REGULAR ARTICLE

Can biological nitrification inhibition (BNI) genes from perennial *Leymus racemosus* (*Triticeae*) combat nitrification in wheat farming?

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Abstract Using a recombinant luminescent *Nitrosomonas europaea* assay to quantify biological nitrification inhibition (BNI), we found that a wild relative of wheat (*Leymus racemosus* (Lam.) Tzvelev) had a high BNI capacity and releases about 20 times more BNI compounds (about 30 ATU g^{-1} root dry weight 24 h^{-1}) than *Triticum aestivum* L. (cultivated wheat). The root exudate from cultivated wheat has no inhibitory effect on nitrification when applied to soil; however, the root exudate from *L. racemous* suppressed NO_3^- formation and kept more than 90% of the soil's inorganic-N in the NH_4^+-form for 60 days. The high-BNI capacity of *L. racemosus* is mostly associated with chromosome Lr#n. Two other chromosomes Lr#J, and Lr#I also have an influence on BNI production. Tolerance of *L. racemosus* to NH_4^+ is controlled by chromosome 7Lr#1-1. Sustained release of BNI compounds occurred only in the presence of

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NH_4^+ in the root environment. Given the level of BNI production expressed in DALr#n and assuming normal plant growth, we estimated that nearly 87,500,000 ATU of BNI activity ha^{-1} day^{-1} could be released in a field of vigorously growing wheat; this amounts to the equivalent of the inhibitory effect from the application of 52.5 g of the synthetic nitrification inhibitor nitrapyrin (one AT unit of BNI activity is equivalent to 0.6 μg of nitrapyrin). At this rate of BNI production it would take only 19 days for a BNI-enabled wheat crop to produce the inhibitory power of a standard commercial application of nitrapyrin, 1 kg ha^{-1}. The synthetic nitrification inhibitor, dicyandiamide, blocked specifically the AMO (ammonia monooxygenase) pathway, while the BNI from *L. racemosus* blocked the HAO (hydroxylamine oxidoreductase) pathway in *Nitrosomonas*. Here we report the first finding of high production of BNI in a wild relative of any cereal and its successful introduction and expression in cultivated wheat. These results demonstrate the potential for empowering the new generation of wheat cultivars with high-BNI capacity to control nitrification in wheat-production systems.

Keywords Biological nitrification inhibition (BNI) · *Leymus racemosus* · Nitrification · Nitrogen use efficiency · *Nitrosomonas europaea* · Root exudate · *Triticum aestivum*

Introduction

Nitrification is a serious problem for agricultural production resulting in costly nitrogen loss, and currently can be reduced only by the application of synthetic nitrification inhibitors (reviewed by Subbarao et al. 2006a). The natural ability of plants to suppress nitrification is not currently recognized or utilized in agricultural production (Subbarao et al. 2006a). The awareness that plants even have the ability to release inhibitory compounds to suppress soil nitrification is only now developing (Ishikawa et al. 2003; Lata et al. 2004; Subbarao et al. 2006a, b; 2007a, b). The in-depth, direct examination of this process has only recently become possible with the development of an assay using recombinant luminescent *Nitrosomonas europaea* that can quantify nitrification inhibitor activity in the root zone (Subbarao et al. 2006b).

Studies using this assay have confirmed that *Brachiaria humidicola* (Rendle) Schweick, a tropical pasture grass, releases significant amounts of biologically active compounds that suppress soil nitrification (Subbarao et al. 2006b). If the high BNI capacity attribute such as found in *B. humidicola* were to be transferred to crop plants it could foster the development of cropping systems with the potential to increase the agronomic efficiency of nitrogen use and decrease the environmental impacts of nitrogen applications in agricultural production.

Evaluation of a number of major crops including wheat showed lack of significant BNI capacity (Subbarao et al. 2007b). However, volga or mammoth wildrye, *Leymus racemosus*, a perennial member of the *Triticeae* and a wild relative of wheat, barley and cultivated rye, showed high BNI capacity during preliminary studies. Relatives of wheat represent a rich gene pool for wheat improvement (Oliver et al. 2005). Genes from alien species have been successfully used to improve genetic resistance of wheat to numerous pathogens (Oliver et al. 2005). Introducing the whole chromosomes from wheat-alien species into cultivated wheat, a strategy widely exploited by wheat breeders to transfer pest and disease resistance from *L. racemosus* into cultivated wheat (Qi et al. 1997; Kishi et al. 2003, 2004; Chen et al. 2005). The present investigation is aimed at assessing the potential for the introduction of the high BNI trait into cultivated wheat using genetic stocks derived from hybridization with its wild relative, *L. racemosus*.

