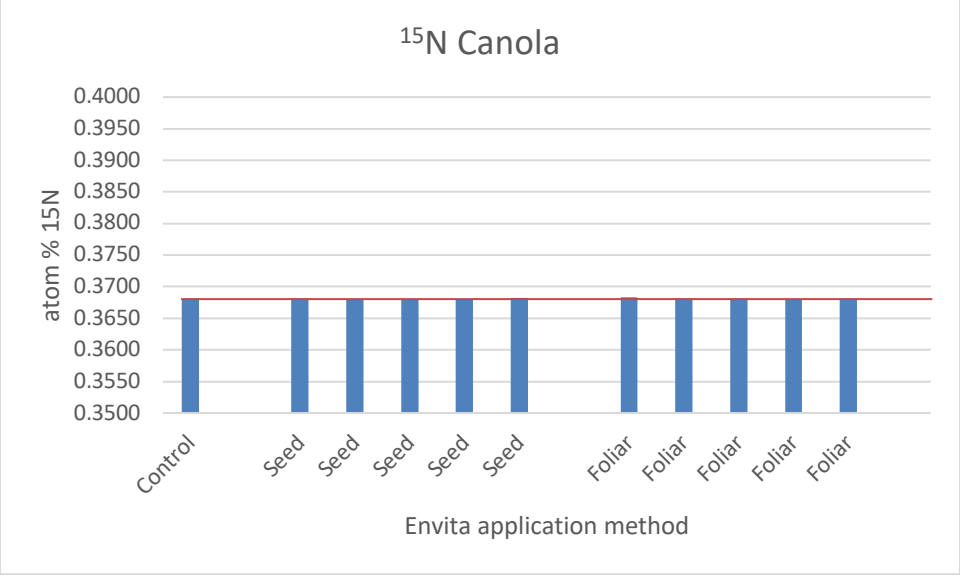


Fig. 8-2. Atom % ^{15}N for **wheat** (top panel) and **canola** (bottom panel) grown in **SOIL** inoculated with Envita™ applied either to the seed (Direct Seed Application – section 6.5.1) or foliarly (section 6.5.3). The red lines indicates the control plants grown without Envita™ inoculation.

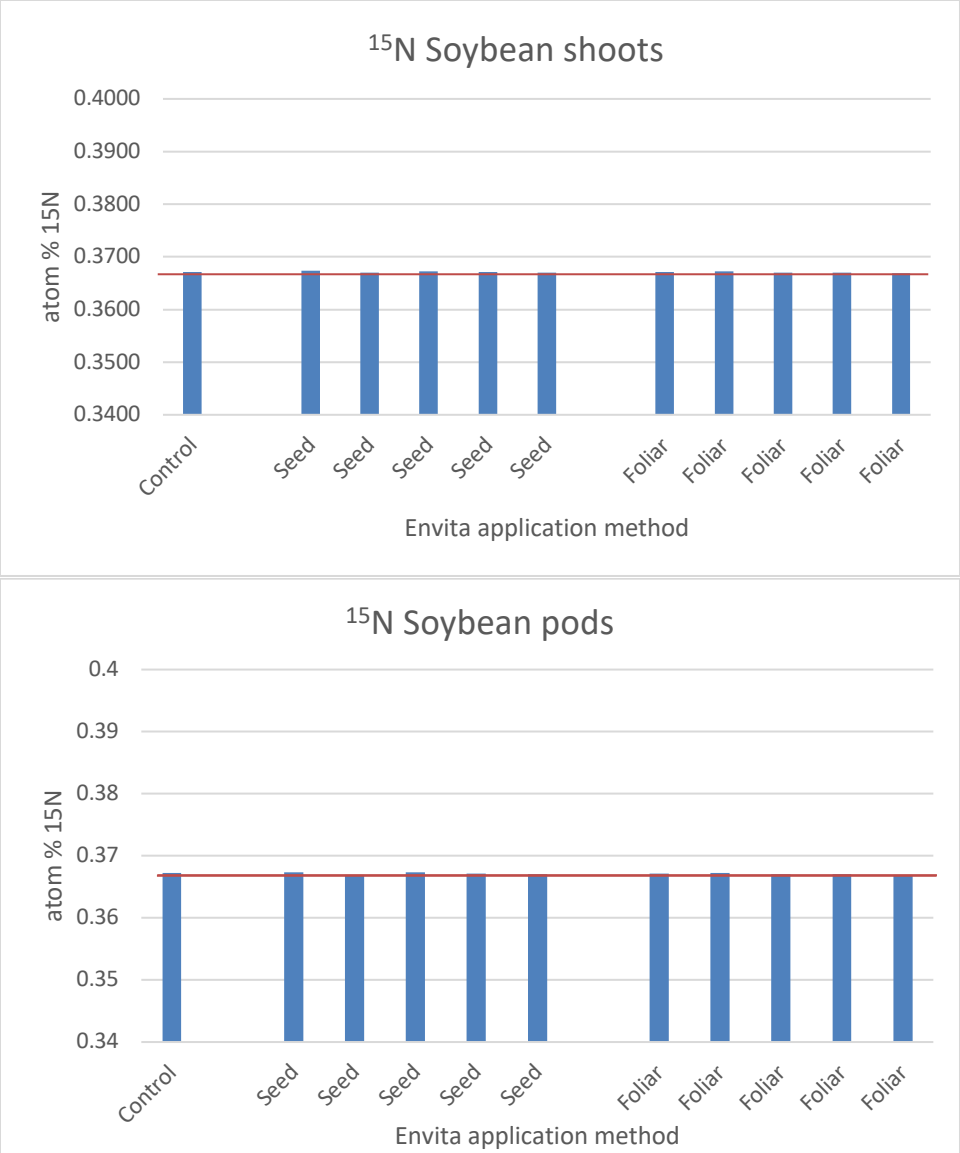


Fig. 8-3. Atom % ^{15}N for **soybean shoots** (top panel) and **pods** (bottom panel) grown in **SOIL** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1). The red line indicates the control plants grown without Envita™ inoculation.

