Learning about the growing habits and reproductive strategy of *Thinopyrum intermedium* through the establishment of its critical nitrogen dilution curve

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Abstract

Context

The perennial grain crop *Thinopyrum intermedium* can provide various ecosystem services and a dual production of grains and forage. Yet, to improve crop management, better knowledges of its physiological behavior and growing habits are required.

Objective

The goal of this study was to characterize *Th. intermedium* nitrogen (N) requirements through the evaluation of its response to N fertilization and the subsequent determination of its critical nitrogen dilution curve (CNDC).

Methods

A field experiment was implemented in Belgium during three growing seasons with various N fertilization schemes. Biomass of the different organs and their N contents were measured at specific phenological stages. To estimate the CNDC, a Bayesian hierarchical model was applied on the assembled dataset. The validity of the curve was assessed on an independent dataset including contrasted N situations.

Results

Globally, N fertilization had a positive impact on the dry matter (DM) of leaves, stems and ears (*p-value*<0.05). The aboveground biomass and N uptake were found maximum with fertilization comprised between 100 and 150kg N/ha applied over the entire growing year. At grain harvest, total DM ranged from 7.0 to 16.4t DM/ha for a fertilization strategy of 100kg N/ha, depending upon the growing season. The N amount of the aboveground biomass was found to decrease during the second phase of the growing cycle. As observed with the proposed CNDC, the aerial N content tended to decrease with the evolution of growing stages and biomass accumulation. Through the low a-coefficient determined for the CNDC, it was confirmed that the crop had reduced need in terms of N nutrition.

Conclusions

The reduced N requirements can be linked to the high N use efficiency and a potential resource-conservative strategy of the crop. This, combined with the observed decrease of the N uptake by the aboveground biomass during the second phase of growth, can be related to the long-term survival strategy of the crop. The latter requires substantial investments in perennial belowground structures coupled with reduced resource allocations to seeds.

Implications

Our study has highlighted that *Th. intermedium* is able to reach a high shoot DM production with low N needs. Our proposed CNDC will be highly helpful to help define N requirements in various pedo-climatic environments and adjust accordingly the soil-crop management, among which the N fertilization. Ultimately, the low N requirements of *Th. intermedium* coupled with a high N use efficiency demonstrated that it could enhance agronomic and environmental benefits.

Keywords

Thinopyrum intermedium, perennial crop, Intermediate wheatgrass, nitrogen dilution curve, nitrogen nutrition index, nitrogen needs

1. Introduction

The intermediate wheatgrass *Thinopyrum intermedium* subsp. *intermedium* (Host) Barkworth & D.R. Dewey is developed as a perennial grain crop that can provide ecosystem services including production and preservation services. Previous research has largely focused on its agronomic performances and analyzed both grain and fodder productions (Dick *et al.*, 2018; Jungers *et al.*, 2018; Tautges *et al.*, 2018; Clark *et al.*, 2019; Favre *et al.*, 2019; Barriball, 2020; Hunter *et al.*, 2020a; 2020b). In the meantime, the crop has proven to be valuable in reducing nitrate leaching (Culman *et al.*, 2013; Jungers *et al.*, 2019), or improving soil food webs, carbon pools and sequestration (de Oliveira *et al.*, 2018, 2020; Bergquist, 2019; Sprunger *et al.*, 2019). *Th. intermedium* is characterized by a recent selection history (DeHaan *et al.*, 2018) and its resource allocation to grains is low and variable (Culman *et al.*, 2013; Zhang *et al.*, 2015; Newell & Hayes, 2017). At the same time, its perenniality leads to large resource allocation to the belowground organs composed of short rhizomes and a deep root system to ensure crop continuity (Ogle *et al.*, 2011; Sainju *et al.*, 2017; Sprunger *et al.*, 2018; Sakiroglu *et al.*, 2020). Consequently, the development of intermediate wheatgrass in cropping system is still impeded by grain yielding capacity and stability, and knowledge gaps about best management practices in fields (Lanker *et al.*, 2020).

To improve yields and crop management, a good description of its physiological behavior and a better understanding of its growing habits are yet required. For instance, recent findings highlighted the ability of using water from deep soil layers and maintaining high water-use efficiency throughout the growing season (de Oliveira *et al.*, 2020; Clément *et al.*, 2021a, 2021b). However, few are known about its nitrogen (N) use, whereas crop N management is a key point by being one of the major limiting factors for agricultural productions (Gastal *et al.*, 2012). Some studies have investigated the impact of N fertilization on *Th. intermedium* performances (Jungers *et al.*, 2017; Frahm *et al.*, 2018), without quantifying the soil N supply. Yet, *Th. intermedium* is characterized by a deep and extensive root system, its soil exploration and resource use are better both in space and time through extended growing period (Culman *et al.*, 2013; Jungers *et al.*, 2019; Duchene *et al.*, 2020). This observation may suggest that external sources of N could be minimized without hampering productions of the crop with the benefit of limiting economic and environmental costs of agriculture. Anyhow, there is a need for research devoted to understanding the impacts of N management on crop ecophysiology which will undoubtedly have consequences on agronomical practices and crop N requirements.

To determine the N status of a plant population, the nitrogen nutrition index (NNI) is frequently used. It corresponds to the ratio between the actual N concentration within aerial plant tissues and the critical N concentration (N_c) required to achieve a non-limiting growth (Lemaire *et al.*, 1997). N_c is derived from the critical N dilution curve (CNDC) and represents the minimal N concentration required in shoots to ensure optimal photosynthesis activity and maximize the total aerial dry matter production (W) (Greenwood *et al.*, 1990). The mathematical description of the curve is provided in Eq.1 linking N percentage and W using the allometric function proposed by Lemaire *et al.* (1984):

$$\%N = aW^{-b} \qquad \text{(Equation 1)}$$

where W is the total shoot biomass expressed in terms of dry matter (t DM/ha), %N is the total N content of shoots (% of W), *a* and *b* are coefficients specific to crop parameters. The *a*-coefficient represents the N concentration in the total aboveground biomass at 1t DM/ha of W, while the *b*-coefficient influences the shape of the curve (Greenwood *et al.*, 1990; Lemaire *et al.*, 1997; Gastal *et al.*, 2002; Ziadi *et al.*, 2010; Santana *et al.*, 2020). The CNDC relies on the principle that under non-limiting soil nitrogen availability, the N concentration in the aboveground biomass is highly related to the crop growth rate and the dry matter accumulation. The CNDC has been determined for many cultivated crops including perennial crops (Table 1) and has been further used as a reference to discriminate N situations that are over (above the curve, NNI>1, i.e. luxury N consumption) or under (below the curve, NNI<1, i.e. N deficiency) the critical curve, thus driving fertilization rate and timing on crop.

The conventional approach to set-up the CNDC consists firstly in identifying the N_c points and then fit the negative exponential curve to these points (Eq.1). Different statistical approaches may be used to identify N_c points: (i) analysis of variance and multiple comparisons (Greenwood *et al.*, 1990), (ii) fitting a linear-plateau curve (Justes *et al.*, 1994), or (iii) hierarchical Bayesian modelling (Makowski *et al.*, 2020). Many studies determined N_c points using the simplified statistical method derived from the study of Greenwood *et al.* (1990). In this approach, ANOVA is first used to identify where variations in W are statistically different under varying N treatments, within each date of sampling. A multiple comparisons analysis is then used to identify the maximal biomass (W_{Max}), the N content recorded under W_{Max} is the critical N_c point. In the event where statistically equivalent W_{Max} are reported under two or more N treatments, the lowest N rate is selected as the N_C. However, N_c points selected using this simplified approach might be biased due to potential deficiencies within the experimental dataset such as the N rates might not be sufficient to reach W_{max} (Fernandez *et al.*, 2022). The second method usually requires dataset sufficiently large enough so that a linear-plateau curve can be identified for each observation set. However, this approach remains difficult to implement as the experimental dataset must meet specific statistical criteria, as described in Justes *et al.* (1994). Finally, more recently, an alternative statistical method based on a hierarchical Bayesian modelling has been proposed by Makowski *et al.* (2020) to relate the N percentage to the W and analyze concomitantly the uncertainty in the fitted CNDC. The hierarchical Bayesian model simultaneously identifies critical points using the linear-plateau method (Justes *et al.*, 1994) while fitting the negative exponential curve which defines N_c. In principle, this model can estimate CNDC from the direct W - %N pair of observations without classifying limiting and non-limiting N data and without assuming that W_{Max} has been reached in all sampling dates (Fernandez *et al.*, 2022). This method has already been successfully used in different study for maize, wheat or tall fescue

(Ciampitti *et al.*, 2021a, 2021b; Fernández *et al.*, 2021; Yao *et al.*, 2021). However, the Bayesian hierarchical method might remain subjected to potential inferential bias due to limitations within experimental datasets in terms of quantity and/or quality of the data (Fernández *et al.*, 2021; Fernandez *et al.*, 2022).

The CNDC is a reliable tool to establish diagnoses of the N status of various crop species growing within different climatic and agronomic conditions and further inform on the crop growing habits (Table 1). Among else, it has allowed differentiating functionally different plants, such as C3 and C4 plants in the study of Greenwood *et al.* (1990). The establishment of the CNDC may also contribute to improve the management practices, such as N fertilization. Therefore, to understand growing habits and N requirements of the newly developed perennial grain crop *Th. intermedium*, our objective was to determine the CNDC associated to its growth.

Table 1 : Coefficients of the critical nitrogen dilution curve (described in Eq.1) of different cultivated species.

Plant species	***a*-coefficient**	***b*-coefficient**	**Statistical method reference**	**Reference**
C3 crops	5.70	-0.50		(Greenwood *et al.*, 1990)
C4 crops	4.09	-0.50		(Greenwood *et al.*, 1990)
Lolium perenne L. (Perennial ryegrass)	6.36	-0.71	(Justes *et al.*, 1994)	(Gislum *et al.*, 2009)
Solanum tuberosum L. (Potato)	5.37	-0.45	(Greenwood *et al.*, 1990)	(ben Abdallah *et al.*, 2016)
Triticum aestivum L. (Wheat)	3.90 [2.08; 5.47]*	-0.41 [0.20; 0.52]*	(Makowski *et al.*, 2020)	(Yao *et al.*, 2021)
Beta vulgaris subsp. *vulgaris* var. alba L. (Fodder beet)	4.9	-0.52	(Greenwood *et al.*, 1990)	(Chakwizira *et al.*, 2016)
Festuca arundinacea Schreb. (Tall fescue)	3.93 [3.59; 4.32]*	-0.42 [-0.35; -0.49]*	(Makowski *et al.*, 2020)	(Fernández *et al.*, 2021)
Linum usitatissimum L. (Linseed)	4.69	-0.53	(Justes *et al.*, 1994)	(Flénet *et al.*, 2006)
Medicago sativa L. (Alfafa)	[4.6; 5.5]	[-0.36; -0.29]	(Lemaire & Salette, 1984)	(Lemaire *et al.*, 1985)
Zea mays L. (Maize)	3.49 [3.25; 3.78]*	-0.38 [-0.33; -0.43]*	(Makowski *et al.*, 2020)	(Ciampitti *et al.*, 2021a)
Miscantus giganteus & *Miscanthus sinensis*	2.70	-0.48	(Greenwood *et al.*, 1990)	(Zapater *et al.*, 2017)
Vitis vinifera L. (Grapevine)	[2.38 ; -3.20]	[-0.17; -0.44]	(Lemaire & Salette, 1984)	(Celette *et al.*, 2013)

Note. "*" indicating a credibility interval set at 95%.