Materials and methods

Experiment 1: Assessment of BNI activity in root exudate

Experiment 1a: Growing plants for collecting root exudate

Seeds of cultivated wheat (*Triticum aestivum* L.) cv. Nobeokabouzu-komugi and Chinese Spring were germinated in a sand–vermiculite mixture (3:1) in trays and watered with distilled water. Plants were grown in a growth chamber with a day/night temperature regime of 24/20°C, a photosynthetic photon flux (PPF) averaging about 300 μmol m^{-2} s^{-1} (at canopy height), and a 13/11 h light/dark

photoperiod. The perennial wild relative, *L. racemosus* was grown from tillers each with two leaves in nutrient solutions. Ten-day-old wheat seedlings were transferred to aerated nutrient solutions where the plants were grown in 50 l tanks on styro-foam blocks having 12 holes with 5 plants per hole supported with sponges; nutrient solution composition and management of culture solutions were similar to described earlier (Subbarao et al. 2006b). There were three replications of two nitrogen treatments consisting of 2 mM N added as $(NH_4)_2SO_4$ or KNO_3. In the $(NH_4)_2SO_4$ treatment, 2 mM K was added as K_2SO_4 to provide a similar K levels in all treatments. The pH of the nutrient solution was adjusted daily to 6.5 using either 1 M NaOH or HCl.

Root exudate was collected from intact plants 40 days after transplantation to the hydroponic system. For collection of the root exudate, 20 plants were removed from the tanks and the roots of the intact plant were immersed in 1 L of 0.5 mM $(NH_4)_2SO_4$ or 1 mM KNO_3 solution for 1 h. This was followed by washing the intact plant roots with de-ionized water, followed by distilled water and then roots were immersed in 1 L of aerated ultra-pure distilled water in which the root exudate was allowed to collect for 24 h. The root exudate solution was then stored at 5°C until extraction of the BNI compounds. After root exudate collection, roots and shoots were separated, dried at 70°C for 48 h in a forced air-circulating oven before determining dry weight. Root exudate is processed for the determination of BNI activity as described earlier (Subbarao et al. 2006b).

Experiment 1b: Effectiveness of BNI compounds on soil nitrification

Collection and preparation of root exudate for soil application Root exudate was collected from cultivated wheat (cv. Chinese Spring) and *L. racemosus* grown with NH_4^+ as the N source. Ninety, 40-day-old wheat plants were used for collecting the root exudate using distilled water. For *L. racemous*, 30 plants grown for 40 days in hydroponics were used for collecting root exudate in distilled water. The growing conditions and collection of the root exudate were similar to those described above. For the incubation experiments with soil, the methanol extracts of root exudate was evaporated and re-extracted with double distilled water following the protocol reported earlier (Subbarao et al.

2006b). The pH of the resulting extract averaged 6.1 for cultivated wheat and 6.7 for *L. racemosus*.

The soil used for the incubation studies was a volcanic ash soil, Typic Hapludands [(pH H_2O) 6.0, clay 54.8%, silt 26.3%, sand 18.9%, total carbon= 29.2 mg g^{-1} soil; total N=2.5 mg g^{-1} soil; and C/N ratio of 11.7], collected from the JIRCAS (Japan International Research Centre for Agricultural Sciences) experimental field site in Tsukuba, Japan. The soil was air-dried, and passed through a 2-mm sieve before use. The soil water status during the experiment was maintained at a level where 60% of the pore space was water filled. This is considered optimum for nitrification (WFPS) (0.36 ml of water was required g^{-1} to give 60% WFPS for this soil) (Mosier et al. 1996). To confirm the effectiveness of BNI compounds in soil, three treatments were setup to determine the degree of soil nitrification inhibition after 30 and 60 days from the application of the aliquots of root exudate collected from *L. racemosus* [containing 30 ATU of activity released during a 24 h period from 1.2 g of intact plant roots (on a dry weight basis)]. Aliquots of root exudate from *T. aestivum* cv. Chinese Spring [containing 9.0 ATU of activity released during a 24 h period from 5 g of intact root (dry weight basis)], and a control where no inhibitor was added. For each treatment 400 μg of N as $(NH_4)_2SO_4$ were added to bottles containing 2 g soil and the remaining details of the soil incubation study were described earlier (Subbarao et al. 2006b). The experiment consisted of two sets of bottles, one incubated for 30 days and the second for 60 days. The experiment was replicated three times. After the incubation period, the soil samples were extracted by shaking with 20 ml of 2 M KCl for 30 min, and then filtered through Wattmann No. 1 filter paper. The filtrate was then analyzed colorimetrically for NH_4^+ (indophenol method) and NO_3^- (sulfanilamide – α – naphthlamine method) using an Auto Ion analyzer (model AA II, Brant+Luebbe, Germany) (Anonymous 1974; Litchfield 1967; Varley 1966). Data were subjected to analysis of variance and least significant differences at $P<0.05$ (Fisher LSD) was determined.

