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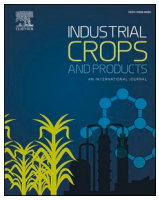

Examining poplar biomass recalcitrance in OrganoCat processing: The impact of genotype, nitrogen fertilization and process severity

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ABSTRACT

Poplar plays a vital role in carbon sequestration and provides valuable raw materials for various industrial applications. This study dissects the effects of genotype and nitrogen fertilization on recalcitrance features in poplar biomass towards OrganoCat pretreatment. Three genetically different poplar hybrids were cultivated for 13 weeks at greenhouse conditions and fertilized with different forms of inorganic nitrogen. Stem material was processed with different degrees of severity. Biochemical data of the biomass and OrganoCat products was used in a multiscale analysis to determine recalcitrance parameters. The relationship between nitrogen fertilization and genotype on the subsequent processing was investigated. The genotype had a greater influence on the recalcitrance of young poplars than nitrogen fertilization. Generally, crystalline cellulose content and enzymatic glucose conversion increased after processing with a higher severity. Moreover, the overall compositional variation decreased after OrganoCat processing. Optimization of nitrogen fertilization, transfer to field experiments and lignocellulose processing techniques are crucial for maximizing economic and environmental benefits of poplar plantations.

1. Introduction

Poplars play a crucial role in the ecological and commercial sectors by contributing to carbon sequestration and providing valuable raw materials. The impact of poplar trees is closely linked to their growth conditions, particularly their fertilization practices. Appropriate nitrogen fertilization substantially enhances growth and biomass production (Baldwin et al., 2017). But nitrogen plays also an important role as a regulator for the biosynthesis of cell wall building blocks (Euring et al., 2014) E.g. lignin deposition and its chemical composition are highly influenced by nitrogen supply in many plant species (Baldwin et al., 2017; Euring et al., 2014; Zhang et al., 2017). The genetic mechanisms underlying nitrogen uptake in poplar have been explored, emphasizing the need for optimization of fertilization to improve growth efficiency (Castro-Rodríguez et al., 2016) and physiological responses to various nitrogen fertilization regimes have been investigated, revealing how nitrogen availability affects the growth rates, biomass accumulation and productivity (Głazowska et al., 2019; Pitre et al., 2007). The interplay between nitrogen supply and environmental stressors highlights the need for tailored nitrogen fertilization strategies to enhance resilience (Poovaiah et al., 2019; Ye et al., 2022).

Poplar biomass is a promising renewable resource for biofuel production but also other value-added products. Efficient processing of the lignocellulosic material is essential to unlock their full potential. However, its complex structure and composition often hamper efficient fractionation. This phenomenon is known as lignocellulose recalcitrance and depends on the heterogeneous multi-scale structure of the cell wall materials and impacts the subsequent conversion process (Holwerda et al., 2019; McCann and Carpita, 2015). Factors affecting lignocellulose recalcitrance are strongly interconnected and often difficult to dissociate (Zoghlami and Paës, 2019). Various pretreatment methods have been used to break down the complex structure of lignocellulose, facilitating enzymatic hydrolysis of cellulose and hemicellulose into fermentable sugars (Magalhães et al., 2019; Singhvi et al., 2014). Organosolv-like technologies are a category of pretreatments that combine effectiveness and low degradation due to their mild reaction conditions (Sakdaronnarong et al., 2016, 2018). The OrganoCat process is one such example that utilizes a biogenic solvent and catalyst in a biphasic system, allowing for *in situ* lignin extraction into a second phase. This process aims to balance the quality and quantity of fractionated components by optimizing the process conditions. This approach allows for the effective fractionation of lignocellulose while minimizing sugar

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degradation to undesirable humins, preserving the native structural features of lignin, and maintaining economic feasibility. The reaction conditions involve temperatures ranging from 125°C to 160°C and durations of 1 to 3 h, which hydrolyze non-cellulosic polysaccharides and extract lignin into 2-methyltetrahydrofuran (2-MTHF). Water, solvents, and catalysts can be recycled (Grande et al., 2015).

This study aims to dissect the effects of nitrogen fertilization on recalcitrance features for different poplar genotypes by investigating the plasticity of the lignocellulosic cell wall and corresponding enzymatic saccharification rates before and after OrganoCat processing with different severities. The intended view of the different dimensions – genetic basis of the plant, environmental influences during cultivation and process conditions – can subsequently provide information for optimized fertilization strategies and lignocellulose processing techniques for poplar maximizing the economic and environmental benefits.