2. Materials and methods

2.1 Experimental sites

To determine the response to N of *Th. intermedium*, a field experiment (C1) was conducted on the experimental farm of ULiège – Gembloux Agro-Bio Tech, Belgium, using a complete randomized split-plot design (2*8m microplots) with four replicates. The first level of randomization is used to assign experimental units to a mowing factor comparing two treatments (not presented in this study). Within these experimental units, different N fertilization treatments (ammonium nitrate granular) were applied on subplots. These treatments differed according to total amount (0, 50, 100 or 150kg N/ha) and timing of application (early-spring (BBCH29), mid-spring (BBCH39), and fall (vegetative stage)) (Table 2). Fertilization levels were chosen according to previous studies on N application (Jungers *et al.*, 2017).

Five French additional field experiments (V1-5) were used to provide validation data and assess the reliability of the CNDC established from the main Belgian experimental site (C1). All detailed information about crop management and experimental designs of the sites used is summarized in Table 3.

Table 2 : Timings and amounts (kg N/ha) of N fertilization treatments of the Belgian experimental C1 site from 2019 to 2021.

Code	**Treatment**	**Total N dose (kg N/ha)**	**Splitting (kg N/ha)**				
			2019		2020 and 2021		
			April BBCH29	**September Vegetative stage**	**April BBCH29**	**May BBCH39**	**September Vegetative stage**
0+0+0N	1	0	0	0	0	0	0
50+0+0N	2	50	50	0	50	0	0
50+0+50N	3	100	50	50	50	0	50
100+0+0N	4	100	100	0	100	0	0
100+0+50N	5	150	100	50	Not applied		
100+50+0N	6	150	Not applied		100	50	0
0+100+0N	7	100	Not applied		0	100	0
50+50+50N	8	150	Not applied		50	50	50

Table 3 : Detailed information about experimental sites, their design and their management.

		Experimental sites					
Site code		***C1***	***V1***	***V2***	***V3***	***V4***	***V5***
Location							
	Country	Belgium	France				
	GPS Long. (DD)	4.7063	5.1251	5.0920	5.143	5.0419	3.5130
	GPS Lat. (DD)	50.5664	45.4250	45.2746	45.3323	45.4350	45.4638
Soil type		Clay loam	Loam	Sandy-loam (stony)	Sandy-loam	Sandy-clay-loam (stony)	Clay-loam
Climate							
	Average annual rainfall (mm)	852	881	984	983	927	628

Average annual min temperature (°c)	7	7.8	6.3	6.3	7.8	6.5
Average annual max temperature (°c)	14.2	16.5	16.1	16.1	16.5	16.7
Type of experiment	Research station (microplots). Randomized split-plot design (4 replicates)		On-farm experiment Strips design (3 replicates)		Research station (microplots). Randomized split-block design (3 replicates)	(4 replicates)
Implementation						
Sowing date	22-09-2017	20-09-2017	15-09-2018	05-09-2017	18-09-2018	19-10-2017
Seed population	Third selection cycle of The Land Institute (TLI-C3)					
Seeding rate (kg/ha)	20		18		25	18
Interrow spacing (cm)	25	25	12	20	15	
Field management						
N fertilization (kg N/ha) BBCH30	See (Table 2)	50	50	50	80	80
BBCH39		0	0	0	40	0
Weeding	Chemical + mechanical	/	/	/	Chemical + hand	
Crop protection	/	/	/	/	/	/
Post-harvest residue management	Chipping or mowing at 5cm from the ground					
Growing season for data collection	2019,2020,2021	2018	2019	2018	2018	2018,2019

2.2 Data collection

The data from the analytical site (C1) used in this study were collected from the second to the fourth growing season after crop implantation. Concerning the validation sites (V1-5), data were collected during the first, the second or both growing season, depending on sites and data availability (Table 3). Aboveground biomasses were sampled through a 50x50cm quadrat, cut at 5 cm above soil surface, oven-dried (72h at 60°C) and weighted to obtain dry matter (DM). Samples were collected at four different main phenological stages, rated with the BBCH scale (Meier, 1997), namely the stem elongation (BBCH30), the flag leaf (BBCH39), the flowering (BBCH65) and the grain maturity (BBCH89) stage. For site C1 only, ears were always separated from straw biomass. Additionally, leaves were separated from stems in 2020 and 2021. During these two years, LAI was also measured at three phenological stages (BBCH30, BBCH39 and BBCH65) by collecting leaves on 50 cm of a row in one replicate of each N treatments. They were then laminated with transparent adhesive cover on paper sheets and scanned. These leaves were beforehand weighted to estimate the specific leaf area (i.e., ratio of leaf area to leaf dry mass) to estimate LAI over the three other replicates. Finally, the leaf area ratio (LAR) was calculated by dividing the LAI by the total aboveground biomass.

For all sites, nitrogen concentrations of samples were measured through the Dumas method (Dumas, 1831); N contents were quantified individually for each replicate (across all sites, cropping seasons and phenological stages). An exception must be notified for the cropping season 2019, where the sole average samples over the four replicates were available to determine N content at the grain maturity stage for C1 site.

When needed, the four phenological stages were translated into development units (sum of degree-days corrected by photoperiodic and cold requirement effects) as proposed in the STICS soil-crop model and described in the study of Duchene *et al.* (2021). The corresponding sum of UPVT (ΣUPVT) is of 191 at BBCH30, 413 at BBCH39, 878 at BBCH65 and 1622 at BBCH89, respectively.

2.3 Analysis of the aboveground biomass, N content and N uptake of Th. intermedium (C1 site)

Analyses of variances (ANOVA) were conducted with the R studio software (R Core Team, 2021). A three-way ANOVA was used, where factors were constituted of i) the growing seasons (year), ii) the N fertilization treatments common to each growing season and iii) the four – or three – phenological stages of the crop at which samples were collected. The total aboveground dry matter, N uptake, N content, leaf/stem ratio as well as LAR were the analyzed variables. Two-way ANOVA's were also performed, within each year and for each plant organ, where factors were constituted of i) the N fertilization treatments and ii) the four phenological stages at which samples were collected. The dry matter, N content and N uptake within plant tissues were the analyzed variables.

Within the different analyses conducted, mixed models were used. The nitrogen fertilization, phenological stage and growing season were considered as fixed effect, while replicates as a random effect. Regarding N fertilization effect, N treatments were considered globally, without dissociating timing or amount effect.

When interactions were observed between the fixed effect (fertilization, phenological stage or year), data were separated by the treatments of one factor to analyze the effects of the other factors. Bartlett's test was used to confirm the homogeneity of variance and Shapiro-Wilk's test was used to confirm that residuals were normally distributed. Following ANOVA analysis, the post-hoc Student–Newman–Keuls test (SNK test) was used to compare treatment means with a significance level set at 0,05.

2.4 Critical nitrogen dilution curve establishment and validation

2.4.1 The Bayesian hierarchical model to estimate CNDC

To estimate the CNDC, a Bayesian hierarchical model (Makowski *et al.*, 2020) was applied on our consolidated C1 dataset. In this model the response of W to N content is considered to follow a linear-plus-plateau function. The variability of this function's parameters across sampling dates is described by a *posteriori* probability distribution function, estimated using Bayesian method, from which the most probable parameter values of CNDC and their credibility intervals are derived (Makowski *et al.*, 2020).

The statistical model was assessed using a Markov chain Monte Carlo algorithm (MCMC) implemented using R (R Core Team, 2021) and its *brms* package (Bürkner, 2017, 2018). As proposed in the study of Bohman *et al.* (2021), the following non-linear *brms* model formula was applied:

$$W \sim min(W_{Max,i} + S_i\left(\%N_{Plant} - \left(a\, {W_{Max,i}}^{-b}\right)\right), W_{Max,i}) \qquad \text{(Equation 2)}$$

where S_i and $W_{Max,\,i}$ are respectively the slope of the linear plateau curve and the maximum value of biomass (i.e., plateau) for a given date [i]. *min* represents the minima function (i.e., the plateau component) and *a*- and *b*-coefficient have the same meaning as previously defined in Eq.1. The parameters *S* and W_{Max} included group-level (i.e., random) effects to fit a linear-plateau curve to each sampling date:

$$W_{Max} + S \sim 1 + (1 \mid index) \qquad \text{(Equation 3)}$$

where *index* represents the unique level of each experimental sampling date [i].

2.4.2 Practical considerations and priors setting

Only data from stem elongation (BBCH30) to flowering stage (BBCH65) and with W above 1t DM/ha were used. Indeed, as explained in the study of Justes *et al.* (1994), N dilution would not be significant for low biomass values (less than 1t DM/ha) as plant canopy is not closed yet. In addition, the theory explaining decline in N percentage with increasing biomass is mostly restricted to the vegetative period, excluding samplings after the flowering stage (BBCH65) (Greenwood *et al.*, 1990; Justes *et al.*, 1994).

Priors were chosen based on expertise and empirical observations (e.g., summary values from our data set, previously reported values for other species) combined with prior distribution boundaries (e.g., if the range of a prior led to biologically or physically impossible predictions, it was narrowed). Values of priors are reported in Table 4.

The MCMC algorithm was run with 4 chains of 10 000 iterations each. A warmup period of 3000 runs was used.

Table 4 : Priors used to fit the critical nitrogen dilution curve (CNDC) with the hierarchical Bayesian model.

Parameter of the CNDC	**Distribution**	**Boundaries**	
		Lower	**Upper**
a	Normal (3; 1)	1	7
b	Normal (0.5; 0.15)	0	1
W_{Max}	Normal (10; 10)	1	30
S	Normal (4; 3)	0	"∞"
σ_{BMax}	Normal (7; 1)	"-∞"	"∞"
σ_S	Normal (2; 1)	"-∞"	"∞"
σ	Student's t (3; 1; 0.1)	"-∞"	"∞"

2.4.3 Evaluating uncertainty on parameters and critical N concentration

The *a*- and *b*-coefficients of the CNDC curves were derived from their respective a *posteriori* distribution. The most probable parameter value was estimated through the median value (centile 0.5) and the 0.025 and 0.975 quantiles were used to determine the 95% credibility interval (CI).

The uncertainty around the CNDC curve was estimated using the following procedure. The *a*- and *b*-coefficients of the 1000 final runs of each of the 4 chains were used to generated CNDC curves. Curves were calculated for a set of discrete values of W ranging from 1t DM/ha to the maximum observed value in the experimental data set. From the population of CNCD curves, quantiles 0.025, 0.25, 0.75 and 0.975 were calculated to determine the 50% CI and 95% CI. As the estimation of *a*- and *b*-coefficients is performed concomitantly by the Bayesian model, this approach allows to account for their correlation and its impact on the generated CNDC curves (Dumont *et al.*, 2014).

