Front. Agr. Sci. Eng. 2022, 9(2): 177–186 https://doi.org/10.15302/J-FASE-2021421

RESEARCH ARTICLE

ROOT EXUDATES FROM CANOLA EXHIBIT BIOLOGICAL NITRIFICATION INHIBITION AND ARE EFFECTIVE IN INHIBITING AMMONIA OXIDATION IN SOIL

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KEYWORDS

ammonia oxidizing microorganisms, biological nitrification inhibition, farming rotations, nitrogen cycling, nitrogen use efficiency

HIGHLIGHTS

- First evidence of BNI capacity in canola.
- BNI level was higher in canola cv. Hyola 404RR than in *B. humidicola*, the BNI positive control.
- BNI in canola may explain increased N immobilization and mineralization rates following a canola crop which may have implications for N management in rotational farming systems that include canola.

Received May 13, 2021;
Accepted July 19, 2021.

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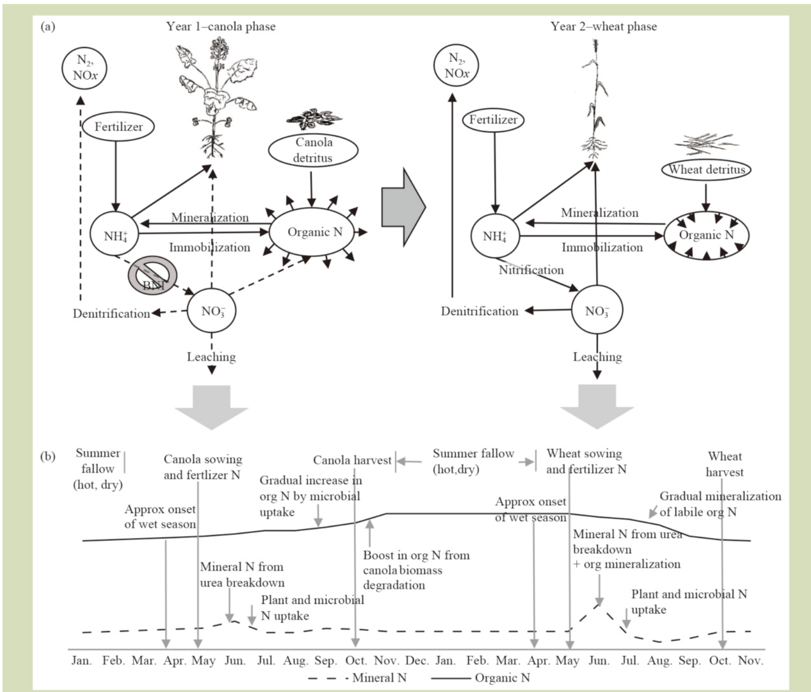
GRAPHICAL ABSTRACT



ABSTRACT

A range of plant species produce root exudates that inhibit ammonia-oxidizing microorganisms. This biological nitrification inhibition (BNI) capacity can decrease N loss and increase N uptake from the rhizosphere. This study sought evidence for the existence and magnitude of BNI capacity in canola (*Brassica napus*). Seedlings of three canola cultivars, *Brachiaria humidicola* (BNI positive) and wheat (*Triticum aestivum*) were grown in a hydroponic system. Root exudates were collected and their inhibition of the ammonia oxidizing bacterium, *Nitrosospira multiformis,* was tested. Subsequent pot experiments were used to test the inhibition of native nitrifying communities in soil. Root exudates from canola significantly reduced nitrification rates of both *N. multiformis* cultures and native soil microbial communities. The level of nitrification inhibition across the three cultivars was similar to the well-studied high-BNI species *B. humidicola.* BNI capacity of canola may have implications for the N dynamics in farming systems and the N uptake efficiency of crops in rotational farming systems. By reducing nitrification rates canola crops may decrease N losses, increase plant N uptake and encourage microbial N immobilization and subsequently increase the pool of organic N that is available for mineralization during the following cereal crops.

