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Effect of the combination of nitrapyrin and gamma-aminobutyric acid on soil nitrogen transformation characteristics and rice yield

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Abstract: When 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin) is applied alone, it typically does not significantly increase crop yield. Therefore, we combined gamma-aminobutyric acid (GABA) with nitrapyrin to address the limitations of nitrapyrin in enhancing yield. We conducted indoor incubation experiments and pot experiments in Chernozem and Calcic Kastanozem, respectively. The results demonstrated that GABA exerted an influence on the effectiveness of nitrapyrin by altering its degradation rate. In Chernozem, GABA accelerated nitrapyrin degradation, whereas, in Calcic Kastanozem, the results were the opposite. The pot experiment results showed that the combination of nitrapyrin and GABA increased rice total biomass by 5%, grain yield by 18 ± 2%, and plant nitrogen (N) uptake by 9 ± 1% compared to nitrapyrin applied alone. The increase in yield was attributed to the combined effect of nitrapyrin and GABA, which elevated root biomass and leaf area. In contrast, the effect of GABA on yield through altering the degradation rate of nitrapyrin was weaker. Therefore, the combination of nitrapyrin and GABA combined with urea increases rice yields in Chernozem and Calcic Kastanozem. The aim of this endeavour was to foster the development of a novel fertiliser product that offers both favourable agronomic outcomes and environmental benefits.

Keywords: yield increase limitations; biostimulant; nutrition; nitrogen use efficiency; agronomic benefits

Food security has emerged as a significant global concern. Projections from the United Nations Department of Economic and Social Affairs indicate that the global population is expected to reach 9.3 billion by 2050. To meet the needs of this growing population, food production must increase by at least

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70% (Tian et al. 2021). The application of nitrogen (N) fertiliser in agricultural production is essential (Cakmak 2002), and urea is the most widely used form of N fertiliser (Zanin et al. 2016). However, to achieve higher production, farmers frequently apply excessive N fertiliser during agricultural production. Liu et al. (2022) demonstrated that N fertiliser is currently overused in approximately half of regions of the world. This excessive amount of N, which is challenging for plants to uptake, is lost to the environment through ammonia volatilisation, nitrogen oxide emissions, and nitrate leaching (Zhu and Chen 2002). These losses pose various environmental risks (Good et al. 2004). Therefore, the method of how to achieve high crop production with reduced environmental risks is a challenge that remains to be addressed by researchers.

Studies have shown that the application of nitrification inhibitors (NIs) is an effective strategy for reducing N losses and has favourable environmental benefits (Wolt 2000). 2-Chloro-6-(trichloromethyl) pyridine (nitrapyrin), which is the first nitrification inhibitor to enter the market (Woodward et al. 2021), has been shown to inhibit ammonia monooxygenase (AMO) enzyme activity (Vannelli and Hooper 1992). The coapplication of nitrapyrin and urea fertilisers reduces N_2O emissions (Burzaco et al. 2013) and nitrate leaching losses (Woodward et al. 2021), thereby reducing the environmental risks associated with N loss (Wolt 2000). However, a meta-analysis by Fan et al. (2022) showed that, although nitrapyrin has favourable environmental benefits, it is difficult to significantly increase crop yield by nitrapyrin application alone (Cerrato and Blackmer 1990, Luo et al. 2017, Niu et al. 2018). Therefore, optimising the formulation of nitrapyrin inhibitors is essential for developing the next generation of nitrapyrin fertiliser products.

The rapid development of the biostimulant industry provides feasibility for the development of new nitrapyrin fertiliser products. The term biostimulant was first proposed by Kauffman et al. (2007). In 2015, EU scientists introduced a new definition of biostimulants. Unlike fertilisers or pesticides, biostimulants do not supply nutrients to plants but can be administered in small doses to enhance plant growth (du Jardin 2015). Gamma-aminobutyric acid (GABA) is an emerging biostimulant that is a protein hydrolysis product and an endogenous signalling molecule involved in plant physiological processes (Li et al. 2016b). Exogenous GABA improves plant stress tolerance (Fait et al. 2008, Krishnan et al. 2013), improves plant growth and establishment (Xie et al. 2020), and enhances plant photosynthesis (Li et al. 2016a). The application of GABA in combination with N fertiliser significantly increased crop biomass and grain yield by approximately 12% (Li et al. 2020), thus exceeding the yield increase achieved through nitrapyrin application. Therefore, we aimed to incorporate the combination of nitrapyrin and GABA into urea to create a novel generation of nitrapyrin fertiliser products that leverage the advantages of both compounds. This initiative seeks to address the shortcomings of nitrapyrin in terms of yield enhancement. Two representative soils (Chernozem and Calcic Kastanozem) were chosen for an indoor incubation experiment to investigate the influence of GABA on the effectiveness of nitrapyrin, and a field rice pot experiment was used to assess the yield efficacy of the new formulation. We addressed the following hypotheses: (1) GABA alters the degradation rate of nitrapyrin, thus consequently influencing nitrapyrin inhibitor efficacy, and (2) the impact of the formulation on crop yield is attributed to the modified effectiveness of nitrapyrin and the influence of GABA on the plant. In previous studies, most researchers have focused on enhancing the effectiveness of NIs for environmental protection, thus overlooking their potential yield enhancement drawbacks. Our study aimed to explore the synergistic yield enhancement effects of combining biostimulants and NIs, thus striving to develop a novel fertiliser that offers agronomic and environmental benefits. This endeavour sets the stage for future research and development in NIs and their applications.

MATERIAL AND METHODS

Preparation of soil samples. The soils for the experiments were collected from regularly fertilised cropland soil: Chernozem at Nong'an (44°43'N, 125°18'E), Jilin Province, China, and Calcic Kastanozem at Chang'Wu (34°59'N, 107°48'E), Shanxi Province, China. The soil samples were thoroughly mixed and air-dried, and fine roots, stones and other debris were removed and kept at room temperature until use. The chemical properties of the two soils are shown in Table 1.

Incubation experiment. An indoor incubation experiment was performed to investigate the influence of GABA on the degradation rate and effectiveness of nitrapyrin. The experiment consisted of five treatments: CK – no urea, nitrapyrin or GABA;

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Table 1. Chemical properties of the two different soils

Soil type	Textural class	Clay	Silt	Sand	pH	Organic carbon	Total N	NH_4^+-N	NO_3^--N	Total P (g/kg)	Available P (mg/kg)	Total K (g/kg)	Available K (mg/kg)
		(%)				(g/kg)		(mg/kg)					
Chernozem	loam	37.4	52.2	10.4	6.23	18.67	1.68	11.15	59.73	0.79	78.88	50.50	322.15
Calcic Kastanozem	loam	10.5	63.1	26.4	8.01	18.13	1.14	9.17	10.70	0.67	11.45	40.45	257.37

U – urea; N – urea + nitrapyrin; GABA – urea + GABA; and N + GABA – urea + nitrapyrin + GABA. N was applied at a rate of 0.8 g N/kg dry soil. To better monitor the degradation rate of nitrapyrin in soil, we increased its application rate. The application rates of nitrapyrin and GABA were 1% and 12‰, respectively, on the *w/w* basis of N. Each treatment had three replications.

