



Effects of nitrogen application rate on phosphorus transformation in an Alfisol: Results from phosphate-oxygen isotope ratios

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ABSTRACT

The effects of N application on P nutrient uptake by plants are well known. However, soil P transformation under the influence of N application and the underlying mechanisms are not yet clearly understood. In this study, we investigated the effect of N application on P uptake by crops and the soil P transformation process using the phosphate oxygen isotope technique combined with the sequential extraction method. The results showed that the rapeseed yield initially increased drastically with N application, but a yield leveling off effect was observed at the highest N level. After applying P fertilizer with a low $\delta^{18}\text{O}_\text{P}$ value (original $\delta^{18}\text{O}_\text{P}$ value of 12.9‰), we observed that P in P fertilizer was transformed primarily into the NaOH-P_i pool. The lowest N rate (75 kg ha⁻¹) and the highest N rate (225 kg ha⁻¹) promoted exchange between the NaOH-P_i pool and the NaHCO₃-P_i pool. The moderate N rate (150 kg ha⁻¹) possibly promoted the transformation of the NaOH-P_o pool to the NaOH-P_i pool. In the highest N treatment, the $\delta^{18}\text{O}_\text{P}$ value of the 1 M HCl-P_i pool significantly increased (19.0‰) and changed toward equilibrium, but the pool size was decreased, suggesting that the highest N rate possibly enhanced the transformation of the otherwise stable P pool. Overall, these results suggested that the specific N application rate could promote the turnover and transformation of soil P, which partly accounts for the enhanced soil P bioavailability and the increased crop yield and possibly leads to a high risk of P losses into the environment.

1. Introduction

Excessive application of N and P fertilizers to agricultural soil resulted in both P and N accumulations in the soil and losses to open water, thus causing serious environmental problems (Heckrath et al., 1995; Geohring et al., 2001; Wang et al., 2016; Powley et al., 2018; Tian et al., 2020). Previous studies have also shown that a large variety of soils are rich in total P but poor in bioavailable P which only accounts for a minor fraction (Schachtman et al., 1998; Chatzistathis et al., 2020). The main reason for this deficiency of bioavailable P in the soil is that a large amount of soil P is fixed onto minerals such as iron oxides, aluminum oxides, and calcium carbonate (Khare et al., 2004; Wang et al., 2013a, 2013b; Yan et al., 2014a, 2014b; Li et al., 2017; Weyers et al., 2017). Therefore, it is critical to improve soil P bioavailability to

meet crop production and sustainable agricultural development requirements.

N is reported to have a promotive effect on the bioavailability of soil P (Smith et al., 1987; Zhou et al., 2013; Emnova et al., 2014; Valadares et al., 2017; Zhang et al., 2018). Several studies have shown that N input and application can increase soil P mobility in the rhizosphere (Apthorp et al., 1987; Peng et al., 2011). Similarly, N deposition input was found to alter soil P uptake in the Inner Mongolia grassland (Zhang et al., 2012). Thien et al. (1972) found that N preconditioning maximally stimulated P uptake and translocation, and at lower concentrations, only uptake was stimulated, while at higher levels, only translocation was stimulated. Moreover, He et al. (1999) reported that the ratio of NaHCO₃-P (NaHCO₃ extractable P) to NaOH-P (NaOH extractable P) released from phosphate rock was increased by adding urea but was

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decreased by adding NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$. On the other hand, the combined application of N and P fertilizers enhanced P uptake, possibly by stimulating root growth (Grunes et al., 1959; Qian and Schoenau 2000; Wen et al., 2016). Increasing the leguminous species proportion was reported to enhance P uptake efficiency (Li et al., 2020). An investigation of physiological mechanisms by which N improves P uptake at the molecular scale indicated that N addition enhanced polyphosphate formation and the expression of alkaline phosphatase and P uptake-related genes (Wang et al., 2018). Field trial experiments have indicated that N impacts P uptake and translocation by regulating the growth of plants and varying photosynthetic allocation in plant organs (Zhang et al., 2018). Taken together, different mechanisms, such as enhanced bioavailability of soil P and P uptake, translocation, and allocation, have been reported to account for the improved P use efficiency with N application. However, the impacts of N application on soil P fractions and their transformation are not well understood.

The phosphate oxygen isotope technique has been increasingly applied to trace sources, migration, and transformation of P in the environment due to the distinct phosphate oxygen isotope ratios in biotic and abiotic reactions (Angert et al., 2012; Tamburini et al., 2012; Jaisi et al., 2010, 2014; Helfenstein et al., 2018; Hacker et al., 2019). According to the principle of phosphate oxygen isotope equilibrium, the rapid exchange of oxygen isotopes between inorganic phosphate and ambient water during biotic processes causes phosphate oxygen isotope equilibrium fractionation (Blake et al., 2005). However, the original signature is maintained in phosphate oxygen isotope abiotic reactions because of negligible isotope exchange under Earth's surface conditions (Lecuyer et al., 1999; O'Neil et al., 2003). Furthermore, from the beginning to the ending values at the same time, the closer to the theoretical equilibrium from the isotope value of a P pool, the faster that the P pool is biologically cycled, thus the higher the bioavailability, and vice versa (Stout et al., 2014; Joshi et al., 2016, 2018). It should be noted that the isotope equilibrium value is independent of the P input source but is determined entirely by the ambient soil environment where biological P cycling occurred. This technique has been widely applied in aquatic environments to trace P sources (Elsbury et al., 2009; Paytan et al., 2011; Granger et al., 2017; Gooddy et al., 2018), in soil-plant systems to reveal the soil P cycle (Frossard et al., 2011; Melby et al., 2013; Pfahler et al., 2012; Pistocchi et al., 2017; Jaisi et al., 2018; Weiner et al., 2018; Hacker et al., 2019; Sun et al., 2020), and in microbial activity studies to investigate microbial activity (Stout et al., 2014; Li et al., 2016; Lis et al., 2019). Previous studies have shown that the $\delta^{18}\text{O}_\text{P}$ value of soil is mainly determined by P sources, biological activity, temperature, and $\delta^{18}\text{O}_\text{w}$ (Angert et al., 2012). In addition, the hydrolysis of organic P also impacts the $\delta^{18}\text{O}_\text{P}$ values. Alkaline phosphatases, phosphodiesterases and nucleotidases, and acid phosphatases hydrolyze organic P, leading to the incorporation of one or two oxygen atoms from water into released P_i (Liang and Blake 2006; von Sperber et al., 2014). This incorporation of oxygen from water is subject to kinetic isotope fractionation with values of -30‰ to $+4\text{‰}$ (Liang and Blake 2006; von Sperber et al., 2014; Bai et al., 2020).

