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Testing Whether Pre-Pod-Fill Symbiotic Nitrogen Fixation in Soybean Is Subject to Drift or Selection Over 100 Years of Soybean Breeding

Malinda S. Thilakarathna [1,2]*, Davoud Torkamaneh [2,3], Robert W. Bruce [2], Istvan Rajcan [2], Godfrey Chu [2], Christopher M. Grainger [2], Krzysztof Szczyglowski [4], Brett Hill [5] and Manish N. Raizada [2]

[1] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada, [2] Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada, [3] Département de Phytologie, Université Laval, Québec, QC, Canada, [4] Agriculture and Agri-Food Canada, London Research and Development Centre, London, ON, Canada, [5] Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, AB, Canada

Soybean [*Glycine max* (L.) Merr.] is the world's leading legume crop and the largest oilseed crop. It forms a symbiotic relationship with rhizobia bacteria residing in root nodules that provide fixed nitrogen to host plants through symbiotic nitrogen fixation (SNF). In soybean, it has been widely reported that the highest SNF occurs at the pod-filling stage, associated with the peak demand for nitrogen. However, the majority of seed nitrogen is derived from remobilizing root/shoot nitrogen, representing cumulative SNF from the seedling stage to the pre-pod-fill stage. Therefore, the question arises as to whether there has also been selection for improved SNF at these earlier stages, or whether pre-pod-fill SNF traits have drifted. To test this hypothesis, in this study, pre-pod SNF-related traits were evaluated in soybean cultivars that span 100 years of breeding selection in the Canadian Province of Ontario. Specifically, we evaluated SNF traits in 19 pedigree-related historical cultivars and 25 modern cultivars derived from the University of Guelph soybean breeding program. Field trials were conducted at Woodstock, Ontario, Canada in 2016 and 2017, and various SNF-related traits were measured at pre-pod-fill stages (R1-R3), including nitrogen fixation capacity. Considerable variation was observed among Canadian soybean cultivars released over the past 100 years for pre-pod-fill nitrogen fixation. The modern soybean cultivars had similar or moderately higher pre-pod-fill SNF compared to the historical lines in terms of the percentage of nitrogen derived from the atmosphere (%Ndfa) and total shoot fixed nitrogen. These findings suggest that, despite no direct selection by breeders, pre-pod-fill nitrogen fixation, and associated SNF traits have been maintained and possibly improved in modern soybean breeding. However, the low level of pre-pod-fill SNF in some modern cultivars, and generally wide variation observed in SNF between them, suggest some level of genetic drift for this trait in some pedigrees. Specific historical and modern soybean cultivars were identified as potential

parents to enable targeted breeding for improved pre-pod-fill SNF. This retrospective study sheds light on our understanding of the impact of decades of recent selective breeding on pre-pod-fill nitrogen fixation traits in soybean in a temperate environment.

INTRODUCTION

Soybean or soya bean [*Glycine max* (L.) Merr.] is the most important legume crop in the world in terms of production, and a major source of human protein, animal feed, and vegetable oil. Soybean oil is now also becoming an important feedstock for biodiesel production (Hartman et al., 2011). Soybean production has increased substantially during the last five decades, mainly due to the expansion of soybean growing areas (Liu et al., 2020). It is widely believed that cultivated soybean originated and was domesticated from wild soybean *Glycine soja* (Sieb. & Zucc.) in East Asia 6000–9000 years ago (Guo et al., 2010; Wang et al., 2016) and then introduced to North America in 1765 (Hymowitz and Harlan, 1983). Soybean was introduced to the Canadian province of Ontario in 1881 (Shurtleff and Aoyagi, 2010), and has now grown into one of the region's most valuable field crops. In Canada, 2.05 million ha of agricultural lands were cultivated with soybean in 2020, producing 6.36 MMT of grain (Soy Canada, 2021).

One of the fascinating features associated with soybean is that its roots can form a mutualistic interaction with specialized soil bacteria termed rhizobia that inhabit underground root nodules; the rhizobia convert atmospheric nitrogen to fixed ammonia (Thilakarathna and Raizada, 2017). Generally, soybean is considered to be a high nitrogen fixing legume, wherein 68% of the plant's nitrogen, on average, can be derived from symbiotic nitrogen fixation (SNF) (Herridge et al., 2008). Because of this high nitrogen fixation ability, soybean is often used in crop rotations, where it facilitates a nitrogen credit to the proceeding crop (Karlen et al., 2006; Ziadi et al., 2014; Attia et al., 2015). However, the percentage of nitrogen derived from the atmosphere (%Ndfa) in soybean can vary from 0 to 95% (Herridge et al., 2008). This high variation in nitrogen fixation can be due to both biotic factors (e.g., genotypes, rhizobia spp.) as well as abiotic factors (e.g., drought, temperature, soil nitrogen availability) (Hungria and Vargas, 2000; Giller, 2001; Thilakarathna and Raizada, 2017, 2018; Santachiara et al., 2019).

Nitrogen fixation in soybean can vary depending on the growth stages of the plant. In general, SNF is lowest during the vegetative stage, then rapidly increases between R1 and R3 growth stages, before peaking then dropping upon pod formation (post R5 stage) (Zapata et al., 1987; Pitumpe Arachchige et al., 2020). In soybean, a significant portion (59%) of seed N is derived from remobilization of vegetative N, which represents N accumulated from SNF and soil uptake at the R5.5 stage (Ortez et al., 2019). In-season SNF provides nitrogen inputs to develop vegetative biomass, and SNF is positively correlated to the aboveground plant biomass in soybean (Córdova et al., 2019). Therefore, SNF inputs during the vegetative stage, flowering stage, and pre-pod-fill stage help to build the plant's N reserves

and subsequently contribute to seed yield and seed protein production (Ortez et al., 2019).

Modern soybean breeding programs such as the program in Ontario have mainly focused on improving yield, oil content and quality, disease resistance, and days to maturity (Morrison et al., 2000; Jin et al., 2010; Bruce et al., 2019a; Liu et al., 2020). By contrast, direct selection for improved symbiotic nitrogen fixation traits has not been a priority. Similar to the practice of farmers, the Guelph soybean breeding program has been conducted in the absence of synthetic nitrogen inputs, instead relying on residual soil nitrogen. We hypothesized that there may have been inadvertent selection for enhanced SNF traits (e.g., nodule number) resulting in improved overall nitrogen fixation. The corollary hypothesis is that modern cultivars are not suffering from genetic drift with respect to SNF traits, as a result of continuous indirect selection.

