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RESEARCH ARTICLE

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# Effects of different fertilization levels on the concentration of high molecular weight glutenin subunits of two spring, hard red bread wheat cultivars

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**Abstract**

**Backgrounds and objectives:** High molecular weight gluten subunits (HMW-GS) are generally considered to play a key role in gluten formation and structure and are closely related to wheat quality. In this study, two spring wheat cultivars (PAN3497 and SST806) with the same HMW-GS composition (1Ax1, 1Bx7, 1By8, 1Dx2, and 1Dy12) were tested in the greenhouse, over 2 years, in order to determine how different genetic backgrounds, and low-nitrogen and low-phosphorus treatments, influenced the expression and quantity of the HMW-GS, as measured by reverse-phase high-performance liquid chromatography (RP-HPLC).

**Findings:** Cultivar effect was highly significant for all HMW-GS, except for 1By8. A large treatment effect was found on subunits 1Ax1, 1Dy12, and 1By8. Cultivar-by-treatment interaction was highly significant and contributed to variation of subunit 1Dx2. Subunits 1Dy12 and 1By8 were highly influenced by low-N conditions. Ratio of HMW-GS x type/y type was higher for PAN3497 than for SST806. Furthermore, a strong treatment effect was observed for the ratio of x type/y type, which was 45% higher in PAN3497 under optimal and low-N conditions, compared to SST806.

**Conclusions:** This study showed that fertilization level had a considerable effect on HMW-GS composition and on FPC, with low-N conditions having the largest influence, followed by a combination of low N and low P.

**Significance and novelty:** The results highlighted the importance of the y-type subunits in the bread wheat quality breeding programs, especially under low-N and low-P growing conditions.

**KEYWORDS**
abiotic stress, glutenin subunits, nutrient deficiency, wheat quality

## 1 | INTRODUCTION

Wheat is one of the most important staple cereal crops in the world. It is grown on 215 million ha, and its world trade is more than that of other crops combined. Wheat production was 757 million metric tons in 2017. It is consumed by 2.5 billion people in 89 countries in many forms, of which bread is one of the most important end-uses (FAO, 2018).

Wheat and flour quality largely determines the quality of bread; therefore, knowledge of the bread-making parameters and the factors influencing them is crucial. Wheat grains contain approximately 75%–80% carbohydrates, and their protein content may vary between 8% and 20% of the total dry matter. Storage proteins play a major role in wheat quality. The two most important storage proteins are gliadins and glutenins (gluten), which constitute about 80%–85% of the total protein content. Glutenin determines the viscoelastic properties that are essential for dough formation, while gliadin influences the viscosity and confers extensibility of the dough. Glutenin is subdivided according to molecular weight of the protein into high molecular weight (HMW) and low molecular weight (LMW) subunits (Payne, Holt, & Law, 1981). About 50% of the storage proteins consist of gliadin, while the other 50% consist of HMW (10%) and LMW (40%) glutenin subunits (GS), respectively (Payne, Holt, Jackson, & Law, 1984).

The HMW-GS are encoded by three loci, *Glu-A1*, *Glu-B1*, and *Glu-D1*, located on the long arms of chromosomes 1A, 1B, and 1D, respectively (Rogers, Payne, & Harinder, 1989). Among HMW-GS subunits, the x types (subunits 1–7) are more important than the y types (subunits 8–12) for baking quality characteristics. In particular, the presence of subunit 1Bx7 was reported to contribute to good wheat quality (Lukow, Forsyth, & Payne, 1992). Quantitative analyses demonstrated that the concentration of HMW-GS varied within a broad range, depending on the genotype and growing conditions. Environmental conditions, such as nutrient deficiency, tend to increase the relative concentration of gliadins and decrease the relative concentration of glutenin in the kernel (Johansson, Prieto-Linde, & Svensson, 2004; Zörb, Ludewig, & Hawkesford, 2018). Furthermore, nitrogen (N) management can influence the metabolic activity within the plant and the protein composition within the kernel (Garcia-Molina & Barro, 2017; Xue et al., 2016).

In this study, the influence of low-N and low-phosphorus (P) application and a combination of the two on concentration of HMW-GS 1Ax1, 1Bx7, 1By8, 1Dx2, and 1Dy12, as determined by RP-HPLC, in two spring wheat cultivars over 2 years was investigated.