Recently, the phosphate oxygen isotope technique combined with the sequential extraction method has provided a new perspective to understand the biological turnover of soil P pools and can be used to better understand bioavailability, speciation, and transformation processes of soil P (Tamburini et al., 2012; Jaisi et al., 2011; Gross et al., 2015; Tian et al., 2020). The variation in the isotopic values of different P pools can provide important information about soil P transformation processes (Joshi et al., 2016). For instance, a continued exchange between the $\text{NaHCO}_3\text{-P}$ pool and the $\text{H}_2\text{O-P}$ pool in field soil was reported by monitoring the $\delta^{18}\text{O}_\text{P}$ values of P pools (Zohar et al., 2010a), and an active transformation from $\text{H}_2\text{O-P}$ and $\text{NaHCO}_3\text{-P}$ into NaOH-P and HCl-P (HCl extractable P) was also identified (Joshi et al., 2016, 2018). In the case of long-term balanced fertilization, the $\delta^{18}\text{O}_\text{P}$ values of $\text{H}_2\text{O-P}$, $\text{NaHCO}_3\text{-P}$, and NaOH-P pools approached equilibrium, implying that the externally applied P was actively mobilized and cycled

and transformed into different P pools (Bi et al., 2018). While the HCl-P pool could be derived from P fertilizer through long reactions and mixed with HCl-P from the rock weathering product (Bi et al., 2018). It was found that the added phosphate was rapidly driven toward isotopic equilibrium with soil water, and this process was mediated by rapid microbial phosphate turnover (Gross et al., 2015). In addition, hydrolyzed products of organophosphorus produced an offset from isotopic equilibrium, which is the result of the strong isotopic fractionation associated with the mineralization process (Gross et al., 2015).

In this study, we analyzed the $\delta^{18}\text{O}_\text{P}$ values of three soil inorganic P (P_i) pools ($\text{NaHCO}_3\text{-P}_\text{i}$, NaOH-P_i , and 1 M HCl-P_i) in a five-year agricultural research field in Hubei, China, with different N application rates. We proposed the following hypotheses: i) With the increase in N uptake by crops, the yield is increased, and the P uptake by crops is also promoted. ii) The crop demand of P imparts changes in soil P pools, such as a decrease in the size of $\text{NaHCO}_3\text{-P}$ and NaOH-P pools and an increase in P_o mineralization. iii) The phosphate oxygen isotope composition reflects the changes and transformation of soil P pools.

2. Materials and methods

2.1. Experiment site and soil sampling

A 5-year crop growth experiment was initiated in 2012 in a winter rapeseed and a summer rice system at the Huazhong Agricultural University Experimental Base, Hubei, China ($30^\circ 28' 10''\text{N}$, $114^\circ 21' 21''\text{E}$). Soil samples were collected from the experimental site, as described in detail in a previous study (Jia et al., 2017). The site is located in a subtropical humid monsoon climate zone with an average annual precipitation of 1269 mm, mostly concentrated from June to August. The yearly average temperature is $15.8\text{--}17.5^\circ\text{C}$, the frost-free period generally lasts 211–272 days, and the annual sunshine duration is approximately 1810–2100 h. The soil is the weathering product of quartz sandstone and is classified as Ferrudalf, a type of Alfisol (USDA) (Jia et al., 2017). Ferrudalf, a typical soil in China, is commonly poor in bioavailable P because of P fixation in high contents of reactive iron (hydr)oxides. In this soil, the organic matter content is 6.72 g kg^{-1} , and the relative mass percentages of clay, silt, and sand are 18.9%, 44.9%, and 36.2%, respectively. After 5 years of agricultural cultivation, the average $\text{pH}_{\text{H}_2\text{O}}$ of the soil was 6.7. The experiment was performed with a randomly distributed design comprising four different treatments (one control and three different N-application rates) in triplicate with each plot measuring $4 \times 5\text{ m}^2$. The application rates of N and P were as follows: 0 N + P + K (0 kg N ha^{-1}), 1 N + P + K (75 kg N ha^{-1}), 2 N + P + K (150 kg N ha^{-1}), and 3 N + P + K (225 kg N ha^{-1}). P_2O_5 and K_2O were applied at rates of 75 kg ha^{-1} and 120 kg ha^{-1} for winter rapeseed, respectively, every year. Potassium chloride was used as potash fertilizer. The P fertilizer used in this study was calcium superphosphate [$\text{Ca}(\text{H}_2\text{PO}_4)_2$], a water-soluble fertilizer. At the end of the rapeseed harvest season (on May 13, 2017), five cores of surface soil (0–20 cm) were retrieved from each treatment plot and mixed thoroughly. Similarly, rainwater ($\delta^{18}\text{O}_\text{w}$ value ranging from -3.2‰ to -3.4‰) was collected during the plant growth season. After removing fine rock pieces and plant roots, each soil sample was partitioned into two parts, with one part centrifuged to extract pore water ($\delta^{18}\text{O}_\text{w}$ value, 0.9‰) and the other part air-dried and sieved ($<100\text{ }\mu\text{m}$) for subsequent analyses. The soil temperature was measured in the field. The daily soil temperature ranged from 3.4°C to 23.6°C during the entire rapeseed growth season.