Experiment 1c: Mode of inhibitory action of inhibitors

The BNI compounds obtained from the root exudate of *L. racemosus* was evaluated as to its inhibitory

mode of action on *Nitrosomonas europaea*. The mode of inhibitory action of the BNI was determined by incubating pure cultures of *N. europaea* in the presence or absence of hydroxylamine in the assay medium using previously reported protocol (Subbarao et al., 2006b). For the water-soluble inhibitor, DCD, 200 μl of the inhibitor solution was added to the 250 μl bacterial culture and it was incubated for 10 min before 200 μl of 1 mM hydroxylamine was added. The total volume of the assay was 650 μl. The mean of the 10 bioluminescence measurements made during the 10 min incubation period was taken as the activity level. Every measurement was repeated two times, and considered as replications for the statistical analysis to determine the significance of treatment effects. The effect of addition of the AMO enzyme product (i.e. hydroxylamine) to the reaction mixture was evaluated. The inhibitory effect of BNI compounds and the synthetic nitrification inhibitor, dicyandiamide, a known AMO inhibitor, was determined in the presence and absence of hydroxylamine.

Experiment 2: Characterization of chromosome-addition lines derived from *L. racemosus* x cultivated wheat for BNI activity

Experiment 2a: Production of wheat–L. racemosus–addition lines

L. racemosus (Lam) Tzvelev (2n=4×=28; genome Ns NsXmXm) was crossed with common wheat (*Triticum aestivum* L. cv. Chinese Spring; 2n=6×=42, AABBDD, accession number KT20-3). The F_1 plant was backcrossed with wheat and embryo-rescued (Kishii et al. 2004). The BC_1F_1 plant was further backcrossed with wheat for seven generations to obtain BC_7F_1 to obtain chromosome-addition lines. Production and characterization of these genetic stocks were described in Kishii et al. (2004). These lines are maintained in NBRP (National Bio-resource Project) in Japan. Confirmation of chromosome-addition lines was done using florescence in situ hybridization following Kishii et al. (2004). Total genomic DNA of *L. racemosus* was used as the probe to detect *L. racemosus* chromosome-addition lines, while two repetitive sequences (TaiI and Afa family) were deployed to identify each of the *L. racemosus* chromosomes.

Experiment 2b: Growth of plants

Seeds of chromosome-addition lines along with Chinese Spring variety were germinated in trays containing a sand–vermiculite mixture (3:1) and watered with distilled water. Plants were grown in a growth chamber with a day/night temperature regime of 22/18°C, a photosynthetic photon flux (PPF), averaging about 300 μmol m^{-2} s^{-1} and a 13/11 h light/dark photoperiod. Ten-day-old seedlings were transferred to aerated nutrient solutions where the plants were grown in 50 l tanks on styro-foam blocks having 20 holes with 2 plants per hole; the plants were kept in place with the support of sponges. Nitrogen was added as 2 mM N in the form $(NH_4)_2SO_4$ to the nutrient solutions. Nutrient solutions and maintenance were to the same as Experiment 1a. Each genetic stock was grown in a separate nutrient solution tank. Plants were grown for 30 days in nutrient solution culture before root exudate was collected. Plants of *L. racemosus* were grown from vegetative cuttings as described earlier for 40 days, before being used for root exudate collection.

Experiment 2c: Root exudate collection and determination of BNI activity

Root exudate was collected from intact plants 30 DAT (days after transplantation). Prior to the collection of root exudate, 36 intact plants of each genetic stock were removed from the hydroponic tanks and the roots pretreated in 1 mM N as $(NH_4)_2SO_4$ for 1 h. Plants from each genetic stock were separated into six sets of six plants per set. The exudates from three sets of each genetic stock were collected for 24 h in 1 L of aerated double distilled water and the other three sets in 1 L of 1 mM NH_4Cl solution. The solution containing the root exudate was stored at 5°C until the extraction of BNI compounds. After the collection of root exudate, the roots and shoots were separated, dried at 70°C for 48 h in a forced air-circulating oven before determining dry weights. Root exudate was processed for the determination of BNI activity using the recombinant luminescent *N. europaea* assay described earlier (Subbarao et al. 2006b). Data were subjected to analysis of variance and least significant differences at $P<0.05$ was determined (Fisher LSD).