## 2 | MATERIAL AND METHODS

### 2.1 | Greenhouse trials

Two commercial South African hard red spring wheat cultivars, PAN3497 and SST806 (which is the commercial standard for spring wheat baking quality in South Africa), with excellent baking quality were used. These cultivars had the same HMW-GS composition of 1Ax1, 1Bx7, 1By8, 1Dx2, and 1Dy12. The cultivars were sown in 2-L pots, filled with 2 kg soil. The soil was collected from 1.5-m-deep subsoil, with very low nutrient content. The trial was conducted in the greenhouse in a randomized complete block design.

Four treatments (optimal, low N, low P, and a combination of low N and low P) were applied to the two cultivars, with three replications, 15 pots per replication in 2016, and 20 pots per replication in 2017. Each pot contained three plants. The trials were carried out from June to the end of October 2016 and the same time in 2017. The day/night temperature in the greenhouse was maintained at 22 and 18°C, respectively, for both years. Low N, low P stress, and a combination of the two were initiated at three-leaf stage according to the protocol given in Table 1. For the control, the plants were optimally fertilized and irrigated with deionized water. Once a week, all pots were flushed with deionized water to prevent salt buildup. Treatments were applied twice a week (250 ml nutrient solution per pot). The electric conductivity was maintained at 1.5 mS/cm$^2$ until tillering and 1.80 mS/cm$^2$ after tillering.

All treatments received the same micronutrient fertilization that consisted of 3.45 mg/L $C_{10}H_{13}FeN_2O_8$, 0.30 mg/L $MnSO_4$, 0.13 mg/L $ZnSO_4$, 0.62 mg/L $H_3BO_3$, 0.05 mg/L $CuSO_4$, and 0.02 mg/L $Na_2MoO_4$. At maturity, the seeds were harvested and milled into whole flour with a laboratory mill (IKA A10 Yellowline analysis grinder; Merck Chemicals Pty Ltd).

**TABLE 1** Fertilization applied during the greenhouse experiment for both seasons for two wheat cultivars

| Chemicals | Optimal | | Low N | | Low P | | Low N and P | |
|---|---|---|---|---|---|---|---|---|
| (mg/L) | UT | AT | UT | AT | UT | AT | UT | AT |
| $KNO_3$ | 261 | 313 | 0 | 0 | 228 | 273 | 0 | 0 |
| $K_2SO_4$ | 210 | 252 | 210 | 252 | 196 | 235 | 196 | 235 |
| KCl | 0 | 0 | 193 | 231 | 56 | 67 | 223 | 268 |
| $NH_4H_2PO_4$ | 87 | 104 | 87 | 104 | 0 | 0 | 0 | 0 |
| $Ca(NO_3)_2$ | 758 | 909 | 0 | 0 | 797 | 956 | 0 | 0 |
| $CaCl_2$ | 0 | 0 | 353 | 424 | 0 | 0 | 446 | 446 |
| $MgSO_4$ | 348 | 418 | 348 | 418 | 369 | 443 | 443 | 443 |

Abbreviations: AT, after tillering; UT, up to tillering.

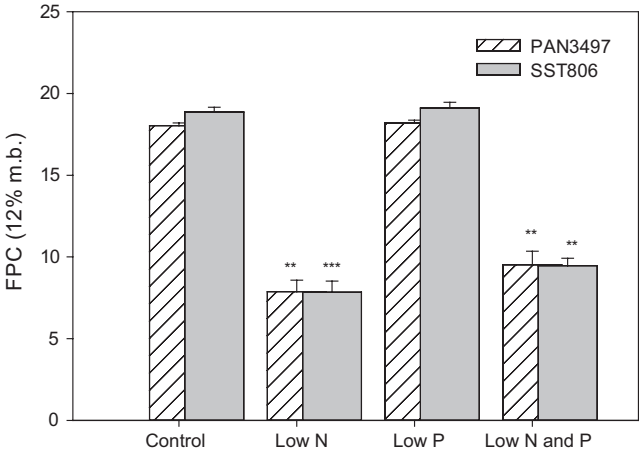


**FIGURE 1** Percentage flour protein content of two cultivars at four different fertilizer treatments for 2 years. ** and *** indicate significant ($p \leq .01$) and highly significant ($p \leq .001$) from the optimal control

