Nitrogen-bonded aromatics in soil organic matter and their implications for a yield decline in intensive rice cropping

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Previous research has shown that long-term intensive cropping of irrigated lowland rice has led to significant grain-yield declines in field trials. The yield decline was attributed to decreased availability of soil nitrogen, which is held mostly in the soil organic matter. By advanced solid-state NMR spectroscopy, we have detected significant amounts of amide nitrogen directly bonded to aromatic rings in a humic acid fraction extracted from a continually submerged, triple-cropped rice soil. Because nitrogen bonded to aromatics is not readily plant-available, this observation can explain the yield decline. Quantitative 13C NMR combined with advanced spectral editing showed that this humic acid is rich in lignin derivatives (>45% of all carbon), whereas the corresponding humic acid fraction extracted from an aerobic, single-cropped rice soil contains less lignin and less nitrogen bonded to aromatics.

Cultivation of two or three rice crops annually in irrigated lowland soils has been the foundation of Asia’s rice supply since such intensive cropping first became possible in the 1960s. Currently, approximately one quarter of global rice production comes from multiple annual cropping of lowland rice, and this central role in the food supply will expand in the future as Asian populations continue to increase. In long-term field trials in which initial yield levels of lowland rice approached the yield-potential ceiling, yields declined by >35% during 20–30 years of double and triple cropping (1). The search for mitigation options that will reverse this yield decline and thus improve the food supply for a significant portion of the world’s population requires an understanding of the underlying processes.

Agronomic data indicate that this yield decline resulted primarily from decreased crop uptake of soil nitrogen (1), which is distinct from fertilizer nitrogen and is held mostly in the soil organic matter. However, total soil nitrogen did not decrease in quantity as yields declined. We propose that the apparent decrease in availability of soil nitrogen is caused by the chemical stabilization of nitrogenous compounds by bonding to aromatic rings in lignin residues, probably via phenolic functionalities. Lignin residues accumulate in soils that are intensively cropped to irrigated lowland rice (1) because of their slow decomposition under the characteristically anaerobic conditions and their high input rates through multiple incorporations annually of crop residues. Nitrogen directly bonded to or in aromatic rings has been found to be less bioavailable than nitrogen in peptides, based on its rate of degradation through soil microbial processes under laboratory conditions (2–4), but 15N NMR spectroscopy has been unable to detect significant fractions of nitrogen bonded to aromatic carbons in the organic matter of these rice soils or any other soils (5), raising doubts as to whether nitrogen can be bonded to aromatic carbons under field conditions.

Recent developments in solid-state NMR pulse sequences have greatly advanced the possibilities for structural identification and quantification in natural organic matter (6–8). In particular, carbons directly bonded to nitrogen have now been observed selectively through saturation-pulse-induced dipolar exchange with recoupling (SPIDER) (7). This selection is achieved through recoupling of 13C–14N dipolar couplings and effective pulsed saturation of 15N under magic-angle spinning (MAS) at spinning frequencies that permit detection of aromatic carbon resonances with sufficient sensitivity. Other NMR spectral-editing methods separate overlapping resonances of various chemical groups (8).

Methods

The mobile humic acid (MHA) fraction was extracted from two similar soils on the research farm of the International Rice Research Institute in Los Baños, Laguna, Philippines (2). Both soils were clays, and their chemical properties were suitable for crop production, including a slightly acidic pH, a dominance of exchangeable cations by calcium, and adequate availability of phosphorus and potassium. One soil had been triple-cropped to irrigated lowland rice for 30 years before sampling, during which time grain yields declined by >30% (9). During such intensive cropping this soil was submerged for 11 months each year. The other soil had been cropped annually to dryland rice under rain-fed conditions for at least 30 years (1) and was largely aerated during this time. The MHA fraction was chemically extracted by shaking soil overnight in 0.25 M NaOH under an N2 atmosphere, then purified through HF incubations and dialysis, and finally freeze-dried (2). This fraction contains less-decomposed organic materials, it responds clearly in quantity and chemical nature to management of lowland rice, and it is active in seasonal nitrogen cycling (10). It contains 13% of the total carbon and nitrogen in the triple-cropped rice soil and 11% of the total carbon and nitrogen in the dryland rice soil.