2.2. Soil physical and chemical properties

The soil water content was measured gravimetrically. The soil pH was measured using a glass electrode with a soil to water ratio of 1.0 : 2.5. The soil organic matter was measured by the potassium dichromate volumetric method through external heating (Nelson et al., 1982). Soil total N was measured by an elemental analyzer (Elementar Vario PYRO

cube). Soil total P was quantified after digestion with concentrated sulfuric acid and perchloric acid (Martin et al., 1999), and the resultant P_i content was measured by using the phosphomolybdate blue colorimetric method (Murphy and Riley 1962).

2.3. Measurement of biomass and nutrient content

After the crop harvest in May 2017, all aboveground parts of the crop from each of the three plots in each treatment were dried at 60 °C. Stalk, pod, and seed were separated and weighed individually. Total N in plants was measured with an elemental analyzer (Elementar Vario PYRO cube). For P measurement, plant samples were first digested with concentrated sulfuric acid and perchloric acid (Martin et al., 1999), and the resultant P_i was measured as before.

2.4. Sequential extraction of P pools

Based on the modified Hedley sequential extraction method (Tiessen and Moir 1993), soil P is classified into different P pools: H_2O -P, $NaHCO_3$ -P, $NaOH$ -P, 1 M HCl-P, 12 M HCl-P, and Residual-P. Among these pools, H_2O -P and $NaHCO_3$ -P are labile and considered bioavailable P (Olsen et al., 1954; Bowman et al., 1978; Tiessen et al., 1983). However, $NaOH$ -P is moderately stable and considered conditionally bioavailable in the long term, but is unavailable directly to plants (Tiessen et al., 1983; Sharpley 1985; Zhang and MacKenzie 1997). 1 M HCl-P and 12 M HCl-P (stable P pool) are stable and not directly bioavailable. Residual P (Residual-P), is the most stable P fraction (Tiessen and Moir 1993).

Soil P was sequentially extracted following the method developed by Hedley et al. (1982) and modified by Tiessen and Moir (1993). In brief, 0.5 g of air-dried, ground, $\leq 100 \mu m$ soil was shaken for 16 h after the addition of 30 ml of sequential extraction reagents. The reagent solutions used were 0.5 M $NaHCO_3$ with a pH of 8.5, 0.1 M NaOH, and 1 M HCl (Tiessen and Moir 1993). After the completion of reactions, the residual soil was extracted for 10 min with 12 M HCl (occluded P) in an 80 °C water bath. After that, the residual soil was extracted with H_2SO_4 and H_2O_2 at 360 °C to quantify residual P. Following each extraction step, the soil-reagent suspension was centrifuged at $25000 \times g$ for 10 min at 0 °C and then filtered (0.45 μm). Supernatant solutions were separated into two aliquots with one aliquot used for the measurement of P_i (as described above) and the other aliquot digested with H_2SO_4 and ammonium persulfate at 121 °C in an autoclave. Each digested suspension was filtered (0.45 μm) for P_t measurement by the standard phosphomolybdate blue method (Murphy and Riley 1962). The P_o content could not be directly measured, thus, it was calculated by the difference between P_t and P_i (Tiessen and Moir 1993). The P_i in the P fertilizer applied to the field was extracted with 1 M HCl.

2.5. Purification of inorganic P and precipitation of silver phosphate

The purification of P_i extracted from 100 g soil followed the protocol described by Joshi et al. (2018). First, the magnesium-induced coprecipitation method (MagIC) (Karl and Tien 1992) was used to concentrate P and to remove humic and fulvic acids, as well as other contaminants (Colman et al., 2005). Then, the MagIC precipitates were dissolved in HNO_3 , and the remaining organic matter was removed by double treatments with amberlite XAD-2 macroporous resin (Sigma, USA) (Tamburini et al., 2010). The contents of total P (P_t) and P_i were determined before and after the treatment to ensure no loss of measurable P_i during the treatment. P_i in the eluent was further purified through ammonium phosphomolybdate precipitation at low pH and then through magnesium ammonium phosphate precipitation at high pH, followed by cation removal using cation-exchange resin (Bio - Rad, USA) (Blake et al., 2005; Tamburini et al., 2010; Joshi et al., 2018). Two milliliters of 1 : 4 $AgNO_3$ reagent ($AgNO_3$: H_2O = 1 : 4) was used for silver phosphate precipitation, and the residual OM (organic matter) in

the silver phosphate precipitate was removed with 15% hydrogen peroxide (Zohar et al., 2010b). Finally, the purified silver phosphate was dried at 105 °C for 48 h to remove the trapped water before phosphate oxygen isotopic analysis. The P_i from P fertilizer was also treated with the same method without the MagIC step (Tian et al., 2016). Due to the very low content of organic matter in Ag_3PO_4 precipitates, the contribution of isotopes due to any trace P from organic matter is considered negligible. Trace contaminants remaining in the sample will be removed by high temperature treatment prior to O isotope testing (McLaughlin et al., 2004).

To measure the oxygen isotopic values of water ($\delta^{18}O_w$), the pore water was first extracted by centrifugation at $10000 \times g$ for 20 min (Hitachi CR21GIII, Japan). The pore water and rainwater samples were filtered through 2 μm filter membrane, sealed, and stored in a 2 ml centrifuge tube until analysis.

2.6. Measurement of phosphate oxygen isotope ratios

To measure the phosphate oxygen isotopic ratio ($\delta^{18}O_p$), approximately 0.3 mg of silver phosphate was packed into a silver capsule and pyrolyzed at 1450 °C. The $\delta^{18}O_p$ value was measured by a Thermo-Chemolysis Elemental Analyzer (TC/EA; Thermo, Germany) coupled with an isotopic ratio mass spectrometer (Thermo Scientific; MAT 253 Plus; IRMS; DELTA V Advantage, Thermo, Germany) at the Third Institute of Oceanography, the State Oceanic Administration, Xiamen. The measured isotope values were calibrated by using the Ag_3PO_4 international standard ($\delta^{18}O_p$ values = 21.7‰ VSMOW, Elemental Microanalysis, UK; $\delta^{18}O_p$ values = 6.44‰, 48.28‰, USA). The $\delta^{18}O_w$ value was determined with gas bench-IRMS by the CO_2 - H_2O equilibrium method, as described by Upreti et al. (2015). The analytical precisions of IRMS measurements for $\delta^{18}O_p$ values and $\delta^{18}O_w$ values were 0.3 delta units and <0.1 delta units, respectively. The $\delta^{18}O_p$ of field replicates in the same treatment displayed an average precision of 0.72 delta units during the entire processing and analytical procedures. The measured $\delta^{18}O_p$ value and $\delta^{18}O_w$ value were presented in the conventional $\delta^{18}O$ notation against Vienna Standard Mean Ocean Water (VSMOW) as the reference.