1 INTRODUCTION

Nitrification, the microbially mediated conversion of ammonium to nitrate, is a key step in the global nitrogen (N) cycle in soils. The oxidation of NH_4^+ to nitrite (NO_2^-), the first and the rate limiting step in nitrification, is of particular interest in agricultural systems because it is the starting point for the subsequent uptake of NO_3^- by crops (as opposed to direct plant assimilation of NH_4^+) and may potentially reduce N loss pathways such as NO_3^- leaching and gaseous N emissions via denitrification[1].

Ishikawa et al.[2] established the term biological nitrification inhibition (BNI) to describe the ability of the tropical grass *B. humidicola* to suppress ammonia oxidation (nitrification) in soils. After a series of studies on this species, Subbarao and coworkers reported that inhibition was caused by several compounds released into the rhizosphere from plant roots[2–6]. It was proposed that by holding N in the NH_4^+ form, BNI-capable plants could limit leaching and denitrification losses of N from soil and potentially increase N use efficiency by providing crops a greater supply of available N over a longer period during the growing season[7,8].

Canola (*Brassica napus*) has become an increasingly popular crop grown in rotation with wheat (*Triticum aestivum*) in Australian farming systems over the past 20 years, with the area sown increasing from ~ 100 kha in the 1980s to > 2.5 Mha by 2014[9]. Growers give several reasons, beyond the value of the oilseed itself, for choosing to grow canola. Most commonly, it provides an important tool in weed management and it is a valuable disease-break crop for cereal production systems[10]. In addition to these benefits, there is evidence that canola may also influence soil N dynamics in ways that lead to the preservation of N in the soil[11]. Australian dryland (rainfed) grain crops, including canola, receive on average ~ 45 $kg \cdot ha^{-1}$ fertilizer-N[12]. While the rate of fertilizer N application is higher in other crops (e.g., sugarcane, *Saccharum officinarum*, horticultural crops), dryland crops are grown over a much greater area (> 20 Mha dryland including ~ 2.5 Mha under canola vs. ~ 350 kha under sugarcane) and, as a result, dryland systems dominate fertilizer N inputs in Australia (1.08 Mt N compared to 0.06 Mt N in sugarcane systems[12]. Therefore, increasing the N use efficiency of dryland cropping systems, including canola/wheat rotational systems, has the potential to have significant impact on total N inputs to agricultural systems in Australia.

There are conflicting data in the literature concerning the impact of canola on soil N availability, with some studies reporting greater mineral-N concentrations in soils following a canola rotation[10,13–15] and others observing little difference in soil mineral N after canola or wheat[10,15–17]. It has been shown that soils amended with Brassica root tissues initially immobilized, and later released, mineral N at a greater rate than soils amended with wheat root tissues[11]. Changes to the N cycling by microbial communities during canola rotations have also been reported which led to increased mineral-N accumulation over a summer fallow following canola compared to cereals or legumes[18]. It is feasible that altered N dynamics in canola crops may be explained, at least in part, by BNI capacity.

Several studies have demonstrated that application of whole meals and stubbles from several different *Brassica* species significantly decreased ammonia-oxidizing microbial populations and associated rates of nitrification[19–21]. However, to date, there have been no reports of BNI activity by growing canola roots. *Brassicas*, including canola, produce glucosinolate (GSL) compounds that break down into isothiocyanates that are released during tissue degradation[22]. These compounds have been shown to influence microbial communities in soils treated with *Brassica* stubbles and meals[22], and are also known to be released by intact *Brassica* roots in soil[23]. Even though the potential role of GSL-related compounds in N cycling remains unclear, Ryan et al.[11] found no relationship between N dynamics and GSL concentrations in plant tissues, suggesting that compounds other than GSL may be involved. Consequently, we investigated the capacity of canola to release compounds into the rhizosphere that have direct effect in decreasing nitrification rates in both culture and soil-based assays. Evidence of BNI activity in canola has implications for both the N uptake efficiency of the canola crop itself, and the subsequent N cycling and N availability for following crops in the sequence.

