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Research Article

Temporal Plant-Height and Tillering Responses of Flooded Rice to Azolla and Urea under Pot Conditions

พลวัตความสูงและการแตกหน่อของข้าวนาน้ำขังภายใต้การใช้แหนแดงและปุ๋ยยูเรีย ในสภาพการทดลองกระถาง

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Abstract

Azolla-based nutrient management was examined in a completely randomized flooded-rice pot experiment with four treatments (T1, control; T2, urea; T3, Azolla; T4, Azolla plus urea), three independent pots per treatment, and five plant subsamples per pot. Pot-level exact treatment-label permutation sensitivity analyses indicated treatment differences in time-averaged height and tillering and in centered temporal profiles (exact $p < 0.05$). Final-assessment height means were 42.03, 86.99, 101.33 and 101.92 cm for T1–T4. The investigator confirmed that manually separated, sun-dried filled-grain mass was recorded in g plant^{-1} and averaged within pots; treatment means were 0.13, 1.40, 3.53 and 3.60 g plant^{-1}. The grain-mass omnibus permutation test gave $p = 0.006169$, but multiplicity-adjusted pairwise comparisons did not establish superiority. Grain moisture was estimated, not standardized. These pot results do not establish field yield, N equivalence or fertilizer replacement.

Keywords: *Azolla microphylla*; flooded rice; plant height; tillering; pot experiment

บทคัดย่อ

แหนแดงเป็นแหล่งธาตุอาหารชีวภาพที่ได้รับความสนใจในการผลิตข้าว การศึกษานี้ประเมินพลวัตความสูงและจำนวนหน่อของข้าวนาน้ำขังภายใต้กรรมวิธี 4 แบบ ได้แก่ ไม่ใส่แหนแดงหรือยูเรีย (T1) ยูเรีย (T2) แหนแดง (T3) และแหนแดงร่วมกับยูเรีย (T4) โดยใช้การทดลองแบบสุ่มสมบูรณ์ 3 กระถางต่อกรรมวิธี และมีต้นข้าวตัวอย่างย่อย 5 ต้นต่อกระถาง วิเคราะห์ข้อมูลการเจริญเติบโตด้วยการทดสอบแบบสับเปลี่ยนป้ายกำกับกรรมวิธีในระดับกระถาง ผลการวิเคราะห์แสดงความแตกต่างระหว่างกรรมวิธีทั้งค่าเฉลี่ยตามเวลาและรูปแบบการเปลี่ยนแปลงตามเวลาของความสูงและจำนวนหน่อ (exact $p < 0.05$) ความสูงครั้งสุดท้ายของ T3 และ T4 เท่ากับ 101.33 และ 101.92 ซม. ตามลำดับ ข้อมูลปลายการทดลองด้านน้ำหนักเมล็ดมีค่าเฉลี่ยจากตารางต้นทางเท่ากับ 0.13, 1.40, 3.53 และ 3.60 สำหรับ T1–T4 โดยเป็นมวลเมล็ดเต็มหน่วยกรัมต่อต้นที่เฉลี่ยภายในกระถางตามคำยืนยันของผู้วิจัย การทดสอบรวมระดับกระถางแบบ

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สับเปลี่ยนให้ p = 0.006169 แต่การเปรียบเทียบรายคู่หลังปรับค่าการทดสอบหลายครั้งไม่พบความแตกต่างที่มีนัยสำคัญ ความชื้นเมล็ดไม่ได้ปรับมาตรฐาน จึงไม่ตีความเป็นผลผลิตต่อพื้นที่ การทดลองนี้สนับสนุนข้อค้นพบด้านพลวัตการเจริญเติบโตในสภาพกระถาง แต่ไม่เพียงพอสำหรับสรุปอัตราทดแทนปุ๋ยยูเรียหรือผลผลิตต่อพื้นที่
คำสำคัญ: แหนแดง; ข้าวนาน้ำขัง; ความสูงต้น; การแตกหน่อ; การทดลองกระถาง

Introduction

Nitrogen (N) management remains a central challenge in flooded-rice production because insufficient N constrains crop development, whereas excessive dependence on synthetic N fertilizer can increase production costs and environmental N losses. Azolla, a floating aquatic fern associated with N-fixing cyanobacteria, has therefore attracted sustained interest as a biologically based nutrient input for lowland rice systems (Marzouk et al., 2023).