The Ontario Agriculture College (OAC) at the University of Guelph began breeding soybeans in 1970, aiming to produce high-yielding cultivars for Ontario growers (Bruce et al., 2019b). A recent study categorized the cultivars released from the University of Guelph into two groups, pedigree-related historical germplasm (1913–1985) and modern cultivars (cultivars developed at the Guelph campus and released between 1983 and 2015) (Bruce et al., 2019a,b). These cultivars have not been systematically analyzed for pre-pod-fill SNF related traits. The objectives of this study were: (1) to evaluate and compare the Guelph cultivars, based on their year of release from the historical group and modern group, for trends in pre-pod-fill SNF-related traits and accumulated vegetative nitrogen fixation as measured during the R1-R3 stages; (2) to understand the relationships between different agronomic traits for pre-pod-fill SNF; and (3) to identify historic and modern soybean cultivars as potential sources for improved nitrogen fixation at this stage.

MATERIALS AND METHODS
Germplasm and Field Trials

Soybean cultivars for this study were selected from a large study that evaluated the changes in soybean traits over 100 years of selection in Ontario, Canada (Bruce et al., 2019a). Among the 44 soybean cultivars selected for this study, 19 cultivars were pedigree related historical cultivars that were released between 1913 and 1985, and 25 cultivars were selected from the Guelph soybean (modern) breeding program, which were released between 1983 and 2015 (**Figure 1**; **Table 1**). The list of published cultivar registration papers with the year of public release is previously described by Bruce et al. (2019a). Pedigrees were visualized using GraphViz 2.38 (www.graphviz.org) allowing

FIGURE 1 | Images of historical (19) and modern-Guelph (25) soybean cultivars used in this study with name and release year.
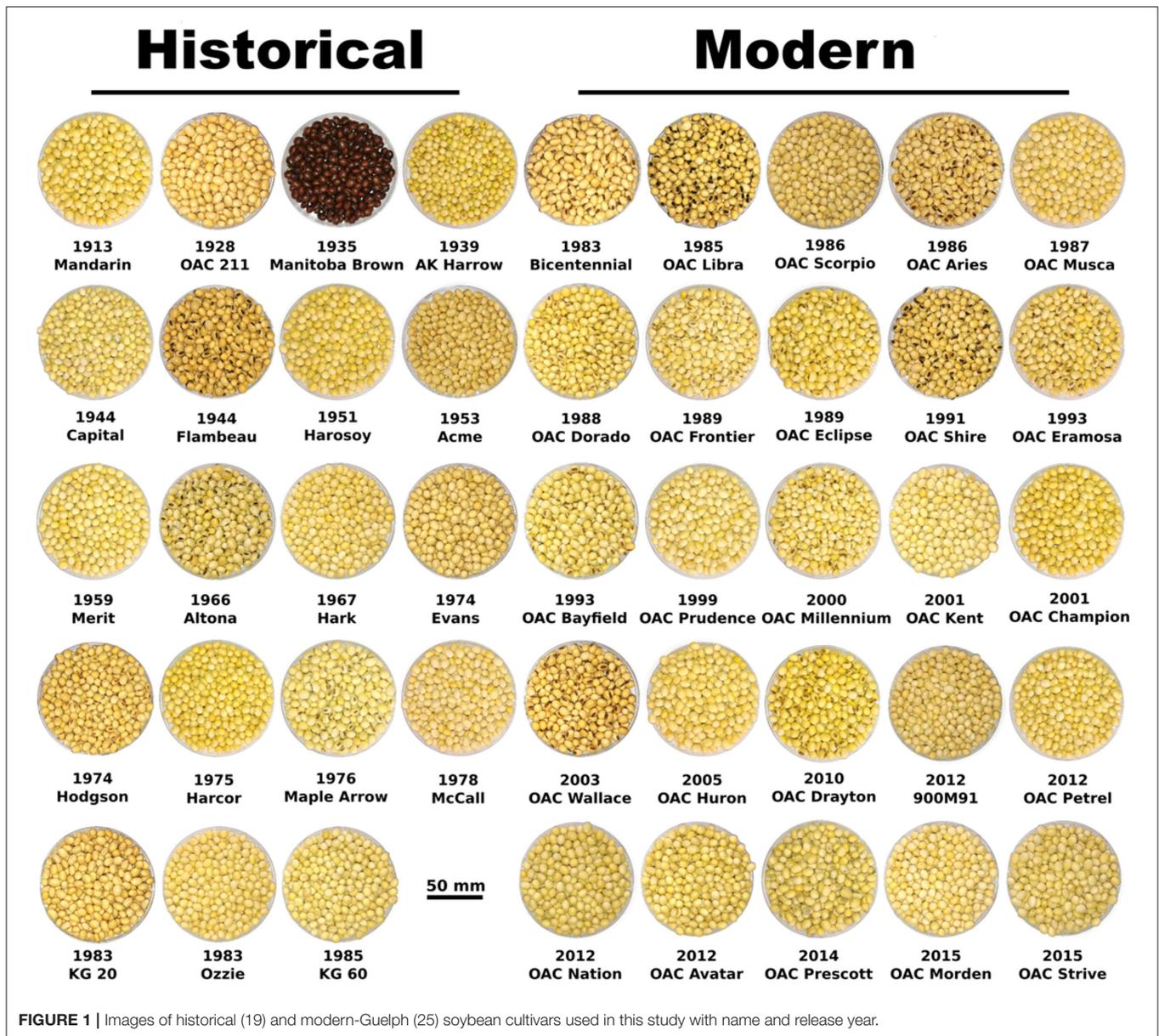
historical connections to be organized according to year-of-release for studied accessions (**Supplementary Figure 1**). All the soybean cultivars were grown at the Woodstock Research Station (Woodstock, ON; 43.145223° N, 80.782389° W; Guelph loam soil type [fine-loamy, mixed, active, mesic Haplic Glossudalfs]) in 2016 and 2017, as part of a larger breeding program study. Each field was previously cultivated with corn in 2015 and 2016. In 2016, the field was supplied with N (10 kg N ha^{-1}), P (40 kg P ha^{-1}), and K (40 kg K ha^{-1}) fertilizer, wherein only K (10 kg ha^{-1}) fertilizer was applied in 2017 before soybean seeding. Broadstrike (RC 86.5 g ha^{-1}) and Dual II Magnum (1.73 L ha^{-1}) were applied as pre-plant herbicides, and Reflex (1 L ha^{-1}), Pinnacle Toss-n-Go (1 pouch ha^{-1}), Venture (2 L ha^{-1}), and Turbocharge (1 L ha^{-1}) were applied as post-emergence herbicides in both production years. Soybean cultivars were planted as a replicated randomized completely block design, with two blocks, wherein each plot consisted of 4 rows, spaced 35 cm apart, and 5 m in length. A 40 cm space between plots and 2 m alley was maintained. Seeds were planted on 8 June in both 2016 and 2017 using a tractor-mounted planter at a seeding rate of 500 seeds per plot. Seeds were not inoculated with commercial rhizobia inoculants as the fields have been used for soybean in the past. Plants were grown under rainfed conditions and plots were not irrigated. Monthly daily maximum, minimum, daily mean temperatures, and precipitation during the 2016 and 2017 growing seasons are shown in **Supplementary Table 1**. Total rainfall received during the two growing seasons (May-June) was 448.8 mm (2016) and 442.9 mm (2017). The field site at

