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Nitrogen Mineralization from Flowering- and Maturity-stage Green Manures under Flooded Conditions after Different Upland Periods

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Abstract

Leguminous green manure (GM) is a nitrogen (N) resource that can replace chemical fertilizers (CFs) in rice production. The pattern of N mineralization from GM, which changes according to species, growth stage, and the timing of incorporation, affects rice growth and yield. Thus, the characteristics of N mineralization should be clarified to promote the appropriate use of GM. The objectives of this study were to clarify the patterns of N mineralization from flowering- and maturity-stage GMs under flooded conditions after different upland periods, and to evaluate the denitrification loss occurring during the upland periods. Nitrogen mineralized from three species of GMs collected at both the flowering and maturity stages was measured through incubation tests. The three species were hairy vetch (HV), crimson clover (CC), and white clover (WC). The incubation tests included five upland periods (0, 1, 2, 3, and 4 weeks) at 20°C, and each upland period involved eight flooded periods (0, 1, 2, 4, 6, 8, 12, and 16 weeks) at 30°C. The percentage of mineralized N to total N input from the GMs after being flooded for four weeks until after 16 weeks was 1% for flowering-stage HV and 3% for flowering-stage CC, whereas the percentage increased to 41% for maturity-stage HV and 58% for maturity-stage CC. The results indicate that maturity-stage HV and CC slowly mineralize N until the late growth stage of rice. The denitrification loss under upland conditions increased markedly when the upland period extended longer than two weeks. Said loss can likely be attributed to the easily mineralizable fraction of N in the GMs, which is mineralized until having been flooded for four weeks. Thus, maturity-stage GMs, which have a higher C/N ratio than flowering-stage GMs, are advantageous in reducing the denitrification loss during the upland period.

Discipline: Agricultural Environment
Additional key words: crimson clover, denitrification, hairy vetch, incubation test, white clover

Introduction

Leguminous green manure (GM) is organic matter that has been conventionally used as a nitrogen (N) source for crops. The use of GMs in agricultural production has rapidly declined since the advent of chemical fertilizers (CFs; Chen 1988, Rosegrant & Roumasset 1988, Yasue 1991). However, GM use has been renewed in agriculture as a sustainable practice with a low required input of energy and fertilizer resources. This is partly because farmers have recently experienced fluctuating prices in CFs caused by the rising cost of fossil fuels and concerns over the depletion of necessary raw materials for CFs (Baligar & Bennett 1986, Rosegrant & Roumasset 1988, World Bank 2019).

Previous studies regarding GM use in Japan have mainly focused on basal application in rice production, traditionally using Chinese milk vetch (*Astragalus sinicus* L.; CMV) as well as hairy vetch (*Vicia villosa* Roth; HV) in recent years (Azuma et al. 2017, Ishikawa 1988, Watanabe 1984). The appropriate incorporation rate and timing have been studied to maximize yield and

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avoid the inhibited growth caused by the decomposition of carbohydrates (Azuma et al. 2017, Ishikawa 1988). In these studies, using GMs as a basal application with topdressing(s) has resulted in the production of a yield equivalent to or greater than that of conventional cultivation using CF (Azuma et al. 2017, Kawase & Kitazima 1994).

On the other hand, the current use of GMs in rice production, which is limited to basal application at the flowering stage, can lower the nitrogen use efficiency (NUE) of rice plants. This is because the N supply from flowering-stage GMs may not be synchronized with the N demand of rice, which changes relative to the growth stage. Flowering-stage GMs rapidly mineralize N during the early growth stage of rice (Kawase & Kitazima 1994), while a report on the improved variety of IR-8 demonstrated a case in which the N uptake of the panicle accounted for 71% of the total uptake (Yoshida 1981). Asagi and Ueno (2009) reported a case in which the NUE of rice plants was 55.6% in hairy vetch, followed by white clover (*Trifolium repens* L.; WC) at 43.5%, crimson clover (*Trifolium incarnatum* L.; CC) at 33.3%, and CMV at 25.7%. In this case, air-dried flowering-stage GMs were incorporated one day before the transplanting of rice. Ko et al. (2021) have estimated that the NUE of rice was 52%, which is 15% lower than that of cultivation using CF, when air-dried flowering-stage CC were incorporated 10 days before flooding. In addition, GMs can supply excessive N that causes lodging and disease because their growth depends on weather conditions (Japan Soil Association 2012). Therefore, excessive N must be reduced through denitrification during an upland period before the transplanting of rice, which will further lower the NUE. Ko et al. (2020) have reported that the NUE of rice plants at the panicle formation stage decreased from 21% to 6% when the upland period was increased from 21 to 45 days, after incorporating crimson clover. The low NUE indicates that N from the GM was lost due to denitrification and leaching (Buresh & De Datta 1991, George et al. 1992), a process that could have an adverse environmental impact.

Supplying N mineralized from GM during the late growth stage of rice can improve its NUE, however, by avoiding an excessive N supply in the early growth stage. Maturity-stage GM, which has a higher C/N ratio than flowering-stage GM, can slowly mineralize N until the late growth stage of rice (Ishikawa 1988, Nagumo et al. 2014, Yasue 1991). Through incubation tests under flooded conditions at 25°C, Nagumo et al. (2014) have demonstrated a case in which 17.9% of the total N (TN) added was mineralized from maturity-stage CMV (C/N ratio: 17.1) in one month. This pattern of N mineralization was slower than the 53%-57.8% for flowering-stage CMV incubated at 23°C for 28 days as revealed by Ishikawa (1963). This difference in the pattern of N mineralization affects the growth, yield, and NUE of rice. Nagumo et al. (2014) and Ko et al. (2021) have both reported that applying maturity-stage GM in rice production can inhibit initial growth, thus reducing yield. One study found the NUE of rice to be 43% under the application of maturity-stage crimson clover, and thus lower than 52% under the application of flowering-stage GM (Ko et al. 2021).