Previous research has established that Azolla can contribute to rice productivity under a range of nutrient-management strategies. Multi-year field experiments have reported improvements in fertilizer-N recovery, N-use efficiency, N uptake, and grain yield when Azolla is integrated with mineral N (Yao et al., 2018a, 2018b), while more recent farmer-field and pot studies have demonstrated productivity responses under reduced-N or Azolla-containing treatments (Ocloo et al., 2025; Ahmad et al., 2024). Related field evidence also includes Azolla with rice-straw incorporation (Marzouk et al., 2024). Other published studies have examined Azolla in combination with nitrogenous fertilizer, incorporation timing, successive green-manure incorporation, and integrated nutrient management (Baktash et al., 2023; Chaudhary et al., 2025; Kimani et al., 2025; Paramanik et al., 2024). Together, these publications provide agronomic context, although their treatment structures and endpoints are not directly equivalent to the present pot experiment.

Relatively few studies have characterized repeated plant-height and tillering responses under contrasting Azolla and urea treatments. Endpoint measurements integrate cumulative crop responses at harvest, whereas longitudinal assessments additionally reveal how treatment responses develop and whether differences persist across successive observations. This study therefore focuses on repeated vegetative measurements, while retaining investigator-confirmed filled-grain mass (g plant^{-1}) as a secondary pot-level endpoint. The grain-mass analysis is interpreted conservatively because grain moisture was not standardized and the original individual weighing records are unavailable. Previous pot and field studies have examined Azolla incorporation timing, nitrogen-fertilizer combinations, and multi-season responses, but their application rates and measurement endpoints differ from those of the present experiment (Bhuvaneshwari and Singh, 2015; Baktash et al., 2023; Paramanik et al., 2024; Chaudhary et al., 2025; Kimani et al., 2025).

The objective of this study was to characterize temporal plant-height and tillering responses of flooded rice under four nutrient-management treatments—an unfertilized control, urea alone, dried Azolla alone, and dried Azolla combined with urea—and to evaluate filled-grain mass (g plant^{-1}) as a

secondary pot-level endpoint. The study was conducted under outdoor pot conditions, and its findings were interpreted within the limitations of the experimental design, without extrapolation to field-scale grain yield or fertilizer-nitrogen replacement.

Material and Method

2.1. Experimental Site and Plant Materials

The historical thesis described sandy clay loam soil (21.44% clay, 53.56% sand and 25.00% silt). Preplant and postharvest soil samples were collected separately from each of the 12 pots and analysed for total N, available P, exchangeable K, organic matter, pH and electrical conductivity. However, only treatment-level summaries survive; pot-level laboratory values cannot be independently audited. Soil chemistry is therefore reported descriptively, without inferential comparisons. The original soil collection site and soil preparation steps beyond those documented in the thesis require source confirmation.

The experiment was conducted from August to early December 2018 outdoors at a university field-training facility in Phitsanulok, Thailand, under natural sunlight and rainfall; temperature, humidity and rainfall were not continuously recorded for this experiment. Rice cultivar Phitsanulok 2 was transplanted as 22-day-old seedlings, five per pot. Azolla microphylla was obtained from the departmental cultivation facility and identified morphologically by a departmental expert; no voucher or molecular confirmation is available. Each of the 12 pots contained 7 kg of sandy clay loam soil; the soil-surface diameter was approximately 16.8 inches (42.7 cm), giving an estimated surface area of 0.1430 m² per pot.