TABLE 1 | Ranking of soybean cultivars based on accumulated nitrogen derived from the atmosphere (%Ndfa) in shoots prior to the pod-filling stage in the 2016 and 2017 seasons.

Cultivar	Origin	Year release	Overall		2016		2017	
			%Ndfa	Rank	%Ndfa	Rank	%Ndfa	Rank
OAC Champion	Modern-Guelph	2001	67.6	1	65.7	2	69.5	1
OAC Frontier	Modern-Guelph	1989	64.5	2	59.7	12	69.2	2
OAC 211	Historical	1928	64.3	3	69.1	1	59.4	12
Hodgson	Historical	1974	63.8	4	65.7	2	61.8	10
OAC Huron	Modern-Guelph	2005	63.7	5	63.9	7	63.4	8
McCall	Historical	1978	61.9	6	57.7	19	66.1	3
OAC Bayfield	Modern-Guelph	1993	61.2	7	58.1	18	64.4	5
OAC Dorado	Modern-Guelph	1988	60.5	8	65.7	2	55.3	21
KG 20	Historical	1983	60.5	9	65.2	5	55.7	18
Hark	Historical	1967	60.4	10	57.2	21	63.6	7
OAC Prescott	Modern-Guelph	2014	60.2	11	56.6	22	63.8	6
OAC Shire	Modern-Guelph	1991	58.6	12	55.9	24	61.3	11
OAC Strive	Modern-Guelph	2015	58.3	13	53.7	25	62.9	9
OAC Avatar	Modern-Guelph	2012	58.0	14	59.7	12	56.3	16
OAC Petrel	Modern-Guelph	2012	56.9	15	58.5	16	55.4	20
OAC Scorpio	Modern-Guelph	1986	56.8	16	48.4	33	65.1	4
KG 60	Historical	1985	56.7	17	64.0	6	49.4	31
Mandarin	Historical	1913	55.4	18	62.4	9	48.4	34
Bicentennial	Modern-Guelph	1983	54.9	19	58.5	16	51.4	26
Harcor	Historical	1975	54.6	20	56.6	22	52.6	23
Altona	Historical	1966	54.6	21	61.5	10	47.6	35
OAC Kent	Modern-Guelph	2001	54.6	22	59.4	14	49.7	30
OAC Wallace	Modern-Guelph	2003	53.9	23	57.4	20	50.3	28
Flambeau	Historical	1944	53.7	24	58.7	15	48.6	33
OAC Prudence	Modern-Guelph	1999	53.1	25	50.7	28	55.5	19
OAC Libra	Modern-Guelph	1985	53.0	26	51.0	27	55.0	22
OAC Millennium	Modern-Guelph	2000	51.6	27	44.7	36	58.5	13
OAC 05-02	Modern-Guelph	2012	50.9	28	63.8	8	38.0	40
OAC Drayton	Modern-Guelph	2010	50.8	29	50.6	30	51.0	27
Acme	Historical	1953	50.1	30	61.3	11	38.8	39
OAC Aries	Modern-Guelph	1986	49.1	31	46.2	35	52.0	25
OAC Morden	Modern-Guelph	2015	48.7	32	42.3	37	50.1	29
Manitoba Brown	Historical	1935	48.1	33	49.6	31	46.6	36
OAC Eclipse	Modern-Guelph	1989	47.2	34	37.0	39	57.4	15
OAC Musca	Modern-Guelph	1987	45.7	35	51.8	26	39.5	38
Ozzie	Historical	1983	45.4	36	34.9	41	55.8	17
OAC Nation	Modern-Guelph	2012	43.5	37	29.2	44	57.8	14
Harosoy	Historical	1951	43.5	38	50.7	28	36.3	42
Merit	Historical	1959	43.0	39	49.5	32	36.5	41
Evans	Historical	1974	42.2	40	35.2	40	49.1	32
Capital	Historical	1944	41.7	41	30.9	43	52.5	24
Maple Arrow	Historical	1976	41.4	42	38.2	38	44.6	37
OAC Eramosa	Modern-Guelph	1993	41.2	43	48.3	34	34.1	43
AK Harrow	Historical	1939	29.9	44	32.9	42	26.8	44

Statistics			Mean	SEM	Mean	SEM	Mean	SEM
Overall			53.2	1.19	53.1	1.58	52.9	1.48
Historical cultivars (*n* = 19)			51.3	2.13	52.7	2.86	49.5	2.31
Modern-Guelph cultivars (*n* = 25)			54.6	1.30	53.5	1.79	55.5	1.80

SEM, standard error mean.

Woodstock, Ontario received 3,421 crop heat units (CHU) in 2016 and 3,408 CHU in 2017.