The measured $\delta^{18}O_w$ values and soil temperature were used to calculate the isotope equilibrium value following the equation developed by Chang and Blake (2015) as:

$$\delta^{18}O_p = (\delta^{18}O_w + 1000) \times e^{\frac{[14.43 \times \frac{1000}{T} - 26.54]}{1000}} - 1000 \quad (1)$$

where $\delta^{18}O_p$ and $\delta^{18}O_w$ refer to the oxygen isotope ratios of P_i and ambient water, respectively, and T denotes the soil temperature in degrees Kelvin (K). Based on the soil temperature during the growth season (3.4–23.6 °C) and the measured oxygen isotopic values of soil water ($\delta^{18}O_w$ = 0.9‰) and rainwater ($\delta^{18}O_w$ = −3.2‰ - 3.4‰), the temperature-dependent equilibrium isotopic value in the field site soil was calculated to be between 18.9‰ and 26.9‰.

2.7. Statistical analysis

Correlation and variance analyses were performed using IBM SPSS Statistics version 20.0. In the same P pool, the P contents and phosphate oxygen isotope ratios at different N application rates were compared and analyzed. In the different P pools, the P contents and phosphate oxygen isotope ratios at the same N application rate were compared and analyzed. The data were presented as the mean \pm SD. In both Duncan's test and Fisher's LSD test (one-way ANOVA), $P < 0.05$ was considered a significant difference (Steel and Torrie 1980).

3. Results and discussion

3.1. Physical and chemical properties of soil

The key soil properties with different N application rates are shown in Table 1. In this study, the pH (approximately 6.70) was constant throughout the experiment. No significant differences in total P (TP) content were observed among the different treatments. The soil total N (TN) with the 225 kg ha⁻¹ N treatment was 0.80 g kg⁻¹, which was significantly higher than those in the other treatments (0.72–0.75 g kg⁻¹), indicating that the soil was enriched with N when the N fertilizer rate reached 225 kg ha⁻¹. It was likely that the highest N application rate could exceed the actual physiological needs of crops. Furthermore, there were no differences in the accumulation of soil organic matter (4.69–5.66 g kg⁻¹) among the four treatments.

3.2. Effect of N application on seasonal rapeseed yield

The rapeseed yields were 1720.9, 2387.9, and 2621.3 kg ha⁻¹ in the 75, 150, and 225 kg ha⁻¹ treatments (Fig. 1 and Table 1S), respectively. Rapeseed biomass showed an increase in stalks, pods, and seeds with N fertilizer application. For example, the biomass in the 225 kg ha⁻¹ N treatment (Table 1S) was over three times as much as that in the control treatment (Table 1S).

As shown in Fig. 1, in three N treatments, the biomass of rapeseed stalks was much greater than those of the pods and seeds. Since the rapeseed plants were tall, leafy, and healthy during a long growth period, they had a high demand for N fertilizer. Our result is consistent with the findings of the study by Sieling et al. (2006) that the application of N fertilizer could significantly increase rapeseed plant height and the numbers of branches and pods, thus eventually leading to a higher biomass yield. To maintain the ratio of N to P, the uptake of soil P also needed to increase proportionally with the increasing N uptake by plants to meet the nutritional requirements.

3.3. Effect of N application on P uptake by crops

The amount of N and P uptake by crops in 2017 is presented in Fig. 2 and Table 2S. The amount of TP uptake by the aboveground parts of the crops in the 5 years (2013–2017) is presented in Table 3S. The TN uptake by the aboveground parts of the crops throughout 2017 gradually increased with N application (174.0–242.4 kg ha⁻¹) (Table 2S). Meanwhile, TP uptake by the crops in the N application treatments (35.2–41.2 kg ha⁻¹) was greater than that in the control treatment (27.0 kg ha⁻¹). We also observed the same trend in Table 3S. In 2013–2017, the TP uptake by the aboveground biomass parts was significantly greater in the N treatments than in the control treatment. These results indicated that N application increased the P content in the rapeseed even if the same amount of P fertilizer was applied. However, the TP content was decreased in the 225 kg N ha⁻¹ treatment (35.2 kg ha⁻¹) compared with that in the 150 kg N ha⁻¹ application treatment (41.3 kg ha⁻¹); therefore, the TP uptake by rapeseed just increased drastically at lowest and moderate N application rates. In contrast, the highest N application rate (225 kg ha⁻¹) led to a weaker promotive effect on P uptake by crops

Table 1

The responses of the key soil properties to long-term N gradient fertilization.

N rate (kg ha ⁻¹)	0	75	150	225
pH	6.86 ± 0.02a	6.70 ± 0.02a	6.89 ± 0.01a	6.52 ± 0.05a
TP (mg kg ⁻¹)	355.87 ± 26.57a	340.49 ± 12.01a	350.97 ± 29.04a	339.54 ± 29.87a
TN (g kg ⁻¹)	0.75 ± 0.02b	0.72 ± 0.00b	0.75 ± 0.01b	0.80 ± 0.02a
OM (g kg ⁻¹)	5.33 ± 0.71a	4.69 ± 0.60a	4.99 ± 0.70a	5.66 ± 1.15a

Different letters following the values in columns indicate significance at $p < 0.05$. TP, Total phosphate; TN, total nitrogen; OM, organic matter.