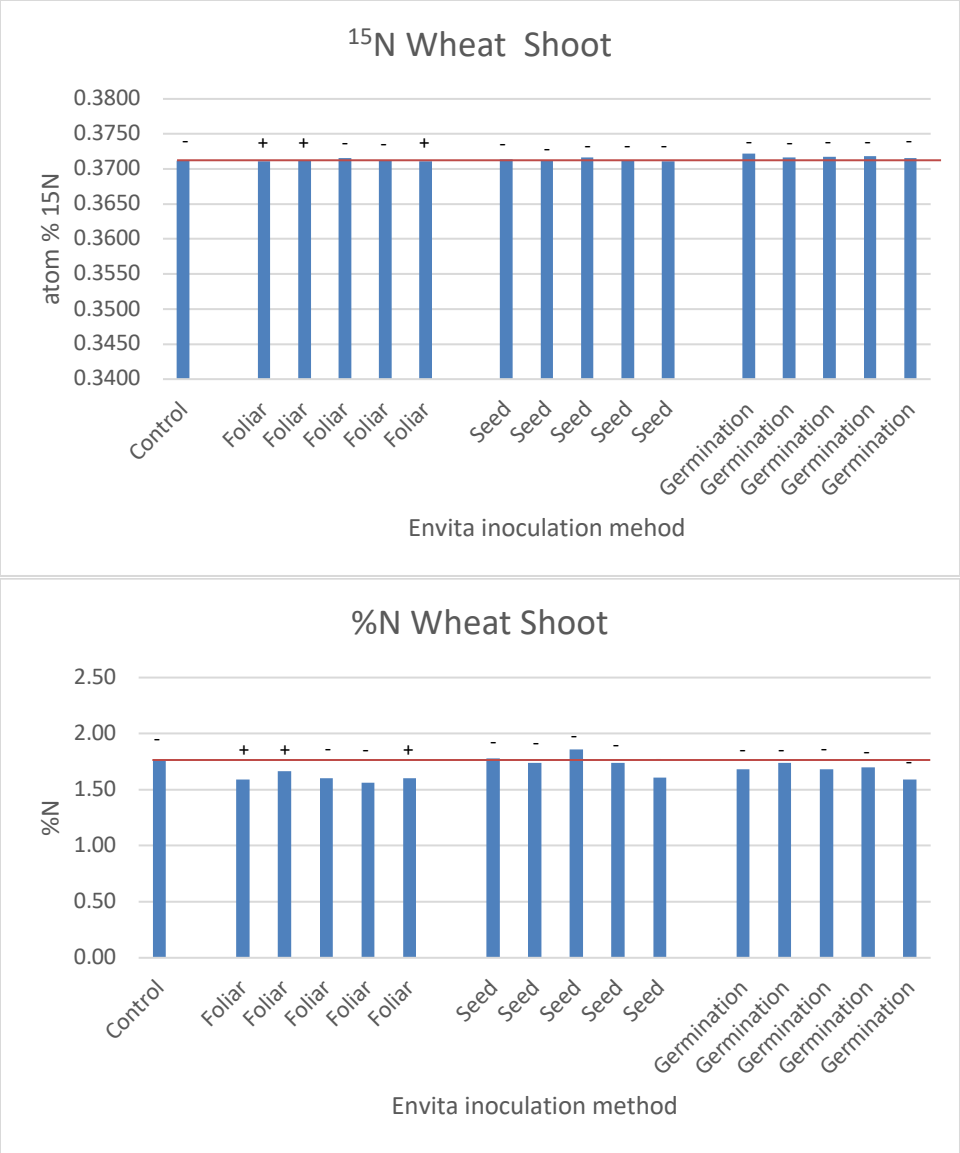
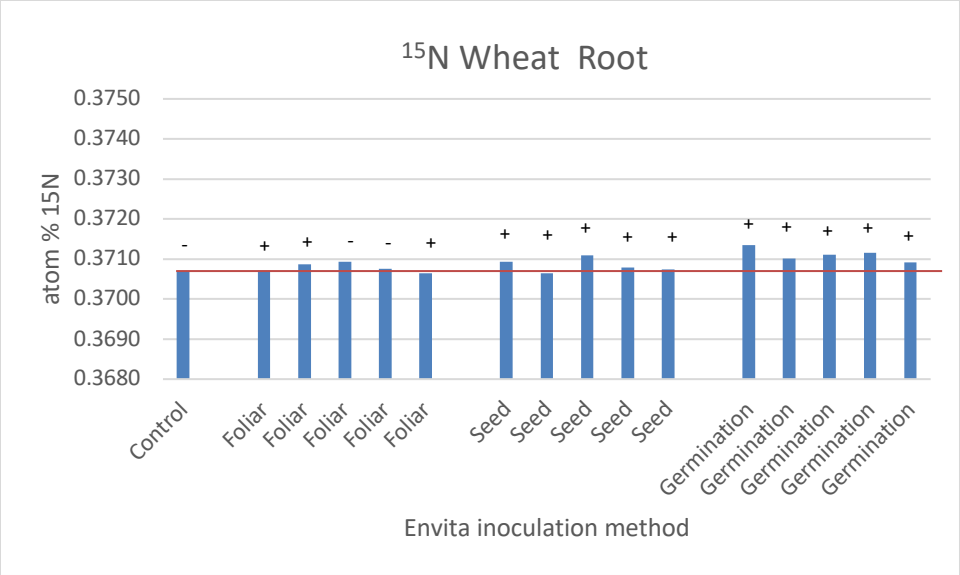