A total of 1.5 kg of air-dried soil was packed in plastic pots (17 cm in diameter and 15 cm in height); afterwards, deionised water was added to maintain the soil water holding capacity (WHC) at 16%, and these pots were placed at 25 °C for 2 weeks to restore soil biological activities. In our formal incubation experiment, we first thoroughly mixed urea with nitrapyrin or GABA before incorporating it into the soil. This process ensures the precision and reliability of our experiments. The soil was incubated in the dark at 25 °C for 110 days. Deionised water was regularly added to maintain the soil's water-holding capacity at 20%.

Soil samples were collected from each pot at specific intervals (1, 3, 5, 7, 10, 14, 21, 28, 35, 50, 65, 80, 95, and 110 days). Approximately 80 g of soil samples were uniformly collected from various locations and depths in plastic pots. From these samples, 1.5 g was used for nitrapyrin residue determination, 10 g was used for urea nitrogen (urea-N) content analysis, 10 g was used for ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) content analysis, 15 g was used for soil potential nitrification rate (PNR) determination, and 20 g was used for soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) analysis.

Pot experiment. A pot experiment was performed to investigate the effect of the specific combination of nitrapyrin and GABA on crop yield increase. The experiment was conducted at the national field observation and research station of Agroecosystems in Shenyang, Liaoning Province (41°31'N, 123°24'E), and the crop that was used for the experiment was Meifeng-1 japonica rice. The mean annual temperature at the experimental site is 7–8 °C, the mean annual precipitation is approximately 600 mm, and the frost-free period is 147–164 days. The pot experiment was composed of five treatments, which was the same as the incubation experiment. Urea, triple superphosphate and potassium chloride were applied at rates of 0.7 g N, 0.05 g P and 0.06 g K per kg soil, respectively, and the application rates of nitrapyrin and GABA were 0.5% and 12‰, respectively, on the *w/w* basis of N.

Six kg of soil were transferred to plastic pots (with a diameter of 26 cm and height of 28 cm), after which water was added to maintain the soil moisture content at approximately 60% of the field capacity. Rice was planted in each pot in 5 caves, and 3 seedlings were planted in each cave. Watering was manually performed during the growth period of the rice plants to ensure consistent watering of each pot. No topdressing was applied during the growth period.

Soil samples were collected at the rice tillering, heading, filling, and maturity stages using a 5-point sampling method to determine the contents of NH_4^+-N and NO_3^--N. To investigate the influence of the formulations on the photosynthetic intensity of the rice plants, the leaf chlorophyll content and leaf area were measured at the end of the heading stage using the YMJ-B and CCM-200 instruments (Nanjing, China), respectively. At maturity, whole rice plants were collected, divided into roots, straw and grain, and air-dried, after which the rice yield was determined.

In both experiments, urea was produced by China National Pharmaceutical Group Corporation and contained 46% N; triple superphosphate was supplied by Yunnan Tianhua Group Co and contained 18% P; potassium chloride was produced by Russia and contained 24% K; nitrapyrin was supplied by Maya Reagent Biotechnology (Jiaxing, China) and had a purity of 98%; and GABA was supplied by Harworld (a Chinese company in Guangxi Province, Nanning, China) and had a purity of 99%.

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Soil and plant analyses. The soil chemical properties, urea-N, NH_4^+-N, NO_3^--N, and PNR, were determined according to the methods of Xiao et al. (2022). The soil MBC and MBN are determined and calculated according to the methods of Liu et al. (2010). The soil nitrapyrin content was determined according to the method of Zhang et al. (2020a). A 1.5 g soil sample was transferred to a glass bottle, and two successive additions of 15 mL of ethanol were ultrasonicated for 10 min each. Subsequently, 5 mL of ethanol was added and ultrasonicated for 10 min. The suspension was centrifuged at 10 000 rpm for 20 min to separate the supernatant, which was then filtered through a 0.45 μm organic membrane and stored at –20 °C for use. The nitrapyrin content in the samples was determined by using high-performance liquid chromatography with ultraviolet detection (HPLC-UV) instrument (Waters e2695, Milford, USA). The HPLC conditions were as follows: wavelength, 285 nm; chromatographic column, C18 column (4.6 × 150 mm, 5 μm, Waters Bridge); column temperature, 25 °C; flow rate, 0.8 mL/min; and mobile phase, acetonitrile/water (4/1). Nitrapyrin eluted at approximately 3.5 min.

A preliminary experiment was conducted to determine the recovery rate of nitrapyrin from soil. The U and N treatments were selected to ensure that the N and nitrapyrin addition rates matched those of the incubation experiment. Nitrapyrin residues in the soil were promptly extracted and quantified following thoroughly mixing fertiliser samples with the soil.

The recovery rate of nitrapyrin in the different soils was calculated as follows:

$$\text{Recovery rate} = (N - U)/NA$$

where: N – nitrapyrin residues in the N treatment; U – nitrapyrin residues in the U treatment; NA – amount of nitrapyrin that was added.

Finally, the recovery rates were calculated to be 86% and 83% in Chernozem and Calcic Kastanozem, respectively.

Calculations and statistical analyses. The nitrogen use efficiency (NUE) was calculated as follows (Xiao et al. 2023):

$$NUE = (Y - YC)/NF$$

where: Y – grain yield with N fertiliser; YC – grain yield with no fertiliser; NF – amount of N fertiliser that was applied.

Statistical analysis was performed using SPSS 21.0 (Chicago, USA) and Microsoft Excel 2010 (Redmond, USA). The differences among treatments were analysed by ANOVA with Tukey's test. The relationships between indicators were assessed using R 4.2.3 (Auckland, New Zealand). The data in the tables are the average value ± standard error.

RESULTS

Incubation experiment

Residues of nitrapyrin in soil. In Calcic Kastanozem, nitrapyrin had a faster degradation rate, with a half-life of approximately 14 days (Figure 1). GABA had different impacts on the degradation rate of

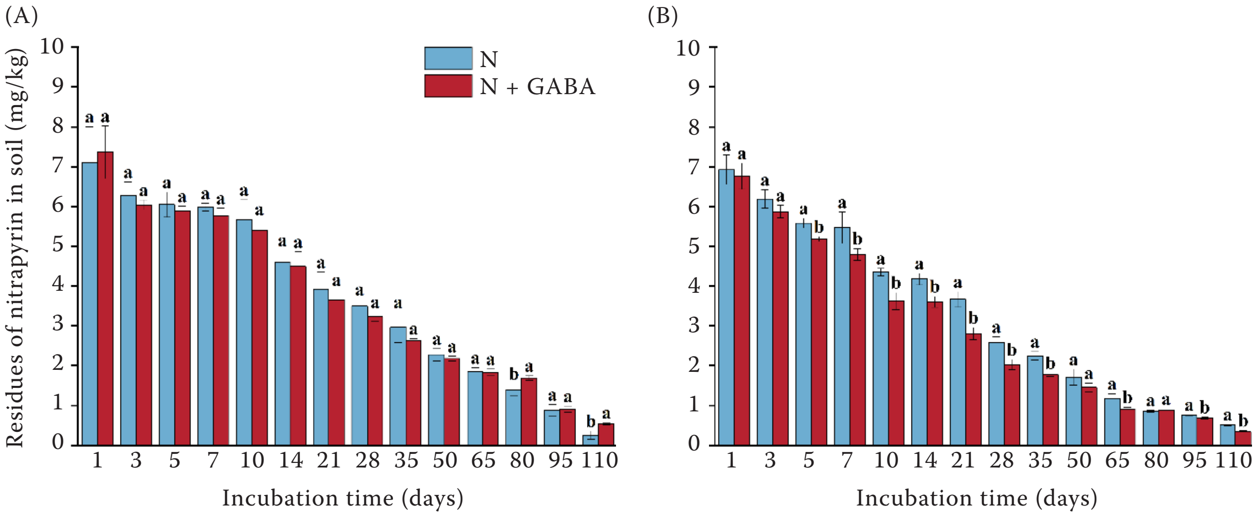

Figure 1. Residues of nitrapyrin in (A) Chernozem and (B) Calcic Kastanozem. Error bars represent the standard deviations (n = 3). Different letters indicate significant differences between different treatments at P < 0.05, according to Tukey's test. N – urea + 2-chloro-6-(trichloromethyl) pyridine; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid

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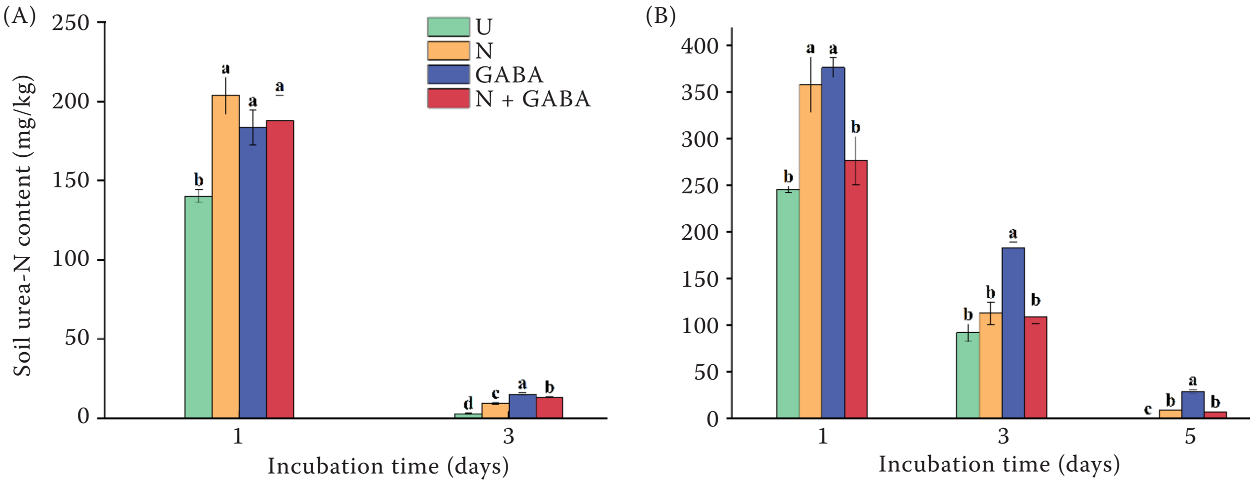

Figure 2. Changes in the urea-N content in (A) Chernozem and (B) Calcic Kastanozem. U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. The vertical bars represent the standard deviations of the means (n = 3). Different letters indicate significant differences between different treatments at P < 0.05, according to Tukey's test

nitrapyrin in both soils. In Chernozem, there were no significant differences between treatments before 50 days. However, this effect changed after 65 days, with GABA significantly increasing the residual amount of nitrapyrin after 80 days (Figure 1A) (P < 0.05). In Calcic Kastanozem, GABA accelerated the degradation rate of nitrapyrin after 5 days (Figure 1B).

Urea-N. The hydrolysis rate of urea-N was greater in the Chernozem. The addition of both nitrapyrin and GABA delayed the hydrolysis process, with GABA having the greatest effect (Figure 2).

Inorganic nitrogen. GABA accelerated the process of soil NH_4^+-N transformation in Chernozem (Figure 3A). However, in the Calcic Kastanozem, the NH_4^+-N content in the GABA treatment was greater than that in the U treatment from 3 to 14 days (Figure 3B). Nitrapyrin had greater effectiveness and longer effective time in Calcic Kastanozem (Figure 3). We were surprised to observe an unexpected phenomenon in both Chernozem and Calcic Kastanozem. In the Chernozem, the NH_4^+-N content in the N treatment began to decrease after 28 days but stabilised again by 80 days. A similar trend was observed in Calcic Kastanozem, thus suggesting the presence of two or more thresholds indicating the inhibitory effect of nitrapyrin on nitrification (Figure 3). GABA had different impacts on the effectiveness of nitrapyrin in different soils. In the Chernozem, the NH_4^+-N content in the N + GABA treatments was greater than that in the N treatments after 10 days; in the Calcic Kastanozem, the NH_4^+-N content in the N + GABA treatment was lower than that in the N treatment after 65 days, and GABA shortened the effectiveness time of nitrapyrin in the Calcic Kastanozem (Figures 3A, B).

Potential nitrification rate. In Chernozem, urea alone significantly increased the PNR within 5 days, and the soil PNR in the U treatment was significantly lower than that in the CK treatment (Figure 4) (P < 0.05). However, in Calcic Kastanozem, the opposite phenomenon was observed. In Calcic Kastanozem, urea alone significantly decreased the PNR within 10 days afterwards, the PNR of the U treatment rapidly increased after 21 days, and from 21 to 95 days, the PNR of the U treatment was significantly greater than that of the CK treatment (Figure 4B) (P < 0.05). GABA had opposite effects on the nitrification process in both soils, which is consistent with the changes in the soil inorganic N content (Figure 3). From days 5 to 10, GABA decreased the PNR in Chernozem, whereas the opposite trend was observed in Calcic Kastanozem (Figure 4) (P < 0.05). In Calcic Kastanozem, nitrapyrin had a stronger inhibitory effect on nitrification and significantly reduced the PNR during the incubation period (Figure 4B) (P < 0.05). In Chernozem, nitrapyrin inhibited the PNR within approximately 21 days (Figure 4A). In Chernozem, GABA improved the effectiveness of nitrapyrin and reduced the PNR after 35 days (Figure 4A) (P < 0.05). In Calcic Kastanozem, GABA reduced