The NMR spectra were obtained on a DSX-400 spectrometer (Bruker Biospin, Billerica, MA) at 100 MHz for 13C (400 MHz for 1H) using 4-mm rotors for direct-polarization (DP) MAS and 7-mm rotors for all other experiments. For the DP MAS experiments at a spinning frequency of 10 kHz, recycle delays of 100 and 20 s were chosen; relaxation factors measured with these delays by using the cross-polarization (CP)/T1 sequence (6) show that effects of differential T1 relaxation are <5%. Dipolar dephasing of signals of immobile protonated carbons after DP and at 10 kHz was achieved by an 87-µs delay with 1H decoupling centered on the 13C 180° pulse that is applied at t1 to generate a Hahn spin echo before detection. The dipolar-dephasing factor of unprotonated carbons has been measured in model compounds to be 0.93 ± 0.04; that of CH3 groups has been measured to be 0.53 ± 0.04.

The signals of CH groups were selected by dipolar distortion-less enhancement by polarization transfer (DEPT) (8). The spinning frequency of 5.8 kHz and all pulse parameters were the...
same as described in ref. 8. In these dipolar DEPT experiments and all experiments with CP, the recycle delays were 0.5 s. The CH peak observed around 53 ppm is due to NCH carbons and possibly also CH carbon signals in pinoresinol and phenylcoumaran units from lignin. (E and F) Spectral editing of unprotonated carbons and methyl groups by 87-μs dipolar dephasing of the DP spectra in A and B. The aliphatic region (<100 ppm) has been scaled up to correct for the dephasing factor of 0.53 ± 0.04 measured for CH₃ signals in model compounds. (G and H) Selection of alkyl-carbon signals by a CSA filter, based on their small ¹³C CSAs, by using 180° recycle delay: 0.5 s. The spectra were obtained on a DSX-400 NMR spectrometer at 100 MHz for ¹³C. Total measuring time for all spectra shown was 102 h.

Table 1. Percentages of carbon in various chemical moieties of two humic acid samples and of the HF-extracted soil organic matter (SOM) of the triple-cropped soil

<table>
<thead>
<tr>
<th>C-O</th>
<th>COO/NCO</th>
<th>Aromatic</th>
<th>Aromatic</th>
<th>Aliphatic</th>
<th>OCO</th>
<th>OCH/OcO</th>
<th>OCH₃</th>
<th>NCH/CCH</th>
<th>CH₃</th>
<th>Nonpolar(aliphatic)</th>
</tr>
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<tr>
<td>ppm</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>220–188.5</td>
<td>188.5–162.6</td>
<td>162.6–142</td>
<td>142–94.5</td>
<td>120–94.5</td>
<td>94.5–65.4</td>
<td>65.4–47.3</td>
<td>50–60</td>
<td>65.4–40</td>
<td>40–10</td>
</tr>
<tr>
<td>Triple-cropped MHA</td>
<td>1.9%</td>
<td>13%</td>
<td>14%</td>
<td>31% (17%)</td>
<td>2.2% (0.5%)</td>
<td>11% (1.0%)</td>
<td>6.3%</td>
<td>4.6%</td>
<td>3.7%</td>
<td>13%</td>
</tr>
<tr>
<td>Dryland MHA</td>
<td>2.1%</td>
<td>17%</td>
<td>10%</td>
<td>28% (20%)</td>
<td>4.2% (0.7%)</td>
<td>14% (1.1%)</td>
<td>2.9%</td>
<td>4.8%</td>
<td>4.6%</td>
<td>13%</td>
</tr>
<tr>
<td>Triple-cropped SOM</td>
<td>1%</td>
<td>12%</td>
<td>9%</td>
<td>28% (19%)</td>
<td>4% (0)</td>
<td>16% (0)</td>
<td>2%</td>
<td>12%</td>
<td>18%</td>
<td></td>
</tr>
</tbody>
</table>