2.4.4 Validation of the critical nitrogen dilution curve (V1-5 sites)

The dataset from validation sites (V1-5) was used to assess the validity of the curve and confirm that it allows to properly distinguishing “limiting” and “non-limiting” N situations according to their biomass and N content. To discriminate situations within the validation sites, the following procedure was applied.

At each phenological stage, a one-way ANOVA was performed to determine if statistical differences existed in W and N percentage between sites. When statistical differences were reported, a *post-hoc* test was performed to group results. The least significant difference (LSD) at the 0,05-significance level (Chakwizira *et al.*, 2016) was calculated to compare and rank means of W and N percentage samples.

Discrimination of the datasets into two groups was made as follows. Samples that were not significantly different from the lowest biomass, were classified as “limiting” N situations, while samples that did not significantly differ from the highest biomass sample, were classified as “non-limiting” N situations. As many points were not categorized, additional information provided by field experts was required for the validation sites: sites with high N fertilization (80 and 120kg N/ha in the spring) and known has being non-water limited were considered as “non-limiting” N situations (V4, V5); sites with relatively low N fertilization (50kg N/ha in the spring), with shallow and stony soils or with a high weed competition were considered as “limiting” N situations (V1, V2 at BBCH30 and V3 at BBCH39).

3. Results

3.1 Impact of N fertilization on crop growth and nutrient uptake (C1 site)

3.1.1 Evolution and partitioning of DM in the aboveground biomass

Significant interactions were found between the fixed factors, namely the growing season (year), the fertilization treatment, and the phenological stage (Table 5). Therefore Table 6 presents detailed results of aboveground biomass within each year and each plant organ (when available). Leaf/stem ratio and LAR are presented in supplementary material (Table S2, Table S3).

As expected, the aboveground dry matter production generally increased along the crop cycle. A sharp increase is observed between the stem elongation (BBCH30) and the flag leave stage (BBCH39), followed by a lower increase of the total aboveground dry matter until the grain maturity stage (BBCH89). The cumulated aboveground DM of the different plant organs in relation with the development stages is illustrated in Figure 1 for the 50+0+50N fertilization level (treatment 3), which was found to best match the plant requirements (see section 3.2 and Figure S3 in supplementary material). The total aboveground biomass was found to be highly variable between growing seasons. It reached, at BBCH89 (ΣUPVT of 1622), 16.4t DM/ha in 2019, only 7.0t DM/ha in 2020 and 10.3t DM/ha in 2021 (Figure 1), indicating the highest final production level in 2019 and the lowest in 2020 (*p-value*<0.001).

Focusing on biomass production of the aboveground organs, the analysis indicated a significant effect of phenological stage. The weight of leaves is generally the highest at BBCH39, before gradually decreasing until BBCH89. Concerning stems, the biomass peak is observed at the flowering stage (BBCH65), except in 2021 where the increase was reported until BBCH89 for some N treatments (Table 6). The biomass of ears was systematically found to statistically increase between BBCH65 and BBCH89 (Table 6).

The comparison between biomass production levels of the different aerial organs indicates a higher amount of leaves than stems at BBCH30. At BBCH39 and after, stems are the most represented organ of the plant. At BBCH30 (ΣUPVT of 191), leaves represented 83.8 ± 2.5% of the total aboveground biomass. Reversely, they accounted for 12.9 ± 0.7% at BBCH89, while stems and ears represented respectively 71.4 ± 1.4% and 15.7 ± 1% of the total aboveground biomass (Figure 1). The leaf/stem ratio seemed to be only influenced by phenological stages. The ratio was found to decrease during the growing season (supplementary material Table S2). The same trend was observed for the leaf area ratio, as it was significantly influenced by phenological stages ($p\text{-}value<0.001$), with a sharp decrease from BBCH30 to BBCH65 (supplementary material Table S3).

N fertilization had generally a positive impact on the aboveground DM production, especially on vegetative organs. Indeed, in 2020, the lowest biomass of leaves and stems were obtained with the reference treatment (0+0+0N) and the high mid-spring fertilization (0+100+0N), regardless of the stage of development. In 2021, the biomass from both stems and leaves was also the lowest with the reference treatment, the high mid-spring fertilization and the low early-spring fertilization (50+0+0N) at early stages of the crop cycle (BBCH30 and BBCH39). Later in the growing season, the biomass of vegetative organs remained broadly equivalent for all N treatments, except in the reference treatment which has always the lowest level of biomass production. Focusing on the biomass of ears, the 100+50+0N treatment showed the highest level in 2020. But apart from this situation, the biomass of ears was not significantly influenced by the different N treatments, with no difference compared to the reference treatment in 2019, and only lower levels of biomass found for the reference treatment in 2021 (Table 6).

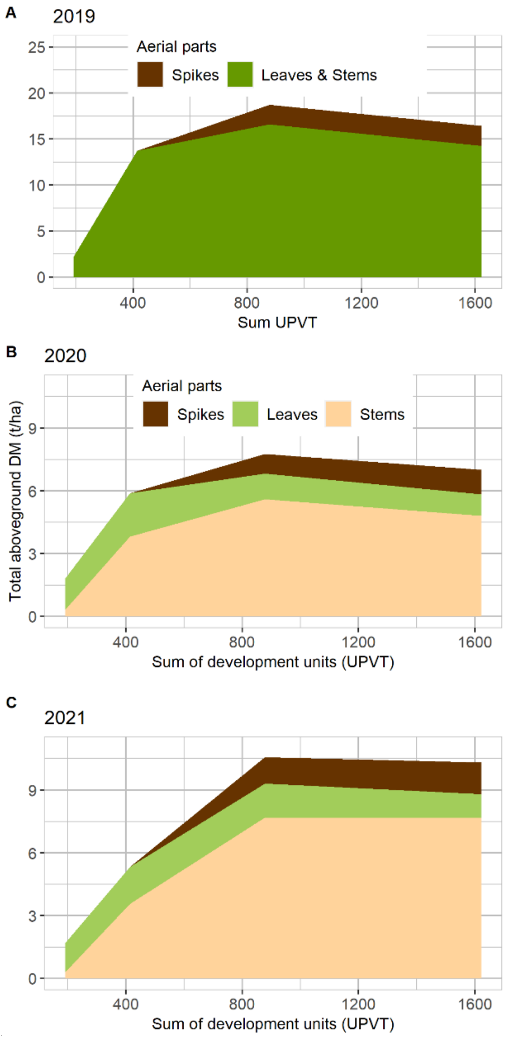

Figure 1 : Aboveground DM (t/ha) partitioning in plant organs according to the accumulation of crop development units (UPVT) in 2019(A), 2020(B) and 2021(C) for the N treatment 3 (50+0+50N).

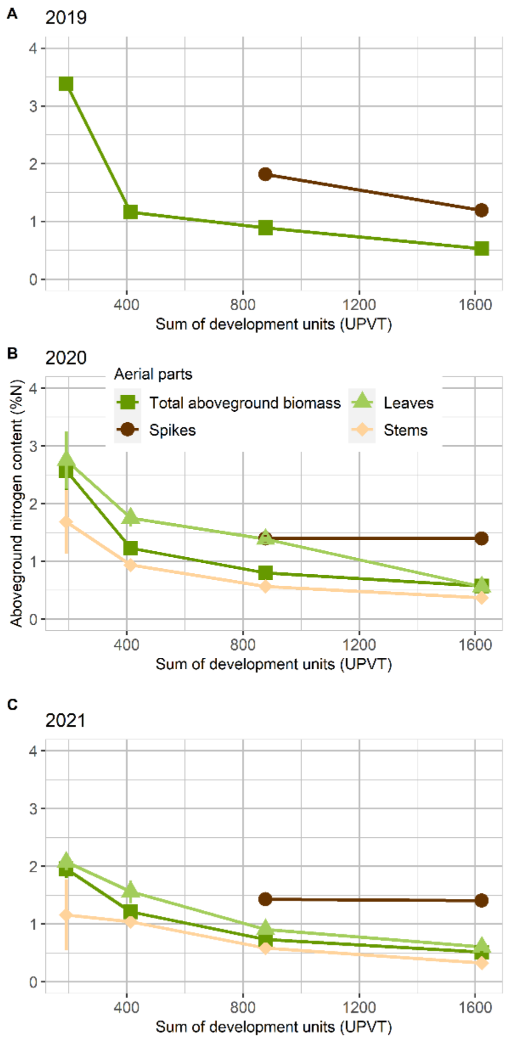

Figure 2 : Aboveground N content (%N) of plant organs according to the accumulation of crop development units (UPVT) in 2019(A), 2020(B) and 2021(C) for the N treatment 3 (50+0+50N).

Table 5 : F-statistics and significance levels from the performed three-way ANOVA.

Source of variation	**Dry matter of the total aboveground biomass**		**N uptake of the total aboveground biomass**		**N content of the total aboveground biomass**		**Leaf/Stem ratio**		**LAR**	
	Df	*F*-value	*Df*	*F*-value	*Df*	*F*-value	*Df*	*F*-value	*Df*	*F*-value
Year (Y)	2	173***	2	122***	1	17***	1	1	1	3
Stage (S)	3	243***	3	51***	3	827***	3	88***	2	3612***
N fertilization (N)	3	17***	3	43***	6	26***	6	2	6	4**
Replicate (R)	3	1	3	1	3	4**	3	2	3	5*
Y*S	6	18***	6	6***	3	22***	3	3	2	27***
Y*N	6	1	6	1	6	3**	6	1	6	4**
S*N	9	2*	9	3***	18	5***	18	2*	12	13***
Y*S*N	18	1	18	1	18	2	18	2	12	5***

Note. “*” indicating statistical significance at *p-value*≤0.05; “**” indicating statistical significance at *p-value*≤0.01; “***” indicating statistical significance at *p-value*≤0.001.

3.1.2 Evolution and partitioning of plant tissues N content

Significant interactions were found between the fixed factors (Table 5). Detailed results within each year and each plant organ (when available) are illustrated in Figure 2, for the treatment 3, and presented in supplementary materials (Table S1).

Overall, the N content of vegetative organs (leaves and stems) decreased along the crop cycle. As illustrated in Figure 2, the highest N content of leaves and stems was obtained at BBCH30 (ΣUPVT of 191). Reversely, the phenological stage of the crop had no significant influence on the N content of ears which was similar between BBCH65 (ΣUPVT of 878) and BBCH89 (ΣUPVT of 1622) stage (Figure 2).

As expected, at each stage of crop development, the N content was higher in leaves than in stems. At BBCH89, the N content was the lowest in stems and the highest in ears (Figure 2).

The N content in aboveground organs increased with the N fertilization. Globally the absence of fall or early-spring fertilization lowered N content in vegetative organs at BBCH30 while the absence of mid-spring fertilization lowered N content of leaves and stems at BBCH65 and BBCH89. Concerning the N content of ears, the SNK’s results showed a globally higher N content with the mid-spring fertilization by increasing it by 0.3% compared to the reference treatment (unshown results – supplementary material Table S1).