Experiment 3: The role of NH_4^+ in the release of BNI compounds from roots

Ten-day-old seedlings of Chinese Spring and chromosome-addition line DALr#n were grown in nutrient solutions supplied with 2 mM N as $(NH_4)_2SO_4$ for 40 DAT in the same conditions as Experiment 1a. Plants from each genetic stock were separated into six sets of six plants per set. Details of nutrient management and growing conditions in the growth chamber were the same as Experiments 1a and 2. Four plants of *L. racemosus* per 20 l tank with three replications were examined to monitor the release of BNI compounds during a 5-day period. Nitrogen was supplied in the nutrient solution as 2 mM N in the form $(NH_4)_2SO_4$ at weekly intervals. Prior to the collection of root exudate, two intact plants from each replication were removed from the nutrient solution tanks, washed with tap water, followed by distilled water and then immersed in 1 l of 0.5 mM $(NH_4)_2SO_4$ solutions for 1 h as part of the pre-treatment. Root exudate was collected over five 24 h intervals for 5 consecutive days by immersing intact plant roots in either 1 l of aerated double distilled water or 1 mM NH_4Cl solution. Following each 24 h collection period, the solution containing the root exudate was immediately stored at 5°C until BNI activity was determined as described earlier. After 5 days of exudate collection, roots and shoots were separated, dried at 70°C for 48 h in a forced air-circulating oven before determining dry weights. Data were subjected to analysis of variance and least significant differences at $P<0.05$ was determined (Fisher LSD).

Results

BNI activity in wheat and *L. racemosus*

The BNI compounds released from cultivated wheat ranged from 1.4 ATU g^{-1} root dry weight 24 h^{-1} for Chinese Spring to 3.4 for Nobeokabouzu-komygi wheat. Nearly 27 ATU g^{-1} root dry weight day^{-1} of BNI was released from *L. racemosus*, which is 10- to 20-fold more than that released from cultivated wheat (Fig. 1). BNI activity was detected in the root exudate of NH_4^+ grown plants, but not from NO_3^- grown plants (Fig. 1). Dry matter production was suppressed in cultivated wheat grown with only NH_4^+ as the sole
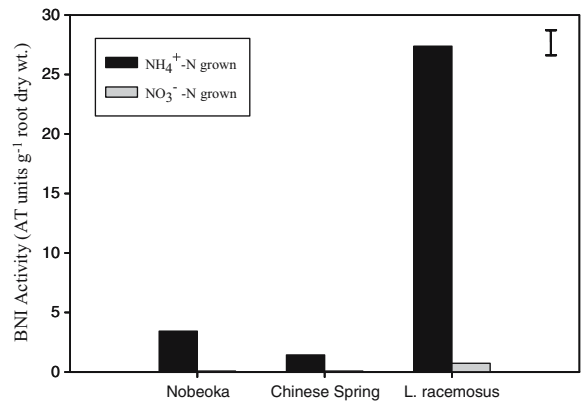

Fig. 1 BNIs (biological nitrification inhibitors) released from roots (i.e. root exudate) of two cultivars of wheat and its wild relative *L. racemosus*. Plants were grown with either NH_4^+ or NO_3^- as the nitrogen source. Root exudate was collected from intact roots in aerated distilled water over a 24-h period. Vertical bar represent Fisher LSD ($P<0.001$) for the interaction term (N source×species)

N source (Fig. 2). For *L. racemosus*, there was no significant difference between dry matter production between NH_4^+ or NO_3^- grown plants (Fig. 2). Cultivated wheat (cvs. Chinese Spring and Nobeokabouze-komygi) showed chlorosis and leaf drooping symptoms, but such toxicity symptoms were not observed in *L. racemosus*.