At present, no noticeable effects caused by the incorporation of maturity-stage GMs on yield and NUE have been reported in rice production. However, the pattern and amount of N mineralization can change due to such factors as the species and biomass amount of the GM, and length of the upland period after incorporation (Azuma et al. 2017, Frankenberger & Abdelmagid 1985, Ko et al. 2020, Oglesby & Fownes 1992). Moreover, these factors depend on various conditions, such as the local weather, cropping system, field drainage, irrigation, and work schedule of the farmer. This suggests that applying maturity-stage GMs could be a favorable option under certain conditions. For example, Ko et al. (2021) suggest that incorporating GMs at the maturity stage is advantageous for reducing the denitrification loss when the dates for transplanting rice are kept separate from the flowering stage of the GMs. Thus, the characteristics of N mineralization should be better clarified to optimize the use of GMs under different conditions in rice production. The objectives of this study were to clarify the patterns of N mineralization from flowering- and maturity-stage GMs under flooded conditions after different upland periods, and to evaluate the denitrification loss occurring during the upland periods.

Materials and methods

1. Cultivation of green manures

We cultivated HV (variety: *Mamesuke*), CC (variety: *Kurenai*), and WC (variety: *Fia*) from October 2018 to June 2019 at the University Farm of Kyushu University in Fukuoka Prefecture, Japan. We prepared a total of 12 planters, four for cultivating each of the three species. The planters were 60 cm in length, 17 cm in width, and 16.5 cm in depth. First, light stones were placed at the bottom of the planters. Then the planters were filled with 7.6 kg of Futsukaichi soil (dry weight basis) that had been collected from Kamigoka, Chikushino city, Fukuoka Prefecture. Futsukaichi soil

was passed through a 2-mm sieve after soybeans had been cultivated. The soil texture was clay loam. The pH, cation exchange capacity, TN, available phosphate (P_2O_5), and exchangeable K were 7.1, 8.35 cmol kg^{-1}, 0.09%, 261 mg kg^{-1}, and 0.23 cmol kg^{-1}, respectively. The date of seeding was October 7, 2018. The seeding rates were 0.5 g $planter^{-1}$ for HV, 0.3 g $planter^{-1}$ for CC, and 0.3 g $planter^{-1}$ for WC. After germination in a greenhouse, six planters were placed outside. The six remaining planters were placed in a phytotron at 15°C, considering possible growth inhibition in the GM cultivated outside during winter. GMs cultivated in the phytotron were moved outside on March 1, as the GMs grew similarly both outside and in the phytotron. The moisture condition of the soil in all planters was adjusted to between 30% and 60% of the maximum water holding capacity during cultivation. Finally, two of the four planters of each species were used to collect the flowering- and maturity-stage GMs, with one having been placed in the phytotron for the first section of GM cultivation, and the other having been cultivated entirely outside.

2. Measurement of physiochemical properties of green manures and physical properties of soil

Flowering- and maturity-stage GMs were collected on March 25 and May 27 for HV, on April 1 and June 12 for CC, and on April 18 and June 24 for WC. We defined the growth stage of GMs collected about two months after the flowering stage as the maturity stage. Maturity-stage GMs were drier than those of the flowering stage. Moreover, the maturity-stage HV and CC were brown, whereas a large portion of the maturity-stage WC was still green. The GM shoots were cut at the soil surface, and the roots were collected by carefully removing the soil. The fresh weights of the shoots and roots were measured separately. The GMs used for incubation tests were preserved in Ziploc® bags and stored in a refrigerator at 4°C, while 5 g of the shoots and 1 g -5 g of the roots were used to measure the moisture content (using oven-drying at 70°C for 48 h), total carbon (TC), and TN. The oven-dried shoots and roots were ground to a fine powder using a sample mill (Cyclotec 1093, Foss, Hilleroed, Denmark). TC was then measured using a CHN coder (MT-5, Yanaco, Tokyo, Japan) with two replications. For TN measurement, the ground shoots and roots were separately digested using the H_2SO_4-H_2O_2 Kjeldahl digestion method with three replications (Ohyama et al. 1991). TN was then measured using the indophenol method (Cataldo et al. 1974).

To measure the soil properties, the soil in two planters (each of the flowering- and maturity-stage GMs) was mixed well and passed through a 2-mm sieve. Then moist soil (approx. 100 g on a dry weight basis) was sampled to measure the moisture content (oven-drying at 105°C for 24 h) and maximum water holding capacity (Kawaguchi & Ojima 1965). The rest of the soil was preserved in a plastic bag for use in incubation tests.

3. Measurement of nitrogen mineralized from green manures

The N mineralized from the GMs was measured through incubation tests using both flowering- and maturity-stage GMs. The tests included five upland periods (0, 1, 2, 3, and 4 weeks) at 20°C, and each upland period involved eight flooded periods (0, 1, 2, 4, 6, 8, 12, and 16 weeks) at 30°C (Fig. 1).

The shoots and roots of the GMs, which were cut into about 1-cm pieces, were mixed well with moist soil (approx. 240 g on a dry weight basis) and then transferred into a 500-mL glass bottle. The N application rate was adjusted to 100 mg kg^{-1} dry soil. The amount of shoots and roots added was determined using the ratio between them on a dry weight basis, as calculated based on the total fresh weight and moisture content of the shoots and roots measured when the GMs were

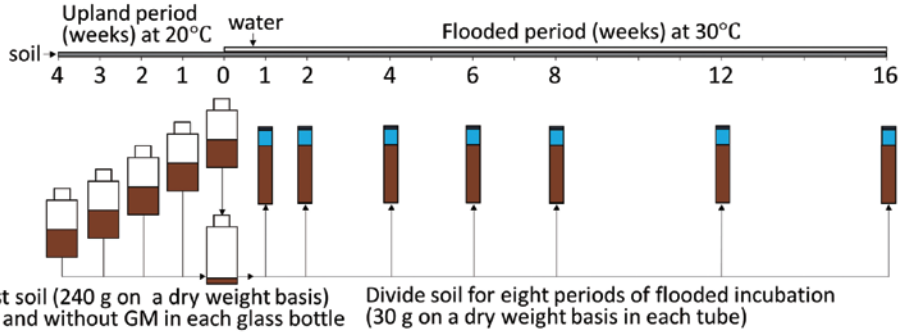

Fig. 1. Incubation test procedure including eight flooded periods (0, 1, 2, 4, 6, 8, 12, and 16 weeks) at 30°C following five upland periods (0, 1, 2, 3, and 4 weeks) at 20°C

collected. Nevertheless, the moisture content of the GMs, especially for shoots with a high moisture content at the flowering stage, decreased during the one-week period of preservation before the incubation tests. Thus, actual N application rates were finally calculated based on the moisture content measured for the shoots and roots when conducting the incubation tests. TN was also measured for pieces of the shoots and roots used in the incubation tests to calculate the N application rates. Three bottles each with and without GM were prepared for each of the five upland periods. The moisture condition of the soil was adjusted to 60% of the maximum water holding capacity during upland incubation.