2 METHODS

2.1 Testing of root exudates for BNI capacity

Three cultivars of spring canola (*B. napus annua*) were grown hydroponically and the root exudates were collected to test their impact on nitrification rates of the common soil ammonia-oxidizing bacterium *Nitrosospira multiformis*. The cultivars were selected to give representatives of current commercially grown canola cultivars, namely: (1) a roundup-ready hybrid (cv. Hyola 404RR) resistant to the herbicide glyphosate, (2) a triazine tolerant hybrid (cv. Hyola 555TT) resistant to triazine herbicides, and (3) an open-pollinated triazine tolerant cultivar (cv. Stingray). *Brachiaria humidicola* cv. Tully which is known to have high BNI capacity, and (4) a wheat cultivar (Janz) which does not have BNI capacity[24] were also included as positive and negative controls, respectively, for comparative purposes.

2.1.1 Growth conditions

Seeds of each of the plant lines were germinated on nutrient-free agar in the dark for 4–7 days to allow rootlets to form and then three replicates were transplanted into a hydroponics system. The hydroponic growth solution contained 0.10 g·L^{-1} KNO_3, 0.05 g·L^{-1} NH_4Cl, 0.04 g·L^{-1} KH_2PO_4, 0.07 g·L^{-1} $MgSO_4{\cdot}7H_2O$, 0.09 g·L^{-1} K_2SO_4, 0.03 g·L^{-1} FeEDTA, 0.11 g·L^{-1} MES, 0.07 g·L^{-1} $CaCl_2{\cdot}2H_2O$, 0.05 mg·L^{-1} $CuSO_4{\cdot}5H_2O$, 0.31 mg·L^{-1} H_3Bo_3, 0.01 mg·L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 0.22 mg·L^{-1} $ZnSO_4{\cdot}7H_2O$, and 0.18 mg·L^{-1} $MnSO_4.H_2O$. The pH of the nutrient solution was adjusted daily with 10 mol·L^{-1} NaOH solution to maintain a pH of 6–7. In the hydroponics setup, 50 L of nutrient solution was recycled through a system of six trays each containing five plants. The nutrient solution was circulated among all six trays in the set and was replaced weekly.

The hydroponic system was installed in a growth cabinet with a 10:14 h L:D photoperiod at 24°C/15°C and 70% RH. The plants were grown for 4 weeks after transplanting to hydroponics. This time period provided sufficient root mass for the collection of root exudates but avoided the root balls becoming so large that they began to interact with the roots of neighboring plants in the hydroponics trays.

2.1.2 Root exudate collection and bioassay to determine BNI levels in root exudates

Root exudates were collected and tested to assess the level of nitrification inhibition as described previously[25]. Briefly, root exudates were collected by immersing the root ball of each plant into a complex nutrient solution designed to support growth of *N. multiformis* over a 24-h period. Each biological replicate was made up of a single plant and exudates were collected from three replicates. The plants were then removed, samples were buffered to pH 7.0 (with $NaHCO_3$) and frozen at −20°C until assayed.

Pure cultures of *N. multiformis* (ATCC 25198) were sourced from the American Type Culture Collection (Manassas, VA). *N. multiformis* is an ammonia-oxidizing bacterium that is commonly isolated from soils[26] and converts NH_4^+ to NO_2^- to gain energy using CO_2 as its carbon source. The culture was maintained and used to assess the impact of root exudates on nitrification rates as described by O'Sullivan et al.[25]. The BNI assay involved growing *N. multiformis* cultures in the presence and absence of the root exudates, and the rates of nitrite production were tracked colorimetrically using the Griess method[27]. BNI was measured as the percentage decrease in NO_2^- production rate in the root exudate-treated cultures relative to the untreated controls.

2.2 Assessment of BNI in soils

The impact of the three canola cultivars on nitrification rates in soil was tested to confirm the BNI activity of the plants observed in the root exudate bioassays. Four replicate plants were grown in separate rectangular root stock pots (7 cm × 7 cm × 20 cm) in bulk potting mix that was known to have a moderate potential nitrification rate under controlled incubation conditions (~ 1 mg NO_3^- formed kg^{-1} dry soil h^{-1}) (i.e., each biological replicate was made up of a single plant). Unplanted control pots were included to give a measure of the background nitrification rates in the soil under the experimental conditions and *B. humidicola* was included as a BNI positive control and wheat (cv. Janz) as a BNI negative control.