2.2 Experimental Design and Treatments

A completely randomized design (CRD) included four treatments with three independent pots per treatment (12 pots): T1, no fertilizer; T2, granular urea (46-0-0) at 20 kg rai^{-1} once at approximately 15 days after transplanting (DAT; approximately 1.79 g per pot); T3, oven-dried ground Azolla microphylla at 100 kg rai^{-1} per application (approximately 8.94 g per pot per application), twice, before transplanting and at approximately 15 DAT; and T4, the same two Azolla applications plus the T2 urea application. Five plants per pot were subsamples, not independent replicates. Rates were converted using 1 rai = 1,600 m² and an estimated pot soil-surface area of 0.1430 m². These application details were confirmed by the investigator; the surviving historical wording was inconsistent.

2.3 Crop Management and Growth Measurements

Azolla was collected from a departmental cultivation stock, washed with water, sun-dried for approximately two days, oven-dried at 50 °C to constant mass (approximately 72 h), ground and passed through a 0.5-mm sieve. The same batch was stored in a sealed container between applications. For T3 and T4, approximately 8.94 g of prepared dry Azolla was applied per pot on each of two occasions (100 kg rai^{-1} per application). The first application was mixed into moist soil 1–3 days before

transplanting; the second was applied at approximately 15 DAT. Azolla tissue N, P and K concentrations were not measured for this batch, so neither N-equivalent doses nor nutrient-release kinetics can be inferred.

All 12 pots were maintained outdoors, exposed to sunlight and natural rainfall. Floodwater was generally maintained at approximately 5 cm above the soil surface, with excess water escaping through overflow openings; tap water was added daily or nearly daily as needed, but its chemical quality was not tested. At approximately 15 DAT, water was drained from every pot and soil disturbed consistently before the assigned second Azolla and/or urea applications; pots were reflooded three days later. Plant height was reported on 17 assessment occasions and tiller number on 15 assessment occasions, approximately weekly. The original dated observation logs are unavailable; exact days after transplanting for individual assessments cannot be verified. Historical statements of 140 g dry Azolla or 5 g fresh Azolla per pot are inconsistent with the investigator-confirmed treatment rates and are not used as application doses.

At harvest, filled grains were separated manually from unfilled grains, sun-dried for two sunny days, and weighed for each sampled plant. Grain moisture was estimated at approximately 13% but was neither instrument-verified nor standardized to a reference moisture content. The five plant values were averaged within each pot; grain mass is expressed as filled grain mass (g plant^{-1}), not field yield. The historical grain dataset contains 60 plant-indexed observations nested in 12 independent pots.

2.4 Data Structure and Experimental Unit

Plant height and tiller number were recorded repeatedly. The EXP-A thesis reports 17 height assessments and 15 tiller assessments. Source-table reconciliation identified height spreadsheet columns 1–17 and tiller columns 3–17 as the corresponding reported series. The growth worksheet records treatment (t) and within-group plant index (r, 1–5), with each five-index sequence repeated three times per treatment. This sequence is consistent with three replicate groups of five plant subsamples, but the growth worksheet contains no explicit pot or replicate identifier. The study investigator confirmed that the first, second, and third consecutive sets of five plants within each treatment correspond to replicate pots 1, 2, and 3, respectively. This mapping is author-confirmed rather than independently traceable to a surviving physical pot-label register. Actual calendar dates and days after transplanting (DAT) are unverified; figures use assessment indices.

2.5 Statistical Analysis

Height (17 occasions) and tiller number (15 source-matched occasions, spreadsheet columns 3–17) were summarized at the author-confirmed pot-group level (three groups per treatment, five plant subsamples per group). Exact treatment-label permutation sensitivity analyses used all 369,600 allocations of 12 investigator-confirmed pots into four groups of three, with two statistics: treatment contrasts in pot-level time averages and treatment differences in pot-mean-centered temporal profiles. These

exact permutation tests use the investigator-confirmed pot grouping conditional on the reported CRD and exchangeability of pot-level treatment assignments. Their randomization-based interpretation assumes that treatment allocation was exchangeable among the 12 pots; the original randomization schedule was not available.

Previously reported Wald chi-square tests were excluded because their analytical provenance could not be reproduced. Exact treatment-label permutation tests at the independent-pot level were used as sensitivity analyses of growth responses. Filled-grain mass was evaluated as a secondary pot-level endpoint using an omnibus ANOVA and exact permutation test; multiplicity-adjusted pairwise comparisons were interpreted conservatively. These tests do not establish fertilizer-N equivalence or field-scale yield.