## 2.2 | Reverse-phase high-performance liquid chromatography

Samples for RP-HPLC were prepared according to Vawser and Cornish (2004) with some modifications. Filtered deionized water (Elix®; Millipore) was used to prepare solvents and eluents. The flour samples (200 mg) were extracted in 50% propanol containing 1% dithiothreitol (DTT) followed by vortexing for 30 s. Samples were incubated in a water bath at 60°C for 30 min (with 5 min of intermittent vortexing) followed by centrifugation (1,700 *g*) for 10 min. Supernatant was transferred into a clean reaction tube, and extraction was done twice. Propanol concentration of the combined supernatants was increased to 600 ml/L to precipitate the HMW-GS. Samples were incubated for 1 hr at 4°C and centrifuged at 1,700 *g* for 10 min. The pellet was then suspended in 600 µl RP buffer containing 50% propanol, 2 M urea, 0.2 M Tris-HCl (pH 6.6), and 1% DTT, followed by incubation at 60°C in a water bath for 1 hr (vortexed every 10 min). Then, 50% propanol containing 71% 4-vinylpyridine was added, followed by vortexing (30 s) and a further incubation at 60°C for 15 min and then centrifugation (1,700 *g* for 4 min). Supernatants were filtered through Acrodisc® syringe filters with a 0.45-µm HT Tuffryn® membrane (Pall®; Life Science), each sample into its own glass vial. Routine HPLC analyses were performed on a Shimadzu 20A HPLC system (Shimadzu Scientific Instruments) fitted with a SPD-M20A Prominence Diode Array detector, equipped with a Class-VP™ chromatography data system for integration events, CTO-10AS VP column oven set at 50°C; and a Gilson FC204 fraction collector (Gilson Inc.). Separation was performed with a Jupiter 300 C18 column (Phenomenex®) over 70 min. Injection volume was 15 µl, and quantification was achieved at 210 nm. Elution system A contained deionized water with trifluoroacetic acid (TFA, 1 ml/L); elution system B consisted of acetonitrile containing 1 ml/L TFA. The linear elution gradient was as follows: 0–3 min 260 ml/L B, 3–49.8 min 260–350 ml/L B, 49.8–50 min 350–500 ml/L B, 50–53.5 min 500 ml/L B, 53.5–54 min 500–260 ml/L B, and 54–70 min 260 ml/L B. Flow rate was 1 ml/min. Absorbance units under different peaks were calculated according to Vawser and Cornish (2004) and expressed as percentages of the total peak area.

## 2.3 | Flour protein content

Flour protein content was determined with AACC procedure 44-15A (AACC (American Association of Cereal Chemists) 2000).

## 2.4 | Statistical analysis

Analysis of variance (ANOVA) and correlation analysis were done with Agrobase (2018) software on the data generated from RP-HPLC. The data represented each HMW-GS peak as a percentage of the total peak area (Vawser & Cornish, 2004) in order to make the data comparable for the four different treatments.

# 3 | RESULTS

The mean values (combined for 2 years) of flour protein content (FPC) varied between 7.87% (low N) and 18.19% (low P) for PAN3497 and ranged from 7.84% (low N) to 19.11% (low P) in SST806 (Figure 1). FPC was significantly lower ($p \leq .01$) under low N than optimal conditions in PAN3497 and SST806. FPC decreased significantly under the combined low-N and low-P treatment ($p \leq .01$) in both cultivars. Low P did not have any detrimental effects on the FPC.

Although the HMW-GS composition was the same for both cultivars, cultivar played an important role in the quantity of each subunit expressed. The average values for HMW-GS 1Ax1, 1Bx7, and 1By8 were higher in SST806 than in PAN3497. The difference between the two cultivars was approximately 18% in the case of 1By8. In addition, a large difference between the cultivars was also observed in the 1Dy2 and 1Dy12 subunits, with values being higher in PAN3497 than in SST806. The concentration of HMW-GS 1Bx8 decreased by 27% due to low N in PAN3497 and by 47% in the case of the combined low-N and low-P treatment. A significant decrease was also detected in 1Dy12 due to the low-P treatment (23%; Table 2).