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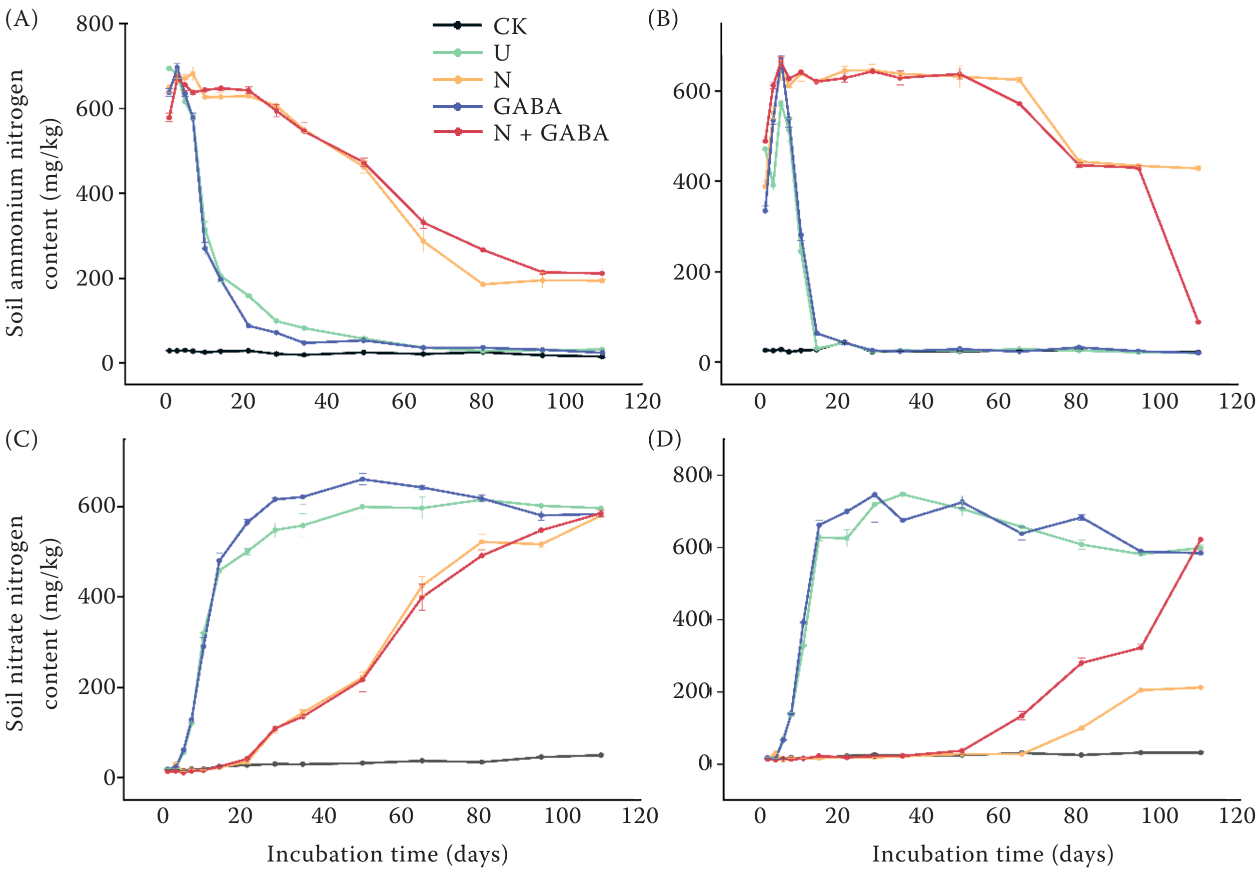

Figure 3. Changes in ammonium and nitrate contents in (A, C) Chernozem and (B, D) Calcic Kastanozem. CK – no urea, nitrapyrin or gamma-aminobutyric acid; U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. The vertical bars represent the standard deviations of the means (n = 3)

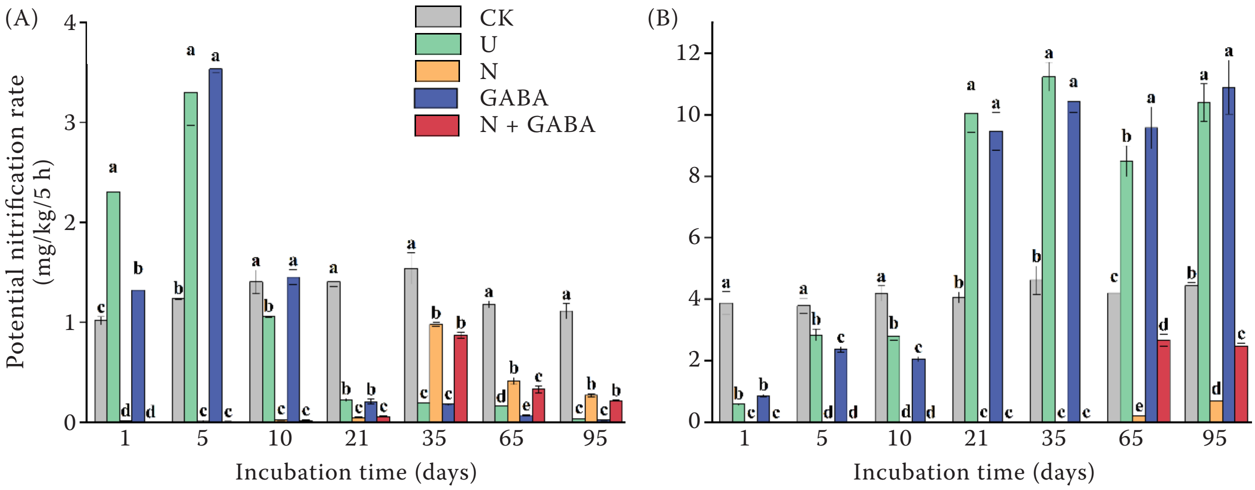

Figure 4. Changes in potential nitrification rate of (A) Chernozem and (B) Calcic Kastanozem. The error bars represent the standard deviations of the means (n = 3). CK – no urea, nitrapyrin or gamma-aminobutyric acid; U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. Different letters indicate significant differences between different treatments at P < 0.05, according to Tukey's test

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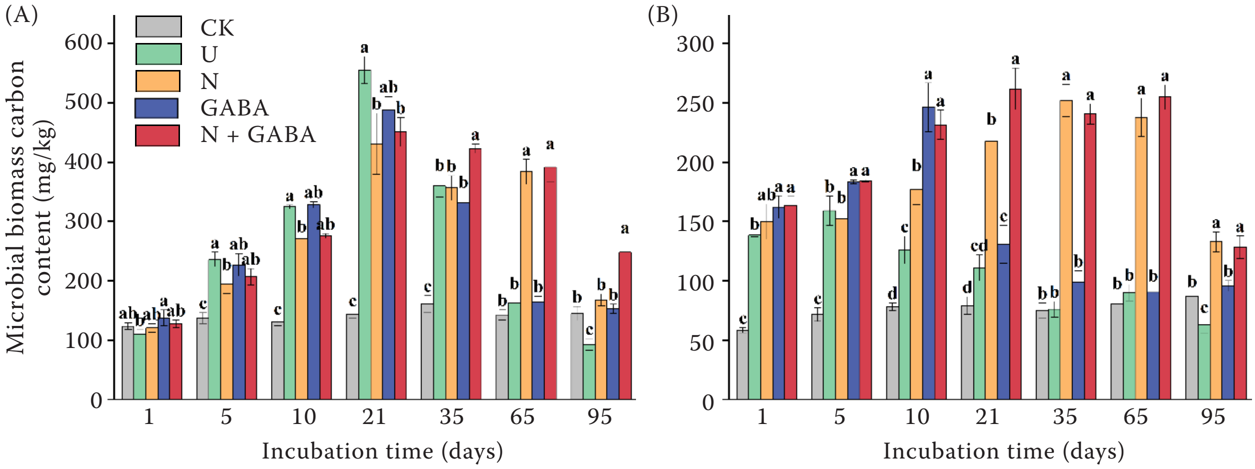

Figure 5. Changes in microbial biomass carbon in (A) Chernozem and (B) Calcic Kastanozem. The error bars represent the standard deviations of the means (n = 3). CK – no urea, nitrapyrin or gamma-aminobutyric acid; U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. Different letters indicate significant differences between different treatments at $P < 0.05$, according to Tukey's test

the effectiveness of nitrapyrin and significantly increased the PNR after 65 days (Figure 4B) ($P < 0.05$). This phenomenon aligns with the degradation rate of nitrapyrin in soil, thus suggesting that variations in the nitrapyrin degradation rate could account for changes in its effectiveness.