Plant Sampling and Measurement of SNF-Related Traits

In 2016 and 2017, three representative plants were collected at the R1-R3 stage from the middle rows at 8 weeks after seeding from each plot representing each soybean genotype. Plants were uprooted using a shovel, where roots and shoots were separated to measure the number of nodules, nodule dry weight, root dry weight, and shoot dry weight. The dry weight of tissue samples was measured after drying samples for 3 days in a forced air oven at 60°C. Shoot samples were ground using a Wiley mill standard model 3 (Arthur H Thomas Co., Philadelphia, PA) to pass through a 1-mm sieve followed by a Bead Ruptor 12 Homogenizer (OMNI International GA) (Thilakarathna et al., 2017). The shoot materials were analyzed for ^{15}N (shoot-δ^{15}N), %N (shoot-N%), and %C using an Isotope Ratio Mass Spectrometer (IRMS) fitted with a Flash 2000 Elemental Analyzer (Thermo Fisher Scientific, Voltaweg, Netherlands) and Conflo IV (Thermo Fisher Scientific, Bremen, Germany) interface between the IRMS and the analyzer at the Stable Isotope Lab, Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre. The proportion of N derived from the atmosphere (%Ndfa) in different soybean cultivars was calculated according to the natural abundance method (Wilker et al., 2019):

$$\%Ndfa = \left(\frac{\delta^{15}N \; reference \; plant - \delta^{15}N \; soybean \; plant}{\delta^{15}N \; reference \; plant - B} \right) \times 100$$

Where δ^{15}N is the ^{15}N enrichment of shoot materials relative to atmospheric nitrogen. The δ^{15}N reference plant was the mean of two reference weeds {Thistle [*Cirsium arvense* (L.) Scop.] and Lamb's quarters [*Chenopodium album* L.]} collected from the same fields, which was used as the non-N-fixing reference plant. The *B* value is the ^{15}N enrichment of the soybean shoots grown solely on atmospheric N, where a *B* value of $-1.674 \; \delta^{15}$N was used. The amount of shoot N derived from nitrogen fixation was calculated based on the shoot N content (mg N plant^{-1}) and %Ndfa (shoot-N content × %Ndfa/100).

Statistical Analysis

Phenotypic data of SNF-related traits [nodule number, nodule dry weight, shoot and root biomass, shoot total N content, shoot ^{15}N abundance (δ^{15}N), %Ndfa, and total shoot N fixed] were analyzed using analysis of variance (ANOVA). Also, a nested *t*-test was performed to compare historical vs. modern-Guelph cultivars within each production year for agronomic traits. Results were expressed at a significance level of $p < 0.05$. Linear regression of different plant traits by year of cultivar release was conducted ($p < 0.05$). Correlation analysis between different plant traits was performed using the Pearson correlation test. All these statistical analyses were performed using GraphPad Prism Software (v9, GraphPad Software, San Diego, CA, USA).

RESULTS

Origins and Phenotypic Characteristics of Soybean Varieties

The panel of 44 soybean cultivars was selected for this study as representative of Central Canadian (Ontario) soybean germplasm diversity over the last 100 years. Of these cultivars, 19 were historical founding accessions as traced through pedigree records (released between 1913 and 1985) while 25 were elite cultivars derived from modern breeding at the University of Guelph (released between 1983 and 2015) (**Figure 1**). As such, this panel represents the current diversity of Ontario soybean germplasm. All these cultivars were grown at the Woodstock Research Station (University of Guelph, ON, Canada) in 2016 and 2017, and phenotypic data for 10 SNF-related traits were measured (**Figure 2**). Significant year × cultivar interaction was observed for all the pre-pod-fill parameters tested, except for shoot dry weight (SDW) (**Supplementary Table 2**). Therefore, data are presented separately for each year.

Diversity for Pre-Pod-Fill SNF-Related Traits

In general, the pre-pod-fill nodule number, nodule dry weight, root dry weight, and shoot dry weight were higher in 2017 compared to the 2016 production year (**Figures 2A–H**). Historical cultivars had higher number of nodules in 2016 (**Figure 2A**). No significant differences were found for pre-pod-fill nodule dry weight, root and shoot dry weight, shoot-N%, shoot-N content, shoot-C/N ratio, and shoot-δ^{15}N among the historical vs. modern-Guelph categories in 2016 (**Figures 2C–P**). However, the root and shoot dry weight were significantly higher in modern-Guelph cultivars compared to the historical cultivars in 2017. The shoot-δ^{15}N content was higher in historical cultivars in 2017 compared to the modern-Guelph cultivars (**Figure 2P**).

We observed a similar level of pre-pod-fill %Ndfa (53%) between 2016 and 2017 (**Figures 2Q,R**). When %Ndfa was analyzed for each year, no significant differences were found between historical and modern-Guelph cultivars in 2016, but modern-Guelph cultivars had higher %Ndfa (55.5%) compared to the historical cultivars (49.5%) in 2017 (**Figure 2R**). However, the amount of shoot-N fixed prior to the pod-fill stage was higher in 2016 (123.7 mg N plant^{-1}) compared to 2017 (114.1 mg N plant^{-1}) (**Figures 2S,T**). Similar to the %Ndfa trend, no significant differences in the amount of total shoot-N fixed were found between historical and modern-Guelph cultivars in 2016 prior to pod fill (**Figure 2S**). However, modern-Guelph cultivars fixed more total shoot N (122 g N plant^{-1}) in 2017 compared to the historical cultivars (105 g N plant^{-1}) (**Figure 2T**).

Shoot Nitrogen Fixation Ranking

All the soybean cultivars were ranked based on their shoot vegetative nitrogen fixation capacity prior to pod fill as measured by the %Ndfa (**Table 1**). Across the two growing seasons, %Ndfa varied from 29.9% to 67.6%, with an average value of 53.2%. In 2016, %Ndfa varied from 29.2% (OAC Nation) to 69.1% (OAC 211), with an average value of 53.1%, whereas in 2017 %Ndfa varied from 26.8% (AK Harrow) to 69.5% (OAC Champion),

FIGURE 2 | Distribution of SNF-related traits in historical and modern-Guelph lines in 2016 and 2017 prior to pod-fill. **(A,B)** Number of nodules per plant. **(C,D)** Nodule dry weight per plant. **(E,F)** Root dry weight per plant. **(G,H)** Shoot dry weight per plant. **(I,J)** Shoot nitrogen percentage. **(K,L)** Shoot total nitrogen content per plant. **(M,N)** Shoot C/N ratio. **(O,P)** Shoot $\delta^{15}N$. **(Q,R)** Percentage nitrogen derived from the atmosphere (%Ndfa). **(S,T)** Shoot nitrogen fixed per plant. Each data point represents the mean value of a single cultivar. The asterisks (*, **) indicate significant difference between the 19 historical and 25 modern-Guelph cultivars at $p < 0.05$ and $p < 0.01$, respectively.

with an average of 52.9%. Although no significant difference was found for %Ndfa between historical and modern-Guelph groups in the 2016 season, when ranked overall, four of the five bottom ranked cultivars (Capital, AK Harrow, Ozzie, Evans) belonged to the historical category (**Table 1**). As mentioned above, a significant difference was found for shoot %Ndfa between historical and modern-Guelph cultivars in 2017; it was

observed that four of the five top-ranked cultivars in 2017 (OAC Champion, OAC Frontier, OAC Scorpio, OAC Bayfield) belonged to the modern-Guelph category (**Table 1**).