Stability and effectiveness of BNI compounds in soil

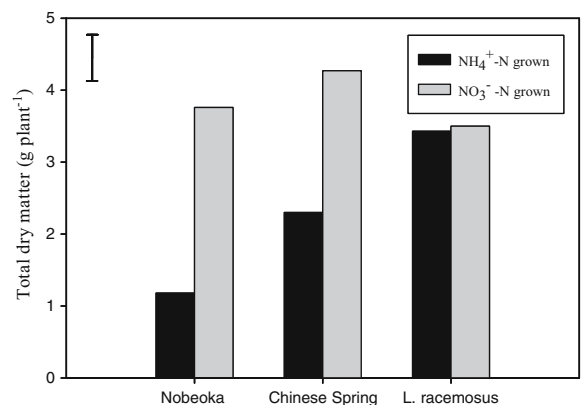The stability and effectiveness of BNI compounds from *L. racemosus* and cultivated wheat (Chinese

Fig. 2 Total dry matter production in two cultivars of wheat and *L. racemosus* after 40 days of growth in solution culture with NH_4^+ or NO_3^- as the sole source of nitrogen. *Vertical bar* represents Fisher LSD ($P<0.001$) for the interaction term (N source×species)

Spring) in inhibiting soil nitrification was evaluated. In soil treated with the root exudate from cultivated wheat, all the NH_4^+ was nitrified within 60 days (Table 1). However, nitrification was inhibited by about 90% in the soil treated with the *L. racemosus* exudate after 60 days incubation (Table 1). The synthetic nitrification inhibitor DCD (100 µg g^{-1} soil) had a substantial inhibitory effect on nitrification (Table 1). After 60 days of incubation, all the added NH_4^+ in the control soil (i.e. no inhibitor added) was nitrified. In contrast, <10% of the added NH_4^+ was nitrified in soil treated with root exudate from *L. racemosus* i.e. a major portion of the added N remained in the NH_4^+ form at the end of the 60 days incubation period (Table 1).

Mode of inhibitory action of BNI compounds

The inhibitory effect of DCD on *N. europaea* was eliminated when hydroxylamine was added to the assay, indicating that the AMO (ammonia monooxygenase) pathway was affected, but not the HAO (hydroxylamino oxidoreductase) pathway (Table 2). In contrast, the inhibitory effect from BNI compounds on *N. europaea* persisted even when hydroxylamine

was added to the assay (Table 2), indicating that the HAO pathway was suppressed (Table 2).

BNI activity in *L. racemosus* chromosome-addition lines of wheat

The extent of the expression of BNI in a wheat genetic background (Chinese Spring) was investigated using *L. racemosus* chromosome-addition lines (Table 3). The high BNI capacity of *L. racemosus* is mostly controlled by chromosome Lr#n (Fig. 3), and is about four times higher than that of Chinese Spring wheat (Table 3). Two other chromosomes Lr#J and Lr#I appears to have some control over BNI compound production as these DAL lines have significantly higher BNI compound released than that of Chinese Spring (Table 3). Chlorosis, a symptom characteristic of NH_4^+ toxicity, appeared in Chinese Spring parental line, and all chromosome-addition lines tested except DtA7Lr#1-1, when grown with NH_4^+ as the sole N source (Table 3). Dry matter production in many of the chromosome-addition lines was significantly lower than of the wheat parent, Chinese Spring. However, in chromosome-addition lines Lr#n, Lr#J, DA5Lr#1, DAlr#k, DtA7Lr#1-2 dry

Table 1 Effectiveness of BNI released from cultivated wheat (cv. Chinese Spring) and wild wheat (*L. racemosus*) in inhibiting nitrate formation in soil, after 30 and 60 days of soil incubation at 20°C

Treatment	NH_4^+ concentration in soil (µg N g^{-1} soil)	NO_3^- concentration in soil (µg N g^{-1} soil)	NO_3^-–N as % ot total N	Inhibition (%) on nitrate formation
30 days incubation period				
Root exudate from *L. racemosus*	199.3 e	18.4 a	8.5	74.7
Root exudate from cultivated wheat cv. Chinese Spring	153.1 c	54.7 b	26.3	24.9
Dicyandiamide® (100 µg g^{-1} soil)	180.0 d	19.0 a	9.6	74.0
Control	125.2 b	72.9 c	36.8	
LSD (0.05)	5.4	2.3		
60 days incubation period				
Root exudate from *L. racemosus*	213.8 d	16.1 a	7.0	91.5
Root exudate from cultivated wheat cv. Chinese Spring	2.4 a	198.6 c	98.8	−5.2
Dicyandiamide® (100 µg g^{-1} soil)	184.9 c	17.3 a	8.6	90.8
Control	0.0 a	188.8 b	100	
LSD (0.05)	9.0	1.0		

All treatments received 200 µg NH_4^+–N g^{-1} soil.

Letters represent values that are significantly different (α=0.05) with the least significant difference, LSD.

Inhibition (%) on nitrate formation is calculated as: [100−((nitrate formation in inhibitor treatment/nitrate formed in control)×100)].