Microbial biomass carbon. In both soils, the addition of urea alone resulted in a trend of increasing and then decreasing soil MBC content (Figure 5). In Chernozem, there were no significant differences in the MBC content between the GABA treatment and U treatment from 5 to 95 days. However, compared with urea alone, GABA addition significantly increased the MBC content at 95 days (Figure 5A) ($P < 0.05$). In contrast to the results in Chernozem, GABA addition significantly increased the MBC content within 10 days compared to urea alone in Calcic Kastanozem ($P < 0.05$) (Figure 5B). In Chernozem, nitrapyrin significantly reduced the MBC content from 5 to 21 days, and the MBC content in the N treatment slowly decreased slowly and was significantly greater than that in the U treatment from 65 to 95 days. In

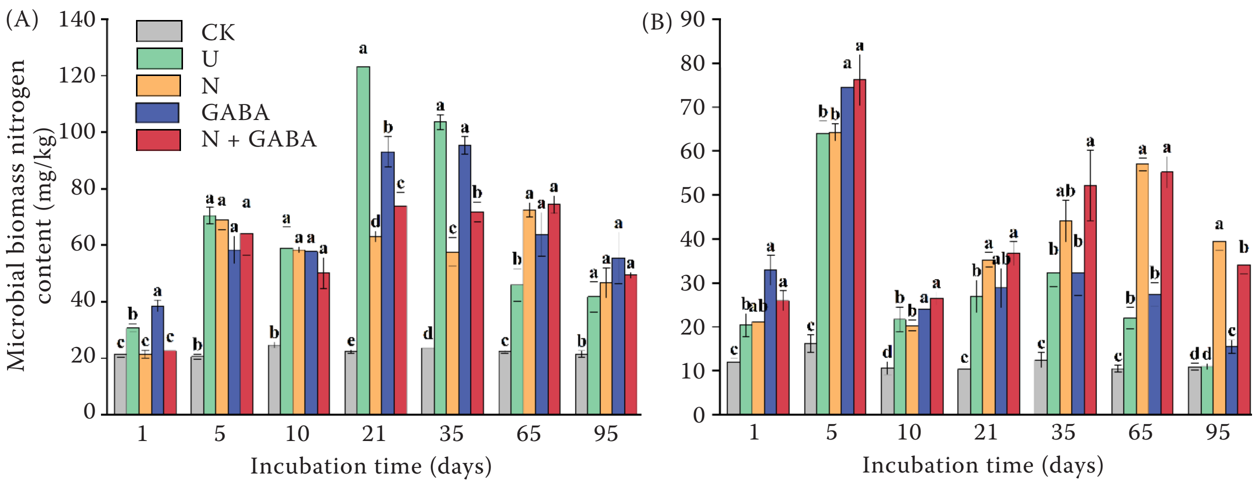

Figure 6. Changes in microbial biomass nitrogen in (A) Chernozem and (B) Calcic Kastanozem. The error bars represent the standard deviations of the means (n = 3). CK – no urea, nitrapyrin or gamma-aminobutyric acid; U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. Different letters indicate significant differences between different treatments at $P < 0.05$, according to Tukey's test

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Table 2. Correlations between the residual nitrapyrin and the potential nitrification rate (PNR), microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) in both soils

	Soil type	PNR	MBC	MBN
Nitrapyrin	Chernozem	–0.544**	–0.489**	–0.457**
	Calcic Kastanozem	–0.573**	–0.323*	–0.197

*$P \leq 0.05$; **$P \leq 0.01$

Calcic Kastanozem, nitrapyrin significantly increased the MBC content after 10 days (Figure 5) ($P < 0.05$). Compared with nitrapyrin alone, GABA addition increased the soil MBC content in both soils during incubation (Figure 5).

Microbial biomass nitrogen. In both soils, urea addition increased the soil MBN content during incubation (Figure 6). Compared with urea alone, GABA addition promoted increased MBN content in Calcic Kastanozem; however, in Chernozem, this phenomenon was observed only from 65 to 95 days (Figure 6). Compared with urea addition alone, nitrapyrin addition increased the soil MBN content in both soils after 21 days. Compared with nitrapyrin alone, GABA addition had different effects on the soil MBN content in both soils. In Chernozem, GABA did not significantly affect the MBN content before 10 days ($P > 0.05$). However, in the Calcic Kastanozem, the N + GABA treatment had significantly greater effects than the N treatment from 5 to 65 days (Figure 6B) ($P < 0.05$).