Plants were grown for five weeks in a glasshouse. This provided time for the roots to develop and explore sufficient soil volume. Soil samples were then collected by upturning the pots, removing the plants by hand and gently shaking the soil off the roots into plastic bags. All soil was collected from each pot so the samples were a combination of rhizosphere and bulk soil surrounding the rhizosphere. These samples were well mixed and 15-g subsamples were extracted as representative samples for the subsequent analysis.

The effect of BNI on nitrification rates in whole soils was assessed using a shaken-slurry potential nitrification rate (PNR) test[28] in which 15 g of soil were placed in 100 mL of medium containing 1 mmol·L^{-1} PO_4^{3-} and 1.5 mmol·L^{-1} NH_4^+ then incubated at 26°C in a shaker incubator rotating at 100 r·min^{-1}. Five-mL slurry samples were collected after 2, 4, 20 and 24 h. The samples were centrifuged, filtered and the NH_4^+ and NO_2^-/NO_3^- contents of the filtrate were determined by continuous flow analysis on an AA1 segmented flow analyzer (Seal Analytical, Norderstedt, Germany). NO_3^- formation was assessed in combination with NO_2^- in the PNR because the conversion of NO_2^- to NO_3^- in soils under the experimental conditions is rapid and NO_2^- is rarely present in significant concentrations. Separate subsamples were analyzed for gravimetric water content and all measurements are reported on a soil dry weight basis. BNI capacity for each species was then calculated by comparing the nitrification rate in the presence of the plant with the nitrification rate in the unplanted controls as described below.

2.3 Statistical analysis

Nitrification rates in both the root exudate assays and the pot trials were calculated from linear regressions of nitrate (NO_2^- and NO_3^-) concentrations versus time as described by Hart et al.[28]. BNI capacities were then calculated as the percentage reduction in the nitrification rate in the root exudate samples relative to the uninhibited controls:

$$\text{Biological nitrification inhibition} = (1-\left(\frac{\text{rate}_{\text{inhib}}}{\text{rate}_{\text{control}}}\right)\times 100 \quad (1)$$

Differences in nitrification rates were statistically tested by the grouped linear regression function in the Genstat statistical package (16th Edition, VSN International, Hemel Hempstead, England, UK).

3 RESULTS

The root exudates from the three canola cultivars tested all inhibited ammonia oxidation by a pure culture of *N. multiformis* to varying degrees (Fig. 1). Of the three canola cultivars, cv. Hyola 404RR produced root exudates with the highest BNI, causing a 56% reduction in ammonia oxidation by *N. multiformis*. Root exudates of cv. Hyola 555TT caused 41% reduction in ammonia oxidation whereas cv. Stingray caused 25% inhibition. Exudates from the positive control, *B. humidicola*, reduced nitrification by 54%. Unexpectedly, the two hybrid cultivars had similar (Hyola 555TT) or superior (Hyola 404RR) BNI values to *B. humidicola* throughout the experiment (Fig. 1).