2. Materials and methods

2.1. Materials

Three genetically different black poplar (*P. nigra*) hybrids—AF6 (*P. nigra* L. x *P.* x *generosa* A. Henry), Max4 (*P. maximowiczii* x *P.* nigra), and Vesten (*P. deltoides* x *P. nigra*)— were used in this study, known for their use as fast-growing Short Rotation Coppice (SRC) crops (Novotná et al., 2020; Sabatti et al., 2014; Verlinden et al., 2013). Clonal material was cultivated and fertilized with three forms of inorganic nitrogen —nitrate (NO_3^-, NO), ammonium (NH_4^+, NH), and nitrate ammonium (NH_4NO_3, NN)— during the summer of 2023 in greenhouse conditions at Forschungszentrum Jülich, Jülich, Germany. Plants were given a nutrient solution four times a week, consisting of 50–100 mL of adjusted Hoagland's solution (Zhao et al., 2016), which contained 10 mM N in the three respective forms. After 13 weeks of growth, the plants were harvested (with 7–13 specimens per fertilization and genotype). Stem material was collected which has been grown during fertilization (approx. 100–150 cm long). The leaves were removed, and the fresh stem was debarked using a razor blade, and the parenchyma was scrapped away. The material was dried for a day at 65°C, ground using a laboratory hammer mill M 400 with two steel ball mills (frequency 30/s, for 2 min, Retsch, Germany) and stored at room temperature for further analysis.

Unless specified differently, chemicals were acquired from Carl Roth and Sigma-Aldrich (Germany) and employed without additional purification.

2.2. Cell wall characterization

Wet chemical analysis was conducted following previously described procedures (Grande et al., 2019; Jablonowski et al., 2017; Martinez Diaz et al., 2023). In brief, alcohol-insoluble residues (AIR) were obtained by sequential extraction with ethanol (70 %, v/v), chloroform:methanol solution (1:1, v/v) and acetone. Subsequently, enzymatic digestion with amyloglucosidase and α-amylase (Megazyme, Ireland) was used to eliminate starch from the residual pellet, resulting in de-starched AIR (dAIR). The dAIR biomass was washed thrice with water and acetone, respectively, and air-dried at room temperature. All analyses of raw biomass were performed using dAIR.

Crystalline cellulose (CrC) was assessed by eliminating non-cellulosic components using Updegraff reagent, followed by sulphuric acid hydrolysis (72 %, v/v), and quantification of the remaining carbohydrate via anthrone spectrophotometric assay. Total acetate content was assessed by dAIR biomass saponification using 0.5 M NaOH, neutralizing it with 1 M HCl, and enzymatically measuring it using an acetic kit (K-ACETRM, Megazyme, Ireland). Acetyl bromide (AcBr) soluble lignin method was used for lignin determination, where acetyl bromide solution (25 %, v/v) was used, followed by incubation at 50°C for 3 h, and subsequent addition of 2 M NaOH, 0.5 M hydroxylammonium chloride, and glacial acetic acid. Then, Acetyl bromide soluble lignin was determined spectrophotometrically by measuring the absorbance of the solution at 280 nm. For quantification of the samples and standardization between different test runs, a standard curve was generated with Kraft lignin (Sigma-Aldrich; CAS No. 8068–05-1). The measurement of non-cellulosic polysaccharides was achieved by hydrolysing with trifluoroacetic acid (TFA) and subsequently separating them using high-performance anion-exchange chromatography with pulsed amperometry detection (HPAEC-PAD).

Enzymatic saccharification rate was determined, previously described (Damm et al., 2017). 10 mg of biomass and 5 µL of Accellerase ® 1500 (60 FPU/mL and 82 CBU/mL, Genencor, The Netherlands) were suspended in a citrate buffer solution (0.1 M, pH 4.5, 20 mg/L buffer solution). Avicel® cellulose was used as an internal control for cellulose hydrolysis. Samples were shaken and incubated at 50°C for 0, 1, and 72 h in an Eppendorf Thermomixer Comfort. Following saccharification, the samples were heated to 99°C for 10 min to inactivate the enzymes, and the remaining glucose concentration was measured by p-hydroxy benzoic acid hydrazide (PAHBAH) assay. PAHBAH reagent was added to the sample supernatant and incubated for 10 min at 100°C, after which the absorbance was measured spectrophotometrically at 410 nm (Lever, 1973).