The effect of the low-N treatment was by far the most severe on HMW-GS 1By8 in SST806, causing a 44% decrease in this subunit. The concentration of 1Dx2 increased by 19% due to the low-N treatment. The same HMW-GS decreased by 45.5% due to the low-P treatment (Table 3).

**TABLE 2** Peak percentage areas of high molecular weight glutenin subunits 1Ax1, 1Bx7, 1By8, 1Dx2, and 1Dy12 in PAN3497 at different nitrogen and phosphorus treatments

| HMW-GS | Optimal | Low N | Low P | Low N and P |
|---|---|---|---|---|
| 1Ax1 | 11.89 ± 0.98 | 11.87 ± 0.21 | 11.87 ± 0.63 | 12.33 ± 0.27 |
| 1Bx7 | 45.75 ± 1.50 | 47.44 ± 1.88 | 49.01 ± 1.83 | 48.04 ± 0.61 |
| 1By8 | 9.84 ± 1.21 | 7.18 ± 1.53* | 10.90 ± 2.41 | 5.24 ± 0.75** |
| 1Dx2 | 19.52 ± 1.61 | 19.95 ± 1.02 | 18.24 ± 0.85 | 22.33 ± 0.97 |
| 1Dy12 | 13.01 ± 0.95 | 13.56 ± 0.48 | 9.97 ± 0.50** | 12.07 ± 0.10 |

*$p > .05$; **$p > .01$; and ***$p > .001$represent significant difference compared to the optimal treatment.

The results of the analysis of variance showed that cultivar effect (as seen from significance of F ratios of mean squares) were highly significant ($p \leq .001$) for 1Ax1, 1Bx7, 1Dx2, and 1Dy12 (Table 4). Cultivar effect contributed the most to variation in HMW-GS 1Dy12. Furthermore, there was a large treatment contribution to variation in this same subunit. Year contributed highly to variation in HMW-GS 1By8 and 1Dy12. HMW-GS 1Dx2 showed large cultivar x treatment interaction.

To get a better understanding of the effect of the treatments on the HMW-GS without the effect of genotype playing a role, values were averaged for the two cultivars (Table 5). Low N and a combination of low N and low P caused a significant increase in the concentration of 1Ax1. The average value for 1Dy12 was highly significantly decreased when plants were grown under low levels of N (57%) and a combination of low N and low P (46.6%). The standard deviation for this subunit was also very high. No treatment effect was detected on 1Dx2, and its concentration remained similar for all four treatments. Low N and a combination of low-N and low-P treatment caused a significant increase in the concentration of subunit 1By8.

An interesting trend was that the low-N treatment caused a significant decrease in FPC, but it caused a significant increase in subunits coded by *Glu-A1* and *Glu-B1* (1Ax1, 1By7, and 1By8) for combined data of the two cultivars, but a significant reduction in subunit 1Dy12. A combination of low N and P also caused a significant reduction in the FPC, although not as severe as in the case of the low-N treatment, and it caused a significant increase in subunits 1Ax1 and 1By8, but not in subunit 1By7. As in the case of the low-N treatment, it caused a significant reduction in subunit 1Dy12.

The x-type HMW-GS concentration was higher than that of the y-type subunits in all samples. The x:y ratio for the *Glu-D1* subunits was more than doubled due to the effect of low N and low N and low P combined (Table 6). For the *Glu-B1* subunits, there was a decrease in the x:y ratio due to especially low N and a combination of low-N and low-P conditions. Overall, the x:y ratio was increased due to low N and combined low-P and low-N conditions, mainly due to the large increase in the ratio of the *Glu-D1* subunits.