Trends in SNF-Related Traits
Regression analysis was performed across different agronomic traits by year of cultivar release to identify trends since 1915.
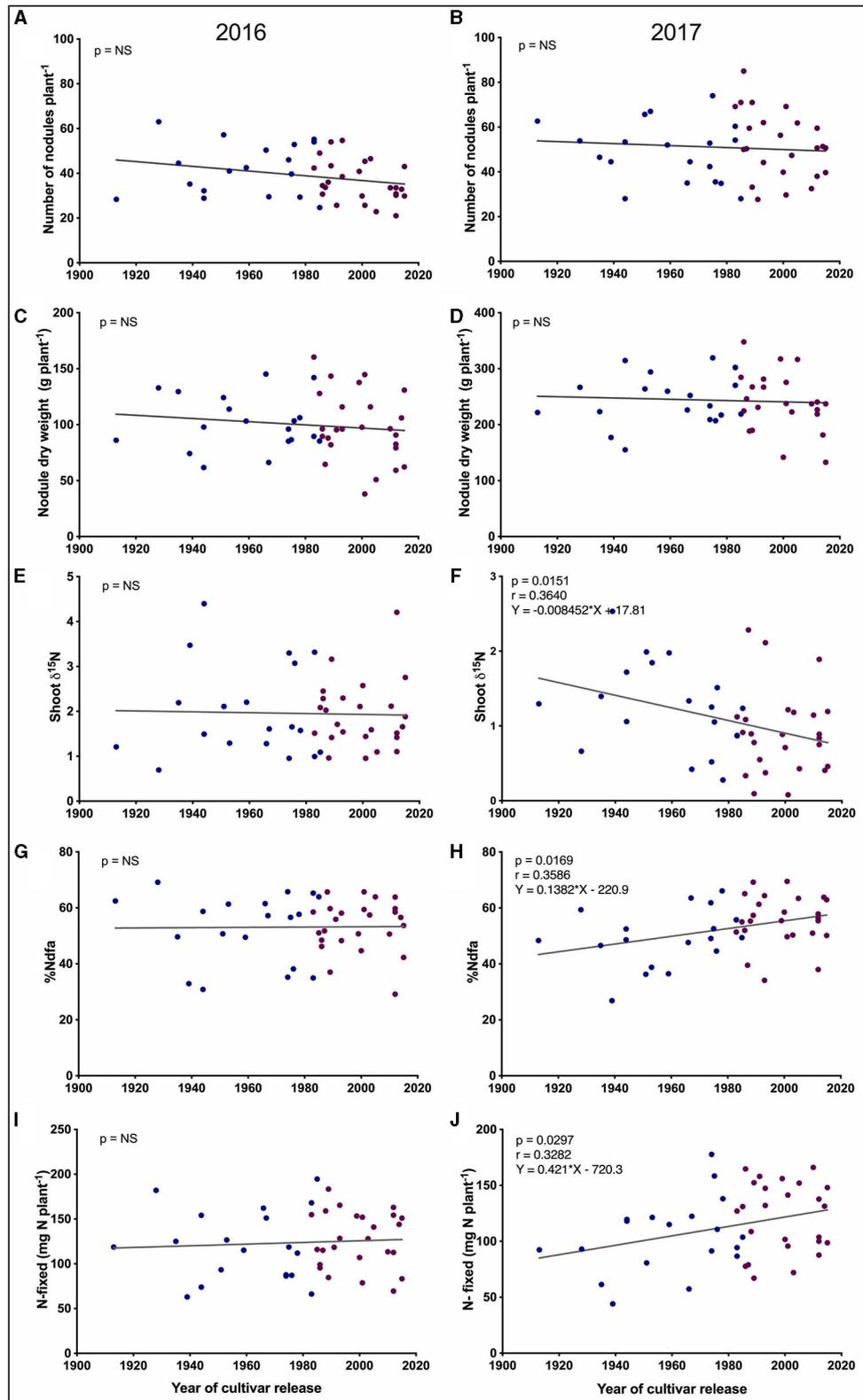
FIGURE 3 | Trait by year of cultivar release regression plots for pre-pod-fill SNF-related traits. **(A,B)** Number of nodules per plant. **(C,D)** Nodule dry weight per plant. **(E,F)** Shoot δ^{15}N. **(G,H)** Percentage nitrogen derived from the atmosphere (%Ndfa). **(I,J)** Shoot nitrogen fixed per plant. Each blue and purple dot represents a historical and modern-Guelph cultivar, respectively. %Ndfa and N-fixed represent the vegetative shoot values. NS, not significantly different at $p < 0.05$.

For nodule number and nodule dry weight prior to pod-fill, no significant trends were identified based on the year of cultivar release either in 2016 or 2017 (**Figures 3A–D**). Similarly, no significant trends were identified for root and shoot dry weights based on the year of cultivar release in both 2016 and 2017 (**Supplementary Figures 2A–D**). Furthermore, no significant trends were observed for shoot N% (**Supplementary Figures 2E,F**), shoot C/N ratio (**Supplementary Figures 2G,H**), and total shoot N content prior to pod fill (**Supplementary Figures 2I,J**) based on the year of cultivar release either in 2016 or 2017.

We found a significant negative trend for pre-pod fill shoot δ^{15}N ($p = 0.0151$, $r = -0.3640$) based on the year of cultivar release in 2017 (**Figure 3F**), but this trend was not observed in 2016 (**Figure 3E**). In 2017, significant positive trends were found for %Ndfa ($p = 0.0169$, $r = 0.3586$) and total shoot-N fixed ($p = 0.0297$, $r = 0.3282$) based on the year of cultivar release (**Figures 3H,J**, respectively), but no significant trends were observed for %Ndfa and total shoot-N fixed in 2016 (**Figures 3G,I**).