2.3. OrganoCat processing

The plant material was processed with a small scale OrganoCat fractionation, previously described (Martinez Diaz et al., 2023). Biological replicates were used with no technical replicates (n = 7–13).

Glass vials (CS-Chromatographie Service, Germany) were loaded with 100 mg of biomass. A mixture consisting of 1 mL of 0.1 M oxalic acid and 1 mL of 2-methyltetrahydrofuran (2-MTHF), along with a micromagnetic stirring bar (length 6 mm, Carl Roth, Germany) was incorporated. A set of ten vials per batch were placed in an oil bath at low and high OrganoCat severities (125°C for 1-hour reaction time and 140°C for a 3-hour reaction time, respectively) and the rotational speed of the magnetic stirring plate was set to 550 rpm. Following the reaction, the vials were allowed to cool to room temperature. To improve phase separation, the reaction products were transferred to an Eppendorf tube and centrifuged for 7 min at 14,000 rpm. Subsequently, 0.5 mL of the organic phase (lignin in 2-MTHF) was collected and evaporated in a rotary evaporator to isolate lignin. The supernatant from the aqueous phase was centrifuged twice for 5 min at 14,000 rpm and stored at −4°C for further analysis. The remaining pulp was washed with distilled water until it reached a neutral pH and was left to dry at 65°C until it reached constant weight.

2.3.1. OrganoCat yields

Given that a small amount of 2-MTHF might be dissolved in the aqueous phase, a 0.5 mL sample of the organic phase was taken to determine gravimetrically the lignin yield, where a 1.92 factor was considered for total lignin yield (Supplementary file, Table A).

$$Lignin\ yield[\%original\ weight] = (Extracted\ lignin\ wt.\ [mg] / Initial\ biomass\ wt.\ [mg]) \times 1.92 \times 100$$

Pulp yield was calculated, as it follows:

$$Pulp\ yield[\%orig.wt.] = (Final\ pulp\ wt.\ [mg] / Initial\ biomass\ wt.\ [mg]) \times 100$$

The procedure for analyzing pulp material followed the same steps as described for the cell wall characterizing and it is referred to as pulp lignocellulosic parameters. Instead of using dAIR biomass, the analysis began with pulp material, and the evaluation of CrC, AcBr lignin, acetate content, and enzymatic saccharification was conducted. Any residual non-cellulosic polysaccharides were cleaved with TFA, and the

monosaccharides were quantified using HPAEC-PAD.

2.4. Statistical analysis

Based on the results of Levene's test for homogeneity of variances and Shapiro-Wilk test to assess normality, parametric analysis was chosen for inferential analysis. Differences in cell wall composition between genotypes and fertilization, as well as their interaction (genotype: fertilization), were assessed for each condition (unprocessed, low, and high OrganoCat severity) by variance analysis of two-way ANOVA type III for an unbalanced size population. Etha squared (η^2) were calculated for the percentage of variance explained by genotype/fertilization/genotype:fertilization. Tukey's Honestly Significant Difference (HSD) test was chosen as a post-hoc test ($p < 0.05$). A t-test was used for differences between low and high OrganoCat severity. Pearson correlations were conducted ($p < 0.5$) in unprocessed and treated variables. Heatmaps were constructed using the mean values of normalized data for all yield and lignocellulosic parameters. A dendrogram generated through hierarchical clustering was incorporated based on these parameters and the values were reordered accordingly. A Principal Component Analysis (PCA) for each condition was performed using cell wall parameters for raw biomass or pulp from OrganoCat. Hierarchical clustering was made for clustering genotypes and fertilization. The statistical analyses were carried out using an open-source programming language R (R: The R Project for Statistical Computing, n.d).

3. Results and discussion

3.1. Cell wall characterization

Stem samples of poplar trees from three hybrids were analyzed for their biochemical cell wall composition under different nitrogen fertilization This includes their crystalline cellulose content (CrC), acetyl groups (Acetyl), acetyl-bromide-soluble lignin (AcBr lignin), and sugars hydrolyzed by Trifluoroacetic acid (total TFA), expressed as a percentage of the de-starched alcohol-insoluble residue (dAIR) of the biomass. Additionally, the ratio of hexoses to pentoses (C5:C6) in the TFA hydrolysate was calculated. Enzymatic saccharification rate was also determined in units of % of glucose released per glucan after 72 h. Average values and their standard deviation are shown in Table 1. Given that the dataset fulfills normal distribution assumptions (homogeneity of variances and normality), significant differences were determined by parametric tests.