After upland incubation, the soil in each bottle was divided for eight periods of incubation tests under flooded conditions (0, 1, 2, 4, 6, 8, 12, and 16 weeks). Approximately 30 g of soil (dry weight basis) was transferred from a glass bottle into a 30-mL polypropylene tube (62.543, Sarstedt, Nümbrecht, Germany). Soil in the tube was mixed well with distilled water, while air in the mixed layer was sucked out for 40-60 sec. under 0.075 MPa by a diaphragm vacuum pump (GM-205, AS ONE, Osaka). This procedure was repeated three times until bubbles did not rise from the mixed layer. Finally, the tube was closed with a polyethylene screw cap after the headspace in the tube was filled with distilled water. The soil in each tube was approx. 6 cm in depth, and water above the soil surface had a depth of approx. 2.5 cm. The flooded incubation was not under a strict anaerobic condition because the tube and cap had some gas permeability.

After each incubation period, soil and water in the tubes were transferred into a 500-mL glass bottle with 150 ml of 10% KCl. For the flooded week 0 sample, approx. 30 g of soil was not transferred into a polypropylene tube and stored in a glass bottle, to which 150 ml of 10% KCl was added. The mineralized N (MN) was then extracted by shaking the bottle for 30 min. The extract was filtered through No. 5B filter paper (Advantec, Tokyo, Japan). Then ammonium (NH_4–N) and nitrate (NO_3–N) in the extract were measured using the indophenol and Cataldo methods, respectively (Cataldo et al. 1975). The nitrate (NO_3–N) from the flowering- and maturity-stage GMs was only measured at weeks 0 and 1, and at week 0 of flooded incubation, respectively. Finally, the N mineralized from the flowering- and maturity-stage GMs was estimated using the difference in MN content between soil mixed with GM and that without GM.

4. Destination of total nitrogen input from green manure

The destination of TN input from the GM after 16 weeks of flooded incubation ($DTN_{f16}(w)$) consists of MN from the GM after 16 weeks ($MN_{f16}(w)$), N loss ($NL(w)$), remaining N in the soil ($RNS(w)$) including recalcitrant fractions of the TN and N immobilized by microorganisms, and measurement errors (ER), as shown in Eq. (1). $NL(w)$ includes the denitrification loss and ammonia volatilization during the upland period and flooded period. Any measurement errors can be attributed to differences in the moisture content and TN of shoots and roots between the samples used for the measurement and incubation tests.

$$DTN_{f16}(w) = MN_{f16}(w) + NL(w) + RNS(w) + ER \quad (1)$$

Here, w in parentheses represents the upland weeks before flooded incubation. We assumed that the amounts of $RNS(w)$ and ER were equal among different upland periods, because the recalcitrant fraction of TN input from the GM, which was a major component in $RNS(w)$, was hardly affected by upland periods. Moreover, ER was considered similar under the same operations prior to the start of upland incubation. Thus, Eq. (2) below calculates the difference in N loss between an arbitrary upland week and upland week 0 when TN input from the GM is the same among different upland periods.

$$NL(w) - NL(0) = MN_{f16}(0) - MN_{f16}(w) \quad (2)$$

Further, the value of the left side ($NL(w) - NL(0)$) was defined as an increase in N loss compared with upland week 0 ($INL(w)$), as expressed in Eq. (3) below.

$$INL(w) = NL(w) - NL(0) \quad (3)$$

Finally, Eq. (4) is derived using Eqs. (1) and (3) as follows:

$$DTN_{f16}(w) = MN_{f16}(w) + INL(w) + NL(0) + RNS(w) + ER \quad (4)$$

In this study, the summation of $NL(0)$, $RNS(w)$, and ER (hereafter denoted as "Others") was an uncertain fraction of the TN input from the GM.

5. Statistical analysis

The difference in the mean values among different upland periods for the same GM collected at the same growth stage was tested using Tukey's method at a 5%

probability level (Yamauchi 2008). The analysis was performed using macros written in Microsoft Excel.

Results

1. Physiochemical properties of green manures and nitrogen application rates

Table 1 lists the physiochemical properties of the GMs and the N application rates. For the shoot portions, the moisture content at the flowering stage exceeded 70%, a value markedly higher than that found at the maturity stage. The maturity-stage CC was markedly dried, with a moisture content of only 14%. The moisture content in the root portions was similar between the flowering and maturity stages. The ratio of shoot/root to total application weight was around 0.5 for the flowering-stage HV and WC. However, the ratios of shoot to total application weight were larger for the maturity-stage HV (0.81) and for CC (flowering: 0.72, maturity: 0.92).

The TC content in the shoots from HV and CC was higher at the maturity stage than at the flowering stage. The TC content of WC was similar between the flowering and maturity stages. The TN content in the shoots was similar between the flowering and maturity stages for CC (1.90% and 1.87%) and WC (3.24% and 3.08%), whereas that for HV was markedly higher at the flowering stage (3.13%) than at the maturity stage (1.96%). Thus, the C/N ratios of shoots in HV had a large difference between the flowering stage (13.3) and the maturity stage (22.2). The C/N ratios of the shoots were 21.6 and 23.1 for CC, and 13.0 and 13.6 for WC at the flowering and maturity stages, respectively. The TC content and TN content of the roots were lower than those of shoots. The TC content in the roots of HV and CC were higher at the maturity stage, resulting in larger C/N ratios of 15.7 for HV and 33.4 for CC at the maturity stage, as compared with 11.6 for HV and 19.5 for CC at the flowering stage.