Table 2 The effects of hydroxylamine on the inhibitory action of BNI activity from *L. racemosus* and dicyandiamide (a synthetic nitrification inhibitor) in a culture of *N. europaea*

DMSO: dimethyl sulfoxide, *ns*: not significantly different from control.

*$P<0.001$

Treatment	Bioluminescence (RLU ml^{-1})	
	Without hydroxylamine	With hydroxylamine
DMSO control	20.4	141.6
BNI from *L. racemosus*	5.8*	12.3*
% inhibition	71.5	91.3
Water control	51.5	357.5
Dicyandiamide (2,200 μM)	6.1*	326.3 ns
% inhibition	88.2	8.7

matter production was similar to that of Chinese Spring (Table 3).

Role of NH_4^+ in the release of BNI compounds

L. racemosus and the chromosome addition line DALr#n, maintained BNI compound release only in the presence of NH_4^+ (in the collection solution) during a 5-day study (Fig. 4). When NH_4^+ was absent, the release of BNI compounds decreased rapidly with time in *L. racemosus*, and was negligible from DALr#n (Fig. 4). BNI compounds released from cultivated wheat in the presence of NH_4^+ was just within the detection limits of the assay, but in the absence of NH_4^+, any BNI compound released from

roots after the first day was below the detection limits (Fig. 4).

Discussion

Production of BNI compounds in cultivated wheat, cvs. Nobeoka and Chinese Spring, is very low, which is in agreement with an earlier report where the wheat cv. Norin-10 was used (Subbarao et al. 2007b). Several cereal (rice, maize, barley and pearl millet) and legume crops (peanut, soybean, beans) were reported to have low or no BNI capacity (i.e. <3.0 ATU of activity g^{-1} root dry weight day^{-1}) (Subbarao et al. 2007b). The BNI capacity of *L.*

Table 3 BNI released from chromosome-addition lines derived from inter-specific crosses of the cultivated wheat Chinese Spring with *L. racemosus*

Genetic stock	*L. racemosus* chromosome introduced	Homologous group to wheat chromosome	BNI released (ATU g^{-1} root dry weight d^{-1})[a]	Total dry matter produced (g plant^{-1})	NH_4^+ tolerance score[b]
DALr#n	Lr#n	Groups 3 and 7	24.57	3.15	S
DALr#J	Lr#J	Group 7	13.47	2.66	S
DALr#I	Lr#I	Group 5	13.02	2.07	S
DALr#l	Lr#l	Group 2	6.40	2.15	S
DALr#k	Lr#k	Group 6	5.50	2.75	S
DALr#F	Lr#F	Group 4	4.12	1.84	S
DALr#H	Lr#H	Group 3	3.65	2.24	S
DA2Lr#1	2Lr#1	Group 2	3.16	2.04	S
DA5Lr#1	5Lr#1	Group 5	6.55	2.72	S
DtA7Lr#1-1	7Lr#1-1	Group 7	6.38	3.38	T
DtA7Lr#1-2	7Lr#1-2	Group 7	4.90	1.64	S
		LSD (0.05)	3.93	0.88	

DA: disomic addition, *Dt*: ditelosomic addition of *L. racemosus* chromosomes to/with Chinese Spring chromosomes.

[a] Root exudate collected using 1 mM NH$_4$Cl.

[b] Tolerance score based on the appearance of chlorosis symptoms, where chlorosis was considered as a sign of sensitivity to assimilation of N in NH_4^+ form.
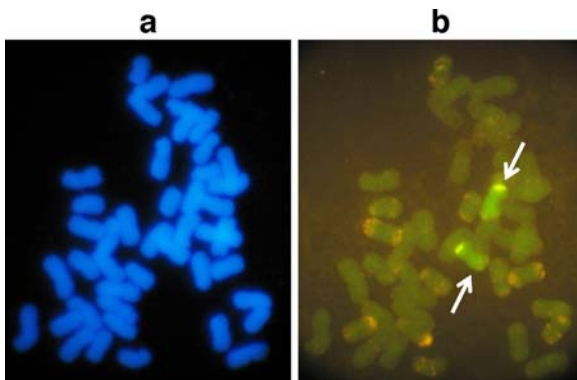
Fig. 3 Karyotype analysis of DALr#n, a chromosome-addition line derived from *L. racemosus* × *T. aestivum*. **a** DAPI staining revealed 44 chromosomes. **b** The probe of *L. racemosus* genomic DNA (*green*) and TaiI and Afa family repetitive sequences showed the presence of two Lr#n chromosomes. The *arrows* indicate Lr#n chromosomes

racemosus is similar to that reported in *B. humidicola* (about 15 to 45 ATU activity g^{-1} root dry weight day^{-1} depending on the genotype), a tropical grass grown extensively in South America (Subbarao et al., 2006b, 2007b). Recent field studies provide further evidence that nitrification and N_2O emissions were suppressed in field plots planted with *B. humidicola* (Subbarao et al. 2007c, d).