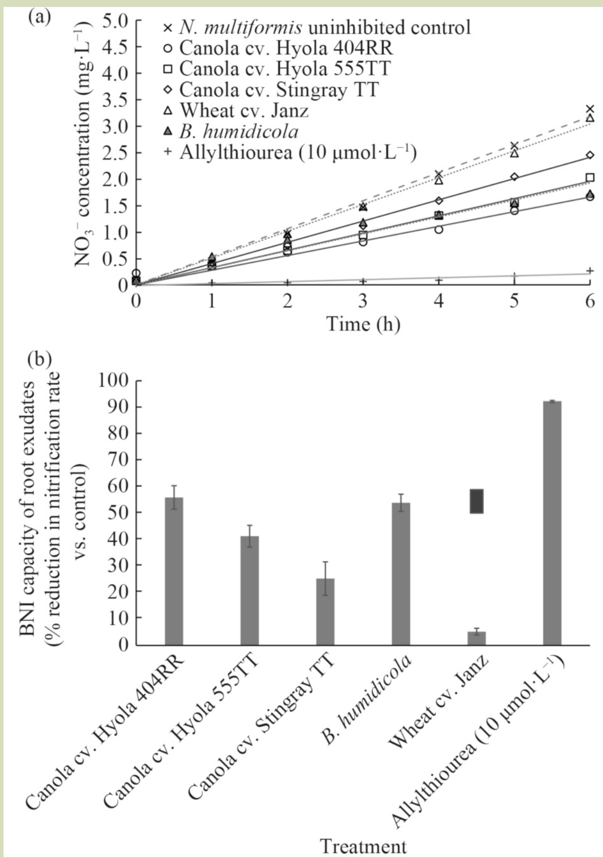


Fig. 1 NO_3^- generation over time (a) and biological nitrification inhibition capacity of root exudates (b) of three canola cultivars (*Brassica napus* cvs Hyola 404RR, Hyola 555TT and Stingray TT), *Brachiaria humidicola* and wheat cv. Janz. Error bars show standard error of the mean of three replicates. Black bar in panel B indicates least significant difference from grouped linear regression analysis at *P* < 0.05. Error bars in panel A are small and often not visible because they sit behind the marker.

The BNI capacities measured in the root exudate tests were confirmed in soil in the pot experiment (Fig. 2). The three canola cultivars inhibited nitrification rates by between 26% and 62%. Consistent with the ranking in the exudate experiment, cv. Hyola 404RR produced the highest level of BNI, followed by cv. Hyola 555TT then cv. Stingray. All three cultivars produced BNI values that were significantly higher than that of wheat cv. Janz. Canola cv. Hyola 404RR inhibited nitrification significantly more than the positive control *B. humidicola* (Fig. 2), whereas the other two cultivars showed BNI values that were equivalent to *B. humidicola.*

4 DISCUSSION

This study indicates that several canola cultivars release root exudates that inhibit nitrification. In the root exudate study the two hybrid cultivars Hyola 404RR and Hyola 555TT produced similar levels of BNI to *B. humidicola* and all three cultivars showed significantly higher levels than the BNI negative control wheat cv. Janz (Fig. 1). The pot study confirms that root exudates were produced and released during early growth of the three cultivars at levels that were sufficient to significantly slow the nitrification rates of a mixed microbial community in unsterilized soil (Fig. 2). Previous studies have shown that *Brassica* stubbles and meals release compounds that inhibit nitrification as they degrade[19–21,29] but this is the first evidence, to our knowledge, that canola releases BNI from its roots as they grow.