3.1.3 Evolution and partitioning of N uptake in the aboveground biomass

Significant interactions were reported between the fixed factors (Table 5). Therefore, Table 7 presents detailed results within each year and each plant organ, when available.

Overall, the N uptake of the total aboveground biomass increased from the BBCH30 to BBCH39 (ΣUPVT of 413) or BBCH65 (ΣUPVT of 878) stages before decreasing until BBCH89 as shown in Figure 3 for treatment 3. The N uptake decrease from leaves is generally more pronounced than in stems, which tend to accumulate N later and conserve it longer (Table 7, Figure 3). Looking at ears, the N uptake generally increased between BBCH65 and BBCH89. However, this increase in ears does not compensate the N uptake decrease in vegetative organs in late growing season, resulting in total N uptake diminution in the aboveground biomass (Table 7).

The N uptake of the aboveground biomass tended to increase with the N fertilization. The lowest N uptake of leaves and stems was always obtained with the reference treatment. At the beginning of the growing season (BBCH30), the N uptake of vegetative organs is increased by high early spring fertilization (100+0+0N and 100+50+0N treatments) and by early spring fertilization coupled with fall fertilization (50+0+50N and 50+50+50N treatments). At BBCH89, the influence of fertilization seemed more limited although the lowest N uptake of leaves and stems is obtained with the reference treatment (Table 7). The N fertilization had no influence on the N uptake by ears in 2019. The reference treatment and the 50+0+0N fertilization seemed to limit N uptake by ears in 2020 and 2021 while the highest N uptake of ears was obtained with the 100+50+0N fertilization in 2020 and with the 50+50+50N fertilization in 2021 (Table 7).

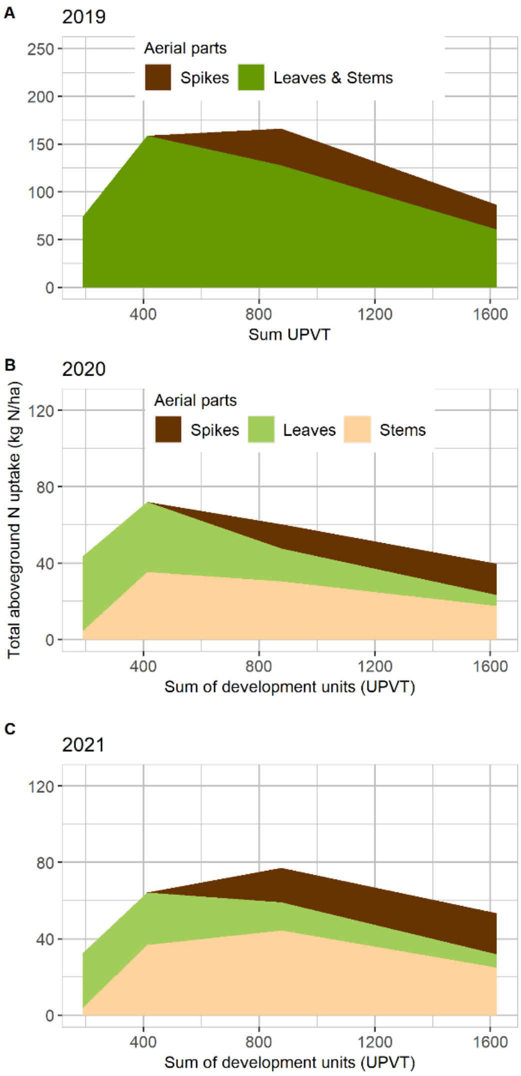

Figure 3: Aboveground N uptake (kg N/ha) partitioning in plant organs according to the accumulation of crop development units (UPVT) in 2019(A), 2020(B) and 2021(C) for the N treatment 3 (50+0+50N).

Table 6 : Aboveground biomass production (t DM/ha) for the different N fertilizations and phenological stages for 2019(A), 2020(B) and 2021(C). Data are presented as average ± standard error.

(A) 2019

Fertilization	Dry matter of leaves and stems ± S.E. (t/ha)					Dry matter of ears ± S.E. (t/ha)		
	BBCH30	**BBCH39**	**BBCH65**	**BBCH89**	**Mean of stages**	**BBCH65**	**BBCH89**	**Mean of stages**
0+0+0N	1.8±0.1	7.8±1.5	12.2±1.2	13.8±2.2	8.9±1.4	1.6±0.2	2.6±0.4	2.1±0.3
50+0+0N	2.1±0.2	11.3±1.5	11.7±0.5	13.4±1.4	9.5±1.3	1.5±0.1	2.3±0.2	1.9±0.2
50+0+50N	2.2±0.2	13.7±3.6	16.6±1.5	14.3±0.5	11.6±1.7	2.1±0.3	2.2±0.5	2.2±0.3
100+0+0N	2.2±0.2	15.6±1.9	13.5±0.7	13.5±0.7	10.6±1.5	1.6±0.2	2.0±0.4	1.8±0.2
100+0+50N	2.7±0.5	12.6±3.4	14.0±1.0	14.2±0.7	10.7±1.5	1.6±0.4	1.9±0.6	1.7±0.4
Mean of fertilizations	2.2±0.1 A	11.7±1.2 B	13.6±0.6 C	13.8±0.5 C		1.7±0.1 A	2.2±0.2 B	

(B) 2020

Fertilization	Dry matter of leaves ± S.E. (t/ha)					Dry matter of stems ± S.E. (t/ha)					Dry matter of ears ± S.E. (t/ha)		
	BBCH30	**BBCH39**	**BBCH65**	**BBCH89**	**Mean of stages**	**BBCH30**	**BBCH39**	**BBCH65**	**BBCH89**	**Mean of stages**	**BBCH65**	**BBCH89**	**Mean of stages**
0+0+0N	0.9±0.1	1.5±0.3	1.2±0.1	0.9±0.2	1.1±0.1c	0.2±0.0	1.9±0.3	3.9±0.1	3.5±0.7	2.4±0.4c	0.5±0.1	0.6±0.1	0.5±0.1c
50+0+0N	1.2±0.1	2.0±0.1	1.3±0.1	1.2±0.1	1.4±0.1ab	0.3±0.1	2.7±0.2	4.8±0.4	5.6±1.0	3.3±0.6abc	0.6±0.1	1.2±0.4	0.9±0.2bc
50+0+50N	1.5±0.2	2.1±0.3	1.2±0.1	1.0±0.1	1.5±0.1ab	0.3±0.1	3.8±0.7	5.6±1.1	4.8±0.5	3.6±0.6ab	0.9±0.1	1.2±0.1	1.1±0.1b
100+0+0N	1.4±0.2	2.3±0.2	1.4±0.1	1.2±0.1	1.6±0.1a	0.4±0.1	3.5±0.2	5.8±0.9	5.7±0.6	3.8±0.6ab	1.0±0.2	1.5±0.2	1.2±0.2b
100+50+0N	1.5±0.2	1.9±0.3	1.5±0.1	1.2±0.2	1.5±0.1a	0.4±0.2	3.5±0.9	6.5±0.9	6.8±0.7	4.3±0.7a	1.2±0.2	2.1±0.3	1.6±0.2a
0+100+0N	1.2±0.1	1.5±0.1	1.3±0.1	0.9±0.1	1.2±0.1bc	0.3±0.1	2.0±0.1	3.6±0.6	4.4±0.4	2.5±0.4c	0.5±0.1	1.1±0.1	0.8±0.1bc
50+50+50N	1.1±0.1	1.9±0.1	1.4±0.1	0.9±0.1	1.3±0.1abc	0.3±0.1	3.1±0.4	4.5±0.7	3.9± 0.2	3.0±0.5bc	0.8±0.2	1.0±0.1	0.9±0.1bc
Mean of fertilizations	1.3±0.1 B	1.9±0.1 A	1.3±0.1 B	1.1±0.1 C		0.3±0.0 A	2.9±0.2 B	4.9±0.3 C	5.0±0.3 C		0.8±0.1 A	1.2±0.1 B	

(C) 2021

Fertilization	Leaves BBCH30	Leaves BBCH39	Leaves BBCH65	Leaves BBCH89	Leaves Mean of stages	Stems BBCH30	Stems BBCH39	Stems BBCH65	Stems BBCH89	Stems Mean of stages	Ears BBCH65	Ears BBCH89	Ears Mean of stages
0+0+0N	0.7±0.1d A	1.2±0.1c B	1.2±0.1b B	1.0±0.1 AB	1.0±0.1	0.1±0.0d A	1.8±0.2d B	4.5±0.4b C	5.80±0.3b D	3.1±0.6	0.7±0.0	1.1±0.1	0.9±0.1b
50+0+0N	0.9±0.1cd A	1.6±0.1bc B	1.5±0.2ab B	1.1±0.1 A	1.3±0.1	0.1±0.0d A	2.7±0.2cd B	6.2±0.8ab C	6.9±0.7ab C	4.0±0.8	1.0±0.1	1.4±0.2	1.2±0.1ab
50+0+50N	1.4±0.3abc	1.8±0.1bc	1.6±0.1ab	1.2±0.1	1.5±0.1	0.3±0.1bc A	3.6±0.2bc B	7.7±0.5a C	7.7±0.5ab C	4.8±0.8	1.3±0.1	1.5±0.2	1.4±0.1a
100+0+0N	1.5±0.1abc AB	1.9±0.1b B	2.0±0.2a B	1.2±0.1 A	1.7±0.1	0.3±0.0bc A	4.0±0.4bc B	8.7±1.0a C	8.1±0.8ab C	5.3±0.9	1.4±0.2	1.6±0.3	1.5±0.2a
100+50+0N	1.7±0.1ab A	2.7±0.3a B	1.6±0.2ab A	1.1±0.1 A	1.8±0.2	0.5±0.1ab A	5.9±0.7a B	7.8±1.2a B	7.9±0.6ab B	5.5±0.9	1.3±0.2	1.7±0.2	1.5±0.2a
0+100+0N	1.1±0.2bcd A	1.7±0.2bc B	1.4±0.2ab AB	1.2±0.0 A	1.4±0.1	0.2±0.1cd A	3.1±0.3cd B	6.4±0.5ab C	8.4±0.4ab D	4.5±0.8	1.2±0.2	1.8±0.2	1.5±0.2a
50+50+50N	2.0±0.1a A	2.2±0.2b A	1.6±0.1ab B	1.3±0.1 B	1.8±0.1	0.6±0.1a A	4.7±0.6ab B	7.1±0.2ab C	9.0±0.9a D	5.4±0.8	1.4±0.1	1.9±0.3	1.6±0.2a
Mean of fertilizations	1.3±0.1	1.9±0.1	1.6±0.1	1.2±0.1		0.3±0.1	3.7±0.3	6.9±0.4	7.7±0.3		1.2±0.1 A	1.6±0.1 B	

Note. Means with a letter differ significantly (*p-value*<0.05). Letters in minuscule represent the result of SNK test of the effect of fertilization and letters in majuscule represent the result of SNK test of the effect of phenological stage. When letters are reported in the 'mean of fertilizations' row or in the 'mean of stages' column, it means no interactions between those factors were reported for that year and plant organ.