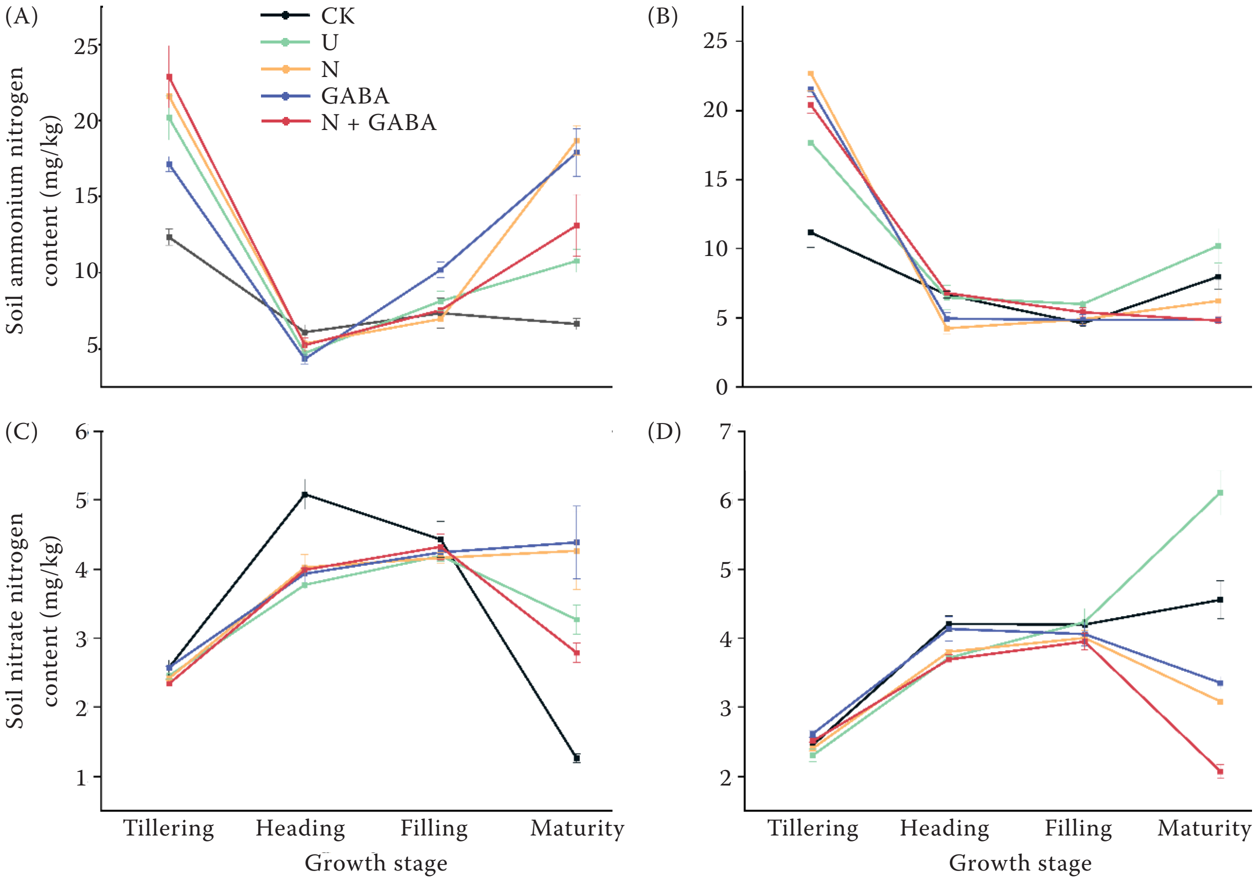

Figure 7. Changes in NH_4^+-N and NO_3^--N content in (A, C) Chernozem and (B, D) Calcic Kastanozem. CK – no urea, nitrapyrin or gamma-aminobutyric acid; U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. The vertical bars represent the standard deviations of the means ($n = 3$). Tillering – rice plants grow side shoots; heading – rice plants start forming panicles; filling – rice grains fill and enlarge; maturity – rice grains fully mature

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Pearson correlation analysis. A Pearson correlation analysis was conducted to investigate the relationship between the degradation rate of nitrapyrin and the microbial population and the soil nitrification intensity. The results showed that the residual amount of nitrapyrin was negatively correlated with the PNR and MBC in both soils (Table 2). There was a negative correlation between the contents of nitrapyrin and MBN in Chernozem; however, this correlation was not detected in Calcic Kastanozem (Table 2).

Pot experiment

Inorganic nitrogen. As the rice growth stage progressed, soil NH_4^+-N decreased, whereas the soil NO_3^--N content decreased in all treatments (Figure 7). Compared with urea alone, GABA addition increased the soil N availability in the Calcic Kastanozem at the tillering stage ($P < 0.05$). The results of the pot experiment showed that nitrapyrin had a greater effect on Calcic Kastanozem and significantly increased the soil NH_4^+-N content at the tillering stage (Figure 7B) ($P < 0.05$). In Chernozem, there was no significant difference between the N and U treatments (Figure 7A), which was the same as the results of the incubation experiment. Compared to nitrapyrin alone, GABA addition to Chernozem significantly increased the soil NH_4^+-N content at the tillering stage (Figure 7A) ($P < 0.05$).

Rice plant physiology and biological indicators. Urea application to Calcic Kastanozem plants promoted rice growth and significantly increased the tiller number, plant height, spike length, chlorophyll content and leaf area ($P < 0.05$) (Table 3). Compared to urea alone, the addition of GABA and nitrapyrin significantly increased the leaf area in both soils (Table 3) ($P < 0.05$). Compared to nitrapyrin alone, the GABA addition significantly increased the leaf area but had no significant effect on the other indicators (Table 3) ($P < 0.05$).

Rice yield, plant N uptake and NUE. Urea application significantly increased the rice yield, root biomass and plant N uptake in both soils (Table 4) ($P < 0.05$). Compared with urea alone, the addition of GABA and nitrapyrin increased the total biomass and plant N uptake of rice to different degrees (Table 4). In Calcic Kastanozem, GABA had a greater effect on increasing the rice yield and significantly increased the total biomass and grain yield by 11% and 19%, respectively (Table 4) ($P < 0.05$). Compared to nitrapyrin alone, GABA addition to Chernozem had a greater effect and significantly increased rice grain yield, root biomass, plant N uptake and NUE (Table 4) ($P < 0.05$). Rice yield, root biomass, plant N uptake and NUE were greater in the N + GABA treatment than in the N treatment, and GABA addition significantly increased root biomass (Table 4) ($P < 0.05$).

Pearson correlation analysis. Pearson correlation analysis demonstrated that rice total biomass and grain yield were positively correlated with leaf area in both soils (Figure 8). Leaf area was positively correlated with root biomass, plant N uptake and NUE in

Table 3. Rice plant physiology and biological indicators

Soil type	Treatment	Tiller number	Plant height (cm)	Spike length (cm)	Grain number per spike	Chlorophyll content	Leaf area (cm^2)
Chernozem	CK	5.27 ± 0.23^b	78.71 ± 1.05^b	16.43 ± 0.14^a	83.69 ± 9.28^a	6.73 ± 1.54^b	20.55 ± 1.16^d
	U	15.47 ± 1.40^a	79.63 ± 2.92^{ab}	15.60 ± 0.11^{ab}	88.09 ± 3.02^a	22.05 ± 0.89^a	29.84 ± 1.09^c
	N	15.93 ± 1.15^a	79.39 ± 2.92^{ab}	15.05 ± 0.93^b	93.82 ± 5.46^a	21.84 ± 1.22^a	33.97 ± 0.38^b
	GABA	17.33 ± 2.48^a	84.78 ± 2.35^a	15.54 ± 0.32^{ab}	90.85 ± 9.03^a	21.16 ± 1.19^a	33.77 ± 0.97^b
	N + GABA	15.93 ± 0.83^a	85.12 ± 1.06^a	15.83 ± 0.21^{ab}	96.16 ± 3.70^a	20.86 ± 0.83^a	37.54 ± 1.17^a
Calcic Kastanozem	CK	3.53 ± 0.50^c	66.07 ± 5.89^b	11.46 ± 0.34^b	63.00 ± 6.82^b	4.41 ± 1.68^b	16.15 ± 1.10^c
	U	13.60 ± 0.69^{ab}	86.51 ± 3.32^a	13.31 ± 0.44^a	85.33 ± 7.64^{ab}	20.67 ± 2.06^a	36.20 ± 1.92^b
	N	12.80 ± 0.87^{ab}	86.43 ± 2.54^a	12.35 ± 1.14^{ab}	100.03 ± 11.66^a	21.26 ± 2.84^a	37.58 ± 1.95^b
	GABA	11.60 ± 1.25^b	87.17 ± 6.60^a	13.71 ± 0.49^a	97.11 ± 10.00^a	21.92 ± 0.41^a	42.75 ± 1.88^a
	N + GABA	14.27 ± 0.42^a	85.53 ± 2.24^a	13.74 ± 0.57^a	99.07 ± 6.97^a	21.45 ± 0.18^a	44.97 ± 1.49^a

The values are the means ± standard deviations (n = 3). CK – no urea, nitrapyrin or gamma-aminobutyric acid; U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. Different letters indicate significant differences between different treatments at $P < 0.05$, according to Tukey's test

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Table 4. Rice yield, plant nitrogen (N) uptake and nitrogen use efficiency (NUE)