The total N application rate from the GMs was around 100 mg kg^{-1} dry soil (equal to our target rate) for HV at the maturity stage and for WC at both stages. Meanwhile, the rates were higher than the target rate for flowering-stage HV (133 mg kg^{-1}), flowering-stage CC (193 mg kg^{-1}), and maturity-stage CC (134 mg kg^{-1}).

2. Nitrogen mineralization from green manure

For flowering-stage HV (Fig. 2 (a)), the MN

Table 1. Physicochemical properties of green manures and nitrogen (N) application rates

Green manure	Growth stage	Plant portion	MC (%)	Ratio to total application weight (dry weight basis)	TC (%)	TN (%)	C/N ratio	N application rate (mg kg^{-1})*	Total N application rate (mg kg^{-1})*
HV	Flowering	Shoot	74.6	0.55	41.55	3.13	13.3	96	133
		Root	44.9	0.45	17.46	1.51	11.6	37	
	Maturity	Shoot	32.6	0.81	43.52	1.96	22.2	85	104
		Root	40.1	0.19	28.43	1.81	15.7	19	
CC	Flowering	Shoot	78.9	0.72	41.03	1.90	21.6	166	193
		Root	58.3	0.28	15.92	0.82	19.5	27	
	Maturity	Shoot	14.0	0.92	43.19	1.87	23.1	130	134
		Root	47.8	0.08	23.39	0.70	33.4	4	
WC	Flowering	Shoot	82.4	0.41	42.18	3.24	13.0	62	109
		Root	65.8	0.59	24.66	1.67	14.8	47	
	Maturity	Shoot	57.6	0.48	41.86	3.08	13.6	60	89**
		Root	64.0	0.52	20.71	1.40	14.8	30	

HV: hairy vetch, CC: crimson clover, WC: white clover, MC: moisture content, TC: total carbon, TN: total nitrogen
Flowering- and maturity-stage GMs were collected on March 25 and May 27 for HV, on April 1 and June 12 for CC, and on April 18 and June 24 for WC, respectively.
* The N application rate (mg kg^{-1}) is on a dry soil weight basis.
** The value is not equal to the summation of each plant portion because the value was rounded to an integer.

following upland week 0 rapidly increased from 14.5 mg kg^{-1} at flooded week 0 to 99.8 mg kg^{-1} at flooded week 2. The MN at flooded week 0 increased in relation to increases in the upland period except for following upland week 4. The MN content was 44.1, 62.4, and 69.6 mg kg^{-1} after upland weeks 1, 2, and 3, respectively. MN then gradually increased and reached its maximum value at flooded week 4. The maximum MN content was 94.7 mg kg^{-1} after upland week 1, followed by 93.0 mg kg^{-1} after upland week 2, and 83 mg kg^{-1} after upland week 3. MN reached a maximum of only 34.1 mg kg^{-1} following upland week 4. For maturity-stage HV (Fig. 2 (b)), MN continued increasing until flooded week 16 under all upland periods, but at slower rates compared with flowering-stage HV. The MN at flooded week 16 decreased in relation to increasing the upland period. The MN content was 99.5 mg kg^{-1} after upland week 0, 94.1 mg kg^{-1} after upland week 1, 87.8 mg kg^{-1} after upland week 2, 80.2 mg kg^{-1} after upland week 3, and 58.2 mg kg^{-1} after upland week 4.

For flowering-stage CC (Fig. 2 (c)), the MN at flooded week 0 was 7.3 mg kg^{-1} following upland week 0, while the values exceeded 20 mg kg^{-1} in upland weeks 1-4. The rates of N mineralization were slower than those observed for flowering-stage HV. MN gradually increased and approached a maximum value at flooded week 4 after upland week 0 (94.7 mg kg^{-1}), and at flooded week 8 following upland week 1 (99.0 mg kg^{-1}), upland week 2 (90.4 mg kg^{-1}), and upland week 3 (81.0 mg kg^{-1}). The MN following upland week 4 reached an approx. maximum value of 43 mg kg^{-1} at flooded week 4. For maturity-stage CC (Fig. 2 (d)), MN kept increasing until flooded week 16 under all upland periods, as observed for HV. MN reached 123.1, 121.6, and 115.7 mg kg^{-1} at flooded week 16 after upland weeks 0, 1, and 2, respectively. The MN at flooded week 16 decreased following upland weeks 3 and 4 to 92.4 and 88.4 mg kg^{-1}, respectively.

For flowering-stage WC (Fig. 2 (e)), the MN content at flooded week 0 was relatively high, though only in the case of a one-week upland period (20.9 mg kg^{-1}). N mineralization for flowering-stage WC was slower than that observed for flowering-stage HV. MN gradually increased and approached its maximum value at flooded week 6 after upland week 0 (94.3 mg kg^{-1}), at flooded week 8 after upland week 1 (93.8 mg kg^{-1}), and at flooded week 16 after upland week 2 (80.8 mg kg^{-1}). The MN content after upland weeks 3 and 4 reached approximate maximums of 46.9 and 39.8 mg kg^{-1} at flooded week 8, respectively. For maturity-stage WC (Fig. 2 (f)), the MN at flooded week 0 had small negative values for upland weeks 1, 2, and 4. MN gradually increased and approached its maximum value (94.3 mg kg^{-1}) at flooded week 8 following upland week 0. MN continued increasing until flooded week 16 following upland weeks 1-4, as observed for HV and CC. MN reached 99.9 and 91.7 mg kg^{-1} at flooded week 16 following upland weeks 0 and 1, respectively. The MN at flooded week 16 decreased after upland weeks 2, 3, and 4 to 72.3, 70.6, and 62.2 mg kg^{-1}, respectively.