Table 7 : Aboveground N uptake (kg N/ha) for the different N fertilizations and phenological stages for 2019(A), 2020(B) and 2021(C). Data are presented as average ± standard error.

Fertilization	N uptake of leaves and stems ± S.E. (kg N/ha)										N uptake of ears ± S.E. (kg N/ha)		
(A) 2019	BBCH30	BBCH39	BBCH65	BBCH89	Mean of stages						BBCH65	BBCH89	Mean of stages
0+0+0N	46.0±1.7b	73.0±13.6	67.5±6.6b	46.9±7.4	58.4±4.9						27.6±3.0	29.1±4.6	28.4±2.5
50+0+0N	61.6±6.5ab A	148.0±19.4 B	73.7±2.9b A	43.5±4.4 A	77.3±10.6						26.5±1.0	25.9±1.7	26.2±0.9
50+0+50N	74.4±6.5ab A	159.0±41.5 B	127.9±11.4a B	60.6±2.2 A	101.9±12.9						38.6±4.6	25.9±5.5	32.2±4.1
100+0+0N	78.9±7.2ab A	218.2±25.9 C	127.6±7.0a B	49.5±2.7 A	104.3±15.8						31.2±4.5	21.9±4.8	26.5±3.5
100+0+50N	91.7±16.1a AB	149.6±39.9 B	98.5±7.0a AB	49.4±2.4 B	93.8±12.1						27.9±7.8	22.5±7.5	25.2±5.1
Mean of fertilizations	70.5±5.0	139.9±16.5	99.0±6.6	50.0±2.2							30.3±2.1	25.1±2.1	
(B) 2020 Fertilization	N uptake of leaves ± S.E. (kg N/ha)					N uptake of stems ± S.E. (kg N/ha)					N uptake of ears ± S.E. (kg N/ha)		
	BBCH30	BBCH39	BBCH65	BBCH89	Mean of stages	BBCH30	BBCH39	BBCH65	BBCH89	Mean of stages	BBCH65	BBCH89	Mean of stages
0+0+0N	19.7±3.0c A	16.5±4.1c AB	10.6±1.1d BC	4.0±0.8c C	12.7±1.9	2.0±0.2 A	14.2±1.0c B	14.5±1.8c B	11.0±1.9b B	10.4±1.5	6.7±1.0	7.4±0.6	7.0±0.6c
50+0+0N	29.2±3.6bc A	26.2±2.0bc A	14.9±1.0cd B	5.9±0.6bc C	19.0±2.6	4.0±0.7 A	21.5±0.2bc B	22. 5±1.3bc B	17.5±3.5b B	16.4±2.1	8.4±1.2	15.9±4.7	12.2±2.7bc
50+0+50N	39.3±3.1ab A	36.6±5.5b A	17.1±1.3bc B	5.7±0.5bc C	24.7±3.9	4.6±1.2 A	35.4±5.6a C	30.5±4.2bc C	17.6±1.2b B	22.0±3.5	12.9±1.5	16.3±1.4	14.6±1.1b
100+0+0N	41.6±5.8ab A	51.2±7.1a A	21.8±1.5ab B	7.6±0.7b B	30.6±4.9	5.9±1.5 A	39.1±4.3a C	39.4±5.4ab C	20.9±1.4b B	26.3±3.9	14.7±3.2	21.0±3.1	17.9± 2.4b
100+50+0N	47.9±7.0a A	41.4±6.8ab AB	28.2±2.5a B	9.9±1.2a C	31.8±4.4	8.1±3.0 A	36.6±6.5a B	51.9±7.2a C	33.3±3.9a B	32.5±4.7	18.7±2.9	35.4±5.3	27.1±4.2a
0+100+0N	23.8±1.8bc A	18.4±0.9c B	24.4±1.6a A	6.7±0.5bc C	18.3±1.9	2.9±0.6 A	14.9±1.5c B	28.1±5.8bc C	20.5±0.6b BC	16.6±2.7	8.8±2.2	18.9±1.5	13.8±2.3b
50+50+50N	30.9±1.2abc A	35.5±1.9b A	24.2±2.4a B	6.2±0.5bc C	24.2±3.0	4.0±0.8 A	30.0±3.3ab C	34.8±6.3b C	18.2±1.5b B	21.7±3.5	12.6±3.0	15.7±1.2	14.1±1.1b
Mean of fertilizations	33.2±2.3	32.3±2.7	20.2±1.3	6.6±0.4		4.5±0.6	27.4±2.3	31.7±2.7	19.9±1.4		11.8±1.1 A	18.7±1.8 B	
(C) 2021													
0+0+0N	12.8±1.1b A	15.5±1.9d A	12.1±2.6b A	5.3±0.7b B	11.4±1.2	0.9±0.5c B	20.3±2.5b A	22.7±1.6c A	19.7±0.9b A	15.9±2.4	9.4±0.8	15.3±1.9	12.4±1.5c
50+0+0N	19.3±2.5b A	23.3±0.9cd A	14.3±2.0b B	5.9±0.5b C	15.7±1.8	1.1±0.4c C	29.0±2.0b AB	35.4±4.3bc A	22.2±1.5ab B	21.9±3.5	13.5±1.7	19.1±3.5	16.3±2.1bc
50+0+50N	28.6±5.4ab A	27.5±0.8bc A	14.7±0.7b B	7.0±0.8ab B	19.4±2.6	4.2±1.8bc C	36.9±1.4b A	44.5±1.4abc A	25.0±3.9ab B	27.6±4.1	18.0±1.0	21.5±3.5	19.7±1.8abc
100+0+0N	37.9±6.9a A	33.0±0.4bc AB	20.2±2.7ab BC	7.7±1.0ab C	24.7±3.5	5.7±0.5ab D	41.5±3.1b B	53.2±5.1abc A	26.7±3.3ab C	31.8±4.8	21.9±3.1	23.7±4.3	22.8±2.5ab
100+50+0N	42.3±3.2a A	52.9±5.2a A	20.4±1.6ab B	10.4±1.5a B	31.0±4.0	7.4±1.4ab B	72.8±9.6a A	72.9±11.5a A	35.5±4.6a B	47.2±7.9	21.8±2.9	26.8±4.3	24.3±2.6ab
0+100+0N	18.3±2.3b B	32.4±4.6bc A	17.7±3.8ab B	8.1±0.7ab C	19.1±2.7	2.3±0.7c C	35.6±3.2b AB	51.1±10.1abc A	29.5±2.6ab B	29.6±5.2	20.3±4.8	26.5±2.8	23.4±2.8ab
50+50+50N	43.3±3.5a	36.6±2.5b	27.0±3.0a	10.4±0.9a	29.3±3.4	8.9±1.0a	61.4±9.5a	64.7±7.2ab	35.7±1.9a	42.7±6.4	25.9±3.1	28.7±4.2	27.3±2.5a

	A	A	B	C	C	A	A	B		
Mean of fertilizations	28.9±2.6	31.6±2.3	18.1±1.2	7.8±0.5	4.4±0.7	42.5±3.8	49.2±3.8	27.8±1.5	18.7±1.4	23.1±1.5
									A	B

Note. Means with a letter differ significantly (*p-value*<0.05). Letters in minuscule represent the result of SNK test of the effect of fertilization and letters in majuscule represent the result of SNK test of the effect of phenological stage. When letters are reported in the 'mean of fertilizations' row or in the 'mean of stages' column, it means no interactions between those factors were reported for that year and plant organ.

3.2 Establishment of the critical nitrogen dilution curve (C1 and V1-5 sites)

Coefficients of the CNDC of *Th. intermedium* with their 95% credibility interval are presented in Table 8 and their posterior densities are graphically represented in Figure 4.

The newly established CNDC is presented in Figure 5-A. It highlights that the N content decreases with the aerial biomass production of *Th. intermedium*. Regarding the dataset used to validate the results (V1-5 sites), the newly developed CNDC seemed to properly separate the "limiting" and "non-limiting" N situations, while some minor errors remained. The expert-based information surimposed to some data might have led to situations where points are not fully N stressed, especially during the early crop growth where they can benefit from residual soil N and early mineralization. Yet, the discrimination of "limiting" and "non-limiting" N situations remains globally very efficient.

Figure 5-B shows the newly developed CNDC of *Th. intermedium* compared with other crops presented in Table 1. With a much lower *a*-coefficient, the CNDC of *Th. intermedium* appeared to be positioned under the curves of *Triticum aestivum* (a C3 annual species) and *Zea mays* (a C4 annual species) obtained by the Bayesian method (Table 1 and Table 8). The closest crops in terms of behavior appeared to be *Miscanthus giganteus* & *sinensis* (Figure 5-B) or Grapevine (Table 1), both perennial plants.

Table 8 : Coefficients of the proposed critical nitrogen dilution curve (CNDC) of Th. intermedium and their 95% credibility interval.

	***a*-coefficient**	***b*-coefficient**	**%N = aMS^{-b}**
CNDC	2.35	0.46	%N = 2.35M$^{-0.46}$
95% credibility interval	[1.25; 4.10]	[0.19; 0.76]	

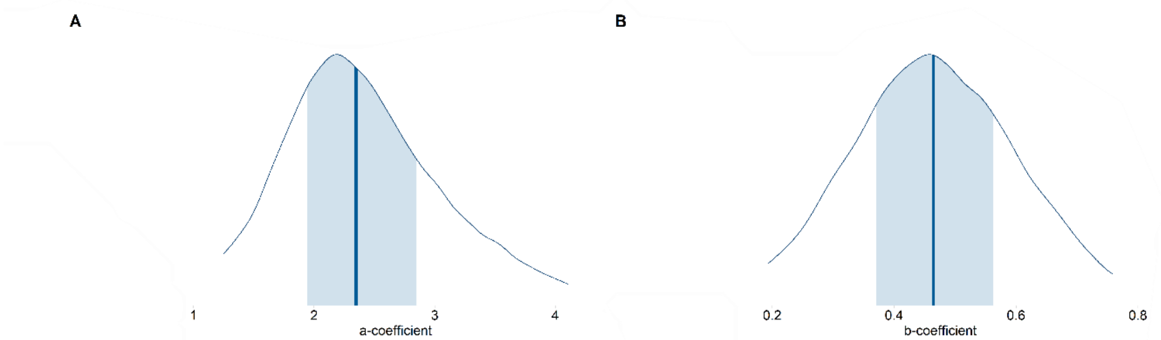

Figure 4 : Posterior densities of a-(A) and b-coefficient(B) of the critical nitrogen dilution curve (CNDC). Distributions are presented over the range of their 95% credibility interval. Dark blue lines represent the median value and light blue zones represent the 50% credibility interval.