For BNI compounds to be functional, ecologically or in agriculture, it must limit or stop the conversion of NH_4^+ to NO_3^- and should also be sufficiently stable

in the soil to be effective during most of the cropping cycle. During this study we have shown that BNI compounds released from *L. racemosus* are very effective in suppressing nitrification and keeping soil nitrogen in NH_4^+ form for at least 60 days. In contrast, all the NH_4^+ in soil samples treated with the root exudate from cultivated wheat was nitrified, indicating either insufficient or ineffective BNI compound production. Recent reports on *B. humidicola* indicate that BNI compounds released from its roots are effective in preventing nitrification, but that a threshold BNI activity level of 5.0 ATU g^{-1} soil needs to be reached before the inhibitory effect becomes evident (Subbarao et al. 2006b).

Analysis of wheat *L. racemosus* chromosome-addition lines revealed that the BNI capacity of *L. racemosus* can be introduced into and expressed in a wheat genetic background. The high BNI capacity of *L. racemosus* roots is mostly controlled by chromosome Lr#n, and the BNI released is about four-fold higher than that from Chinese Spring wheat. Two other chromosomes, Lr#J, and Lr#I appeared to have some control over BNI compound production. It is interesting to note that the chromosome that has a major control on BNI compound production from roots Lr#n does not also provide tolerance to NH_4^+, which appears to be under the control of chromosome 7Lr#1-1. Since cultivated wheat lacks the ability to

Fig. 4 BNI released in the presence and absence of NH_4^+ in the solution used to collect the root exudate from NH_4^+-grown plants (*LR – L. racemosus*, *CS* – wheat cv. Chinese Spring, and DALr#n) during a 5-day monitoring study. *Vertical bar* represents Fisher LSD (*P*<0.001) for the interaction term (genetic stock×day of exudate collection)
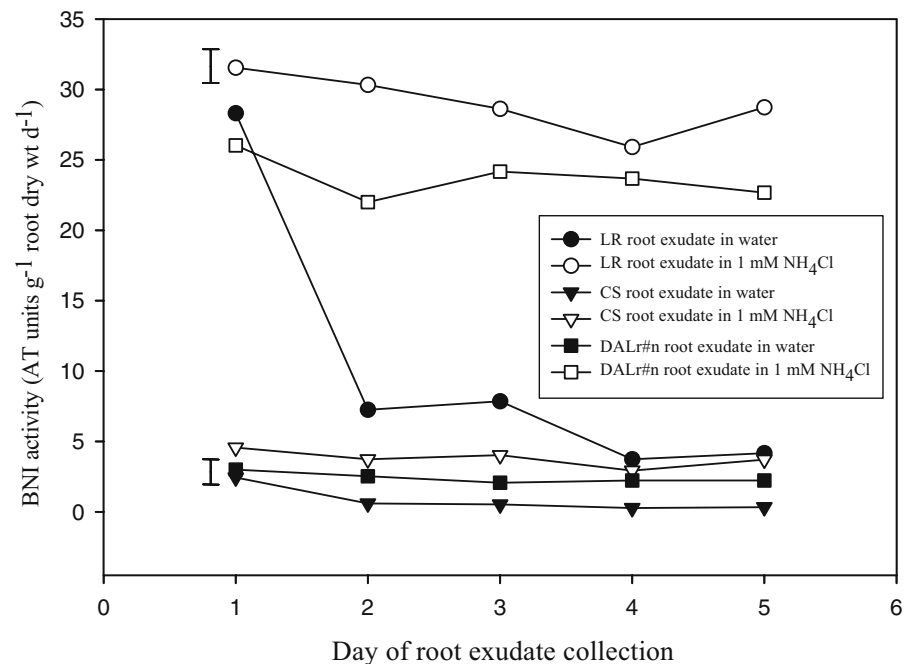
tolerate and utilize NH_4^+ as the sole N source (Britto and Kronzucker 2002), it is crucial that introduction of genes that provide tolerance to NH_4^+ is a prerequisite for the introduction of BNI capacity into cultivated wheat. Capacity for BNI compound production and tolerance to NH_4^+ assimilation are two attributes associated with the genetic exploitation of BNI compound production in cultivated wheat, where *L. racemosus* could be used as a genetic source for both attributes. Wheat breeders have widely utilized the strategy of using chromosome -addition and -substitution lines as a bridge to transfer several characters from *Leymus* spp. into cultivated wheat (Kishi et al. 2004; Chen et al. 2005). However, some of these chromosome-addition lines contain genes that could be agronomically undesirable, but they can be avoided by producing translocation lines in which only small segments with the desirable gene/s (i.e. BNI gene/s) are transferred into the wheat chromosome (Friebe et al. 1993).