The objective of this initial characterization was to establish a baseline for assessing the inherent variation present in the dataset prior to processing by the OrganoCat system.

Among several biochemical cell wall parameters, CrC showed the most significant variation among all poplar genotypes and N fertilization, ranging from 34.5 % in AF6 to 49.4 % in Max4. As the main constituent of lignocellulosic biomass, CrC had the highest concentration, which led to more apparent and significant differences in the dataset, resulting in greater variations compared with other biochemical cell wall parameters. A similar trend in fertilization was found in AF6 and Max4, where NN showed the highest content compared to NH and NO. A similar level of cellulose was reported in other poplar species by du Pasquier et al. (2024). The acetyl content did not show much variation, especially between fertilization. However, a contrast was observed in Max4, which had the lowest content, whereas AF6 and Vesten displayed similar content. Notably, high acetylation tended to result in low cellulose content, as can be seen in Max4, which has the highest CrC content and lowest acetyl content. The contents of AcBr lignin and TFA hydrolysate, had a minor variation in the dataset, with no significant differences. Among all poplars, xylose was the most predominant sugar in the TFA fraction (Supplementary file), ranging from 11.7 % in AF6 to 15.5 % in Max4. This is consistent with the results reported by du Pasquier et al. (2024), where Poplar hybrids (*P. tremula x P. alba*) had around 17 % of xylose. Enzymatic saccharification showed significant variation in the dataset, fluctuating from 21.3 % in AF6 to 36.4 % in Vesten, where AF6 differed significantly from the other genotypes. However, no systematic differences were found between the fertilization.

Overall, Max4 showed the highest CrC, AcBr lignin, and total sugar (TFA fraction) contents, while it exhibited the lowest acetyl content. In contrast, AF6 had the lowest CrC and AcBr lignin contents. As for total sugar content, Vesten had the lowest content. Among the fertilization within a genotype, differences were observed in CrC, enzymatic saccharification, and sugars from the TFA fraction, whereas NN in Max4 differed significantly from NH and NO.

3.2. Biomass OrganoCat processed at different severities

Biomass from the different genotypes and fertilization was processed using the OrganoCat system to evaluate its yield and lignocellulosic parameters, and to study its recalcitrance.

A general examination of the obtained yield data for all variants (processing severity, genotypes, and fertilization) showed only a modest level of variation (Table 2).

The average OrganoCat yields showed that under low severity, the lignin yield was 6.97 % of the original biomass, the pulp yield was 74.03 %, and the sugar yield was 6.67 %. Under high OrganoCat severity, the lignin yield increased to 12.63 % and the sugar yield increased to 32.89 %, resulting in a decrease in the pulp yield to

Table 1
Content of cell wall characterization of 3 genotypes of poplar under 3 different N fertilization. Values are presented as mean ± standard deviation. CrC: crystalline cellulose; AcBr lignin: acetyl bromide soluble lignin; total TFA: total content of hydrolyzed sugars; C5:C6: ratio between pentoses and hexoses; enzymatic saccharification rate after 72 h of reaction time. Glucan: sum of CrC and glucose from TFA hydrolysis. NH: ammonium (NH_4^+), NN: ammonium nitrate (NH_4NO_3), NO: nitrate (NO_3). AF6 NH, NN, NO n= 9; Max4 NH n=12, Max4 NN n=13, Max4 NO n=11; Vesten NH, NN n=9, Vesten NO n=7. Significance differences are indicated by letters (a, b, c) based on two-way ANOVA type 3 followed by Tukey's HSD test ($p < 0.05$).