3. Destination of total nitrogen input from green manure after 16 weeks of flooded incubation

For flowering-stage HV (Fig. 3 (a)), MN_{f16} ranged from 82.2 (62% of TN input from HV) to 89.6 mg kg^{-1} (67%) following upland weeks 1-3, and the values decreased from 98.8 mg kg^{-1} (74%) after upland week 0. Meanwhile, $MN_{f16}(4)$ sharply declined to 33.9 mg kg^{-1} (25%), and the amount of INL(4) showed a high value of 64.9 mg kg^{-1} (49%). "Others" that include NL(0), RNS(*w*), and ER accounted for 26%. For maturity-stage HV (Fig. 3 (b)), MN_{f16} decreased as the upland period increased. MN_{f16} was 96% of the TN input from HV after 0 weeks, 91% after one week, 85% after two weeks, 77% after three weeks, and 56% after four weeks. In contrast, INL increased as the upland period increased from 1-4 weeks. After weeks 1-4, INL was 5.4 (5%), 11.6 (11%), 19.3 (19%), and 41.2 mg kg^{-1} (40%), respectively.

For flowering-stage CC (Fig. 3 (c)), MN_{f16} was found to decrease in relation to increasing upland period, similar to the trend observed for flowering-stage HV. $MN_{f16}(4)$ sharply declined to 39 mg kg^{-1} (20%). The amount of INL increased from 0.7 (0.3%) to 60.8 mg kg^{-1} (31%) as the upland period increased from one to four weeks. "Others" accounted for 48%. For maturity-stage CC (Fig. 3 (d)), $MN_{f16}(3)$ and $MN_{f16}(4)$ declined to 92.4 (69%) and 88.4 mg kg^{-1} (66%), respectively, compared with 123.1 mg kg^{-1} (92%) for $MN_{f16}(0)$. INL(3) and INL(4) were at relatively high levels of 30.7 (23%) and 34.7 mg kg^{-1} (26%), respectively.

For flowering-stage WC (Fig. 3 (e)), $MN_{f16}(2)$ (80.8 mg kg^{-1}, 74%) decreased, while $MN_{f16}(3)$ (47.5 mg kg^{-1}, 44%) and $MN_{f16}(4)$ (44.2 mg kg^{-1}, 41%) markedly decreased compared with $MN_{f16}(0)$ (99.7 mg kg^{-1}, 91%). INL(3) and INL(4) were high at 52.2 mg kg^{-1} (48%) and 55.5 mg kg^{-1} (51%), respectively. For maturity-stage WC (Fig. 3 (f)), $MN_{f16}(2)$, $MN_{f16}(3)$, and $MN_{f16}(4)$ sharply declined to 72.3 (81%), 70.6 (79%), and 62.2 mg kg^{-1} (70%), respectively. INL(2), INL(3), and INL(4) were high at 27.6 mg kg^{-1} (31%), 29.3 mg kg^{-1} (33%), and 37.7 mg kg^{-1} (42%), respectively. "Others" accounted for −12%.

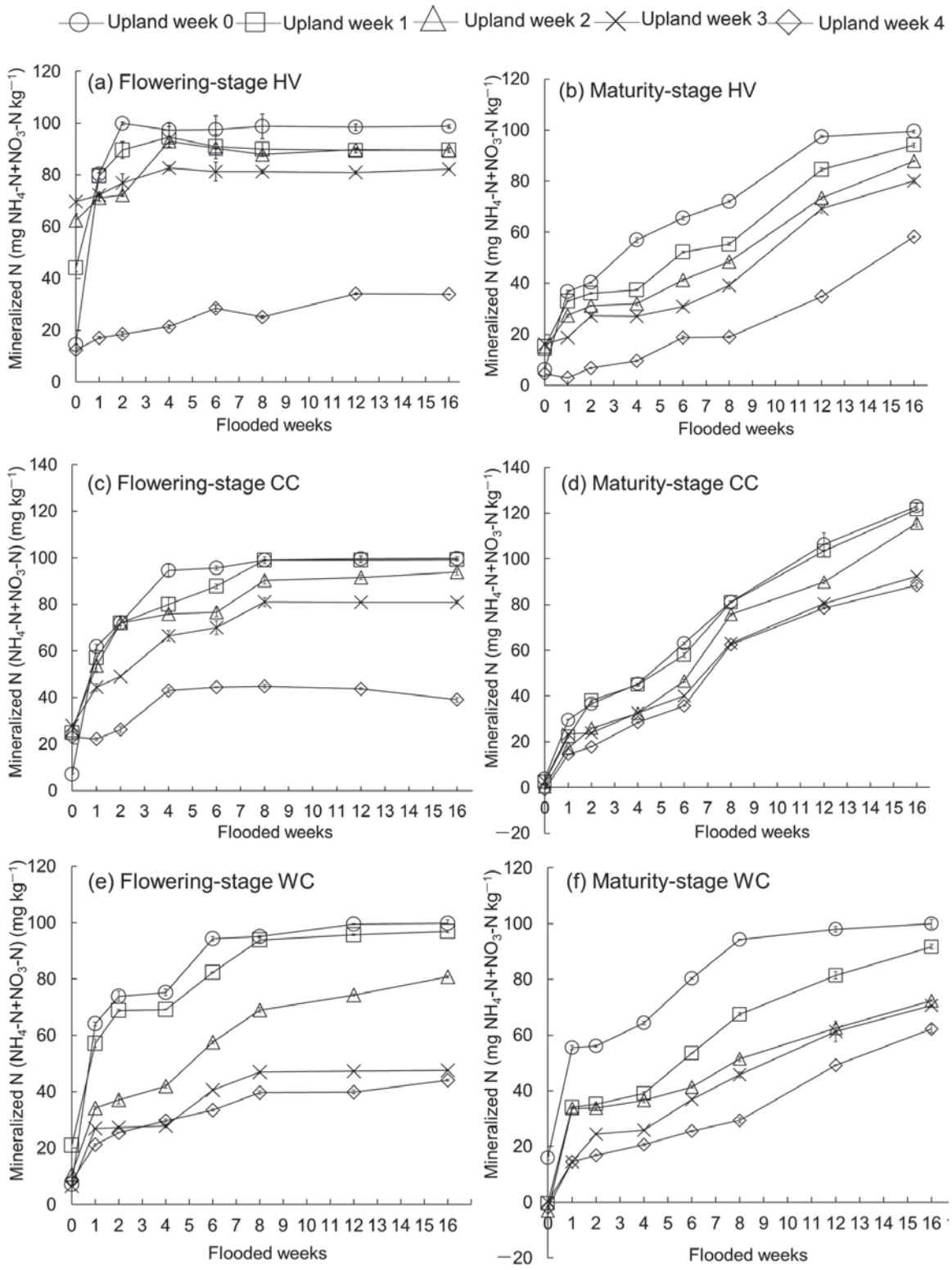

Fig. 2. Nitrogen (N) mineralized from (a) flowering-stage hairy vetch (HV), (b) maturity-stage HV, (c) flowering-stage crimson clover (CC), (d) maturity-stage CC, (e) flowering-stage white clover (WC), and (f) maturity-stage WC estimated through incubation tests at 30°C under flooded conditions

Incubation tests under flooded conditions involved one of five upland periods (0, 1, 2, 3, and 4 weeks) at 20°C. The N mineralized from green manure (GM: HV, CC, or WC) was estimated using the difference in mineralized N between soil mixed with GM and soil without GM.