The mode or modes of inhibition of BNI compounds on *Nitrosomonas* appears to differ from that of the synthetic nitrification inhibitors. The inhibitory effect of DCD on *N. europaea* was eliminated in the presence of hydroxylamine, indicating that only the AMO pathway was affected, which agrees with its reported mode of action (Powell and Prosser 1986; McCarty 1999). In contrast, the inhibitory effect of BNI compounds (from *L. racemosus*) on *N. europaea* persisted in the presence of hydroxylamine suggesting that the HAO pathway was blocked. It has been hypothesized that the BNI compounds released from *B. humidicola* roots is likely to be a cocktail of as many as ten organic compounds each of which may have different mode/site of action on *Nitrosomonas* spp (Subbarao et al. 2007a). Monoterpenes, a group of terpenoids released from conifers, suppress nitrification through blocking the AMO pathway, similar to the commonly used synthetic nitrification inhibitors (McCarty 1999; Ward et al. 1997). Recently two nitrification inhibitors (methyl-*p*-coumarate and methyl ferulate) were isolated from *B. humidicola* roots (Gopalakrishnan et al. 2007). Most synthetic nitrification inhibitors rely on a single mode/site of action on AMO, thus they could be vulnerable to genetic changes in the nitrifier populations or natural genetic diversity in ammonia-oxidizing organisms (Belser and Schmidt 1981; Norton et al. 2002; Leninger et al. 2006). However if the BNI compound/s from *Leymus*

is composed of a cocktail of inhibitors with multiple sites of action, they could be more effective over a much wider range of environmental conditions.

We reported that the presence of NH_4^+ in the root environment is critical for the synthesis and sustained release of BNI compounds from *B. humidicola* roots (Subbarao et al. 2007a). In *L. racemosus* and the chromosome-addition line DALr#n, the release of BNI compound/s was maintained only in the presence of NH_4^+ in the root environment. The successful evolution of BNI capacity as an adaptation mechanism in nitrogen-limiting environments would need some environmental signal such as NH_4^+ to make it responsive to the environment (Subbarao et al. 2007a). Based on the root mass reported for wheat at the heading stage being about 3.5 t ha^{-1} (Savin et al. 1994), with an expressed BNI activity of 25 ATU g^{-1} root dry weight 24 h^{-1} in the *Leymus*-wheat chromosome-addition line DALr#n, we estimate that nearly 87,500,000 U of BNI ha^{-1} day^{-1} can be released from a BNI-enabled wheat under favorable field conditions; this is based on the assumption that BNI capacity similar to those found in DALr#n are incorporated into some of the elite breeding lines of wheat. This BNI activity (i.e. 87,500,000 U) is equivalent to a nitrapyrin application of 52.5 g ha^{-1} (one ATU unit of BNI activity is equivalent to 0.6 μg of nitrapyrin). Considering that the recommended nitrapyrin application levels is 1 kg ha^{-1} (Hughes and Welch 1970), it would require only about 19 days for plants of this size to release sufficient BNI to be the equivalent of a commercial application of nitrification inhibitors. However, the projected BNI release capacity is an optimistic scenario based on maximal root mass reported under best growing conditions at heading stage; the inhibitory effect from roots during the initial phase of growth will be far below these projections, thus will require much longer-times than mentioned above to control nitrification. Characterization of BNI-enabled wheat that has good agronomic and high-yield potential (that are yet to be developed and will require several years of breeding effort) will have to be addressed at a later stage to determine the BNI potential at various growth stages under field conditions.

The BNI attribute is a natural mechanism some plants utilize to restrict nitrification, which could be genetically exploited to increase NUE in agricultural production. In this manuscript we demonstrate that

BNI attribute exists in a wild wheat relative, it can be moved into cultivated wheat, and that BNI compounds can be produced in sufficient quantities to be effective under controlled laboratory conditions. Utilizing a genetic approach that combines the natural ability of wild wheat to combat nitrification in intensive agricultural systems is a novel strategy that has the potential to reduce nitrogen pollution from wheat production systems.

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