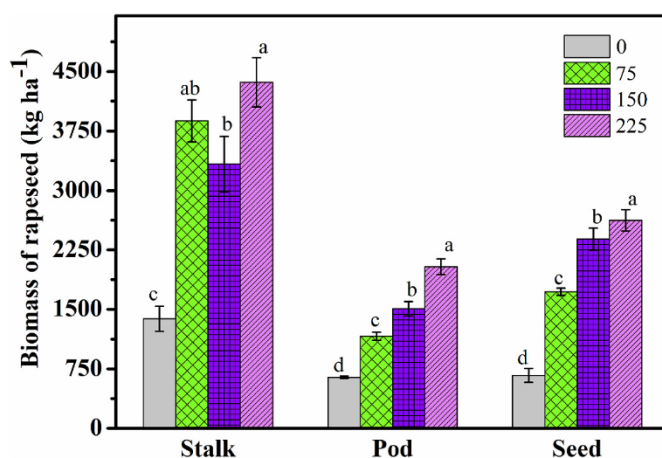


Fig. 1. Biomass yields of rapeseed under different N application rates. The error bars represent standard deviations among triplicate analyses.

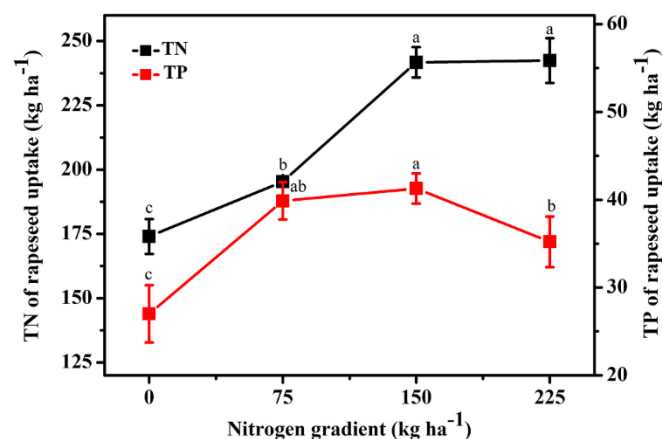


Fig. 2. The content of N and P uptake by crops in 2017. The error bars represent standard deviations among triplicate analyses.

in 2017 (Table 2S). Likewise, in 2013 and 2016, the TP uptake by crops in the 225 kg N ha⁻¹ treatment was also lower than that in the 150 kg N ha⁻¹ treatment. In 2014 and 2015, no significant difference in TP uptake was observed between these two N treatments (Table 3S). This trend in biomass production was consistent over the five years (Fig. 1).

Compared to both the control treatment and the highest N treatment (225 kg ha⁻¹), the N:P ratio of the crops was decreased in the lowest and moderate N treatments, also indicating a promotive effect of an appropriate N application on P uptake (Table 2S). It was also reported that the N:P ratio could be maintained by a simultaneous increase in N and P uptake and that more effective P uptake was achieved via greater root mass (Qian and Schoenau 2000). Therefore, the application of N fertilizer promoted P uptake, but it led to a weaker effect at the highest N rate, which was consistent with previous reports by Koper et al. (2008) and Lemanowicz et al. (2010). The synergistic effect of N application on P uptake most likely resulted from soil P transformation or changes in the distribution of soil P pools.

3.4. Effect of N application on P fractions

The size of the NaHCO₃-P_i pools (biologically available P pool) (25.3–8.4 mg kg⁻¹) in the N treatments was significantly decreased compared to that in the control treatment (Fig. 3 and Table 4S). And that in the 225 kg ha⁻¹ treatment markedly decreased compared with those in the other N treatments (Fig. 3). These indicated that the NaHCO₃-P_i pool was preferentially used up by the plants (Table 3S) and was not

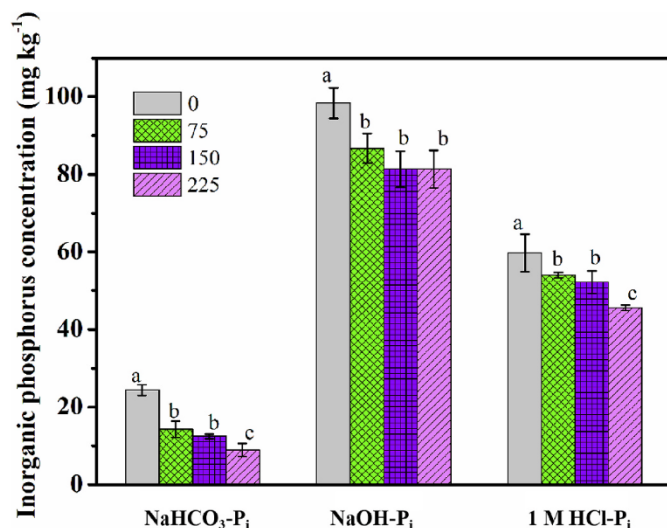


Fig. 3. Inorganic phosphorus concentrations in different P pools. The NaHCO₃-P_i pool extracted by 0.5 M NaHCO₃ (pH 8.5); the NaOH-P_i pool extracted by 0.1 M NaOH; and the HCl-P_i pool extracted by 1 M HCl. The error bars represent standard deviations among triplicate analyses.

replenished at the time of the growing season. These results were consistent with the findings in several previous studies that soil bioavailable P fractions (NaHCO₃-P_i and NaHCO₃-P_o) were significantly decreased with N addition in agricultural cropping systems (Singh et al., 2001; Chen et al., 2018; Lourenzi et al., 2021). However, this is in contrast with the above observation that the P uptake by crops in the highest N treatment actually decreased with respect to the lowest and moderate N treatment (Fig. 2, Tables 2S and 3S). The possible reasons for the decreasing P uptake might be the higher P runoff and leaching due to the most mobilized P pool in the highest N treatment (McDowell and Sharpley 2001; Cui et al., 2020). Our results further showed a significant decrease in the sizes of the NaOH-P_i pool (from 86.6 to 81.3 mg kg⁻¹) and 1 M HCl-P_i pool (from 59.7 to 45.6 mg kg⁻¹) in the N application treatments compared to the control treatment (98.4 and 59.7 mg kg⁻¹) (Fig. 3). In addition, the 1 M HCl-P_i pool in the 225 kg ha⁻¹ treatment exhibited the lowest P content (Table 6S). The decrease in relatively less bioavailable and more stable P pools, such as the NaOH-P_i and HCl-P_i pools, indicated that the NaHCO₃-P_i pool size in the soil might no longer meet the crop P demand in these treatments. Under these circumstances, these relatively less bioavailable and more stable P pools was subjected to mobilization to supplement the NaHCO₃-P_i pool and supply enough P for crop growth (Hassan et al., 2012).