## 4 | DISCUSSION

Liu et al. (2016) concluded that dough strength and baking performance of wheat cultivars are related to allelic variation in HMW-GS. Vasil and Anderson (1997) stated that the HMW-GS alleles 1Ax1 and 1Ax2* and the 1Dx5 + 1Dy10 subunit pair are associated with stronger dough and better baking properties, and the 1Dx2 + 1Dy12 pair with weaker dough. Sabine, Oberfoster, Werteker, Grausgruber, and Lelly (1997) reported that the *Glu-B1* alleles 7 + 9 and the *Glu-D1* alleles 5 + 10 occurred more frequently in cultivars with better bread-making quality, and no cultivar with good quality contained subunits 6 + 8 and 2 + 12. Both PAN3497 and SST806 had subunits 2 + 12, but they both have excellent baking quality. Moreover, SST806 is the commercial standard for spring wheat baking quality in South Africa. According to several studies, the expression of storage proteins and the quantity of HMW-GS are strongly associated with genotype (Plessis, Raval, Bordes, Balfourier, & Martre, 2013; Rodriguez-Nogales, Garcia, & Marina, 2006). In the current study, a significant cultivar effect was found in the case of 1Dy12, 1Dx2, 1Bx7, and 1Ax1. HMW-GS composition was found to be genetically determined (Johansson, Henriksson, Svensson, & Heneen, 1993; Payne, Nightingale, Krattiger, & Holt, 1987), but the concentration of subunits is largely determined by environmental

**TABLE 3** Peak percentage areas of high molecular weight glutenin subunits 1Ax1, 1Bx7, 1By8, 1Dx2, and 1Dy12 in SST806 at different nitrogen and phosphorus treatments

| HMW-GS | Optimal | Low N | Low P | Low N and P |
|---|---|---|---|---|
| 1Ax1 | 14.03 ± 1.88 | 16.09 ± 1.55 | 12.17 ± 0.69 | 18.47 ± 2.39 |
| 1Bx7 | 47.34 ± 6.24 | 45.17 ± 7.22 | 48.98 ± 0.67 | 40.72 ± 7.43 |
| 1By8 | 12.02 ± 0.53 | 6.82 ± 0.69*** | 6.55 ± 0.88*** | 11.37 ± 1.97 |
| 1Dx2 | 15.64 ± 2.52 | 19.23 ± 3.15 | 21.63 ± 1.16* | 16.18 ± 0.73 |
| 1Dy12 | 10.96 ± 2.08 | 12.70 ± 2.85 | 10.67 ± 0.70 | 13.24 ± 2.36 |

*$p > .05$; **$p > .01$; and ***$p > .001$ represent significant difference compared to the optimal treatment.

**TABLE 4** Analysis of variance for high molecular weight glutenin subunits in a trial of two wheat cultivars with four treatments over 2 years

| Mean squares | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cultivar (*C*) | Treatment (*T*) | Season (*S*) | *C* × *T* | *C* × *S* | *T* × *S* | *C* × *T* × *S* |
| 1Ax1 | 12.37** | 9.80** | 6.40 | 3.48 | 0.21 | 0.99 | 1.38 |
| 1Bx7 | 600.24** | 20.42 | 21.63 | 21.72 | 5.19 | 20.91 | 15.91 |
| 1By8 | 12.68 | 40.00** | 45.70** | 0.86 | 3.00 | 4.04 | 6.04 |
| 1Dx2 | 111.29** | 2.49 | 0.06 | 25.65** | 3.32 | 6.94 | 5.24 |
| 1Dy12 | 1,121.72** | 178.94** | 114.52** | 45.68 | 1.09 | 17.48 | 2.73 |

**$p \leq .01$.

**TABLE 5** Average peak percentage areas of high molecular weight glutenin subunits 1Ax1, 1Bx7, 1By8, 1Dx2, and 1Dy12 in PAN3497 and SST806 for all four treatments combined

| | PAN3497 | SST806 | LSD (0.05) |
|---|---|---|---|
| 1Ax1 | 10.72 ± 1.35 | 11.73 ± 1.53 | 0.39 |
| 1Bx7 | 40.62 ± 4.67 | 33.55 ± 3.89 | 1.53 |
| 1By8 | 13.59 ± 2.38 | 12.56 ± 2.71 | 0.64 |
| 1Dx2 | 30.61 ± 2.57 | 27.56 ± 1.71 | 0.69 |
| 1Dy12 | 4.92 ± 6.86 | 14.59 ± 6.33 | 1.35 |

*Note:* Values are followed by standard deviations.

factors such as N in the soil (Cho, Kang, Kang, Cho, & Park, 2018; Graybosch, Peterson, Shelton, & Baenziger, 1996). A significant treatment effect was evident for 1Ax1, 1Dy12, and 1By8 in the current study. Low N and a combination of low-N and low-P treatments had a very large influence on the y subunits, specifically 1Dy12 and 1By8.