Error bars represent standard deviation.

$n = 3$

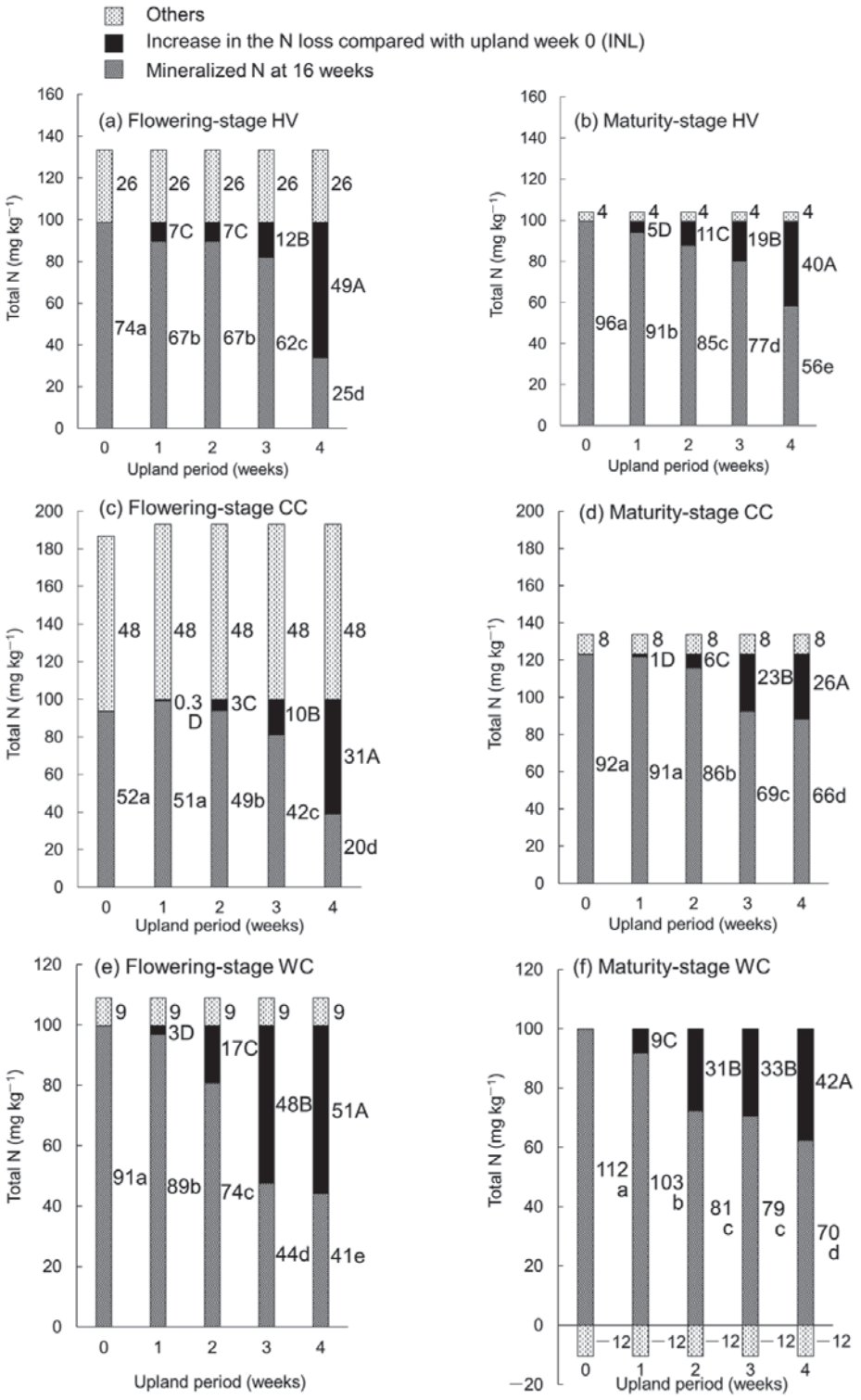

Fig. 3. Destination of total nitrogen (TN) input from (a) flowering-stage hairy vetch (HV), (b) maturity-stage HV, (c) flowering-stage crimson clover (CC), (d) maturity-stage CC, (e) flowering-stage white clover (WC), and (f) maturity-stage WC after 16 weeks of flooded incubation

"Others" include N loss during incubation under upland 0 week, remaining N in the soil, and measurement errors.
Numbers in the graphs represent percentages of each component relative to TN input.
The summation of percentage is not always 100 because the percentage of each component is rounded to an integer.
Different letters of each item indicate that mean values among different upland periods are significantly different ($P < 0.05$) according to Tukey's method.
$n = 3$.