Fig. 8.4. Atom % ^{15}N (top panel) and %N (bottom panel) for **Wheat Shoots** grown in **SOIL** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1) or at the time of germination (section 6.5.2). The red line indicates the control plants grown without Envita™ inoculation. The symbol above each bar is the result of the ddPCR for G. diazotrophicus: 0=borderline (5-8 copies); +=positive (>8 copies); - = negative (<5 copies).



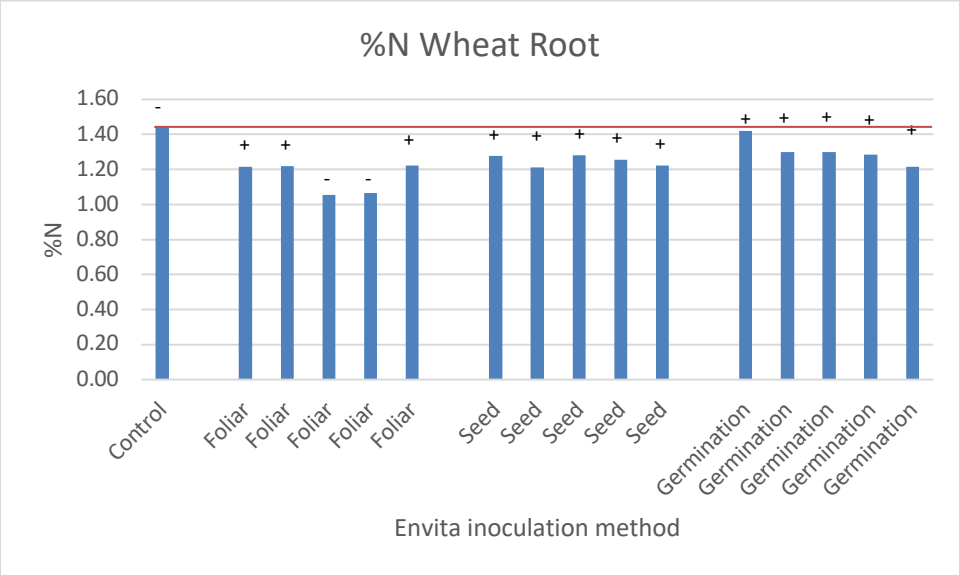
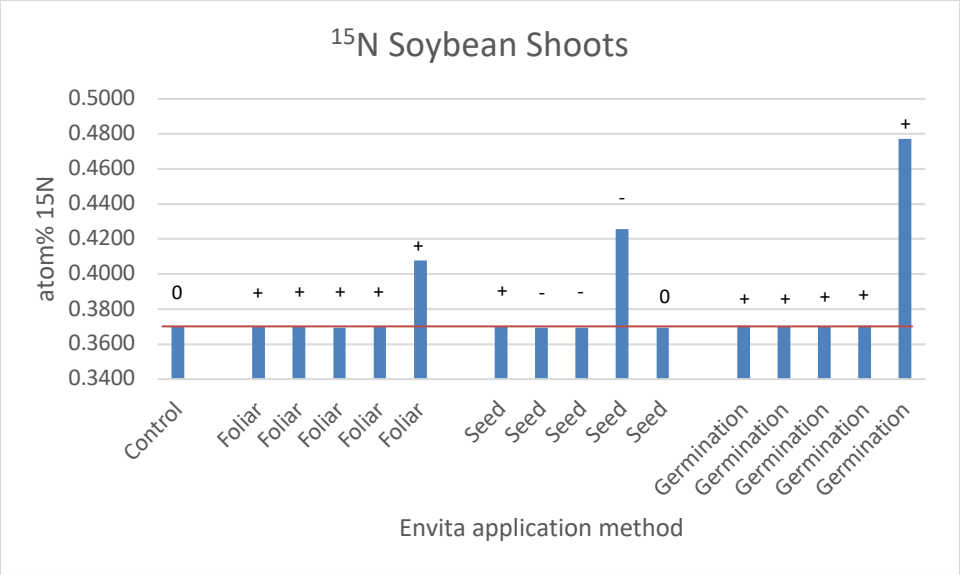


Fig. 8.5. Atom % ^{15}N (top panel) and %N (bottom panel) for **Wheat Shoots** grown in **SOIL** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1) or at the time of germination (section 6.5.2). The red line indicates the control plants grown without Envita™ inoculation. The symbol above each bar is the result of the ddPCR for G. diazotrophicus: 0=borderline (5-8 copies); +=positive (>8 copies); - = negative (<5 copies).