The size of the 12 M HCl-P_i pool in the 75 kg N ha⁻¹ treatment (27.0 mg kg⁻¹) was significantly reduced, while that in the 225 kg N ha⁻¹ treatment was remarkably increased (63.6 mg kg⁻¹), compared with other N application treatments (P contents of 27.0–45.0 mg kg⁻¹) and the control treatment (P content of 50.2 mg kg⁻¹) (Table 4S). The possible reason for this trend could be because the 12 M HCl-P_i pool in the 225 kg N ha⁻¹ treatment might be partially supplemented by other P pools. Moreover, the size of the Residual-P pool showed no significant difference among all treatments (Table 4S), indicating that N fertilizer application had little effect on the residual P, at least during the investigation time.

For the size of NaHCO₃-P_o pools, no significant differences were observed among the four treatments (Fig. 4 and Table 5S). While the size of the NaOH-P_o pool in the 150 kg N ha⁻¹ treatment was significantly smaller than that in the other two N treatments. It could be presumed that more hydrolysis of the NaOH-P_o pool occurred in this treatment than in the other treatments (Fig. 4 and Table 5S). The inorganic P generated from hydrolysis of the NaOH-P_o pool was utilized by crops or transformed into other P pools such as the NaHCO₃-P_i or NaOH-P_i pools.

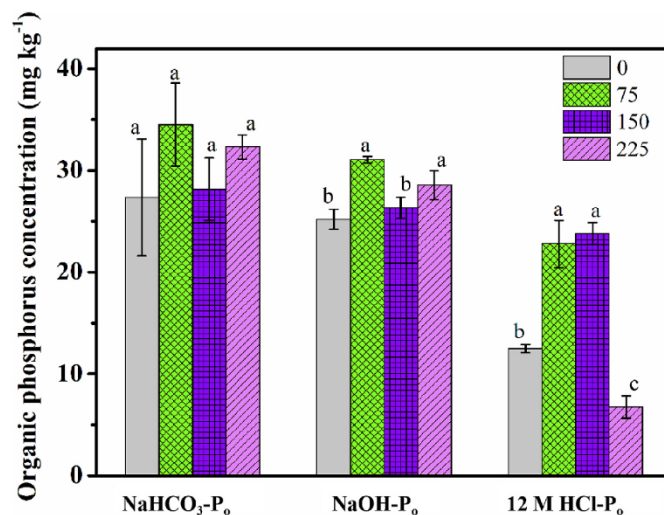


Fig. 4. Organic phosphorus concentrations in different P pools. The NaHCO₃-P_o pool extracted by 0.5 M NaHCO₃ (pH 8.5); the NaOH-P_o pool extracted by 0.1 M NaOH; and the HCl-P_o pool extracted by 12 M HCl. The error bars represent standard deviations among triplicate analyses.

In addition, the size of the 12 M HCl-P_o pool was smaller in the 225 kg N ha⁻¹ treatment than in the other treatments (Table 5S), suggesting hydrolysis of this pool. Therefore, it was likely that the highest N application rate could promote the hydrolysis of rather recalcitrant P_o pool. The size of the 12 M HCl-P_i pool was larger in the 225 kg N ha⁻¹ treatment than in the other treatments (Table 4S), indicating that part of the hydrolysis products of the 12 M HCl-P_o pool might have transferred into the 12 M HCl-P_i pool. The hydrolyzed P from 12 M HCl-P_o pool or NaOH-P_o pool would transfer directly to the NaHCO₃-P_i pool first. However, if the hydrolyzed free P or the directly formed NaHCO₃-P_i cannot be absorbed by crops or microorganisms in time, those parts of P close to the surface of the original mineral particles, could readily be readsorbed and fixed again and merged into 12 M HCl-P_i or NaOH-P_i pool. In our previous work (Yan et al., 2014c), it was found that desorbed P was easily readsorbed to the oxide surface after 20 h. Therefore, the hydrolysis of P from the 12 M HCl-P_o pool or NaOH-P_o pool might lead to indirect formation of the 12 M HCl-P_i or NaOH-P_i pool. Therefore, the transformation of soil P pools and the increase of soil P bioavailability could also occur via hydrolysis of moderately stable and stable soil organic P (Figs. 3 and 4).

Although the 1 M HCl-P and 12 HCl-P pools are stable soil P pools, our results suggest that their mobilization could occur under the highest N application rate (Figs. 3 and 4). These results are consistent with previous reports of enhanced P mobilization under high P deficiency stress conditions (Alamgir et al., 2013; Amelung et al., 2015; Roberts et al., 2015; Polain et al., 2018). Although the highest N rate promoted the hydrolysis of 12 M HCl-P_o, the P uptake of the plants was similar to or even lower than that at the moderate N rate (41.3 kg ha⁻¹) (Table 2S). This might be related to attenuated P uptake due to the higher P runoff and leaching at the highest N application rate, as mentioned above.

3.5. N application effect on phosphate oxygen isotope compositions of soil P pools

A history of 5 years of fertilization and agricultural use changed the phosphate oxygen isotope compositions of different inorganic P pools (Fig. 5). To better understand the transformations of P among different P pools, the temperature-dependent isotope equilibrium values were calculated based on the soil temperature during the entire rapeseed growth season and the measured $\delta^{18}\text{O}_{\text{w}}$ values of soil water and rainwater (Eq. (1) above, Chang and Blake 2015) and are shown in the gray zone in Fig. 5.