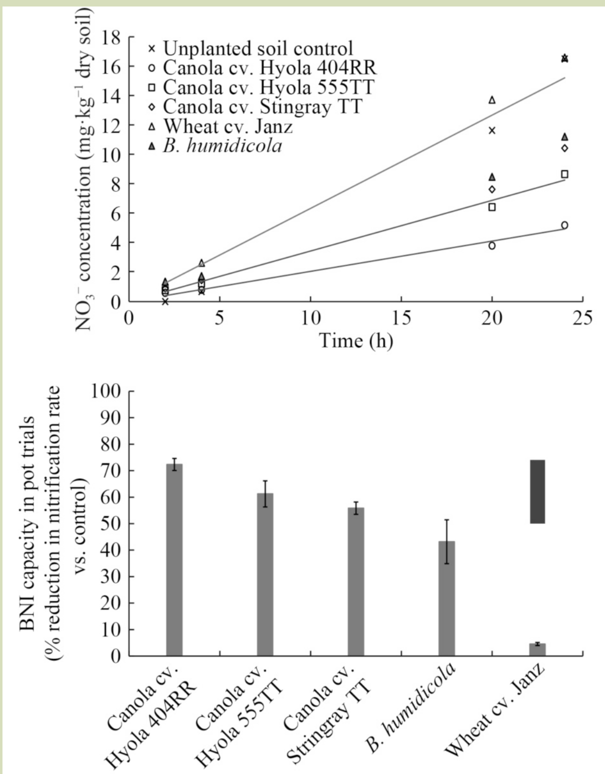


Fig. 2 NO$_3^-$ generation rates (a) and biological nitrification inhibition (BNI) in soils (b) from the rhizospheres of three canola cultivars, *Brachiaria humidicola* and wheat cv. Janz. LSD indicates least significant difference from grouped linear regression analysis at $P < 0.05$. Error bars show the standard error of the mean over four replicates.

B. humidicola is the most studied species of BNI-producing plants. Various research groups, particularly the group led by Gunter Subbarao, have documented evidence of BNI in this species at both laboratory and field scales[8,30–32]. These studies have explored the underlying mechanisms of BNI in root exudates[6,30,31] and have investigated the impact on downstream N cycling in soils and the nutrition of following crops[33].

The ecological driver for BNI evolution has not been studied in detail but it is hypothesized that species with BNI capacity gain a competitive advantage by altering the N cycle, leading to accumulation of NH_4^+ in the soil, allowing them to outcompete neighboring species that have lower affinity for NH_4^+ assimilation[34,35]. Modeling studies have shown that BNI can lead to increased primary production in some species under certain conditions[36]. There is some evidence that canola can assimilate NH_4^+ more efficiently than NO_3^-[37] and several other *Brassica* spp. have been shown to use a mix of both NH_4^+ and NO_3^- for growth[38,39]. It might be anticipated that plants with high BNI capacity would have a preference for NH_4^+, or a mix of NH_4^+ and NO_3 because slower nitrification rates would lead to an increased proportion of NH_4^+-N in the root zone, although it is unlikely that the production of NO_3^--N would be totally excluded.

It has also been hypothesized that BNI may have evolved as a competitive response to low N inputs in some ecosystems[40]. Plant species composition has been shown to influence N cycling in natural ecosystems[33,41–43]. It has been suggested that BNI may be responsible for the low nitrification rates that tend to occur in climax ecosystems and are often regarded as an indicator of ecosystem maturity[40,42].

The results of our laboratory study indicate that root exudates from three canola cultivars contain compounds that slow nitrification by a pure culture of *N. multiformis* to varying degrees (Fig. 1). Similar differences in levels of BNI between genotypes have been shown in several species including *B. humidicola*, rice (*Oryza sativa*), sorghum (*Sorghum bicolor*) and wheat[5,24,44–46]. The high and varied levels of BNI we observed in the three canola cultivars relative to *B. humidicola* suggest that further screening of canola germplasm is warranted to investigate the full extent of genotypic variation for the BNI trait in canola. Canola has been shown to have significant genetic variation in other root compounds such as glucosinolates, and these traits are under relatively simple genetic control[47,48]. If this is also the case for BNI, it may be possible to select and breed for BNI and to use the trait to increase N uptake and N use efficiency. In addition to understanding the genotypic variation in the production of BNI compounds, further understanding of BNI activity in relation to differences in root morphology and localized effects due to root structure is warranted. In this experiment BNI has been measured on a per plant basis rather than per unit of root mass or length. Studies with other root exudate compounds and other plant species have shown that root morphology can affect both exudation and microbial community structure, both of which may impact BNI capacity[49].

4.1 Implications in canola-cereal farming rotations

It has been shown that BNI-positive pastures (*B. humidicola)* and crops (sorghum) can have positive impact on following crops. Karwat et al.[33] used a combination of laboratory-based soil incubations and field experiments with ^{15}N to show that BNI from *B. humidicola* altered the N cycle leading to an accumulation of organic N during the pasture phase which was subsequently mineralized, providing an additional N source during the next maize (*Zea mays*) cropping phase. The residual BNI effect of the preceding pasture phase also decreased nitrification during cropping, further reducing the loss of N which was later available for mineralization and maize N uptake. Zhang et al.[50] showed that N-fertilized vegetable crops grown after sorghum hybrid variety sorgo had higher yields, significantly increased agronomic N use efficiency and significantly lower emissions of the potent greenhouse gas nitrous oxide (N_2O) compared to vegetables following other crops.