Correlation Among SNF-Related Traits

Across all 44 cultivars, we found that pre-pod-fill nodule number was positively correlated to nodule dry weight ($r = 0.68$, 0.66), root dry weight ($r = 0.31$, 0.35), shoot dry weight ($r = 0.34$, 0.30), and total shoot-N content ($r = 0.30$, 0.32) in both 2016 and 2017, respectively (**Figure 4**). Additionally, there was a negative correlation between nodule number and shoot-C/N ratio ($r = -0.32$) in 2016. Nodule dry weight was positively correlated to root dry weight ($r = 0.56$, 0.48), shoot dry weight ($r = 0.70$, 0.59), and total shoot-N content ($r = 0.47$, 0.39) in 2016 and 2017, respectively (**Figure 4**). We also found that root dry weight was positively correlated with shoot dry weight ($r = 0.53$, 0.71), and total shoot-N content ($r = 0.39$, 0.61) in both 2016 and 2017, respectively (**Figure 4**). Across the cultivars, there was no significant correlation between the pre-pod-fill nodule number and %Ndfa or amount of shoot-N fixed either in 2016 or 2017. However, nodule dry weight was positively correlated to %Ndfa in 2016 ($r = 0.30$) and amount of shoot-N fixed in 2016 ($r = 0.50$) and 2017 ($r = 0.37$) (**Figure 4**). Additionally, the amount of shoot-N fixed prior to pod fill was positively correlated to root dry weight ($r = 0.31$, 0.44), shoot dry weight ($r = 0.64$, 0.54), total shoot-N content ($r = 0.75$, 0.73), and %Ndfa ($r = 0.71$, 0.39) in 2016 and 2017, respectively (**Figure 4**).

DISCUSSION

Modern Breeding Has Maintained SNF in Soybean Prior to the Pod Fill Stage

Accumulated symbiotic nitrogen fixation starting from the seedling stage to the pre-pod fill stage is important to fulfill part of the N demand for soybean grain yield and seed protein. Despite peak nitrogen demand occurring at the pod fill stage in soybean, overall the results of this study highlight that modern soybean breeding in the Canadian province of Ontario has maintained, and possibly modestly improved, the absolute rates of pre-pod-fill symbiotic nitrogen fixation, based on 2 years of field trials.

Three lines of evidence support this statement. First, in the 2017 trial, across the 44 cultivars, the pre-pod-fill shoot %Ndfa was significantly higher amongst the modern cultivars, though no significant difference was observed in 2016. Based on the cultivar year of release, the total nitrogen fixed and shoot %Ndfa prior to pod fill showed a significant but modest positive trend in the 2017 trial with no trend in 2016. Among the bottom ranked 10 cultivars for %Ndfa, seven cultivars were historical cultivars, suggesting modern cultivars may have improved SNF; perhaps breeding has removed some but not all of the worst SNF traits prior to pod fill. Improvement in pre-pod-fill SNF has been modest: the average nitrogen fixation capacity (averaging both trial years) was 51.3% for the historical cultivars compared to 54.6% for the modern cultivars. These SNF results align with the recently published average soybean %Ndfa values (56%) (Ciampitti and Salvagiotti, 2018).

Despite the above trends, a critical observation is that there was wide variation amongst the modern Guelph lines for %Ndfa (41–68%) and other SNF traits prior to pod fill, suggesting that in some pedigrees, SNF may have suffered from some level of genetic drift because of a lack of direct selection for SNF traits prior to the peak demand for nitrogen at the pod fill stage. To the best of our knowledge, soybean breeding in Ontario has not directly selected for improved SNF rates either before or during pod fill. This was consistent with the results of this study that showed no significant trends for nodulation parameters and shoot nitrogen based on the year of cultivar release. In soybean, nodulation and SNF rates vary depending on the growth stage (Zapata et al., 1987; Yinbo et al., 1997; Gan et al., 2003). In general, nitrogen fixation sharply increases starting at the flowering stage (R1) and peaks at R4-R5; the pre-pod SNF provides substantial N inputs to develop the aboveground biomass (Zapata et al., 1987; Yinbo et al., 1997; Ortez et al., 2019). These nitrogen reserves in the vegetative biomass help to meet subsequent seed nitrogen demand in soybean, especially since SNF drops post- R5.5, during the pod filling stages and/or under limited soil nitrogen supply (Córdova et al., 2019). It would be interesting to explore whether changes in alleles responsible for vegetative nitrogen remobilization to pods (Masclaux-Daubresse et al., 2010; Zhao et al., 2014) have an inverse correlation with alleles that promote SNF traits late in development. In soybean, variation in N remobilization from leaves during pod fill has been observed (Locke and Ramirez, 2020). Limited soil nitrogen during pod-filling is relevant to Canadian growing conditions, since farmers do not apply nitrogen fertilizer for soybean production. Therefore, it may be helpful to identify soybean varieties that have high SNF prior to the pre-pod-fill stage to meet the subsequent nitrogen demand of the seeds.