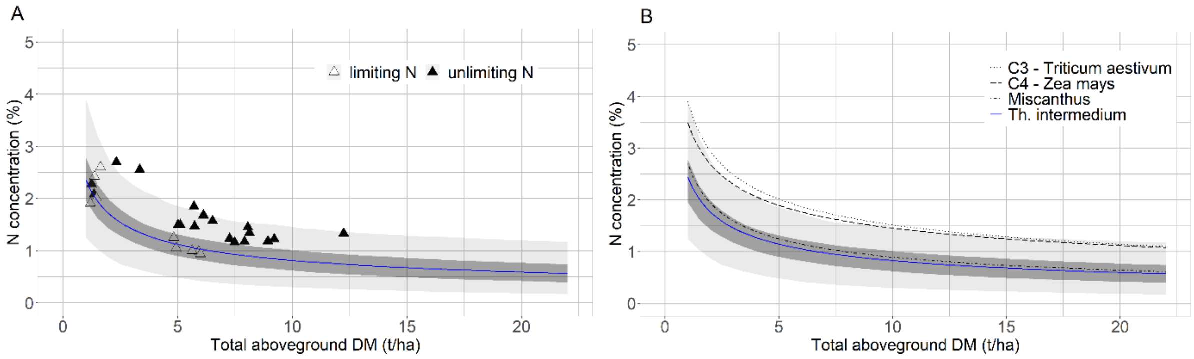

Figure 5 : Newly developed critical nitrogen dilution curve (CNDC) of Th. intermedium set up over the C1 dataset and plotted against the V1-5 validation dataset (A). Comparison of the CNDC of Th. intermedium, Tritcum aestivum (C3 annual plant), Zea mays (C4 annual plant) and Miscanthus (C4 perennial plant). Blue line represent the CNDC generated with median parameter value, dark grey area represented the 50% CI and light grey area the 95% CI of CNDC of Th. intermedium.

4. Discussion

4.1 Understanding nitrogen needs through the dilution curve

Globally, the maximization of the aboveground production of DM and the N uptake was obtained with a N application comprised between 100 and 150kg/ha applied over the entire growing year (including fall fertilization). However, depending upon the year and the phenological stage, the increase might not always be significant compared to reduced fertilizations strategies (i.e., 50kg N/ha over the entire growing year). Jungers *et al.* (2017) found the optimum to range between 61 and 96kg N/ha applied in spring to maximize yields with an average of 10.8t DM/ha at the grain maturity stage. Using our proposed N dilution curve, the N content for this level of biomass production is averaging 0.79% corresponding to a N uptake of 85kg/ha. If we consider our maximizing N treatments (application ranging between 100 and 150kg/ha), the Belgian mean biomass yield at grain maturity stage is around 11.5t DM/ha over the three cropping seasons. It corresponds to an average N content of 0.76% resulting in a N uptake of 88kg/ha. Production levels are relatively similar, corresponding to comparable N requirements. Regarding these N needs, N fertilizations should be adjusted to the yield targets, the different soil and climatic conditions of the field including soil N availability to be at the optimum. In addition, to further optimize N fertilization, we recommend studying the response of the crop to finer fertilization amount intervals. Yet, the newly proposed CNDC provides a robust characterization of the N critical status and needs for *Thinopyrum intermedium*, as a response to crop management practices or environmental conditions (Lemaire *et al.*, 2019). However, as *Th. intermedium* has a recent selection history, the potential development of new genotypes or cultivars should be studied in the future as it could lead to different growing patterns, therefore influencing N requirements or the CNDC (Lemaire *et al.*, 2019).

Concerning the N fertilization timings of *Th. intermedium*, a late summer or fall fertilization could be integrated into the N management strategy of the multi-annual *Th. intermedium* crop. Indeed, a fall N application combined with an early-spring application resulted in relatively similar aboveground production levels of the crop as a full early-spring nitrogen application if we compare treatment 3 and 4 of our study. In addition, as Cattani *et al.* (2017) highlighted, N applied in fall could enhance fertile tiller initiation and N applied in spring during pre-reproductive induction could allow a better N use by inducing larger fertile tillers, larger panicles, and greater seed set. Therefore, spring applications could be reduced by transferring a part of these applications in late summer or fall or in very early-spring before reproductive induction. This strategy could also prevent risks of lodging at the end of the growing cycle, lodging that has been observed in the study of Jungers *et al.* (2017) under high spring N fertilizations.

During the crop cycle of *Th. intermedium,* N is diluted in the aboveground biomass, which results in a reduction of the N percentage. Furthermore, data reported in this study indicated that the leaf area ratio (LAR) of *Th. intermedium* decreased along the growing season. As explained in the study of Ratjen *et al.* (2018), as aboveground biomass increases, growth becomes more vertical and leaves are organized in leaf-layers which progressively differentiate leaf declination, specific leaf area and vertical N distribution. The fraction of structural biomass which has a low N concentration is known to increase at a higher relative rate than the metabolic fraction which is characterized by a high N concentration. In that way, the metabolic fraction varies with the photosynthetic surface of the plant while the structural fraction varies with the canopy height and leaf thickness. Therefore, it has been shown that, in a large range of crops, the decline of N percentage is strictly parallel to the decline of the leaf area ratio (Ratjen *et al.*, 2018; Lemaire *et al.*, 2020). Our results provided evidences that the dilution of N within *Th. intermedium* tissues might respond to the same mechanisms.

4.2 Storing nutrients in perennial structures to ensure survival strategy

After an initial increase until BBCH39 or BBCH65, the N uptake within the aboveground biomass was found to decrease during the second part of the growing season as shown in Figure 3. For some treatments we observed, a decrease of up to 50% of the N uptake within the aboveground biomass during the second phase of the growing season. Surprisingly, N fertilization appeared to not really influence this N disappearance, not allocated to grains. Neither was the addition of N as fertilizer associated to any substantial increase in the allocation of N towards the grains. While it has no impact in 2019, the fertilization of 150kg N/ha increased the N uptake of ears by only around 20kg in 2020 and 12kg in 2021 in comparison to the reference treatment. Allocation of N to ears seems much lower compared to other cultivated crops such as wheat (Hussain *et al.*, 2006). A first explanation lies in the low allocation of DM to grains. We observed that ears, including grains and vegetative biomass such as seed hulls and rachis, represented approximatively 16% of the aboveground biomass at grain harvest for the treatment 3. A study conducted by Culman *et al.* (2013) reported that grains represented only 10% of total aboveground dry matter of *Th. intermedium*, compared to 50% for annual winter wheat that allocates much more resources to seeds.

We believe that the low seeds production and the associated low N uptake in the grains are related to a long-term survival strategy of the plant, which can translocate nutrients to belowground organs at the expense of grains production. This has been previously underlined in the study of Nassi *et al.* (2013) for *Arundo Donax* L., a C3 rhizomatous grass. The plant experienced a peak nutrients level in shoots over the summer period followed by a decline and a simultaneous increase in belowground rhizomes' level. At the end of the growing season, the crop exhibited relatively low nutrient contents in shoots. The same trend was reported for C4 crops (*Miscanthus* & *Spartina cynosuroides*), which translocates nutrients to rhizomes at the end of each growing season, with a mean N content within the aboveground biomass declining respectively by 83% and by 77% for *M. x giganteus* and S. *cynosuroides* (Beale *et al.*, 1997). The latter species produce larger quantities of rhizomes than *Th. intermedium*, approximately 50% of the belowground biomass for *Arundo donax* (Quinn *et al.*, 2007) and between 60 to 80% at shallow depth considering different *Miscanthus* species and growth years (Dohleman *et al.*, 2012; Christensen *et al.*, 2016) compared to 17% for *Th. intermedium* (Sakiroglu *et al.*, 2020). As reported in the study of Sakiroglu *et al.* (2020), nutrients are stored in rhizomes of *Th. intermedium* suggesting that this organ could play an important role in spring regrowth and plant survival. In addition, in this latter study, it was hypothesized that the storage of reserves in rhizomes for spring regrowth would be significant in the first few years and would then decrease with the age of the crop. Another belowground storage organ could be represented by the root system, where N might be stored and then remobilized to the shoot after defoliation to support leaf regrowth. This was observed for alfalfa or ryegrass. The N reserves stored in alfalfa roots and that contributed to shoot regrowth reached 30kg N/ha in the study of Lemaire *et al.* (1992).

Finally, ground-level stem's bases might be another storage organ used by *Th. intermedium* in its survival strategy. As proposed in the review of White (1973) about perennial grasses, we also believe that the lower region of the stems (i.e., the stem's bases) could also be a storage area of most carbohydrate reserves for *Th. intermedium* that could be used as an energy source to initiate new growth following herbage removal.

Considering all these aspects, it is not unlikely that *Th. intermedium* would have similar internal mechanisms for the reallocation of nutrients toward belowground organs (i.e., roots and short rhizomes) or ground-level organs (i.e., stem's bases). During regrowth, *Th. intermedium* could use the nutrients stored in these organs to develop plants already established (from reserves of roots and/or stem bases) or produce the shoot and root systems of new plants (from rhizomes - as hypothesized by Sakiroglu *et al.* (2020)).

4.3 Linking nitrogen use efficiency with resource-conservative strategy

The newly proposed CNDC of *Th. intermedium* seemed very different from other annual crop species' CNDC, such as *Triticum aestivum* L. or *Zea mays* L. (Figure 5-B), with globally much lower needs in terms of N nutrition translated in a *a*-coefficient of 2.35. Based on the estimations of *a*- and *b*-coefficients, it seems that the N amount needed for intermediate wheatgrass would be approximatively 60% of the N needed by *Triticum aestivum* at a production of 1t DM/ha, and 53% at a production of 15t DM/ha. These differences in N use efficiency - mostly related to a lower *a*-coefficient (Lemaire *et al.*, 1981) - have been highlighted in the pioneer work of Greenwood *et al.* (1990) who identified clear differences between C3 and C4 metabolic groups. The lower *a*-coefficient reported for *Th. intermedium* can be associated to aerial tissues with lower N content. At low levels of W (~1t DM/ha), aboveground biomass is mainly composed of leaves; this would reflect a lower leaves' N content. For treatment 3, the leaves' N content was about 2.8% in 2020 and 2.1% in 2021 at the beginning of the growing season for a biomass of 1.5 and 1.3t DM/ha respectively.

In addition, we found out that the CNDC of *Th. intermedium* is relatively close from the one reported by Zapater *et al.* (2017) for *Miscanthus* (Figure 5-B). In this study, such low N needs have been related to several life traits. Potential explanation lies in the work of Beale *et al.* (1997) who reported (i) a higher nitrogen use efficiency (ii) a high nutrient uptake efficiency thanks to a deep and extensive root system and (iii) an efficient nutrient recycling through translocation from shoots to rhizomes and through remobilization from rhizomes to shoots the following growing season. As shown in the study of Sprunger *et al.* (2018) and considering the simplest NUE definition – the N content of the whole plant (roots included) divided by the N available – the nitrogen use efficiency of *Th. intermedium* is very high, and the plant seems to be able to assimilate large quantities of N, even greater than what has been applied. This same study reported that *Th. intermedium* allocated between 23 to 50% of biomass to roots. Its deep and dense root system allows an extensive exploration of the soil profile which can further increase the nitrogen use efficiency while at the same time reducing nitrate leaching (Jungers *et al.*, 2019).