	AF6			Max4			Vesten		
Parameters	NH	NN	NO	NH	NN	NO	NH	NN	NO
CrC [% wt. dAIR]	34.7 ± 3.1^{b}	39.6 ± 5.4^{b}	34.5 ± 4.5^{b}	41 ± 5.6^{a}	49.4 ± 3.8^{a}	42.2 ± 5.9^{a}	38.7 ± 4.4^{ab}	35.7 ± 5.4^{b}	44.5 ± 7.6^{a}
Acetyl [% wt. dAIR]	4.7 ± 0.6^{a}	4.7 ± 0.4^{a}	4.6 ± 0.3^{a}	3.7 ± 0.4^{b}	3.7 ± 0.3^{b}	3.8 ± 0.4^{b}	4.6 ± 0.3^{a}	4.3 ± 0.8^{a}	4.7 ± 0.5^{a}
AcBr lignin [% wt. dAIR]	18.6 ± 2.1^{b}	19.7 ± 2.7^{a}	21.4 ± 1.8^{a}	22.8 ± 3.4^{a}	21.4 ± 2.8^{a}	22.1 ± 3^{a}	20.7 ± 1.5^{ab}	19.8 ± 1.6^{a}	20.5 ± 3.1^{a}
Total TFA [% wt. dAIR]	17.8 ± 1.4^{b}	18.6 ± 2.1^{b}	16.9 ± 1.1^{b}	21.0 ± 2.5^{a}	22.1 ± 2.1^{a}	21.1 ± 1.8^{a}	16.3 ± 2.7^{b}	17.3 ± 1.7^{b}	16.8 ± 1.1^{b}
C5:C6	4.0 ± 0.3	4.2 ± 0.7	4.2 ± 0.4	4.2 ± 0.4	3.9 ± 0.4	4.0 ± 0.3	4.3 ± 0.4	3.8 ± 0.4	3.8 ± 0.4
Enzymatic [%Glc/Glucan]	21.3 ± 3.8^{b}	23.4 ± 4.5^{b}	23.5 ± 6.2^{b}	32.1 ± 6.1^{a}	27.7 ± 3.2^{b}	35.1 ± 6.4^{a}	28.2 ± 4.7^{a}	36.4 ± 8.4^{a}	31.9 ± 4.7^{a}

Table 2
Yield of poplar biomass (genotype and N fertilization) at low and high OrganoCat severities, in % wt. of original biomass. Values are presented as mean ± standard deviation. Pulp yield refers to the solid residue after OrganoCat processing. Hydrolysate (monosaccharide) yield is determined as the content of reducing sugars present in the aqueous phase. Extractives (lignin) yield was determined as the weight of the remaining residues after 2-MTHF evaporation of the organic phase. Mild OrganoCat: 125°C, 1 h; high OrganoCat: 140°C, 3 h. AF6 NH, NN, NO n= 9; Max4 NH n=12, NN n=13, NO n=11; Vesten NH, NN n=9, NO n=7. Significance differences are indicated by letters (a, b) based on t-test ($p < 0.05$).

Yield [% w. orig. b.]	Low OrganoCat	High OrganoCat
Extractives (Lignin)	7 ± 1.8[a]	12.6 ± 1.4[b]
Pulp	74 ± 2.2[a]	51.6 ± 1.4[b]
Hydrolysate (Monosaccharides)	6.7 ± 1.2[a]	32.9 ± 8.4[b]

51.65 %. These findings are consistent with our earlier research (Martinez Diaz et al., 2023; Schrey et al., 2023), exhibiting an increase in sugar and lignin yield in the same order of magnitude.

The obtained OrganoCat product streams pulp and hydrolysate were further analyzed on their chemical composition to investigate whether qualitative differences between the genotypes (AF6, Max4, Vesten) and fertilizer regimes (NO, NH, NN) could be identified. The remaining solid pulp was analyzed based on its lignocellulosic composition, determining the same biochemical parameters as for the unprocessed biomass, including for the pulp monosaccharide composition after TFA hydrolysis, CrC, AcBr lignin, acetyl content, and enzymatic saccharification, and for the OrganoCat hydrolysate the monosaccharide composition (Supplementary file). To visualize the obtained results, heatmaps were created (Fig. 1), and clustered by parameters (rows-dendrogram). For the data obtained at low severity (Fig. 1A), the lowest residual sugar values in the hemicellulose fraction (TFA) were observed in Max4 NO and NN, whereas they showed the highest values for enzymatic saccharification and CrC. At high severity (Fig. 1B), Vesten NO had the lowest values for the monosaccharides from the hydrolysate. Moreover, Vesten NO also had the lowest values in almost all parameters, except for enzymatic saccharification rate, CrC, and xylose content in the pulp, where it had the highest values among all poplars, indicating low recalcitrance.

This analysis already showed which parameters exhibited statistical similarities and which poplar hybrid grown under which N regime were more processable under OrganoCat severities. At low severity, the parameter clustering was sectioned and ordered. CrC, enzymatic saccharification, and glucose were grouped together, whereas the remaining lignocellulosic parameters were classified into another cluster, and OrganoCat yields comprised a latter cluster. In contrast, at high severity, monosaccharides from the hydrolysate are not clustered together. Nevertheless, under both severities, the total monosaccharide content in the hydrolysate, including glucose and xylose, remained grouped together. Similarly, CrC, enzymatic saccharification, and residual glucose in the pulp cluster, implying that these factors remain interconnected regardless of the process severity and, that the hydrolysate yield and its sugar composition vary based on the OrganoCat parameters.