Modern soybean breeding programs, including in Ontario, mostly focus on yield and seed oil quality improvement, not nitrogen fixation as already noted (Bruce et al., 2019a; Liu et al., 2020). However, as soybean breeding trials in Ontario have been conducted in the absence or minimum of nitrogen inputs, breeding for improved grain yields may have maintained or improved biological nitrogen fixation even at the pre-pod-fill stage—despite breeders not directly selecting for improved SNF rates. Previous studies have shown positive correlations between
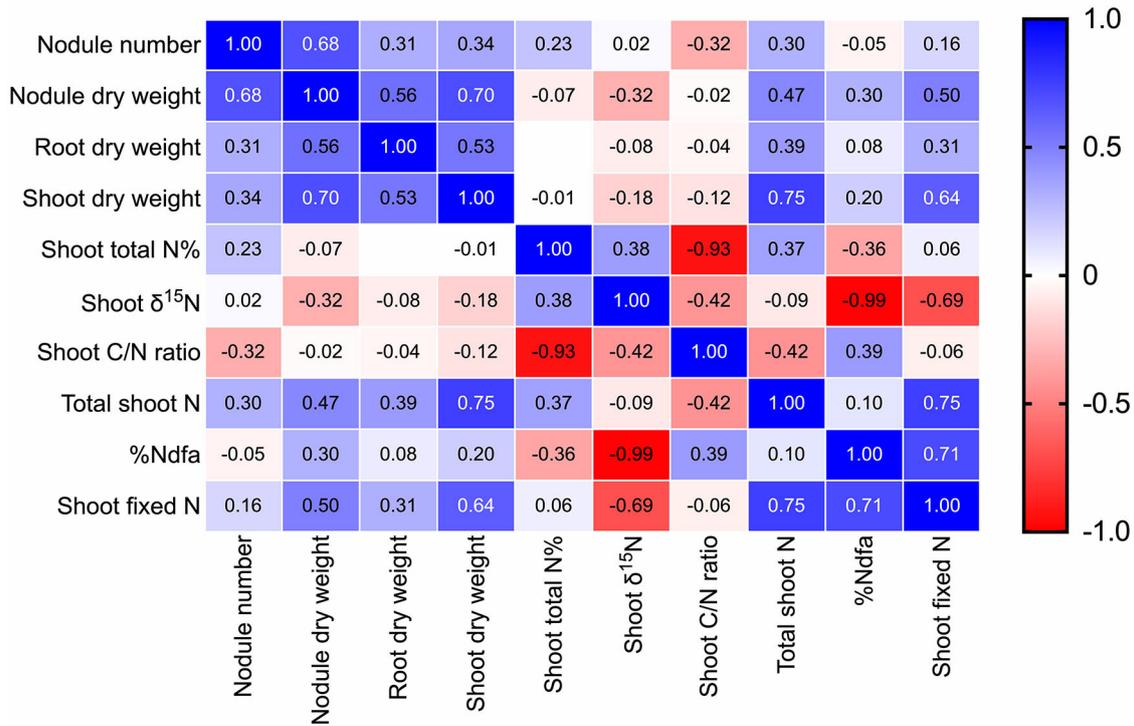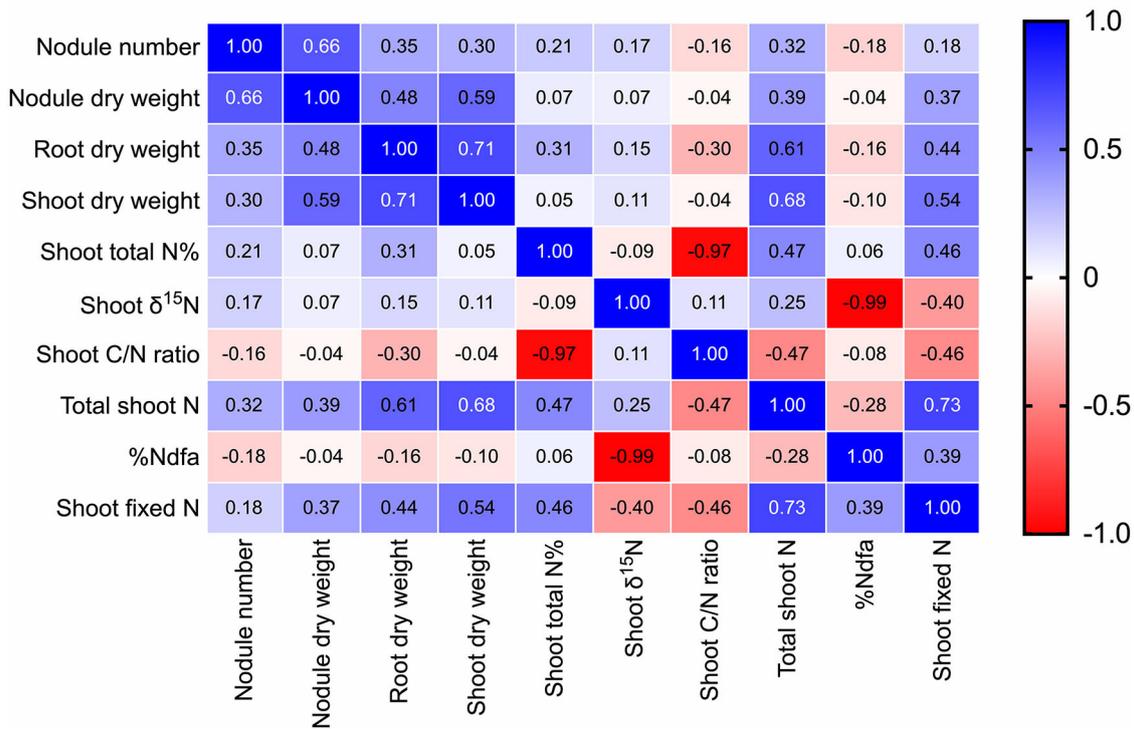
FIGURE 4 | Pearson correlation matrix for pre-pod-fill nodulation parameters (nodule number and dry weight), plant growth (root and shoot dry weight), shoot nitrogen (nitrogen concentration and total nitrogen), shoot $\delta^{15}N$, shoot C/N ratio, and shoot nitrogen fixation (%Ndfa and total nitrogen fixed) in soybean in 2016 and 2017 (using mean data). Correlation coefficients (*r*) in individual cells represent each correlation between variables. The boldness of the color represents the strength of the relationship between variables, with stronger correlations having bolder colors.

grain yield and SNF in soybean (Salvagiotti et al., 2008; Collino et al., 2015; Ciampitti and Salvagiotti, 2018).

Nodules are the major nitrogen source tissues that support vegetative and reproductive sink development. It is highly likely that selecting for improved soybean yield (i.e., reproductive sink development) has inadvertently impacted the source (nodule) tissue strength, including source-to-sink allocation processes. This could account for the observed stability or slight improvement in the symbiotic nitrogen fixation trait. In addition to impacting nodule mass, a positive effect on nodule metabolism may have been at play here, a notion supported by the observation that the increased nodule to shoot nitrogen allocation leads to improved seed yield (Carter and Tegeder, 2016).

This study did not extend into the pod-fill stage; it would be interesting to know the extent to which the SNF-related trends identified in this study correlate with later stages of soybean development. At the molecular level, it would be interesting to know if loci that regulate SNF traits during vegetative stages always control these traits during pod-fill, or whether different loci act at different growth phases. In pea, a genotype was identified wherein the majority of its nodulation occurred during the reproductive stage (Depret and Laguerre, 2008).

Furthermore, seeds were not inoculated with rhizobia strains, as the field sites have been used for growing soybean many times in the past and hence there would be a reservoir of compatible rhizobia in the soil. However, new soybean varieties might be more adapted to newer rhizobia strains that are available through commercial inoculants—in this case, this study may be underestimating improvements in SNF made by modern breeding. Soybean breeding under low N inputs and inoculation with effective rhizobia strains was the paradigm in Brazil for their success in improved biological nitrogen fixation in soybean (Alves et al., 2003), helping Brazil to become the world's highest soybean producer in 2019.