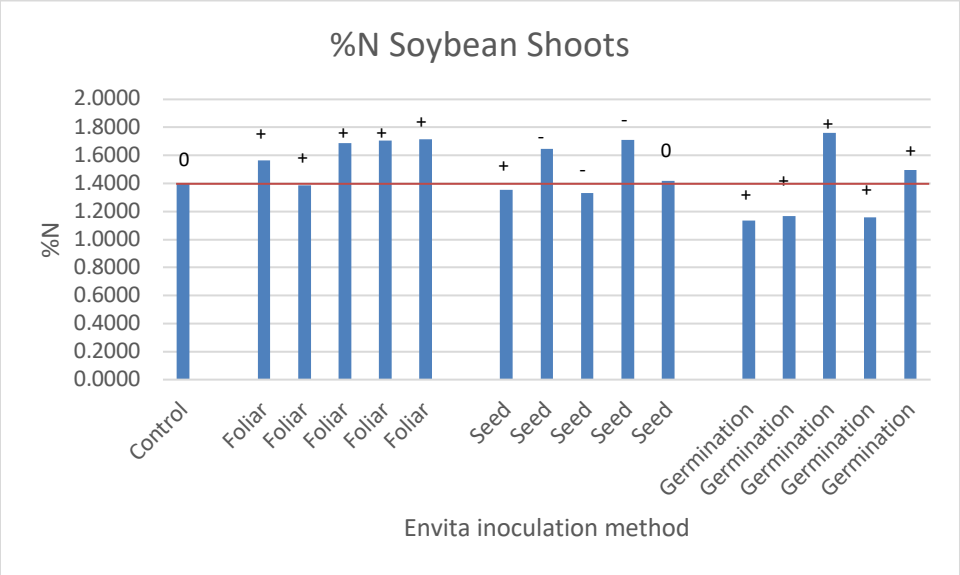
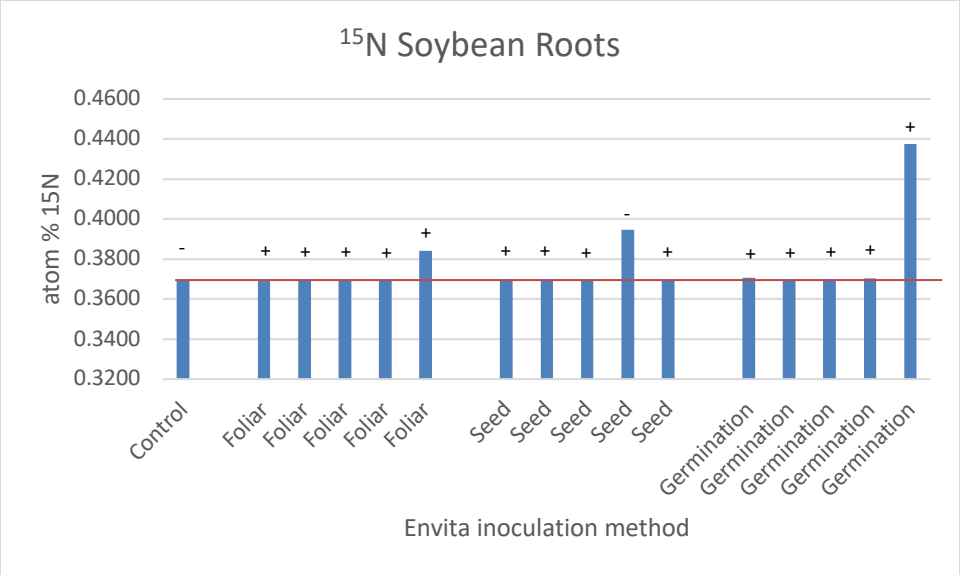


Fig. 8.6. Atom % ^{15}N (top panel) and %N (bottom panel) for **soybean shoots** grown in **SOIL** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1) or at the time of germination (section 6.5.2). The red line indicates the control plants grown without Envita™ inoculation. The symbol above each bar is the result of the ddPCR for G. diazotrophicus: 0=borderline (5-8 copies); +=positive (>8 copies); - = negative (<5 copies).