Soil type	Treatment	Total biomass (g/pot)	Grain yield (g/pot)	Root biomass (g/pot)	Plant N uptake (g/pot)	NUE (%)
Chernozem	CK	180.39 ± 5.23[c]	57.63 ± 3.97[c]	33.39 ± 1.71[d]	1.28 ± 0.07[d]	–
	U	425.82 ± 4.59[b]	124.17 ± 3.95[b]	60.82 ± 0.72[c]	4.25 ± 0.18[c]	70.60 ± 4.22[c]
	N	434.69 ± 9.07[ab]	123.48 ± 7.10[b]	62.69 ± 1.09[bc]	4.45 ± 0.17[bc]	75.51 ± 4.12[bc]
	GABA	448.25 ± 3.04[ab]	127.17 ± 10.29[b]	66.25 ± 3.54[b]	4.64 ± 0.02[ab]	79.91 ± 0.56[ab]
	N + GABA	456.61 ± 14.60[a]	148.43 ± 9.06[a]	73.28 ± 1.63[a]	4.82 ± 0.12[a]	84.30 ± 2.81[a]
Calcic Kastanozem	CK	89.39 ± 0.74[c]	28.33 ± 1.53[c]	29.06 ± 1.71[c]	0.63 ± 0.01[c]	–
	U	412.83 ± 5.91[b]	108.30 ± 7.33[b]	67.16 ± 4.33[ab]	3.95 ± 0.20[b]	79.10 ± 4.80[b]
	N	436.77 ± 8.31[ab]	117.16 ± 9.84[ab]	66.11 ± 4.09[b]	4.25 ± 0.31[ab]	86.10 ± 7.37[ab]
	GABA	457.55 ± 13.05[a]	128.55 ± 10.03[a]	71.88 ± 4.55[ab]	4.09 ± 0.13[ab]	82.33 ± 3.20[ab]
	N + GABA	458.13 ± 18.74[a]	136.34 ± 3.56[a]	76.46 ± 2.65[a]	4.58 ± 0.19[a]	94.01 ± 4.62[a]

The values are the means ± standard deviations (n = 3). CK – no urea, nitrapyrin or gamma-aminobutyric acid; U – urea; N – urea + 2-chloro-6-(trichloromethyl) pyridine; GABA – urea + gamma-aminobutyric acid; N + GABA – urea + 2-chloro-6-(trichloromethyl) pyridine + gamma-aminobutyric acid. Different letters indicate significant differences between different treatments at $P < 0.05$, according to Tukey's test

Chernozem (Figure 8A). Root biomass was positively correlated with grain yield in Calcic Kastanozem (Figure 8B), which indicates that the increase in leaf area and root biomass was the critical factor for the increase in yield and plant N uptake in both soils.

DISCUSSION

In both soils, nitrapyrin (NI) effectively inhibited soil nitrification, consistent with previous studies (Li et al. 2021a, Pokharel and Chang 2021). Adsorption of nitrapyrin by soil clay reduces the effectiveness of nitrapyrin (Hendrickson and Keeney 1979, Zhang et al. 2020b) and provides some degree of abiotic protection to nitrapyrin, thus reducing its volatilisation losses (Zhang et al. 2020b). In our study, Chernozem had a higher clay content than Calcic Kastanozem (Table 1), and a higher clay content led to a lower effectiveness and longer half-life of nitrapyrin in Chernozem (Figure 1). Chibuike et al. (2023) measured the threshold concentrations of nitrapyrin in soil, and the results showed that soil nitrification was completely inhibited when the concentration of nitrapyrin in the soil was greater than 5 mg/kg. However, our experimental results showed that the threshold values were approximately 4 mg/kg and 2 mg/kg in Chernozem and Calcic Kastanozem, respectively. The effectiveness and half-life of nitrapyrin can be affected by soil texture, pH and organic carbon content (Zhang et al. 2020a, b), and the differences in the selected soils for the two experiments led to the different results.

Typically, the half-life of NIs determines their effective period (Hendrickson and Keeney 1979); therefore, we also proposed the hypothesis that GABA would alter the degradation rate of nitrapyrin, thus consequently influencing its efficacy. In Chernozem, GABA slowed nitrapyrin degradation (Figure 1A), thus enhancing nitrification inhibition during incubation. Conversely, in Calcic Kastanozem, GABA accelerated nitrapyrin degradation (Figure 1B), thus significantly increasing the PNR after 65 days (Figure 4B) ($P < 0.05$). Thus, our results support the hypothesis that GABA impacts the efficacy of nitrapyrin by influencing its degradation rate. The soil MBC content is commonly used to evaluate microbial abundance (Koerner et al. 2011), and Pearson correlation analysis demonstrated that nitrapyrin degradation was influenced by the soil MBC content. Microorganisms can efficiently utilise available amino acid molecules in the soil (Henry and Jefferies 2003). Consequently, the application of GABA to the soil resulted in noticeable increases in microbial biomass carbon content in both soils, although to different degrees. This increase was more significant in the Calcic Kastanozem (Figure 5). The soil carbon-to-nitrogen (C/N) ratio is a crucial factor influencing the structure and abundance of soil microbial communities (Dijkstra et al. 2018). In this experiment, the inorganic N content of the selected Calcic Kastanozem was lower than that of the Chernozem (Table 1). Consequently, the application of fertiliser N affected the Calcic Kastanozem C/N

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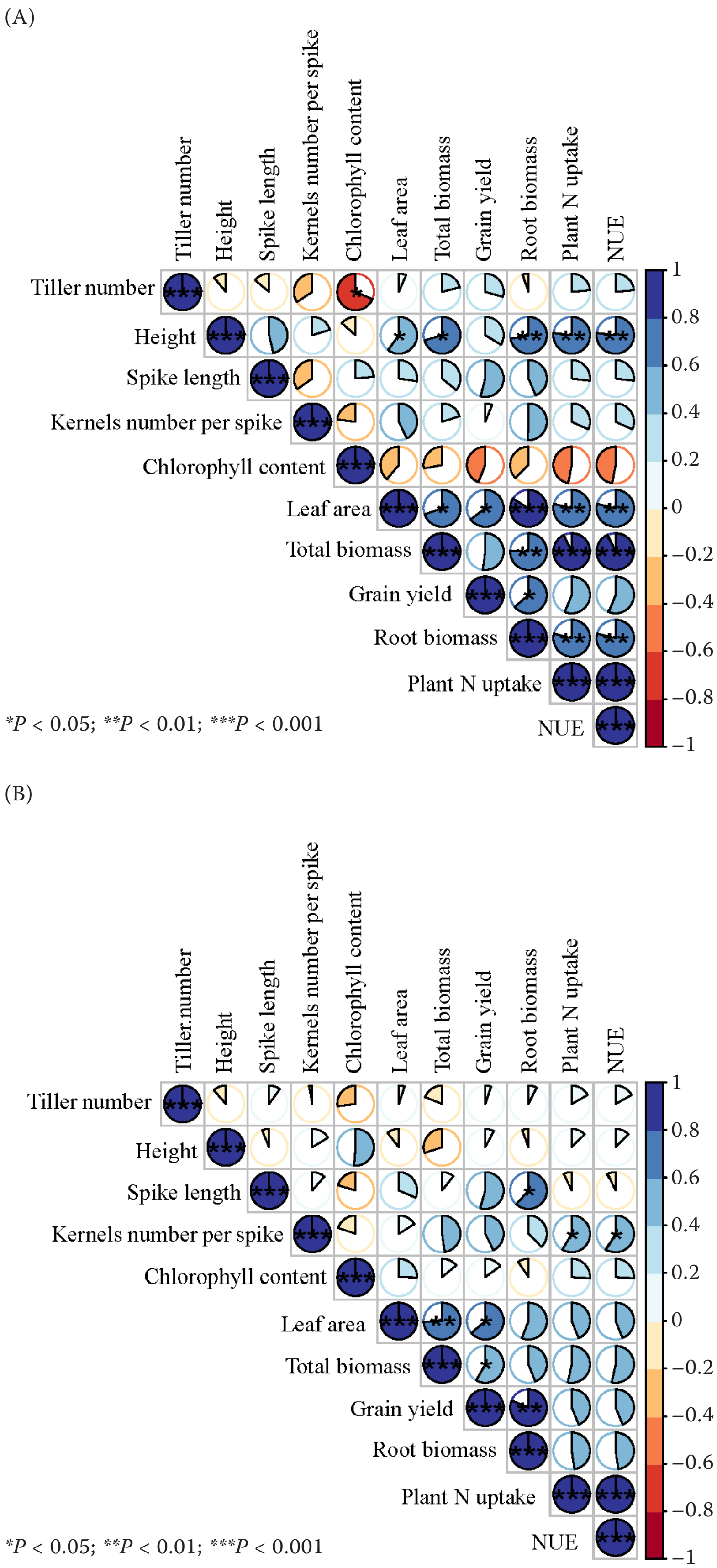