Traits that Predict Symbiotic Nitrogen Fixation in Ontario Soybean

We did not find any positive correlation between the number of nodules per plant and %Ndfa. Although nodule number has been used as a major indicator of nitrogen fixation capacity (%Ndfa) in the literature, it has also shown limited proxy value in some studies (Hungria and Bohrer, 2000; Hungria et al., 2001; Thilakarathna and Raizada, 2017). However, in this study, nodule dry weight was positively correlated with the total shoot-N fixed. Among these two nodulation traits, nodule dry weight had a positive correlation with the amount of shoot-N fixed; thus, it can be used as a better proxy for effective nitrogen fixation, at least for Ontario soybean breeding. Hungria and Bohrer (2000) also found that nodule mass was a better indicator of nitrogen fixation than nodule number in terms of shoot-N accumulation. In soybean, various studies have identified loci that contribute to nodule size including GmINS1 (Hwang et al., 2014; Li et al., 2018), so it would be interesting to know if they have been under selection in breeding programs. We also found positive correlations between nodule number/dry weight, and shoot and

root dry weights, similar to the recent findings (Torkamaneh et al., 2020), where 297 African soybean cultivars were screened for nitrogen fixation related traits. In the current study, shoot N fixed also had a consistent positive correlation with the total shoot N content, suggesting that nitrogen fixation is critical for building up nitrogen reserves even during the pre-pod formation stage. Recent findings by Córdova et al. (2019) suggest that biomass production is the best predictor of SNF in Midwest US soybean. Our correlation data also showed that shoot biomass production was a good predictor of total N fixed but not a good predictor of %Ndfa. At the molecular level, it would be interesting to explore the relationship between alleles that regulate hormone pathways or other pathways involved in shoot growth promotion including branching (Ongaro and Leyser, 2008; Haq et al., 2017) with those that regulate SNF traits. For example, in soybean, GmKIX8-1 has been shown to increase the size of aerial plant organs including leaves as well as seeds (Nguyen et al., 2021).

Identification of Historical and Modern Cultivars for Improved SNF in Soybean

The results of this study show that considerable variation exists in Canadian soybean cultivars released over the past 100 years for nitrogen fixation prior to pod fill. The above noted drifting among modern cultivars, especially the low level of SNF in some modern cultivars (e.g., OAC Morden), and wide variation available in modern lines, suggests opportunities for deliberate selection for SNF prior to pod fill, and possibly during pod fill. The best cultivar from this study derived ∼70% of its nitrogen from the atmosphere prior to pod fill, which would be considered average based on previous studies in soybean around the world (Herridge et al., 2008). Either this represents an upper limit for SNF in the generally fertile soils of Southern Ontario, where the soil itself can contribute N, or this limit can be raised, given that previous studies have shown that up to 95% of soybean nitrogen can come from the atmosphere (Herridge et al., 2008). On average, among the top-10 ranked cultivars for %Ndfa, five belonged to the modern-Guelph category (OAC Champion, OAC Frontier, OAC Huron, OAC Bayfield, OAC Dorado), and five belonged to the Guelph-Historical group (OAC 211, Hodgson, McCall, KG 20, Hark). This observation suggests that some of the historical and modern cultivars may be good sources of SNF alleles for future breeding programs, especially if similar results are obtained during pod fill. None of these best 5 historical cultivars are close parents of modern cultivars in Ontario, suggesting the best historical lines have been under-utilized. This study has also identified historic and modern cultivars with low %Ndfa (e.g., AK Harrow, OAC Eramosa), suggesting that these cultivars may possess alleles with poor absolute performance for pre-pod-fill SNF. An important caveat to these statements is that the trials in this study were conducted at one site (used for soybean breeding), whereas the breeding historically has been undertaken at multiple sites within Southern Ontario. Perhaps some of the poor performing lines for %NDFA might do better at other locations, having a sandy soil, cooler/warmer temperature, different organic matter, higher/lower soil N or specific micronutrients, and/or local/introduced rhizobia strains.

CONCLUSION AND FUTURE DIRECTIONS

Overall, this study has revealed that pre-pod-fill nitrogen fixation capacity and associated symbiotic nitrogen fixation traits, in modern soybean cultivars (post-1983 year of release) from Ontario, Canada, are similar or modestly higher as a group compared to earlier historical lines (starting in 1913). This data supports the hypothesis that, despite a lack of direct selection, there has been indirect selection pressure to maintain and possibly improve pre-pod-fill nitrogen fixation traits in modern soybean breeding. Cultivars with higher pre-pod-fill SNF may significantly contribute to the nitrogen reserves in vegetative parts and meet the subsequent seed nitrogen demand during the pod filling stage. However, the wide variation observed for SNF among modern cultivars, in the context of the study limitations, suggests some level of genetic drift for pre-pod-fill SNF in the absence of direct selection for this trait. As some historical and modern soybean cultivars possess high nitrogen fixation capacity, they may have the potential to improve this trait in future breeding programs especially if these genotypes behave similarly at later stages.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MT, DT, and MR conceived of the manuscript. MT, MR, RB, and IR conceptualized the study. MT, RB, GC, and CG conducted all experiments. KS and BH involved in sample analysis. MT wrote the manuscript. DT and MR edited the manuscript. All authors discussed the results, commented on the manuscript, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.725813/full#supplementary-material

Supplementary Figure 1 | Pedigree relationships of the 44 historical and modern-Guelph accessions tracing back to historical founder accessions, plotted by year of release (cultivars). Blue shows historical cultivars, while red shows modern-Guelph cultivars. Data from: (Bruce et al., 2019b).

Supplementary Figure 2 | Trait by year of cultivar release regression plots for pre-pod-fill agronomic traits. **(A,B)** Root dry weight per plant. **(C,D)** Shoot dry weight per plant. **(E,F)** Shoot N%. **(G,H)** Shoot C/N ratio. **(I,J)** Total shoot nitrogen content. Each blue and purple dot represents a historical and modern-Guelph variety, respectively. NS, not significantly different at $p < 0.05$.

Supplementary Table 1 | Monthly daily maximum, minimum, daily mean temperatures, and total precipitation at Woodstock, Ontario during the 2016 and 2017 growing seasons.

Supplementary Table 2 | Analysis of variance (ANOVA) table for the plant parameters tested across 44 soybean cultivars in two production years (2016 and 2017).

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