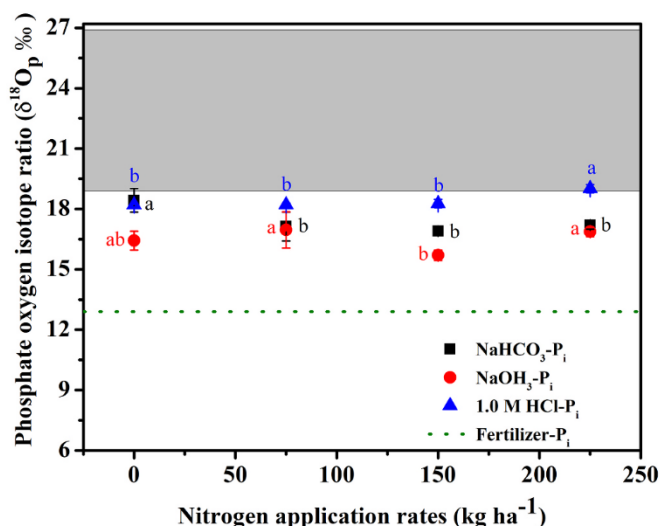


Fig. 5. The oxygen isotope ratios in phosphate ($\delta^{18}\text{O}_\text{P}$) of soil inorganic P pools among different nitrogen gradient fertilized treatments. The error bars represent standard deviations among triplicate analyses.

The $\delta^{18}\text{O}_\text{P}$ values of the $\text{NaHCO}_3\text{-P}_\text{i}$ pools in the N treatments (from 17.2‰ to 16.9‰) deviated from the equilibrium range, and there were no significant differences in the $\delta^{18}\text{O}_\text{P}$ values among the different N treatments (Fig. 5 and Table 6S). Even though the $\text{NaHCO}_3\text{-P}_\text{i}$ pool was the most bioavailable P pool, the $\delta^{18}\text{O}_\text{P}$ value significantly dropped in this pool and deviated from the equilibrium zone. This might be due to the low $\delta^{18}\text{O}_\text{P}$ value of the applied P fertilizer (12.9‰), which is consistent with past findings that part of the applied P fertilizer supplements into the $\text{NaHCO}_3\text{-P}_\text{i}$ pool (Joshi et al., 2016). The $\delta^{18}\text{O}_\text{P}$ value of the $\text{NaHCO}_3\text{-P}_\text{i}$ pool in the control treatment was close to the equilibrium range (Fig. 5), indicating that the biological turnover of P in the $\text{NaHCO}_3\text{-P}_\text{i}$ pool in this treatment was nearly complete. Studies on $^{18}\text{O}_\text{P}$ values in plants have shown that crops enrich $^{18}\text{O}_\text{P}$ (Pfahler et al., 2013). In the presence of soil biota alone, biological turnover and cycles of P drive the isotope values of $\text{NaHCO}_3\text{-P}_\text{i}$ pool toward equilibrium (Blake et al., 2005; Tamburini et al., 2012; Helfenstein et al., 2018). Meanwhile, the $\delta^{18}\text{O}_\text{P}$ values of the $\text{NaHCO}_3\text{-P}_\text{i}$ pool were lower in the N treatments (16.9‰–17.2‰) than in the control (18.4‰). With the addition of N fertilizer, the $\text{NaHCO}_3\text{-P}_\text{i}$ pool was no longer able to meet the needs of crops for P, leading to a partial transformation of the NaOH-P_i pools into the $\text{NaHCO}_3\text{-P}_\text{i}$ pools. This was also confirmed by the fact that the size of the $\text{NaHCO}_3\text{-P}_\text{i}$ pool significantly decreased among the different N treatments (Fig. 3).

The $\delta^{18}\text{O}_\text{P}$ values of the NaOH-P_i pool deviated from the equilibrium range, and no significant differences in the $\delta^{18}\text{O}_\text{P}$ values were observed among three treatments except the moderate N treatment (Fig. 5 and Table 6S). Contrastly, the $\text{NaHCO}_3\text{-P}_\text{i}$ pool approached isotope equilibrium, indicating its more rapid biological turnover due to its higher bioavailability (Joshi et al., 2016; von Sperber and Stallforth 2017). Therefore, the NaOH-P_i pool underwent less biological turnover likely due to its large size and relatively less availability to plants (Wang et al., 2013a, 2013b; Yan et al., 2014a, 2014b). For example, the size of the NaOH-P_i pool was 1.5- to 9.0-fold as much as that of the $\text{NaHCO}_3\text{-P}_\text{i}$ pool in all treatments including the control treatment (Table 7S), and there was no significant difference in the size of the NaOH-P_i pool among the three different N treatments (Fig. 3). Furthermore, the $\delta^{18}\text{O}_\text{P}$ values of the NaOH-P_i pools were significantly lower (15.7–17.0‰) than those of the 1 M HCl- P_i pools, especially in the 150 kg ha⁻¹ treatment (Fig. 5, Table 8S). This implied that the applied P fertilizer (with a light $\delta^{18}\text{O}_\text{P}$ value of 12.9‰) was mainly transformed into the NaOH-P_i pool. Furthermore, the lower $\delta^{18}\text{O}_\text{P}$ value of the NaOH-P_i pool in the 150 kg ha⁻¹ treatment (Fig. 5) than in the other treatments. This might be due