The use of canola in rotation in mixed cropping systems has similarly been shown to yield benefits for the growth of following cereal crops[10,51]. In addition to benefits for weed management and disease-break effects of a canola crop, changes in N immobilization, mineralization and nitrification rates have been observed in soils amended with Brassica residues[11]. Shifts in N cycling by microbial communities during a canola rotation have also been observed and are associated with increased mineral-N accumulation over a summer fallow following canola[18].

BNI from canola may initiate a series of changes to the N cycle that may explain these observations (Fig. 3). A decrease in nitrification due to BNI may decrease losses of mineral N through NO_3^- leaching or denitrification. The NH_4^+ retained can then be assimilated by plants, consumed by the soil microbial community[52], or held in the upper soil layers. These N pools would subsequently be available throughout the crop growing season and potentially contribute to increased crop N use efficiency. Since it has been demonstrated that soil microorganisms absorb NH_4^+-N preferentially over NO_3-N[52], the elevated rates of N immobilization reported by Ryan et al.[11] are consistent with the presence of high concentrations of NH_4^+-N for longer during the canola growing season supporting a larger microbial community. Increased uptake and assimilation of NH_4^+-N by microorganisms will promote a larger soil organic N pool and provide a potentially greater source of N that can be subsequently mineralized. Kirkegaard et al.[18] showed that more mineral N accumulated in the field following canola than following legumes, suggesting that there is significant conservation of N during the growth of a canola crop. Also, canola crops drop large quantities of leaves and petals onto the soil after flowering compared to cereals, and this biomass may further contribute to soil organic pools (Fig. 3).

To explain the higher mineral-N availability and altered N dynamics following canola we propose a mechanism where BNI from canola roots slows nitrification rates during the growth of the crop thereby retaining more N as NH_4^+ for more of the growing season and decreasing N losses in two ways. First, decreased NO_3^--N production would lead to lower N losses due to leaching and denitrification[53]. Secondly, and concurrently, elevated NH_4^+ concentrations would allow for greater N uptake by the canola crop and/or microbial community leading to immobilization of N in plants and microbial organic matter[52,54]. Collectively, these two processes would retain a greater proportion of N in the organic fraction of the soil after the canola crop is harvested (Fig. 3). In Mediterranean climates, as in Western Australia, low soil water contents over summer generally mean that little microbial activity occurs thereby retaining N in organic forms until the break of the season the following year[55]. The onset of rain in the following winter season would allow the microbial community to become active, mineralizing the organic N pool leading to a release of stored organic N.

5 CONCLUSIONS

This study confirms that canola has significant BNI capacity that was similar to or exceeded that of *B. humidicola*, as