2.6 Evidence Boundaries

Height, tiller number and investigator-confirmed filled-grain mass (g plant^{-1}) were evaluated using the pot as the experimental unit. Soil chemistry was summarized descriptively because pot-level laboratory results were not recoverable. No inference was made regarding biological N fixation, plant N uptake, N-use efficiency, fertilizer replacement or field-scale yield.

Results

3.1 Treatment-Dependent Temporal Variation in Rice Growth

For the 17-assessment height series, author-confirmed pot-level exact permutation sensitivity analyses indicated treatment differences in time-averaged height ($F[3,8] = 78.548$; exact $p = 0.000130$) and centered height trajectories (exact $p = 0.000065$). Reanalysis of the source-matched 15-occasion tiller series yielded a time-averaged treatment contrast ($F[3,8] = 6.306$; exact $p = 0.009156$) and a centered-profile contrast ($SS = 27.621$; exact $p = 0.022078$). All four results use the investigator-confirmed pot grouping; inference assumes the reported completely randomized design and exchangeability of treatment labels. No occasion-specific statistical claims are made.

Note: Exact treatment-label permutations were performed at the pot level (12 pots, three per treatment); repeated observations and plants within pots were not permuted independently.

3.2. Plant-Height Trajectories

Plant height increased across the recorded measurement occasions in all treatments (Figure 1). The unfertilized control (T1) remained lower, while the Azolla treatments (T3 and T4) showed higher final means. At the final recorded height assessment, mean heights were 42.03, 86.99, 101.33, and 101.92 cm in T1, T2, T3, and T4, respectively. The precise DAT of the final spreadsheet column is not independently established.

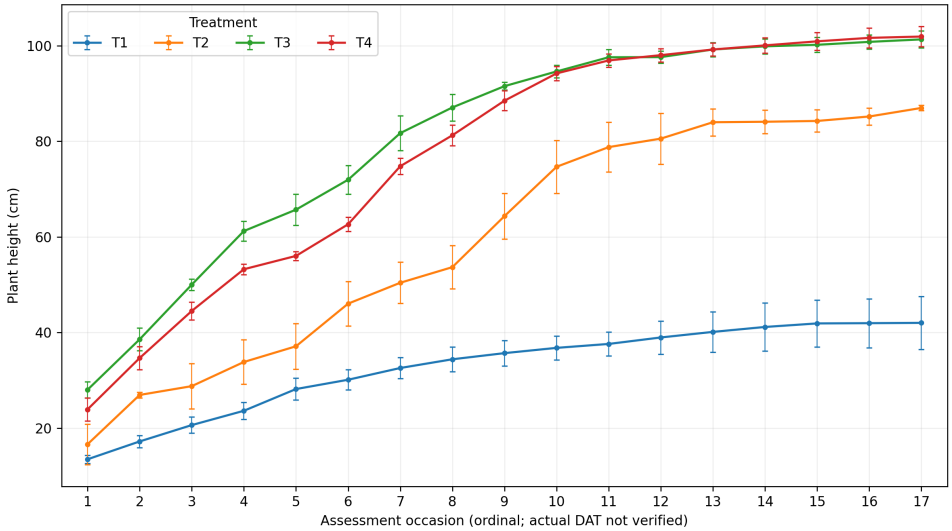


Figure 1. Rice plant height across recorded assessment occasions under four nutrient-management treatments. Points indicate means ± SE of three independent pot means per treatment (five plant subsamples per pot). Assessment occasions are shown by ordinal index because the original days-after-transplanting labels could not be verified.

Table 1. Nutrient-management treatments in the flooded-rice pot experiment.