Beyond these aspects, the higher N use efficiency of *Th. intermedium* could be discussed in link with different growing habits and with a resource-conservative strategy of the crop. As explained by the theory of Tilman (1982), in low soil fertility conditions, the rate of acquisition of nutrients would be low and plants would grow very slowly. The plants having the more efficient uptake capacity for the more limiting resource, and/or the ability to store and to conserve this resource through efficient internal recycling mechanisms will be more competitive. The concept of 'resource conservation' within the plant has thus been highlighted: 'as the time of residence of one resource within a plant increase, this resource becomes more efficient and in consequence it can be acquired in lower quantity for maintaining the plant alive' (Lemaire, 2001). Therefore, species with long leaf life span should have a lower demand for N resources and should persist better in a poor soil condition than species with short leaf life span. The ability for acquiring and conserving resources, for most herbaceous plant species, can be described by leaf traits (i.e., specific leaf area, dry matter content of leaf, leaf N% and leaf life span), allowing a rapid classification between slow- and fast-growing species (Lemaire, 2001). In the study of Maire *et al.* (2009) this N conservative strategy has been related to different physiological traits. Indeed, some tall grass species with high N-yields (i.e., N uptake of shoots) and high root and shoot biomass can display more conservative traits such as a high leaf N use efficiency combined with a low leaf N concentration and a low root uptake capacity which is the case of *Dactylis glomerata* or *Festuca arundinacea*. Furthermore, Duchene *et al.* (2020) hypothesized that some root traits of *Th. intermedium* were also linked to a resource-conservative strategy of the plant, namely the higher average root diameter and tissue density, suggesting an enhanced root storage functions with a higher residence time of nutrients in tissues.

5. Conclusion

Our study has highlighted that *Th. intermedium* perennial grain crop is able to reach a high shoot dry matter production with low N needs. This is most likely associated to its long-term survival strategy that implies an important investment in perennial structures coupled with a weaker resource allocation to reproductive seeds. Some growing patterns of the crop were put in relation with mechanisms observed in plant with similar strategies, such as the N recycling through the storage of nutrients in the perennial organs or an extensive exploration of soil with a dense and deep root system allowing for a certain efficiency at extracting nutrients. In the future, N contents of roots, rhizomes and stem's bases of *Th. intermedium* should be studied to confirm the possible translocation of nutrients to these belowground or ground-level organs during the second part of the growing season. Overall, the CNDC found in this study will be highly helpful to help define N requirements in various pedo-climatic environments and adjust accordingly the soil-crop management, and more precisely the management of the N fertilization. Ultimately, the low N requirements of *Th. intermedium* coupled with a high N use efficiency demonstrate that the crop can enhance agronomic and environnemental benefits such as (i) the N cycling and accumulation in soil by its belowground and/or storage organs, (ii) the reduction of nitrate leaching or (iii) the potential to produce high aboveground biomass in N limited environnements.

6. Competing interests

The authors declare that they have no competing interests.

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8. Author contribution

L.F., B.D.: conceptualization and planning of the experiments. Formulation of research goals and aims.

L.F.: carrying out the samplings, data curation, formal analyses (statistical and mathematical).

B.D.: Supervision.

B.D., L.F.: Development and design of methodology.

F.C., C.D., O.D., J.B., B.D.: help provided for data presentation and visualization.

L.F., B.D., F.C., O.D., C.D. contributed to the interpretation of result.

L.F.: Writing. Original Draft Preparation.

F.C., C.D., O.D., J.B., B.D.: critical review, commentary and revision, validation.

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10. List of tables

11. List of figures

12. Supplementary materials

Table S1 : N content (%N) within the aboveground biomass for the different N fertilizations and phenological stages for 2019(A), 2020(B) and 2021(C). Data are presented as average ± standard error. In 2019 at the BBCH89 stage no statistical analyses were performed, as the N content was measured on averaged samples.

(A) 2019

Fertilization	N content of leaves and stems ± S.E. (%)					N content of ears ± S.E. (%)		
	BBCH30	BBCH39	BBCH65	BBCH89	Mean of stages	BBCH65	BBCH89	Mean of stages
0+0+0N	2.40±0.04c	0.91±0.01b	0.50±0.02b	0.34	1.04	1.71±0.04	1.13	1.42
50+0+0N	2.90±0.01b	1.08±0.08b	0.56±0.03b	0.32	1.22	1.89±0.09	1.15	1.52
50+0+50N	3.23±0.05a	0.99±0.06b	0.54±0.08b	0.43	1.30	1.95±0.05	1.19	1.57
100+0+0N	3.35±0.07a	1.16±0.08ab	0.72±0.08a	0.37	1.40	1.96±0.02	1.10	1.53
100+0+50N	3.46±0.03a	1.32±0.04a	0.80±0.03a	0.35	1.48	1.89±0.03	1.19	1.54
Mean of fertilizations	3.09±0.08	1.09±0.04	0.62±0.03	0.36		1.88±0.03	1.15	

(B) 2020

Fertilization	N content of leaves ± S.E. (%)					N content of stems ± S.E. (%)					N content of ears ± S.E (%)		
	BBCH30	BBCH39	BBCH65	BBCH89	Mean of stages	BBCH30	BBCH39	BBCH65	BBCH89	Mean of stages	BBCH65	BBCH89	Mean of stages
0+0+0N	2.13±0.09c A	1.09±0.10b B	0.90±0.05e B	0.46±0.01d C	1.14±0.16	1.19±0.05bc A	0.78±0.09a B	0.38±0.06c C	0.32±0.02b C	0.67±0.09	1.37±0.04	1.30±0.11	1.33±0.06b
50+0+0N	2.40±0.13bc A	1.34±0.11b B	1.18±0.05d B	0.48±0.01d C	1.35±0.18	1.39±0.09abc A	0.80±0.04a B	0.48±0.04bc C	0.31±0.01b D	0.74±0.11	1.43±0.08	1.32±0.03	1.37±0.05b
50+0+50N	2.75±0.25ab A	1.75±0.08a B	1.38±0.02c B	0.56±0.02cd C	1.61±0.21	1.68±0.28ab A	0.94±0.04a B	0.57±0.03b BC	0.37±0.01b C	0.89±0.14	1.39±0.03	1.39±0.03	1.39±0.02b
100+0+0N	3.01 ±0.12a A	2.19±0.13a B	1.61±0.02b C	0.65±0.04bc D	1.86±0.23	1.74±0.12ab A	1.11±0.11a B	0.68±0.04a C	0.37±0.02b D	0.98±0.14	1.47±0.04	1.46±0.04	1.46±0.03b
100+50+0N	3.26 ±0.18a A	2.15±0.15a B	1.86±0.10a C	0.81±0.06a D	2.02±0.23	1.92±0.24a A	1.12±0.12a B	0.80±0.04a BC	0.49±0.02a C	1.09±0.15	1.57±0.08	1.73±0.05	1.65±0.05a
0+100+0N	2.02 ±0.06c A	1.25±0.03b C	1.83±0.03a B	0.73±0.04ab D	1.46±0.13	1.11±0.08c A	0.76±0.06a B	0.78±0.05a B	0.48±0.03a C	0.78±0.06	1.65±0.04	1.72±0.06	1.69±0.03a
50+50+50N	2.98 ±0.27a A	1.91±0.19a B	1.75±0.04ab B	0.72±0.03ab C	1.84±0.22	1.73±0.20ab A	1.00±0.11a B	0.76±0.03a BC	0.46±0.02a C	0.99±0.13	1.61±0.07	1.55±0.06	1.58±0.04a
Mean of fertilizations	2.65±0.10	1.67±0.09	1.50±0.07	0.63±0.03		1.54±0.08	0.93±0.04	0.64±0.03	0.40±0.02		1.50±0.03	1.50±0.04	

(C) 2021

0+0+0N	1.81±0.07b A	1.31±0.03c B	1.01±0.15b C	0.53±0.02c D	1.17±0.13	1.10±0.18	1.15±0.10	0.51±0.01	0.34±0.01	0.77±0.10c	1.34±0.11	1.37±0.07	1.35±0.06b
50+0+0N	2.09±0.05b A	1.48±0.02bc B	0.94±0.04b C	0.53±0.01c D	1.26±0.15	1.19±0.20	1.07±0.04	0.57±0.01	0.33±0.02	0.79±0.10c	1.33±0.03	1.40±0.04	1.37±0.02b
50+0+50N	2.07±0.07b A	1.56±0.10abc B	0.90±0.02b C	0.60±0.04bc D	1.29±0.15	1.16±0.31	1.04±0.05	0.58±0.02	0.32±0.04	0.78±0.11c	1.43±0.06	1.41±0.05	1.42±0.04ab
100+0+0N	2.47±0.22a A	1.73±0.05ab B	0.99±0.06b C	0.66±0.03bc C	1.46±0.19	1.71±0.12	1.05±0.02	0.61±0.03	0.33±0.03	0.92±0.14abc	1.60±0.05	1.47±0.04	1.53±0.04ab
100+50+0N	2.51±0.08a A	1.94±0.07a B	1.30±0.12b C	0.90±0.07a D	1.66±0.16	1.69±0.06	1.26±0.18	0.94±0.04	0.45±0.05	1.08±0.13a	1.76±0.16	1.57±0.11	1.66±0.10a
0+100+0N	1.77±0.13b A	1.85±0.17ab A	1.21±0.18b B	0.70±0.06bc C	1.38±0.14	1.20±0.12	1.16±0.05	0.79±0.14	0.35±0.03	0.88±0.10bc	1.61±0.20	1.49±0.11	1.55±0.11ab
50+50+50N	2.16±0.16b A	1.72±0.11ab A	1.78±0.27a A	0.79±0.07ab B	1.61±0.15	1.47±0.02	1.31±0.17	0.91±0.08	0.41±0.06	1.03±0.11ab	1.84±0.12	1.56±0.05	1.70±0.08a

Mean of fertilizations	2.13±0.07	1.66±0.05	1.16±0.07	0.67±0.03		1.36±0.07	1.15±0.04	0.70±0.04	0.36±0.02		1.56±0.05	1.46±0.03
						A	B	C	D			

Note. Means with a letter differ significantly (*p-value*<0.05). Letters in minuscule represent the result of SNK test of the effect of fertilization and letters in majuscule represent the result of SNK test of the effect of phenological stage. When letters are reported in the 'mean of fertilizations' row or in the 'mean of stages' column, it means no interactions between those factors were reported for that year and plant organ.

Table S2 : Leaf/Stem ratio of the aboveground biomass for the different N fertilizations and phenological stages from 2020(A) to 2021(B). Data are presented as average ± standard error.