Cₐ refers to unprotonated carbons, the fractions of which are given in parentheses. The error margins are approximately ±7% (relative error, i.e., the given percentage multiplied by 0.07) of each percentage given for the MHAs and ±2% (absolute error) for triple-cropped SOM because of its lower signal-to-noise ratio.

SPIDER (¹⁴N–)¹³C NMR experiments (7) were performed at a spinning frequency of 5 kHz. In the center of a 1.6-ms period with pulsed recoupling of ¹⁴N–¹³C dipolar interactions, pulsed dephasing of ¹⁴N was applied for 1.6 ms in 60% of scans and 0.8 ms in 40% of scans to excite coherences of ¹⁴N sites with different quadrupolar couplings more evenly. The resulting partially dephased carbon signal S is compared with the signal S₀ obtained without ¹⁴N pulses and thus without ¹⁴N-induced dephasing. The difference S – S₀ shows signal exclusively of carbons near ¹⁴N, mostly from directly bonded species. More details on the SPIDER method can be found in ref. 7. For each MHA sample, eight pairs of spectra (S and S₀) were block-averaged to minimize spectrometer-drift effects. The S/S₀ ratio of the COO/NCO signal, combined with the carbon fraction represented by this peak, enables us to estimate the number of nitrogens per 100 carbons represented by this signal.

Results and Discussion

Fig. 1 A and B show quantitative ¹³C NMR spectra (6) of the MHA fraction from the triple-cropped soil and dryland soil, respectively. Fig. 1 also shows partial deconvolutions of the ¹³C spectra for both MHA samples by CH spectral editing (8) (Fig. 1 C and D), by selection of quaternary- and methyl-carbon signals through dipolar dephasing of the DP spectrum (Fig. 1 E and F), and by selection of sp³-hybridized carbons through CSA filtering (Fig. 1 G and H). These partial deconvolutions permit assignment of peaks to specific structural units such as lignin derivatives, peptides, and carbohydrates. The resulting compositions are compiled in the first two rows of Table 1. For instance, there are <4.8 NCH carbons per 100 carbons, which is less than two thirds of the >7.2 nitrogens per 100 carbons in these humic acid samples (1), indicating that peptides do not account for all of the nitrogen. Based on the aromatic C–O–CH₃ ether signals at 55 ppm, we estimate that at least 45% of all carbon in the MHA of the triple-cropped soil is part of lignin-derived residues, corresponding to >4.5 lignin aromatic rings per 100 carbons. In the dryland MHA, by contrast, the lignin content estimated in the same way is only ~20% (~2 lignin rings per 100 carbons). The total aromatic-carbon fraction differs much less: 45% and 38% in the triple-cropped and dryland MHA, respectively.

Fig. 2 A shows the spectrum of carbons directly bonded to nitrogen (N–C) obtained by the SPIDER NMR technique (7) for the MHA of the triple-cropped soil. The spectrum is dominated by the amide (NCO) peak at 174 ppm. In addition to aliphatic NCH resonances of peptides at 55 ppm, the (N–C) spectrum exhibits clear signals of nitrogen-bonded aromatic carbons around 134 ppm, accounting for approximately one quarter of the spectral area, and a smaller peak near 150 ppm. The nitrogen-bonded aromatic carbons observed here are mostly unprotonated, because their peak areas are only slightly diminished by C–H dipolar dephasing added after the SPIDER selection (Fig. 2B).
good agreement with the observed peak position in the spectrum of Fig. 2A, and its $^{15}$N chemical shift is well within the range conventionally assigned to amides and peptides (5). Anilides, with CO–NH groups bonded to aromatic carbons, could form through reaction of lignin-derived phenolic compounds with ammonia (13) and the COOH or NCO groups of amino acids or peptides (14, 15). Amine groups might also be expected to bond to aromatic carbons (CH–NH–C$_{arom}$) through condensation of phenolic OH groups with NH$_2$ moieties of amino acids, but the abundance of this nitrogen form seems negligible based on the low intensity of amine nitrogen in $^{15}$N spectra of these samples (5).