Discussion

1. Mineralized nitrogen at flooded week 0

The MN at flooded week 0 is discussed here as an indicator that can affect the initial growth of rice (Ando et al. 1988). A large amount of MN at flooded week 0, which increased after upland weeks 1-3, was observed only in flowering-stage HV (Fig. 2 (a)). This result indicates that low C/N ratios of shoots (13.3) and roots (11.6) in flowering-stage HV promoted N mineralization during the upland period. The C/N ratios in flowering-stage HV were lower than those in maturity-stage HV and other GMs, except in the shoots of flowering-stage WC (Table 1). In flowering-stage HV, MN increased after upland weeks 1-3 because the amount of N mineralization was greater than the denitrification loss during the upland period. Meanwhile, there was a low level of MN in flowering-stage HV after upland week 4. This result indicates a marked increase in the denitrification loss during the upland period after three to four weeks for flowering-stage HV as suggested by large INL(4) (Fig. 3 (a)). Although INL(4) is determined by the denitrification loss and ammonia volatilization during both upland and flooded periods, we considered the denitrification loss during the upland period to be a major contributor to INL(4) for the following reasons: The mineralized N did not decrease sharply at the later weeks of flooded incubation, unlike the results of waterlogged incubation reported by Kanda (2000). In addition, relatively small amounts of nitrate that were lost due to denitrification or immobilization were detected only at 0 weeks of flooded incubation: 5 mg kg^{-1} (3.7% of TN input from HV), 8.5 mg kg^{-1} (6.4%), and 11.2 mg kg^{-1} (8.4%) for upland weeks 2, 3 and 4, respectively. Ammonia volatilization during the upland period was assumed to be negligible (Mitsui et al. 1954). Ammonia volatilization during the flooded period was also considered a small fraction of INL(4) from the experimental results by Iwata & Okuda (1937), where the fractions of ammonia volatilization were at most about 5% under the N application rate of 200 mg kg^{-1}.

The MN at flooded week 0 showed relatively low levels for GMs other than flowering-stage HV (Fig. 2 (a)-(f)). One reason for this is that the amount of N mineralization from maturity-stage HV and CC was probably small during the upland periods for higher C/N ratios (Table 1). Another reason is that the denitrification loss increased after upland week 2 or 3 in WC, as suggested by large INL (Fig. 3 (e) and (f)), which is a shorter period than that for flowering-stage HV. Moreover, measurement errors rather than the immobilization of MN probably caused small negative values at flooded week 0 observed for maturity-stage WC (Fig. 2 (f)), considering the relatively low C/N ratio measured as compared with the other GMs (Table 1).

2. Pattern of nitrogen mineralization from flowering- and maturity-stage green manures

When considering the use of GMs in rice production, we need to discuss the supply of MN by first separating that used for vegetative growth and that used for reproductive growth. Thus, the percentage of MN relative to TN input from the GMs was compared between the flooded period until four weeks and that after four weeks until 16 weeks (Fig. 4 (a)-(f)). For the flowering-stage GMs, the percentage until four weeks of flooding was 73% for HV (C/N ratio: 13.3 in shoots, 11.6 in roots), 49% for CC (C/N ratio: 21.6 in shoots, 19.5 in roots), and 69% for WC (C/N ratio: 13 in shoots, 14.8 in roots) following upland week 0 (20°C; Fig. 4 (a), (c), and (e)). In contrast, the percentage after being flooded for four weeks until 16 weeks was 1% for HV, 3% for CC, and 22% for WC. This pattern of rapid N mineralization is in agreement with the results obtained in past studies. For example, Ishikawa (1963) reported that MN from flowering-stage CMV (dried powder, C/N ratio: 9) was 53%-57.8% in 28 days under flooded conditions at 23°C. In another study, MN from flowering-stage CMV (air-dried small pieces, C/N ratio: 17.3) was approx. 60% in one month under flooded conditions at a mean temperature of 28.5°C (Saeki & Azuma 1956). In a further study, MN from flowering-stage HV (small fresh pieces less than 1 cm) was approx. 60% in four weeks under flooded conditions at 30°C, following an upland period of one week at 14°C (Azuma et al. 2017). In the present study, the percentage of MN relative to TN input tended to increase when the C/N ratio was low. However, the percentage for flowering-stage CC (49%) may have involved some measurement errors, considering that the percentage of "Others" was as large as 48%, which was larger than that in maturity-stage CC (8%) with higher C/N ratios (Table 1; Fig. 3 (c) and (d)). The moisture of samples after the series of incubation operations might have been lower than the actual moisture when samples were weighed for incubation tests, probably due to evaporation during operations before the upland incubation or a variation of moisture content in the shoots and roots. If lower moisture content is measured as compared with the samples used for the incubation tests, the TN input from GM is overestimated, resulting in an increase in the percentage of "Others."

For maturity-stage GMs, N mineralization until four weeks of flooding was lower in HV and CC as compared with the flowering-stage plants. The