(A) 2020	***Leaves/stems ratio***				
	BBCH30	***BBCH39***	***BBCH65***	***BBCH89***	***Mean of stages***
0+0+0N	5.58±0.46	0.76±0.05	0.31±0.02	0.25±0.03	1.72±0.59
50+0+0N	4.41±0.55	0.73±0.03	0.27±0.03	0.23±0.02	1.41±0.47
50+0+50N	6.02±1.41	0.56±0.04	0.24±0.03	0.21±0.01	1.76±0.71
100+0+0N	4.68±0.98	0.66±0.05	0.24±0.02	0.21±0.01	1.45±0.53
100+50+0N	4.76±1.15	0.61±0.08	0.25±0.03	0.18±0.02	1.45±0.56
0+100+0N	4.95±0.50	0.76±0.05	0.40±0.04	0.21±0.02	1.58±0.52
50+50+50N	5.00±0.92	0.64±0.06	0.32±0.03	0.22±0.01	1.54±0.56
Mean of fertilizations	5.06±0.32	0.67±0.02	0.29±0.01	0.22±0.01	
	A	B	B	B	
(B) 2021					
0+0+0N	15.70±9.81	0.66±0.02a	0.26±0.01	0.17±0.01	3.43±2.33
	A	B	B	B	
50+0+0N	9.09±0.30	0.59±0.05ab	0.24±0.01	0.16±0.01	2.08±0.94
	A	B	B	B	
50+0+50N	7.64±3.44	0.50±0.03b	0.21±0.01	0.15±0.01	2.13±1.13
	A	B	B	B	
100+0+0N	4.49± 0.36	0.49±0.05b	0.24±0.01	0.14±0.01	1.34±0.48
	A	B	B	B	
100+50+0N	4.18±0.66	0.47±0.02b	0.21±0.02	0.15±0.01	1.25±0.46
	A	B	B	B	
0+100+0N	6.00± 0.99	0.57±0.03ab	0.22±0.02	0.14±0.01	1.73±0.68
	A	B	B	B	
50+50+50N	3.40±0.24	0.46±0.03b	0.22±0.02	0.15±0.01	1.06±0.36
	A	B	B	B	
Mean of fertilizations	6.82±1.29	0.53±0.02	0.23±0.01	0.15±0.01	

Note. Means with a letter differ significantly (*p-value*<0.05). Letters in minuscule represent the result of SNK test of the effect of fertilization and letters in majuscule represent the result of SNK test of the effect of phenological stage. When letters are reported in the 'mean of fertilizations' row or in the 'mean of stages' column, it means no interactions between those factors were reported for that year.

Table S3 : Leaf area ratio of the aboveground biomass for the different N fertilizations and phenological stages from 2020(A) to 2021(B). Data are presented as average ± standard error.

(A) 2020	***Leaf area ratio***				
	BBCH30	***BBCH39***	***BBCH65***	***BBCH89***	***Mean of stages***
0+0+0N	0.0084±0.0009c A	0.0066±0.0008a B	0.0016±0.0003b C	0.0055±0.0031	0.0084±0.0009c A
50+0+0N	0.0105±0.0008b A	0.0054±0.0003bc B	0.0016±0.0003b C	0.0058±0.0038	0.0105±0.0008b A
50+0+50N	0.0133±0.0014a A	0.0051±0.0003bc B	0.0017±0.0002b C	0.0067±0.0051	0.0133±0.0014a A
100+0+0N	0.0129±0.0012a A	0.0057±0.0006ab B	0.0016±0.0003b C	0.0068±0.0049	0.0129±0.0012a A
100+50+0N	0.0129±0.0017a A	0.0042±0.0008c B	0.0020±0.0004b C	0.0064±0.0050	0.0129±0.0017a A
0+100+0N	0.0103±0.0006b A	0.0052±0.0006bc B	0.0026±0.0001a C	0.0060±0.0034	0.0103±0.0006b A
50+50+50N	0.0116±0.0007ab A	0.0050±0.0006bc B	0.0021±0.0001b C	0.0062±0.0042	0.0116±0.0007ab A
Mean of fertilizations	0.0114±0.0020	0.0053±0.0009	0.0019±0.0004		0.0114±0.0020
(B) 2021					
0+0+0N	0.0116±0.0012ab A	0.0056±0.0005 B	0.0006±0.0001b C	0.0060±0.0047	0.0116±0.0012ab A
50+0+0N	0.0129±0.0008ab A	0.0051±0.0005 B	0.0010±0.0001a C	0.0064±0.0052	0.0129±0.0008ab A
50+0+50N	0.0124±0.0008ab A	0.0051±0.0002 B	0.0008±0.0001ab C	0.0061±0.0050	0.0124±0.0008ab A
100+0+0N	0.0123±0.0011ab A	0.0047±0.0007 B	0.0009±0.0001ab C	0.0060±0.0050	0.0123±0.0011ab A
100+50+0N	0.0133±0.0009a A	0.0049±0.0006 B	0.0008±0.0003ab C	0.0063±0.0055	0.0133±0.0009a A
0+100+0N	0.0112±0.0005b A	0.0055±0.0004 B	0.0011±0.0002a C	0.0060±0.0044	0.0112±0.0005b A
50+50+50N	0.0117±0.0004ab A	0.0046±0.0005 B	0.0006±0.0001b C	0.0056±0.0048	0.0117±0.0004ab A
Mean of fertilizations	0.0122±0.0010	0.0051±0.0006	0.0008±0.0002		0.0122±0.0010

Note. Means with a letter differ significantly (*p-value*<0.05). Letters in minuscule represent the result of SNK test of the effect of fertilization and letters in majuscule represent the result of SNK test of the effect of phenological stage. When letters are reported in the 'mean of fertilizations' row or in the 'mean of stages' column, it means no interactions between those factors were reported for that year.

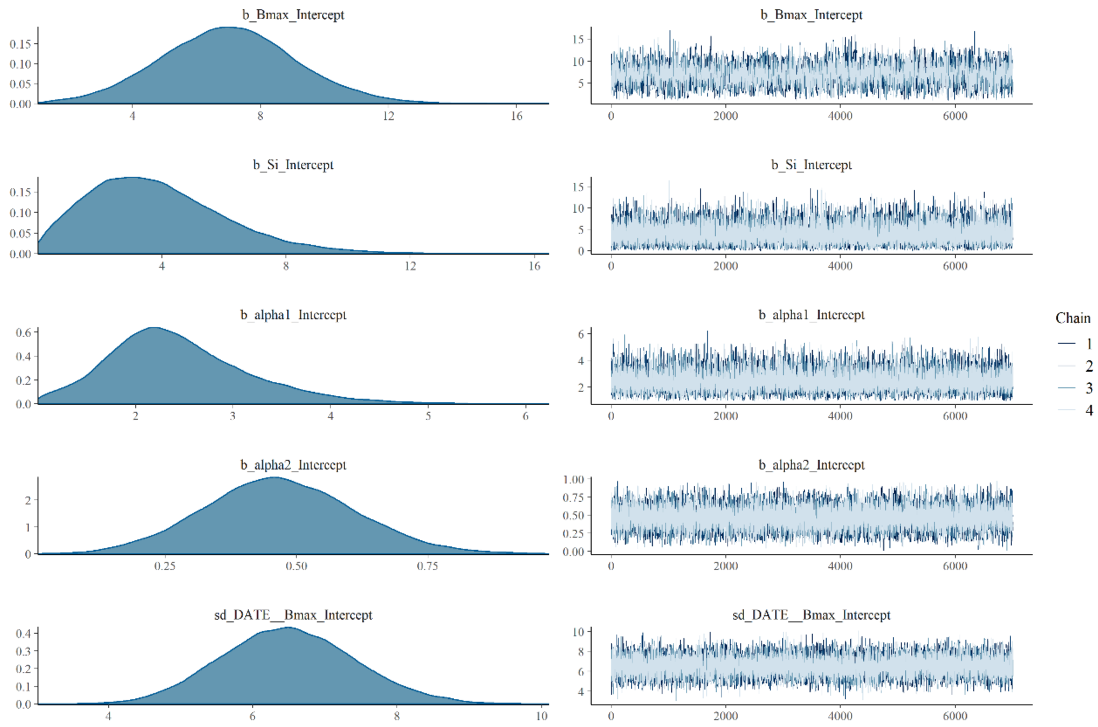

Figure S1 : Posterior distribution and the post-warmup samples from all 4 Markov chains of 10 000 iterations of Wmax, S, a-coefficient, b-coefficient and $\boldsymbol{\sigma_{BMax}}$, obtained with the Bayesian approach.

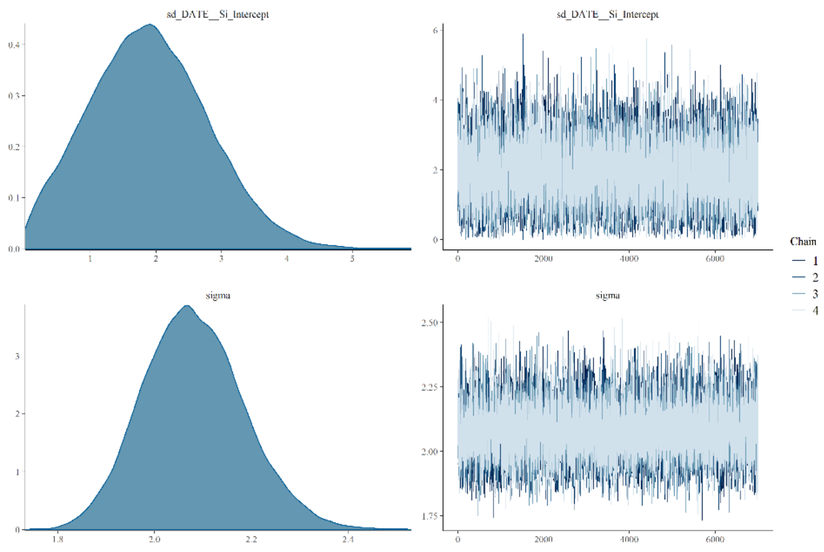

Figure S2 : Posterior distribution and the post-warmup samples from all 4 Markov chains of 10 000 iterations of $\boldsymbol{\sigma_S}$ *and* $\boldsymbol{\sigma}$ obtained with the Bayesian approach.

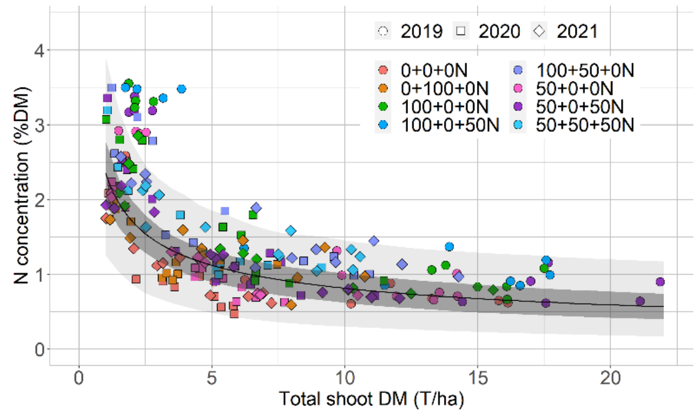

Figure S3 : Relationship between nitrogen concentration of the total aboveground biomass (%) and dry matter production of Th. intermedium from the Belgian experimental C1 dataset used to set up the critical nitrogen dilution curve (CNDC). Black line represent the CNDC generated with median parameter value, dark grey area represented its 50% CI and light grey area its 95% CI of CNDC.