Based on their characteristic chemical shift, the $^{13}$C NMR bands at $\approx$150 ppm in the SPIDER spectra can be assigned to nitrogen in pyridinic six-membered aromatic rings (12, 16). Note that these unprotonated nitrogens, which have large CSAs, are difficult to detect in CP-MAS $^{13}$C NMR spectra (17). Based on the dipolar dephased SPIDER spectrum of Fig. 2B, we also conclude that most of the nitrogen-bonded carbons resonating near 150 ppm are unprotonated; this conclusion is confirmed by the CH-only spectra of Fig. 1 C and D, which show no significant protonated-carbon signals near 150 ppm.

Table 2 compiles the nitrogen-containing functional groups per 100 carbons, as estimated from the SPIDER $\{{^{14}}N\}^{13}$C spectra and the quantitative $^{13}$C NMR results presented here, combined with the $^{15}$N NMR and elemental-composition data (5) for the same samples. Peptides (CO–NH–CHR) account for approximately half of all nitrogen in both samples. According to the $S_{CH}/S_{N}$ ratio of the amide peak in the SPIDER experiment (see Methods) and the carbon fraction of the COO/NCO (amide) peak (Table 1), amides (CO–NH) account for $\approx$6 nitrogens per 100 carbons, or approximately four fifths of all nitrogen, given that there are 7.2 nitrogens per 100 carbons in the triple-cropped MHA. They include CO–NH–C$_{arom}$ anilides, which are the second largest fractions in these samples, with 25% of all nitrogen in triple-cropped MHA versus 11% of all nitrogen in dryland MHA. These percentages differ by a factor of $\approx$2 between the two samples, which reflects the different aromatic-carbon peak areas in the SPIDER NMR spectra of Fig. 2. Their ratio for triple-cropped MHA/dryland MHA (2.3) matches the corresponding ratio of lignin-derived residues (2.3), further suggesting that these anilides formed through reaction of lignin-derived residues. According to our quantitative $^{13}$C NMR analysis, there are $\approx$4.5 aromatic rings of lignin per 100 carbons in the triple-cropped MHA and 2 lignin rings per 100 carbons in the dryland MHA. Comparison with the amounts of aniline (1.8 and 0.9 per 100 carbons, respectively) shows that there is approximately one aniline group per two lignin rings. Overall, in the

### Table 2. Nitrogen per 100 carbons in various functional groups of the two humic acids

<table>
<thead>
<tr>
<th>X nitrogens per 100 carbons:</th>
<th>CO–NH–</th>
<th>Six-membered</th>
<th>Five-membered</th>
<th>CO–NH–C$_{arom}$</th>
<th>CH–NH–C$_{arom}$</th>
<th>NH$_3$</th>
<th>CO–NH$_2$</th>
<th>Total nitrogens per 100 carbons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triple-cropped MHA</td>
<td>3.4 (47%)</td>
<td>0.7 (10%)</td>
<td>0.6 (8%)</td>
<td>1.8 (25%)</td>
<td>0.3 (4%)</td>
<td>0.2 (3%)</td>
<td>0.9 (11%)</td>
<td>7.2 (100%)</td>
</tr>
<tr>
<td>Dryland MHA</td>
<td>4.3 (53%)</td>
<td>0.7 (9%)</td>
<td>0.7 (9%)</td>
<td>0.9 (11%)</td>
<td>0.2 (2%)</td>
<td>0.4 (5%)</td>
<td>0.9 (11%)</td>
<td>8.1 (100%)</td>
</tr>
</tbody>
</table>