Treatment	*Azolla*	Urea (46-0-0)	Description
T1	None	None	Unfertilized control
T2	None	20 kg rai^{-1} once (~1.79 g pot^{-1})	Urea only; applied at ~15 DAT
T3	100 kg rai^{-1} per application × 2 (~8.94 g pot^{-1} per application)	None	Oven-dried, ground Azolla; two applications
T4	100 kg rai^{-1} per application × 2 (~8.94 g pot^{-1} per application)	20 kg rai^{-1} once (~1.79 g pot^{-1})	Azolla twice plus urea once

Note. Three independent pots per treatment and five sampled plants per pot. T2 received urea (46-0-0), 20 kg rai^{-1} once (~1.79 g/pot at ~15 DAT). T3 received oven-dried ground Azolla, 100 kg rai^{-1} per application (~8.94 g/pot), twice (before transplanting and ~15 DAT). T4 received both T3 Azolla and T2 urea. Converted using soil-surface area ~0.1430 m²/pot and 1 rai = 1,600 m². Rates are investigator-confirmed; Azolla N concentration was not determined.

3.3. Tiller-Number Dynamics

The source-matched 15-occasion tiller series (spreadsheet measurement columns 3–17) is shown in Figure 2. At the final reported assessment, descriptive treatment means were 1.07, 1.73, 4.73, and 3.87 tillers per plant for T1, T2, T3, and T4, respectively. Two differences of approximately 0.03 tillers between spreadsheet and thesis table remain documented; original observations were not altered. The 15-occasion exact permutation sensitivity tests identified between-treatment contrasts in pot-level averages and centered temporal profiles (Table 2), using the investigator-confirmed grouping of five sampled plants per pot.

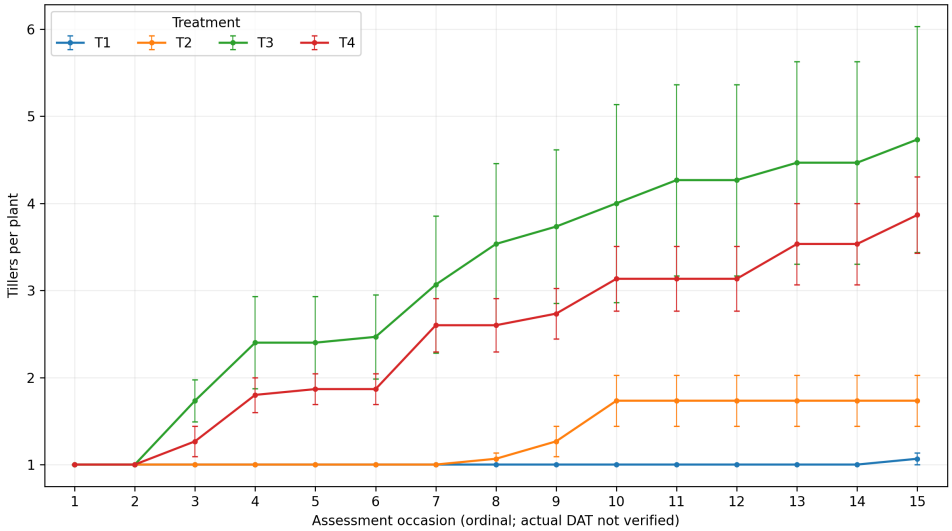


Figure 2. Rice tiller number across 15 reported assessment occasions, matched to original spreadsheet columns 3–17. Points indicate means ± SE of three investigator-confirmed replicate pots per treatment, each based on five sampled plants. The five-plant grouping within each replicate pot was confirmed by the study investigator; the original physical pot-label register was unavailable. Horizontal-axis indices are not verified DAT. Descriptive figure only; no occasion-specific significance is asserted.

Table 2. Pot-level exact permutation tests for rice growth responses (investigator-confirmed pot grouping)

Outcome	Pot-level test	Statistic	Exact p
Plant height	Time-averaged treatment contrast	F(3,8) = 78.548	0.000130
Plant height	Centered trajectory shape	SS = 10209.403	0.000065
Tiller number	Time-averaged treatment contrast	F(3,8) = 6.306	0.009156
Tiller number	Centered trajectory shape	SS = 27.621	0.022078

Note. Height analyses use 17 assessments; tiller analyses use the source-matched 15 assessments (spreadsheet columns 3–17). Exact p-values enumerate all 369,600 allocations of 12 author-confirmed pot groups into four treatments of three groups. Centered-profile tests compare trajectories after subtracting each pot group's across-occasion mean. The five-plant-to-pot mapping was confirmed by the study investigator, although an independent historical pot-label register and randomization record were not available. No inference about occasion-specific treatment differences or field-scale responses is made.