High yield and good bread-making quality are the most important characteristics in the wheat industry. Both can be improved through N fertilization strategies, such as the rates and timing of N fertilization (Li et al., 2016; Zhong et al., 2019). Yu et al. (2018) reported that grain protein content was more sensitive to N application than grain yield and that protein content was mainly determined by genotype.

Several studies have shown that the increase in flour protein content resulting from N application can lead to changes in protein composition (Gupta, Khan, & MacRitchie, 1993; Saint Pierre et al., 2008; Xue et al., 2016). Studies reported previously were based on various timings of N fertilization, when the application at an early stage increased yield, and at a later stage increased the amount of protein, and improved the baking quality properties (Jia, Fabre, & Aussenac, 1996). In the current study, the low-N treatment decreased FPC significantly, but it changed the protein composition in the sense that HMW-GS coded by *Glu-A1* and *Glu-B1* were increased, but those coded by *Glu-D1* were decreased, especially HMW-GS 1Dy12. On average, the low-P treatment did not have a significant effect on either FPC or the HMW-GS.

Robert, Peterson, Shelton, and Baenzicar (1996) noted that flour protein concentration and the percentage of protein present as gliadin and nongluten proteins were most sensitive to environmental stress conditions, although in the current study it was seen that the HMW-GS were also significantly influenced by low-N and low-P conditions. Glutenin composition was reported to be almost totally genotype dependent (Graybosch et al., 1996), which was also the case in the current study.

## 5 | CONCLUSIONS

This study showed that fertilization level had a considerable effect on HMW-GS composition and on FPC, with low-N conditions having the largest influence, followed by a

**TABLE 6** Average peak percentage areas for PAN3497 and SST806 of high molecular weight glutenin subunits 1Ax1, 1Bx7, 1By8, 1Dx2, and 1Dy12 for four treatments

| | Control | Low N | Low P | Low N and P | LSD (0.05) |
|---|---|---|---|---|---|
| 1Ax1 | 10.33 ± 0.711 | 11.73 ± 1.47 | 10.61 ± 0.63 | 12.23 ± 1.43 | 0.54 |
| 1Bx7 | 36.22 ± 4.62 | 38.87 ± 3.81 | 36.02 ± 4.38 | 37.23 ± 5.72 | 1.53 |
| 1By8 | 11.22 ± 1.10 | 14.68 ± 2.19 | 11.80 ± 1.37 | 14.61 ± 2.31 | 0.90 |
| 1Dx2 | 29.76 ± 3.78 | 28.95 ± 2.02 | 28.86 ± 2.86 | 28.77 ± 0.98 | 0.99 |
| 1Dy12 | 13.40 ± 7.42 | 5.76 ± 4.35 | 12.71 ± 7.30 | 7.15 ± 5.04 | 1.91 |
| x:y ratio (B) | 3.23:1 | 2.65:1 | 3.05:1 | 2.53:1 | |
| x:y ratio (D) | 2.22:1 | 5.03:1 | 2.27:1 | 4.02:1 | |
| x:y ratio (total) | 2.68:1 | 3.32:1 | 2.65:1 | 3.03:1 | |

*Note:* Values are followed by standard deviations.

combination of low N and low P. A strong cultivar effect was also evident. The 1Dy12 subunit was the most influenced by low N and a combination of low N and P fertilization, where it was highly significantly decreased. Subunits 1Ax1, 1Bx7, and 1By8 were significantly increased due to low N levels. Subunits 1Ax1 and 1By8 were also significantly increased under a combination of low N and low P levels. Subunits 1Ax1, 1Bx7, and 1By8 were significantly increased due to low N levels. Subunits 1Ax1 and 1By8 were also significantly increased under a combination of low N and low P levels. Although there was a large decrease in FPC due to the low-N treatment, the same treatment had a very different effect on the HMW-GS, where the subunits coded for by *Glu-A1* and *Glu-B1* generally were increased, while those coded by *Glu-D1* were generally decreased, especially subunit 1Dy12. The same pattern was seen in the combined low-N and low-P treatment, although to a lesser extent than in the low-N treatment.

The current results were obtained under controlled greenhouse conditions, and results may be different under field conditions, where effects such as N leaching may occur and where other factors could affect results.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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