Data were calculated from $\{{^{14}}N\}^{13}$C SPIDER NMR (Fig. 2), $^{15}$N NMR (5), CH-only dipolar DEPT (see Fig. 1 C and D), and the C/N ratio. The data give NCO/N$_{C_{arom}}$/NCH$_e$ carbon ratios of 1:0.83:0.70 and 1:0.62:0.79 for triple-cropped and dryland MHA, respectively. Error margins are approximately $\pm$6% (absolute error) or $\pm$0.4 nitrogens per 100 carbons.
soil (1) and an assumption of 0.8 g cm⁻¹ deep plow layer, based on the abundance of MHA in this nitrogen in the MHA of the triple-cropped soil. This quantity is composition established by¹⁵N NMR analysis of these relationship between heterocyclic nitrogen and degree of de-

Based on the enriched lignin levels of the MHA and the inverse and substantially less than 25% of the soil heterocyclic nitrogen, contains substantially more than 25% of the soil anilide nitrogen because of the weak signal for whole organic matter. We speculate that the MHA signal for whole organic matter.

The lignin-enriched MHA fraction, which accounts for 13% of organic carbon and nitrogen in this soil (1), contains ~25% of the aromatic-carbon-bonded nitrogen in the soil. The aromatic-carbon-bonded nitrogen could not be partitioned reliably into anilide nitrogen and heterocyclic nitrogen because of the weak signal for whole organic matter. We speculate that the MHA contains substantially more than 25% of the soil anilide nitrogen and substantially less than 25% of the soil heterocyclic nitrogen, based on the enriched lignin levels of the MHA and the inverse relationship between heterocyclic nitrogen and degree of decomposition established by ¹⁵N NMR analysis of these samples (5).

Significant agronomic implications arise from the finding that nitrogen bonded to aromatic carbons constitutes >40% of the nitrogen in the MHA of the triple-cropped soil. This quantity is equivalent to at least 150 kg of N per hectare (ha) in the 15-cm-deep plow layer, based on the abundance of MHA in this soil (1) and an assumption of 0.8 g cm⁻³ bulk density for a puddled rice soil. The aromatic-carbon-bonded nitrogen in whole organic matter of the triple-cropped soil corresponds to ~600 kg of N per ha. The 14% excess of aromatic-bonded nitrogen in triple-cropped vs. dryland MHA, mostly representing anilide nitrogen, corresponds to ~55 kg of N per ha. By comparison, the cumulative yield decline of ~3 tons of grain per ha per cropping season represents a decrease in crop nitrogen uptake of ~60 kg ha⁻¹ per season, assuming a physiological efficiency of 50 kg of grain per 1 kg of nitrogen uptake (19). Hence, the excess quantity of anilide nitrogen detected by NMR in the triple-cropped soil is agronomically significant to the yield decline.

Conclusions

A significant (>40%) fraction of nitrogen bonded to aromatic carbons was detected by advanced solid-state NMR in a humic acid fraction extracted from a continually submerged, triple-cropped rice soil. Most of this aromatic-bound nitrogen seems to be anilide nitrogen. This humic acid fraction was also shown to be rich in lignin-derived residues (>45%). The quantitative analysis indicates that, on average, amides are bonded to every other aromatic ring of lignin. In contrast, the corresponding humic acid fraction extracted from a comparable aerobic rice soil has less than half the content of lignin and anilide nitrogen. Its NMR spectrum of carbons bonded to nitrogen showed peak intensities more characteristic of easily degradable peptides. Because nitrogen bonded to aromatic carbons is known to be less biodegradable, the agronomically significant excess of anilide nitrogen detected by NMR in the humic fraction of the triple-cropped soil can explain a yield decline observed in this intensively cropped soil.

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