3.4. Filled-grain mass as a secondary endpoint

The investigator confirmed that the endpoint was manually separated, sun-dried filled-grain mass, expressed as g plant^{-1} and averaged over five plants per pot. The treatment means were T1 = 0.13, T2 = 1.40, T3 = 3.53 and T4 = 3.60 g plant^{-1} (three independent pot means per treatment). The pot-level omnibus ANOVA gave F(3, 8) = 9.641, and the exact treatment-label permutation test gave p = 0.006169. Multiplicity-adjusted pairwise comparisons did not establish significant differences between individual treatments. Grain moisture was estimated at approximately 13%, not measured or standardized; these values must not be converted to yield per unit land area.

Discussion

4.1. Temporal divergence is the primary response signal

The main contribution of this study is the demonstration that nutrient-management effects were dynamic rather than constant through time. Pot-level exact permutation analyses identified differences in mean-centered temporal trajectory shapes across treatments, conditional on treatment-label exchangeability under the reported CRD. These results do not constitute conventional treatment-by-occasion interaction tests. The unfertilized control remained consistently constrained, whereas Azolla-containing treatments increasingly separated from the control during later observations. This temporal pattern complements the cumulative information captured by an end-point comparison and is therefore the strongest distinctive feature of the present experiment.

At the final height assessment, T3 and T4 reached approximately 101 cm, compared with about 87 cm under urea alone and 42 cm in the unfertilized control. Tillering showed a similar directional pattern, with T3 having the highest numerical final mean; no tiller-specific pairwise significance is claimed. Previous studies have examined Azolla in rice production (Marzouk et al., 2023; Yao et al., 2018a, 2018b; Ahmad et al., 2024). In contrast, the present pot experiment describes how growth differences developed across repeated observations; it does not establish field productivity effects, N recovery, or fertilizer substitution.

4.2. Secondary filled-grain mass and interpretation limits

Filled-grain mass was numerically greater in Azolla-containing treatments, with an omnibus pot-level treatment difference, but no multiplicity-adjusted pairwise superiority. The experimental unit was the pot, and the moisture content of the sun-dried grains was estimated rather than standardized. Consequently, the grain-mass results are secondary pot-level observations, not field-yield estimates.

The investigator confirmed the physical endpoint as filled-grain mass (g plant^{-1}), obtained after manual separation and sun-drying. Original individual weighing sheets are unavailable, although the surviving dataset reproduces treatment means. In contrast, field research has directly evaluated rice grain yield and agronomic nitrogen-use efficiency under rice-straw and Azolla management (Marzouk et al., 2024). Such field endpoints and N-use indices were not established in this experiment.

4.3. The Azolla-plus-urea contrast was inconclusive for an additional benefit

The secondary filled-grain means in T3 and T4 (3.53 and 3.60 g plant^{-1}) were numerically similar. This does not demonstrate treatment equivalence or an additive effect of urea; the study was not powered for equivalence testing, and adjusted pairwise comparisons were not significant.

This distinction is important when placing the study within the current literature. Experiments designed to quantify fertilizer substitution use graded N rates, direct N measurements, or field validation (Ocloo et al., 2025; Yao et al., 2018a, 2018b). The present experiment lacks those design elements. Its contribution is therefore not an estimate of N equivalence: the observed vegetative trajectories do not

establish an equivalence between Azolla alone and Azolla plus urea, and the secondary filled-grain endpoint, although investigator-confirmed in g plant^{-1}, cannot be interpreted as field-scale grain yield because moisture was not standardized and original individual weighing sheets are unavailable. The broader literature also documents Azolla-related changes in grain traits and field-scale environmental outcomes (Xu et al., 2017; Bazihizina et al., 2025; Yang et al., 2025); these findings provide context, not measurements of nitrogen cycling, grain quality, or emissions in the present pots.