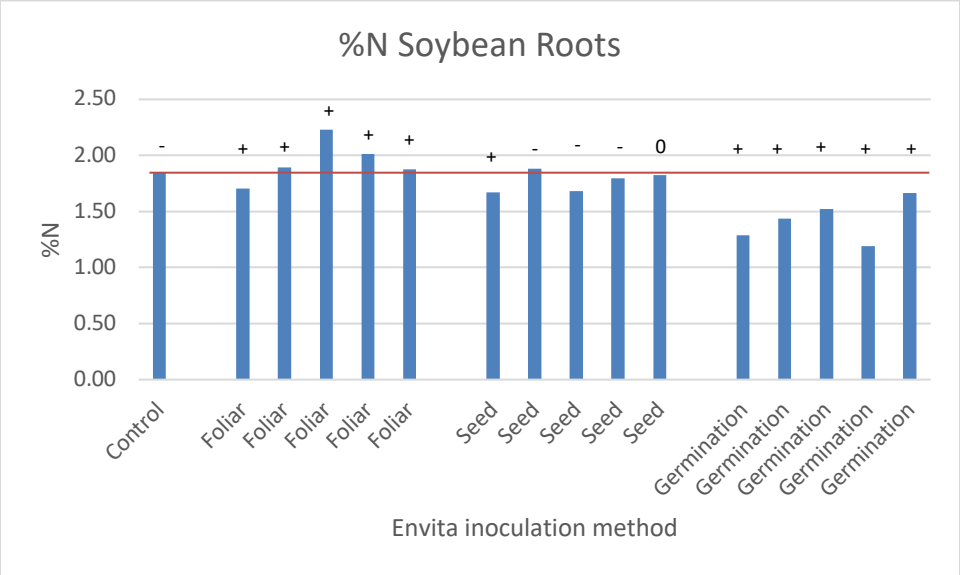
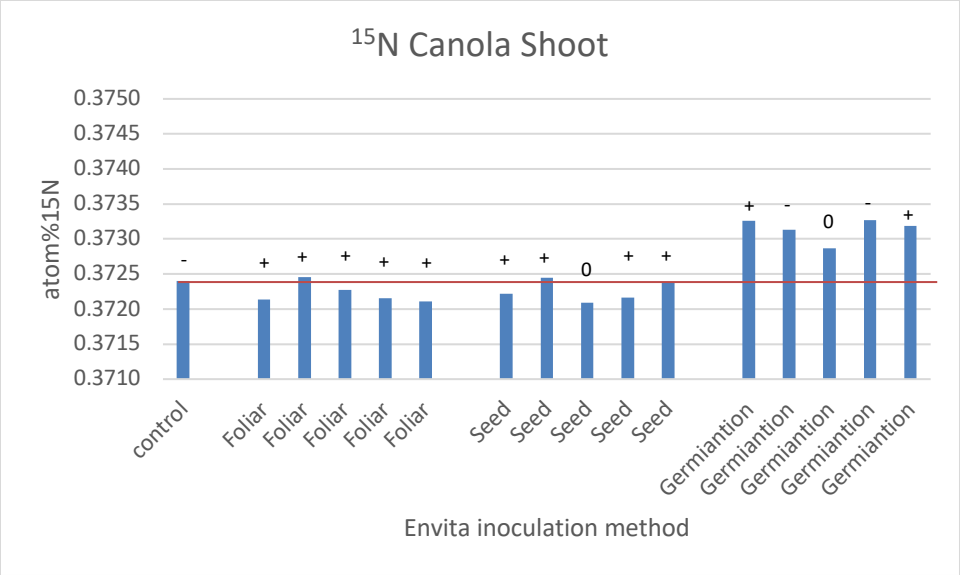


Fig. 8.7. Atom % ^{15}N (top panel) and %N (bottom panel) for **soybean roots** grown in **SOIL** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1) or at the time of germination (section 6.5.2). The red line indicates the control plants grown without Envita™ inoculation. The symbol above each bar is the result of the ddPCR for G. diazotrophicus: 0=borderline (5-8 copies); +=positive (>8 copies); - = negative (<5 copies).



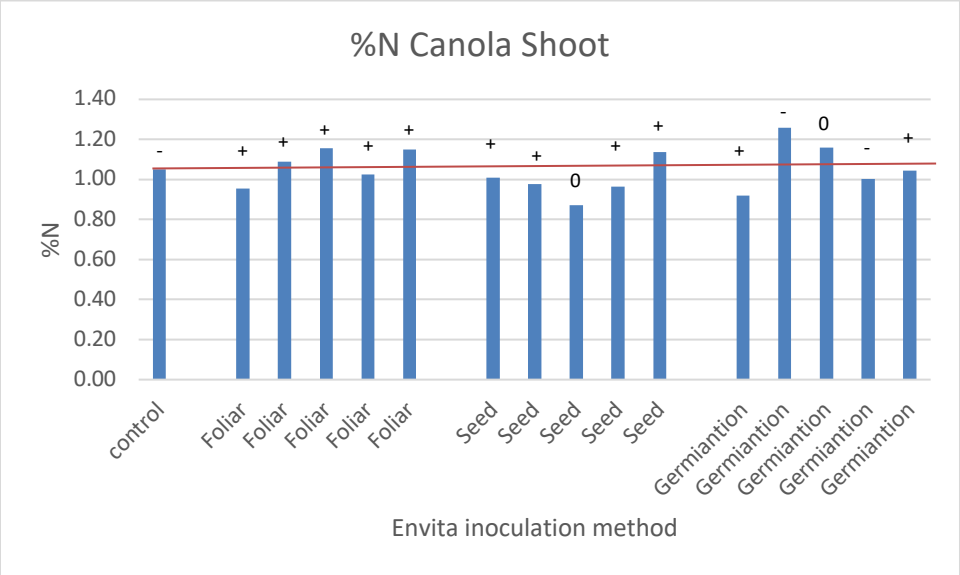
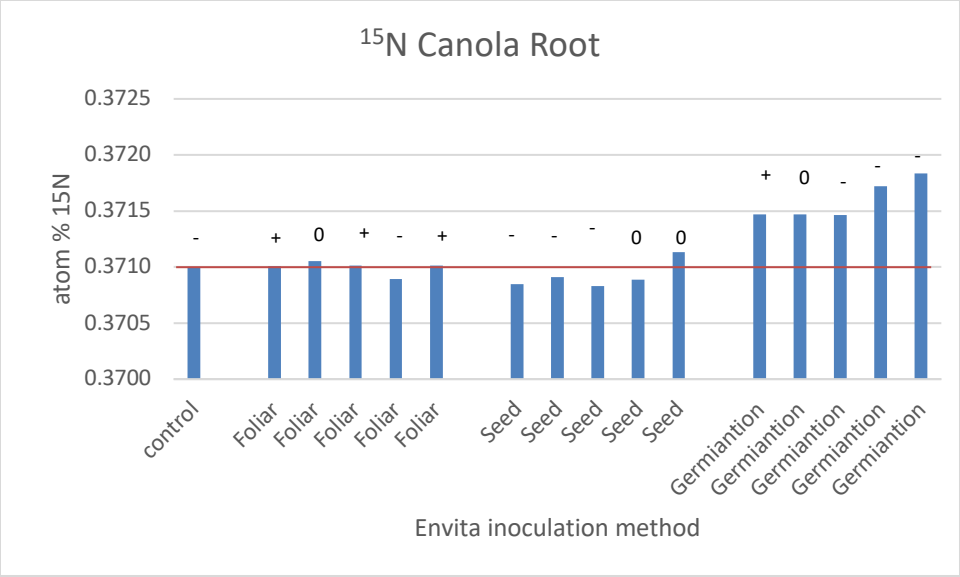