Figure 8. Pearson correlation analysis among rice physiological and biological indicators, plant nitrogen (N) uptake, and nitrogen use efficiency (NUE) in (A) Chernozem and (B) Calcic Kastanozem

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ratio more significantly, thus resulting in a differing pattern of change in the MBC content between the two types of soil. The rapid increase in the MBC content in Calcic Kastanozem indicates that nitrapyrin is subjected to stronger microbial decomposition at the early stage of incubation, thereby shortening the half-life of nitrapyrin. Compared to the addition of nitrapyrin alone, the addition of GABA to Chernozem resulted in a greater soil MBC after 35 days; by this time, most of the nitrapyrin had decomposed. Therefore, an increase in the MBC did not significantly impact the rate of nitrapyrin degradation ($P > 0.05$). Typically, soil MBC only accounts for less than 5% of soil organic C (Xu et al. 2013, Liang 2020); however, this amount of C plays a pivotal role in the mineralisation of soil organic C. A higher soil MBC content represents a higher organic C mineralisation rate (Creamer et al. 2015, Guo et al. 2019). Consequently, we conjectured that elevated soil MBC content at the later stages of incubation contributes to the partial mineralisation of organic matter. This process leads to the release of nitrapyrin that was previously adsorbed by organic matter into the soil, thereby extending the effective duration of nitrapyrin. Unfortunately, no corroborative research has been conducted to substantiate our conjecture. The mechanism by which GABA delays nitrapyrin degradation in Chernozem requires further investigation in subsequent studies.

Remarkably, GABA plays a regulatory role in N transformation. The contact area of urea with the soil impacts its hydrolysis rate (Shah and Wolfe 2003). In our experiment, urea was initially mixed with either nitrapyrin or GABA before being introduced into the soil. This approach resulted in a reduction in the contact area between urea and the soil and consequently delayed the urea hydrolysis process to some extent. Additionally, Shao et al. (2000) reported that the presence of potential hydrogen bonding centres in urea enables it to form complexes with amino acid molecules. Consequently, we speculated that GABA may exhibit a similar property, thus further delaying urea hydrolysis. GABA accelerates nitrification in Chernozem, which is in direct contrast to its behaviour in Calcic Kastanozem. At present, no research has reported the ability of GABA to regulate soil N transformation, and its mechanism of action requires further investigation.

GABA is a non-protein amino acid commonly found in living organisms, containing both amino and carboxyl groups (Shelp et al. 1999), and the concentration of γ-aminobutyric acid in plants is usually approximately 0.05 μmol/g (Wallace et al. 1984). Moreover, exogenous GABA treatment increases endogenous GABA levels, which correspondingly promotes physiological and metabolic processes in plants (Dabravolski and Isayenkov 2023), such as stimulating plant N assimilation (Li et al. 2021b). Therefore, we proposed a second hypothesis that the effect of the nitrapyrin + GABA formulation on yield is influenced by changes in nitrapyrin effectiveness and the effect of GABA on the plant.

In both soils, nitrapyrin applied alone enhanced rice yield and plant N uptake to various degrees, however, this effect was not significant (Table 4). This result was also observed in other studies (Cerrato and Blackmer 1990, Luo et al. 2017, Niu et al. 2018), which is why we conducted this study. In both soils, the application of GABA resulted in slightly greater yield increases than did the application of nitrapyrin (Table 4) ($P < 0.05$). Compared to nitrapyrin alone, the combination of nitrapyrin + GABA had a greater effect on Chernozem than on Calcic Kastanozem (Table 4), which was different from the results of our incubation experiment. Pearson correlation analysis demonstrated that the key factor for increased rice yield was increased root biomass and leaf area (Figure 7). Additionally, compared with nitrapyrin alone, GABA significantly increased the root biomass and leaf area in both soils (Tables 3 and 4) ($P < 0.05$). Greater root biomass indicates a heightened capacity for N uptake in rice plants (Guo et al. 2017), and an increase in leaf area indicates that the rice plant captures more light, which is beneficial for the accumulation of dry matter in the plant (Usuda 2004). Our hypothesis was confirmed; specifically, GABA affects crop yield not only by modifying the degradation rate and efficacy of nitrapyrin but also by promoting both plant root growth and leaf extension. This consequently leads to an increase in rice yield. During the early stages of crop growth, plant N uptake affects organ differentiation and morphology (Teng et al. 2022). Notably, research on N uptake in rice has primarily focused on the tillering stage (Yuan et al. 2010). After the tillering stage, the inorganic nitrogen content in both soils rapidly decreased and remained low, as shown by our results (Figure 7). The application of the nitrification inhibitor nitrapyrin can reduce fertiliser N loss (Burzaco et al. 2013), which is consistent with our result describing increased N retention in the soil during the early growth stage. Additionally, the addition of GABA facilitates N assimilation by plants (Li et al. 2021b), thus promoting

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plant body development (Xie et al. 2020); this likely contributed to the observed increase in root biomass and leaf area of rice in our experiment. The primary factor leading to increased rice yield is the effect on rice growth resulting from the coaction of nitrapyrin and GABA, rather than the impact of GABA on the nitrapyrin degradation rate and its effectiveness. Therefore, in Calcic Kastanozem rice-growing regions, the combination of nitrapyrin and GABA can be employed to achieve significant yield increases. Furthermore, the application of this formulation in Chernozem rice-growing regions led to a moderate increase in yield. To gain a better understanding of the environmental benefits associated with the combination of nitrapyrin and GABA, it is crucial for subsequent investigations to evaluate the effects of the combination of nitrapyrin and GABA on N dynamics in field experiments.

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