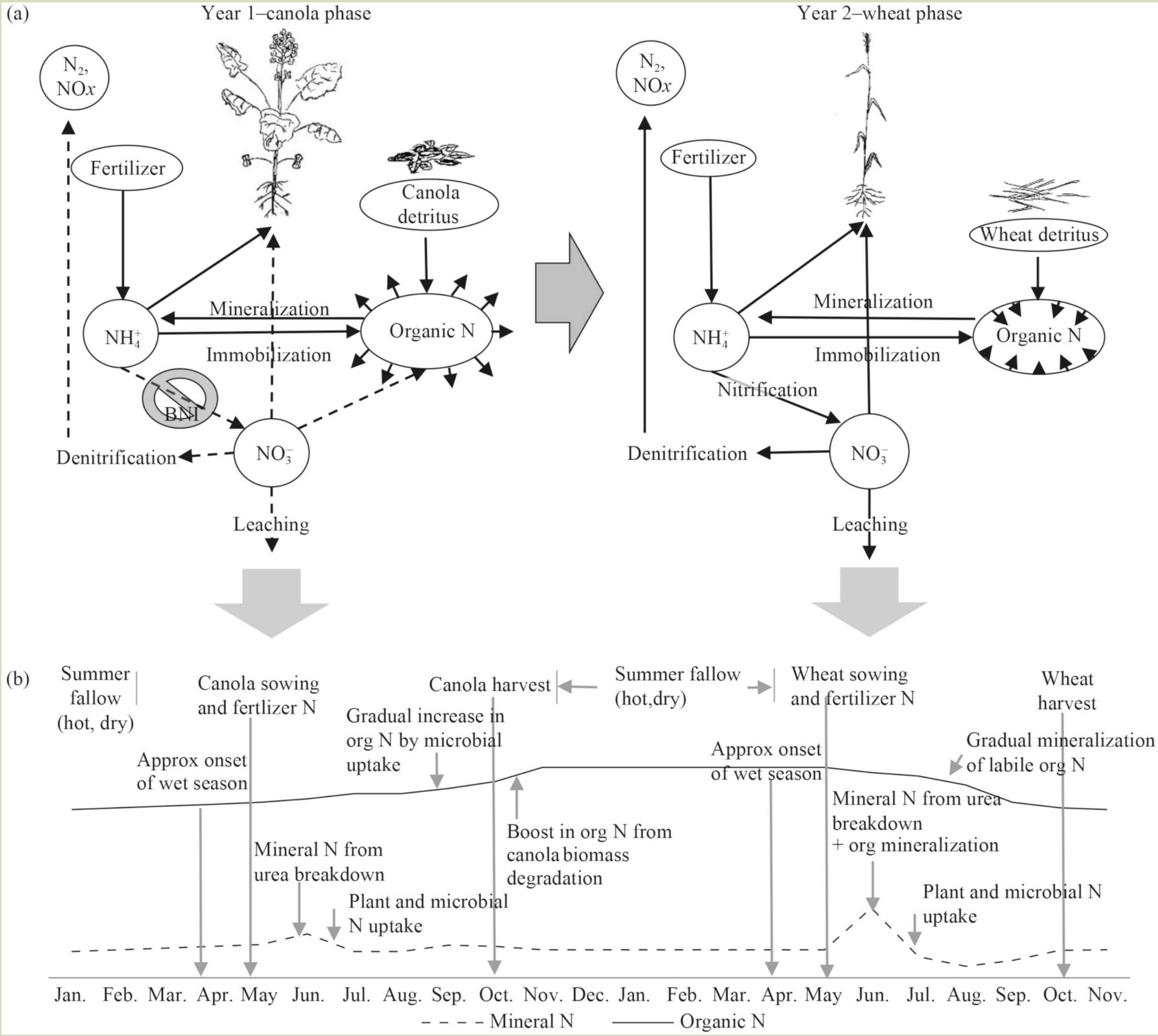


Fig. 3 Conceptual diagram of the impact of BNI from a canola crop on the immobilization and mineralization of N during the course of a canola-wheat cropping sequence. (a) The mechanistic differences in soil N cycle of the two crops with size of arrows indicative of N flows. (b) Hypothesized changes in soil pools of mineral (dashed) and organic N (solid).

demonstrated by BNI assay in both laboratory assays with *N. multiformis* cultures and nitrification rates under non-sterile soil conditions. There were also significant differences between the three canola cultivars that were consistent in both the non-soil and soil environments. Additional work to further evaluate genetic variation in BNI in canola is warranted. Exploitation of BNI in canola has implications for the N dynamics of the canola crop itself and for the availability of N in subsequent rotation crops leading to more efficient use of N in agricultural systems. Further investigations are required to confirm BNI values in canola grown under field conditions and to quantify the links between BNI, increased immobilization to organic N and subsequent mineralization of organic N to supply the subsequent crop.

Compliance with ethics guidelines

Cathryn A. O'Sullivan, Elliott G. Duncan, Margaret M. Roper, Alan E. Richardson, John A. Kirkegaard, and Mark B. Peoples declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any study with human or animal subjects performed by any of the authors.

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