to the produced P_i from the hydrolysis of P_o pools (Fig. 4, Table 5S) because the P_i derived from the hydrolysis of NaOH-P_o possessed the lower isotope values (Liang and Blake 2006; von Sperber et al., 2014; Joshi et al., 2015; Sun et al., 2019). Moreover, the $\delta^{18}\text{O}_\text{P}$ values of the NaOH-P_i pools and the $\text{NaHCO}_3\text{-P}_\text{i}$ pools in both the 75 kg ha⁻¹ N treatment (17.0‰ and 17.1‰) and those of the 225 kg ha⁻¹ N treatment (16.9‰ and 17.2‰) were almost the same. This implied the close transformation relationship between NaOH-P_i and $\text{NaHCO}_3\text{-P}_\text{i}$, and relatively higher bioavailability of NaOH-P_i with N application. It might be the hydrolysis of NaOH-P_o causing the different trend in the moderate N treatment. Considering that crop yield was very high at N rates (Table 2S), the demand was proportionally increased following N: P of the crop (Qian and Schoenau 2000). In this case, P was released from the NaOH-P_i pool and was transformed into the $\text{NaHCO}_3\text{-P}_\text{i}$ pool to meet the P demand of crops. Therefore, the variation in $\delta^{18}\text{O}_\text{P}$ values of the $\text{NaHCO}_3\text{-P}_\text{i}$ pool and NaOH-P_i pool in different N treatments suggested P transformation in response to the increasing P demand by crops.

The $\delta^{18}\text{O}_\text{P}$ values of the 1 M HCl- P_i pools were close to the equilibrium range (18.9–26.9‰) (Fig. 5) and showed no significant differences among the lowest N treatment, the moderate N treatment, and the control treatment. Therefore, considering the stability of 1 M HCl- P_i pools, their $\delta^{18}\text{O}_\text{P}$ values might represent the original isotope signature of the soil (Angert et al., 2012; Tamburini et al., 2012). The experimental field soil derived from the fallow area in Shizi Hill of Huazhong Agricultural University has been cultivated since spring 2012. A few studies reported that the $\delta^{18}\text{O}_\text{P}$ values of 1 M HCl- P_i were likely to remain unchanged after years of cropping (Angert et al., 2011; Bi et al., 2018). The other two P pools ($\text{NaHCO}_3\text{-P}_\text{i}$ and NaOH-P_i) were influenced by the fertilizer P application, resulting in changing their isotope values followed by isotope excursion toward equilibrium.

The $\delta^{18}\text{O}_\text{P}$ value of the 1 M HCl- P_i pool in the 225 kg ha⁻¹ treatment was the highest of all (19.0‰) (Fig. 5, Table 8S), but its pool size was the smallest (Fig. 3). This indicates that at the highest N application rate, the turnover and transformation of the generally stable 1 M HCl- P_i pool could also occur. As mentioned above, in rapeseed, rhizosphere acidification-induced stable P dissolution (Bertrand et al., 1999) increases the bioavailability of total P. The root architecture and related performance might also affect P utilization by rapeseed (Strong et al., 1974; Wen et al., 2016). Under neutral or alkaline conditions, the 1 M HCl-P pool might be involved in the bidirectional exchange of P_i , including the precipitation of Ca-P minerals (Helfenstein et al., 2020). The limited treatments and isotope data do not provide sufficient evidence to interpret the effect of N application on P uptake, but our results provide a perspective to tap more P from stable P (such as HCl- P_i) or promote the hydrolysis of organic P.

In summary, the synergistic effect of N application on P uptake by plants is most likely to be achieved through P_i transformation and/or hydrolysis of P_o . The N fertilizer addition facilitated the transformation of different soil P fractions which could be mainly responsible for the increased bioavailability of soil P. This synergistic effect became even greater at the highest N rate. N application promoted the growth of crops (Fig. 1). The demand for P increased with crop growth (Fig. 2), and the demand could be satisfied through the transformation of soil P pools (Figs. 3–5). Rapid uptake of the $\text{NaHCO}_3\text{-P}_\text{i}$ pool by crops and the mobilization of NaOH-P_i and 1 M HCl- P_i pools might occur when the $\text{NaHCO}_3\text{-P}_\text{i}$ pool could not meet crop growth demand with N application (Fig. 3). In addition, soil P mobilization continuously occurred via the transformation of stable P pools and moderately stable P into bioavailable P pools, such as the transformation of 12 M HCl- P_o and NaOH-P_i to $\text{NaHCO}_3\text{-P}_\text{i}$ (Fig. 5, Table 4S, 5S and 7S). However, the underlying mechanisms of P activation under N application are very complicated and may involve complicated interactions of plants and microorganisms with soil P (Turan et al., 2007; Khalili-Rad et al., 2015). Several important pathways of P activation by plants and microbes include the fact that plant roots (1) secrete phosphatase to increase the hydrolysis of organophosphorus (Tadano and Sakai 1991), (2) secrete organic acids

and/or protons to dissolve refractory soil P (Dinkelaker et al., 1989; Lemanowicz and Koper 2010; Wang et al., 2015), and (3) recruit a large number of phosphate-solubilizing microorganisms to mobilize P (Khan et al., 2007).

4. Conclusions

Our results showed that the rapeseed yield and P uptake significantly increased with the increased N fertilizer application rate. However, the highest N application rate (225 kg N ha⁻¹) did not stimulate P uptake as moderate N application did, suggesting that these processes are concentration dependent. The NaHCO₃-P_i pool was more sensitive to N fertilizer application and was more quickly taken up by crops. P fertilizer with a low δ¹⁸O_P value (12.9‰) was mainly transformed into the NaOH-P_i pool. In the lowest N and the highest N treatments, moderately stable P was transformed into bioavailable P. The moderate N treatment promoted organic P transformation to inorganic P. Furthermore, the highest N application rate enhanced the mobilization of stable P. Overall, these results indicate that N fertilizer application can promote the turnover and transformation of soil P, which partly accounts for the enhanced soil P bioavailability and P uptake and possibly causes increased P losses through runoff and leaching. In addition, the phosphate oxygen isotope technique can provide novel insight into the P transformation processes in soils and various other geochemical environments. Further experiments are needed to reveal the detailed mechanisms underlying the mobilization of soil P, including that of moderately stable (NaOH-P) and stable P (HCl-P) upon N application.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apgeochem.2021.105094>.

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