Fig. 8.8. Atom % ^{15}N (top panel) and %N (bottom panel) for **Canola Shoots** grown in **SOIL** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1) or at the time of germination (section 6.5.2). The red line indicates the control plants grown without Envita™ inoculation. The symbol above each bar is the result of the ddPCR for G. diazotrophicus: 0=borderline (5-8 copies); +=positive (>8 copies); - = negative (<5 copies).



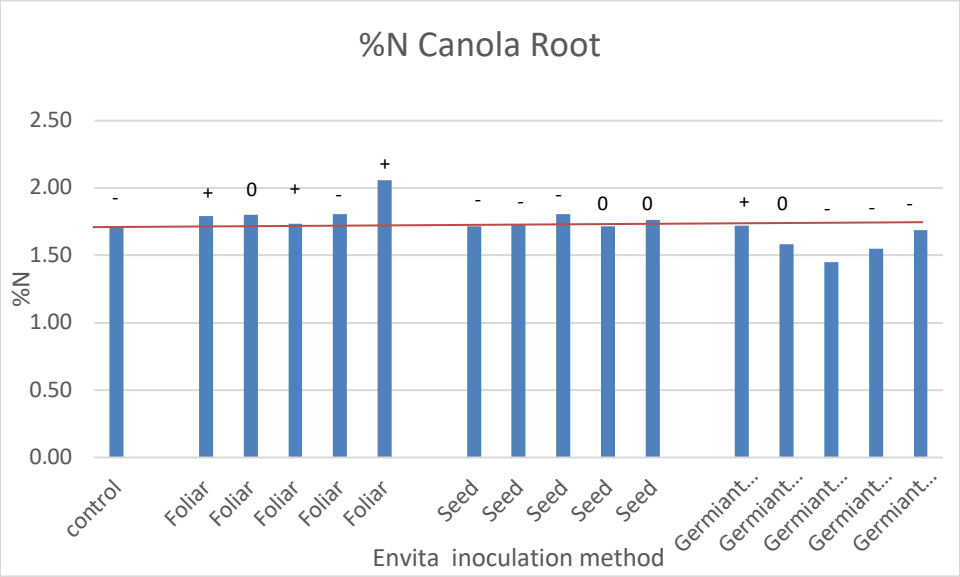


Fig. 8.9. Atom % ^{15}N (top panel) and %N (bottom panel) for **Canola Roots** grown in **SOIL** inoculated with Envita™ applied either foliarly (section 6.5.3) or to the seed (Direct Seed Application – section 6.5.1) or at the time of germination (section 6.5.2). The red line indicates the control plants grown without Envita™ inoculation. The symbol above each bar is the result of the ddPCR for G. diazotrophicus: 0=borderline (5-8 copies); +=positive (>8 copies); - = negative (<5 copies).

Table 8-1. Dry weights and *G. diazotrophicus* colonization in shoots and roots of **Wheat** grown in soil, inoculated with EnvitaTM inoculant using different inoculation methods. Details of inoculation methods: Foliar (Section 6.5.3), Seed (Section 6.5.1) and Germination (Section 6.5.2). Gd status is the result of the ddPCR protocol (section 6.7.1) with 0=borderline (5-8 copies); - = no copies (<5) and +=> 8 copies. There were no statistically significant different in shoot or root dry weights due to the different inoculation methods (p>0.05)