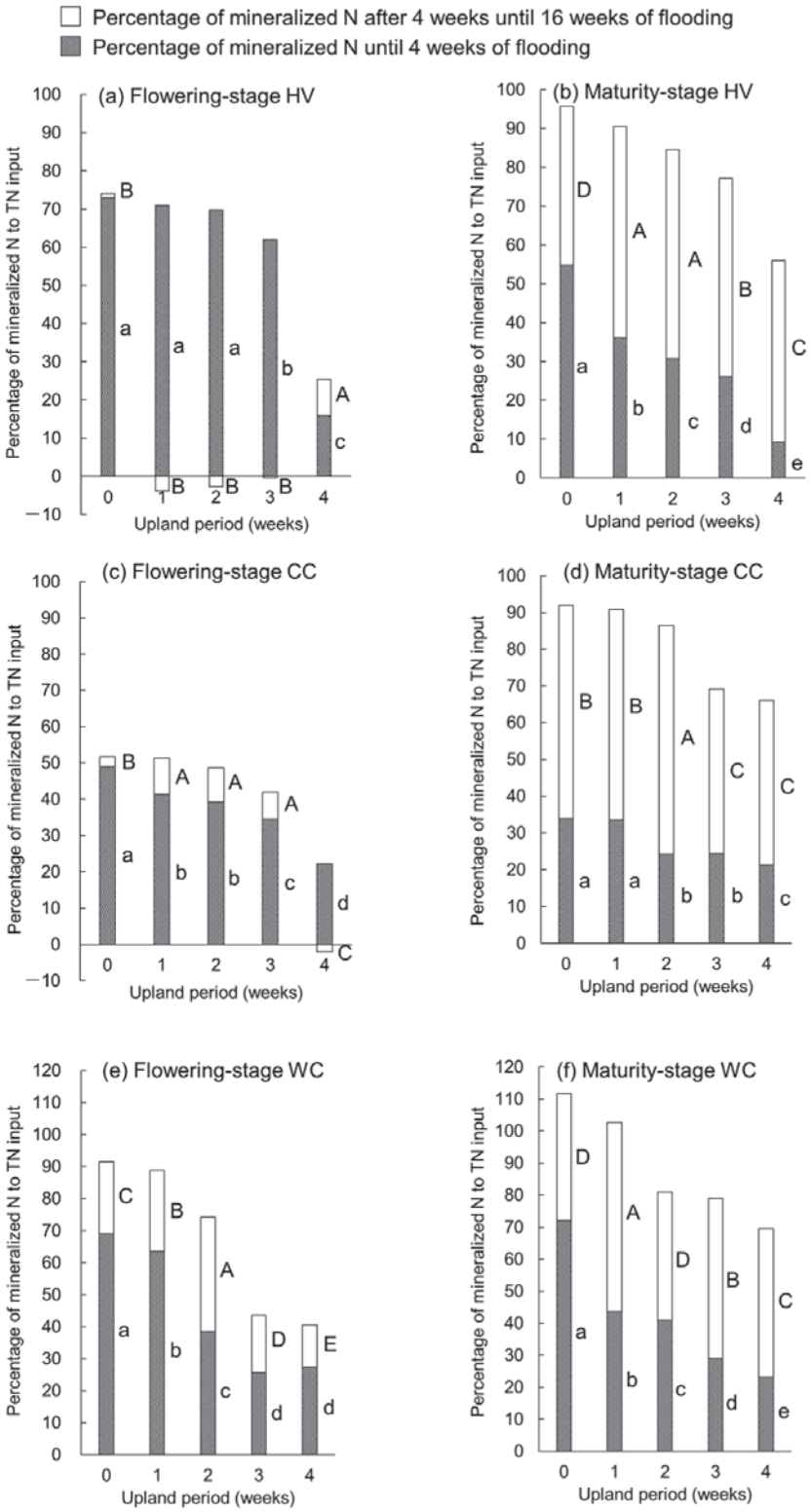

Fig. 4. Comparison of the percentage of mineralized nitrogen (N) relative to total N from (a) flowering-stage hairy vetch (HV), (b) maturity-stage HV, (c) flowering-stage crimson clover (CC), (d) maturity-stage CC, (e) flowering-stage white clover (WC), and (f) maturity-stage WC between the flooded periods until 4 weeks and that after 4 weeks until 16 weeks

Different letters indicate that mean values among different upland periods are significantly different ($P < 0.05$) according to Tukey's method.

$n = 3$.

percentage of MN until four weeks of flooding was 55% for HV (C/N ratio: 22.2 in shoots, 15.7 in roots) and 34% for CC (C/N ratio: 23.1 in shoots, 33.4 in roots) following upland week 0 (Fig. 4 (b) and (d)), whereas the percentage after four weeks until 16 weeks increased more significantly compared with the flowering-stage plants (41% for HV and 58% for CC). These slower rates of N mineralization in HV and CC were caused by higher C/N ratios in the maturity stage compared with the flowering stage. Nagumo et al. (2014) have also reported a case where maturity-stage CMV (C/N ratio: 17.1) had a slow N mineralization of 17.9% of the total N input in one month under flooded conditions at 25°C. This smaller percentage compared with our results is partly due to the fact that Nagumo et al. (2014) conducted their incubation tests at a lower temperature. For WC, the percentage of MN until four weeks of flooding was similar between the flowering- (69%) and maturity-stage WC (72%; Fig. 4 (e) and (f)). This is probably because their C/N ratios were almost identical (Table 1). The higher percentage of MN after four weeks until 16 weeks for the maturity-stage WC might have been due to overestimation, considering that the percentage following upland week 0 was greater than 100%. The overestimation might have been caused by differences in the moisture content and TN of shoots and roots between the samples used for the measurement and incubation tests.

The dates of sample collection between the flowering- and maturity-stage GMs were separated by about two months. Meanwhile, the changes in C/N ratios were different among the species. The C/N ratio of the shoots from HV increased to 22.2 at the maturity stage due to a decrease in TN (1.96%) of the shoots from 3.13% at the flowering stage (Table 1). This large change in C/N ratio changed the N mineralization pattern markedly in HV. N mineralization of the maturity-stage CC also changed markedly compared with the flowering-stage CC (Fig. 4 (c) and (d)), although the increase in the C/N ratio was not as large as that observed in HV. It is possible that higher cellulose levels, which were expected to increase as the GM matured (Ishikawa 1988, Yasue 1991), might have lowered the N mineralized from maturity-stage CC until four weeks, resulting in an increased percentage of MN after four weeks until 16 weeks.

3. Denitrification loss during the upland period

The amount of INL increased as the upland period increased (Fig. 3 (a)-(f)). We considered the denitrification loss during the upland period to be a major contributor to INL as discussed above. The decreases in the percentage of MN that occurred relative to increases in the upland period were larger until four weeks of flooding than in the period after four weeks until 16 weeks (Fig. 4 (a)-(f)). Thus, the denitrification loss during the upland period can likely be attributed to the easily mineralizable fraction of N in the GM. This result indicates that maturity-stage GMs are advantageous in reducing the denitrification loss during the upland period for species having a higher C/N ratio in the maturity stage than in flowering stage, such as HV and CC.

The upland periods during which INL sharply increased were generally longer than two weeks, except for the case of maturity-stage WC (Fig. 3 (a)-(f)). The results indicate that the denitrification loss under upland conditions, in which the moisture condition of the soil was 60% of its maximum water holding capacity, increased markedly when the upland period extended longer than two weeks. However, this period might vary considering the result for maturity-stage WC, for which INL sharply increased at upland week 2 (Fig. 3 (f)).

Conclusions

The objectives of this study were to clarify the patterns of N mineralization from flowering- and maturity-stage GMs under flooded conditions after different upland periods, and to evaluate the denitrification loss occurring during the upland periods. The following conclusions were drawn:

1. Maturity-stage HV and CC slowly mineralize N until the late growth stage of rice.
2. Denitrification loss under upland conditions increased markedly when the upland period extended longer than two weeks.
3. Maturity-stage GMs, which have a higher C/N ratio than flowering-stage GMs, are advantageous in reducing the denitrification loss during the upland period.

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