4.4. Evidence hierarchy, limitations, and interpretation

The study has several constraints that define the strength of inference. Only three independent pots were available per treatment, limiting statistical power and the precision of treatment comparisons. The five plants within each pot increased within-unit observation density but did not increase treatment replication. The relatively small pots may have constrained Azolla establishment, surface coverage, decomposition dynamics, and rice root development; these possibilities were not measured and could affect extrapolation to field conditions. In addition, chlorophyll content (SPAD), total biomass, panicles per hill, grains per panicle, and filled-grain percentage were not measured, limiting physiological and yield-component interpretation of the secondary filled-grain mass endpoint. No source-verifiable measurements of plant N uptake, N-use efficiency, biological N fixation, N loss, or replicate-level soil chemistry were available. The investigator confirmed that pots were maintained outdoors under natural sunlight and rainfall, but instrument-recorded temperature, humidity and rainfall data are unavailable. Tap water was used, with a target floodwater depth of approximately 5 cm; water chemistry and continuously monitored water depth are unavailable. Nominal urea and oven-dried Azolla application rates and calculated per-pot equivalents are reported, but original pot-level weighing records are unavailable. Azolla tissue nutrient concentrations were not measured, precluding nutrient-equivalent dose comparisons. The historical designation as a CRD is retained, but the original randomization sequence cannot be independently audited.

These limitations mean that mechanistic explanations must remain literature-supported hypotheses rather than conclusions from this experiment. Azolla-associated N supply and nutrient cycling are biologically plausible mechanisms (Marzouk et al., 2023; Yao et al., 2018a, 2018b; Yang et al., 2020), but they were not measured directly here. The absence of these measurements is especially relevant because contemporary higher-strength Azolla studies often combine yield with N-recovery, N-loss, or soil-process measurements (Yao et al., 2018a, 2018b; Yang et al., 2020). The present manuscript therefore occupies a narrower evidence tier: a retrospectively reconstructed pot experiment with investigator-confirmed subsampling groups describing temporal vegetative responses; the investigator-confirmed filled-grain mass is retained as a secondary pot-level endpoint (g plant^{-1}), but cannot support field-yield or fertilizer-replacement inference because moisture was not standardized and original weighing sheets are unavailable.

4.5. Agronomic implication and next test

The results justify further testing of Azolla-based nutrient management, but they point to a specific next question rather than a fertilizer recommendation. Field experiments should determine whether the temporal divergence observed here is reproducible across environments and whether the similarity between Azolla alone and Azolla plus the recorded nominal urea rate persists when N inputs are quantified on an equivalent basis. Such studies should include plot-level yield, plant N uptake, soil mineral N, N-use-efficiency indices, and sufficient replication to estimate treatment differences with greater precision. This would allow the response pattern identified here to be linked directly to fertilizer substitution and N-cycling mechanisms.

Conclusion

Azolla-associated treatments differed in the temporal vegetative development of flooded rice under pot conditions. Pot-level exact permutation sensitivity analyses identified differences in time-averaged height and tiller number and in centered trajectory shapes; these are not conventional treatment-by-time interaction tests. Final-assessment heights were numerically highest in T3 and T4. Secondary filled-grain mass differed in the omnibus pot-level test, but adjusted pairwise comparisons did not establish individual treatment superiority. With only three pots per treatment, estimated rather than standardized grain moisture, and no direct N measurements or field validation, fertilizer-replacement and field-yield recommendations are not supported.

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Author Contributions

Kamonchanok Huangmee: Investigation, data curation, methodology, and writing – original draft. Suphannika Intanon: Conceptualization, methodology, supervision, and writing – review and editing. Panisara Thepkusol: Investigation, data curation, and validation. Parwat Wijarat: Investigation, resources, and validation. Tepsuda Rungrat: Methodology, visualization, and writing – review and editing. Anupong Wongtamee: Conceptualization, formal analysis, validation, supervision, and writing – review and editing. All authors reviewed and approved the final version of the manuscript.

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