Species	Tissue	Replicate	Inoculation	Dry wt (g)	Dry wt average (g)	Gd status
Wheat	Shoot	1	Control	13.66	13.57	-
Wheat	Shoot	2	Control	13.49		-
Wheat	Shoot	3	Control	13.49		-
Wheat	Shoot	4	Control	13.73		-
Wheat	Shoot	5	Control	13.47		-
Wheat	Root	1	Control	13.15	13.36	-
Wheat	Root	2	Control	13.4		-
Wheat	Root	3	Control	13.39		-
Wheat	Root	4	Control	13.44		-
Wheat	Root	5	Control	13.4		-
Wheat	Shoot	1	Foliar	13.75	13.65	+
Wheat	Shoot	2	Foliar	13.64		+
Wheat	Shoot	3	Foliar	13.61		-
Wheat	Shoot	4	Foliar	13.63		-
Wheat	Shoot	5	Foliar	13.6		+
Wheat	Root	1	Foliar	13.37	13.39	-
Wheat	Root	2	Foliar	13.39		-
Wheat	Root	3	Foliar	13.41		0
Wheat	Root	4	Foliar	13.46		0
Wheat	Root	5	Foliar	13.32		-
Wheat	Shoot	1	Seed	13.63	13.60	-
Wheat	Shoot	2	Seed	13.63		-
Wheat	Shoot	3	Seed	13.62		-
Wheat	Shoot	4	Seed	13.49		-
Wheat	Shoot	5	Seed	13.61		-
Wheat	Root	1	Seed	13.32	13.33	+
Wheat	Root	2	Seed	13.3		+
Wheat	Root	3	Seed	13.23		+
Wheat	Root	4	Seed	13.39		+
Wheat	Root	5	Seed	13.39		+
Wheat	Shoot	1	Germination	13.60	13.50	-
Wheat	Shoot	2	Germination	13.46		-
Wheat	Shoot	3	Germination	13.45		-
Wheat	Shoot	4	Germination	13.48		-
Wheat	Shoot	5	Germination	13.52		-
Wheat	Root	1	Germination	13.53	13.60	+
Wheat	Root	2	Germination	13.65		+
Wheat	Root	3	Germination	13.43		+
Wheat	Root	4	Germination	13.63		+
Wheat	Root	5	Germination	13.72		+

Table 8-2. Dry weights and *G. diazotrophicus* colonization in shoots and roots of **Soybean** grown in soil, inoculated with EnvitaTM inoculant using different inoculation methods. Details of inoculation methods: Foliar (Section 6.5.3), Seed (Section 6.5.1) and Germination (Section 6.5.2). Gd status is the result of the ddPCR protocol (section 6.7.1) with 0=borderline (5-8 copies); - = no copies (<5) and +=> 8 copies. There were no statistically significant different in shoot of dry weights due to the different in0culation methods (p>0.05)

Species	Tissue	Replicate	Inoculation	Dry wt (g)	Dry wt average (g)	Gd status
Soybean	Shoot	1	Control	15.79	15.68	0
Soybean	Shoot	2	Control	15.60		-
Soybean	Shoot	3	Control	16.03		-
Soybean	Shoot	4	Control	15.56		0
Soybean	Shoot	5	Control	15.42		0
Soybean	Root	1	Control	13.80	13.85	0
Soybean	Root	2	Control	13.85		-
Soybean	Root	3	Control	13.78		-
Soybean	Root	4	Control	13.77		-
Soybean	Root	5	Control	14.05		-
Soybean	Shoot	1	Foliar	15.75	15.46	+
Soybean	Shoot	2	Foliar	15.51		+
Soybean	Shoot	3	Foliar	15.53		+
Soybean	Shoot	4	Foliar	15.38		+
Soybean	Shoot	5	Foliar	15.15		+
Soybean	Root	1	Foliar	13.79	13.89	+
Soybean	Root	2	Foliar	14.11		+
Soybean	Root	3	Foliar	13.94		+
Soybean	Root	4	Foliar	13.91		+
Soybean	Root	5	Foliar	13.72		+
Soybean	Shoot	1	Seed	15.94	15.75	+
Soybean	Shoot	2	Seed	15.39		-
Soybean	Shoot	3	Seed	15.60		-
Soybean	Shoot	4	Seed	16.01		-
Soybean	Shoot	5	Seed	15.82		0
Soybean	Root	1	Seed	13.98	13.83	+
Soybean	Root	2	Seed	13.57		+
Soybean	Root	3	Seed	13.83		+
Soybean	Root	4	Seed	13.91		-
Soybean	Root	5	Seed	13.86		+
Soybean	Shoot	1	Germination	14.95	14.71	+
Soybean	Shoot	2	Germination	14.87		+
Soybean	Shoot	3	Germination	14.33		+
Soybean	Shoot	4	Germination	14.31		+
Soybean	Shoot	5	Germination	15.08		+
Soybean	Root	1	Germination	13.61	13.70	+
Soybean	Root	2	Germination	13.87		+
Soybean	Root	3	Germination	13.63		+
Soybean	Root	4	Germination	13.61		+
Soybean	Root	5	Germination	13.77		+

Table 8-3. Dry weights and *G. diazotrophicus* colonization in shoots and roots of **Canola** grown in soil, inoculated with EnvitaTM inoculant using different inoculation methods. Details of inoculation methods: Foliar (Section 6.5.3), Seed (Section 6.5.1) and Germination (Section 6.5.2). Gd status is the result of the ddPCR protocol (section 6.7.1) with 0=borderline (5-8 copies); - = no copies (<5) and +=> 8 copies. There were no statistically significant different in shoot of dry weights due to the different in0culation methods (p>0.05)

Species	Tissue	Replicate	Inoculation	Dry wt (g)	Dry wt average (g)	Gd status
Canola	Shoot	1	Control	13.50	13.50	-
Canola	Shoot	2	Control	13.40		-
Canola	Shoot	3	Control	13.55		-
Canola	Shoot	4	Control	13.55		-
Canola	Shoot	5	Control	13.50		-
Canola	Root	1	Control	13.37	13.47	-
Canola	Root	2	Control	13.53		-
Canola	Root	3	Control	13.47		-
Canola	Root	4	Control	13.50		-
Canola	Root	5	Control	13.48		-
Canola	Shoot	1	Foliar	13.52	13.52	+
Canola	Shoot	2	Foliar	13.52		+
Canola	Shoot	3	Foliar	13.48		+
Canola	Shoot	4	Foliar	13.49		+
Canola	Shoot	5	Foliar	13.60		+
Canola	Root	1	Foliar	13.48	13.50	+
Canola	Root	2	Foliar	13.56		0
Canola	Root	3	Foliar	13.43		+
Canola	Root	4	Foliar	13.43		-
Canola	Root	5	Foliar	13.62		+
Canola	Shoot	1	Seed	13.60	13.49	+
Canola	Shoot	2	Seed	13.37		+
Canola	Shoot	3	Seed	13.50		0
Canola	Shoot	4	Seed	13.53		+
Canola	Shoot	5	Seed	13.43		+
Canola	Root	1	Seed	13.50	13.47	-
Canola	Root	2	Seed	13.60		-
Canola	Root	3	Seed	13.41		-
Canola	Root	4	Seed	13.41		0
Canola	Root	5	Seed	13.41		0
Canola	Shoot	1	Germination	13.45	13.48	+
Canola	Shoot	2	Germination	13.41		-
Canola	Shoot	3	Germination	13.47		0
Canola	Shoot	4	Germination	13.53		-
Canola	Shoot	5	Germination	13.52		+
Canola	Root	1	Germination	13.48	13.46	+
Canola	Root	2	Germination	13.39		0
Canola	Root	3	Germination	13.55		-
Canola	Root	4	Germination	13.38		-
Canola	Root	5	Germination	13.50		-

9.0 Conclusions and Recommendations:

The Envita™ was able to deliver the free-living, N-fixing, *Gluconobacter diazotrophic* bacteria to roots and shoots of wheat, canola and soybean, and the bacteria was able to colonize the tissues in many, but not all cases. However, the mere presence of the *G. diazotrophicus* in the shoot and root tissues did not often translate into $^{15}N_2$ being fixed into the plant tissues. Foliar application of the inoculant in soybean (3 of five plants) grown in the semi-sterile Leonard jars provided the strongest evidence of the inoculant inducing biological N fixation. But when inoculation was performed in non-sterile soil systems where other soil-borne microorganisms presumably compete with the introduced *G. diazotrophicus*, evidence of BNF was generally lacking. To further complicate matters, when tissues were slightly enriched in ^{15}N indicating probable BNF, the %N in the tissues tended to be unchanged or reduced compared to the control with no change in biomass production. In these cases it seems that the *G. diazotrophicus* is preferentially using N from the system possibly at the expense of the plants.

Of the three inoculation methods evaluated soaking the seed in inoculant during germination was the most effective at introducing the *G. diazotrophicus* for tissue colonization, followed by foliar application and finally applying the inoculant to the seed. Unfortunately the germination soaking method is also the least practical to implement on a large scale. The parent company has recently suggested a soil soaking method where a liquid formulation of the inoculant is applied to the seed row after planting which might prove successful. However, given the time restrictions that farmers are faced with during seeding and spraying, it is difficult to imagine that this could be implemented on a wide-scale.

Better methods of inoculating crops as well as better translation of inoculant colonization with BNF is needed.

10.0 Success stories/practical implications for producers or industry:

Prior studies have demonstrated that inoculation with *G. diazotrophicus* improve tissue N status of corn. However transferring that information/technology to wheat, soybean and canola has proven challenging. There is clear evidence that the introduced organism colonizes tissues of wheat, soybean and canola, but colonization did not generally translate to enhanced biological nitrogen fixation in these western Canadian crops.

11.0 Patents/IP generated/commercialized products:

None to report

12.0 Technology transfer activities:

Chi, W. 2020. Verify and characterize the effects of new N_2-fixing inoculant for use with wheat, canola and soybean. College of Agriculture and BioResources Seminar, April 3, 2020.

Chi, W., **J.D. Knight** & **R. Farrell**. 2022. Verify and Characterize the effects of a N_2-fixing inoculant for wheat, canola, and soybean. 2022 Annual Soils and Crops Workshop. Virtual Meeting March 8-9, 2022.

Chi, W., **J.D. Knight** & **R. Farrell**. 2022. Verifying and characterizing the effects of a N2-fixing inoculant for wheat, canola and soybean. Canadian Society of Soil Science Annual Meeting, May 23-27, 2022. Hybrid conference, virtual presentation.

13.0 Industry contributions or support received

Azotic technologies Ltd provide all of the inoculant used in the project. They also provided invaluable advice on inoculation strategies and alerted us to the work of Dr. Sean Hemmingsway at NRC- Saskatoon that enabled us to have the ddPCR test done in the later stages of the study.

14.0 Is there a need to conduct follow up research?

Given the economic and environmental constraints on N fertilizer application, the idea of developing free-living endophytic inoculants for non-legume crops continues to be very enticing. Such inoculant products have be marketed in South America for years and are still sold, so must have convincing positive effects for growers to continue using them. We need to be open and available to evaluate new technologies.

15.0 Acknowledgements:

Funding for this project was provided by Western Grains Research Foundation and SaskCanola. We are grateful for the funding and have acknowledged the funding bodies for their support in all presentations, interviews etc. The following statement was used to acknowledge support.

"Financial support was provided by Western Grains Research Foundation and SaskCanola. Technical support was provided by the Saskatchewan Ministry of Agriculture Strategic Research Program–Soil Biological Processes".

3. Appendices:

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