These analyses were conducted using normalized data to provide a comprehensive overview and identify the statistical relationship between the investigated parameters.

3.2.1. *Multivariate analysis of the lignocellulosic recalcitrance*

To analyze the impact of specific patterns among cell wall composition of different poplar genotypes and fertilization on the recalcitrance towards the OrganoCat processing, Principal Component Analyses (PCA's) were performed using normalized data from the lignocellulosic characterization (Fig. 2).

For unprocessed biomass (Fig. 2A), the clustering of poplars was primarily based on their genotype, with no significant differences observed among fertilization. However, an exception was noted for Max4, which did not cluster with the other Max4 when fertilized with NN, indicating that fertilization with NN, NO, and NH may have distinct effects on this particular genotype.

Variability in the unprocessed biomass data is mainly explained by the first two components, which collectively explain 81.5 % of the total

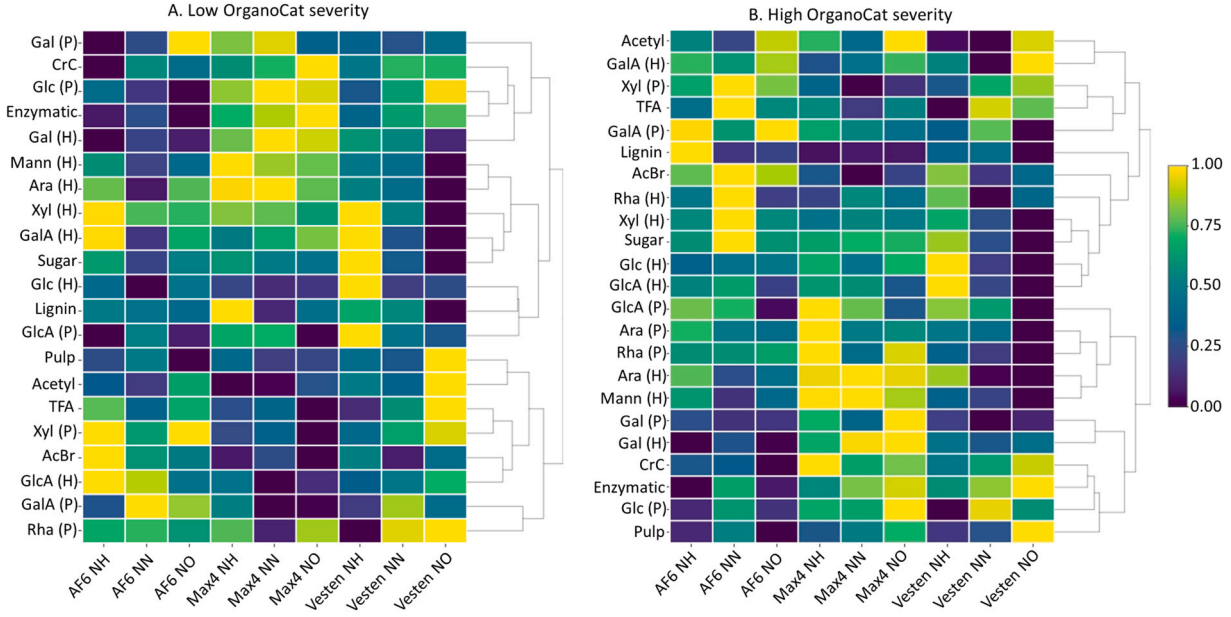

Fig. 1. Heatmap of OrganoCat processability for 3 genotypes of poplar with different fertilization under low and high OrganoCat severity. Normalized data from mean values of OrganoCat yields and lignocellulosic parameters by color gradient. Pulp: solid residue after OrganoCat processing. Sugar: content of reducing sugars in the aqueous phase. Lignin: amount of lignin extracted from the organic phase. CrC: crystalline cellulose in pulp, AcBr lignin: acetyl bromide lignin in pulp, Acetyl: acetyl content in pulp, Enzymatic: enzymatic saccharification rate in pulp, TFA: total content of sugars from the pulp, Xyl: xylose, Glc: glucose, Ara: arabinose, Mann: mannose, Gal: galactose, Rha: rhamnose, GalA: galacturonic acid, GlcA: glucuronic acid in pulp (P) or in hydrolysate (H).

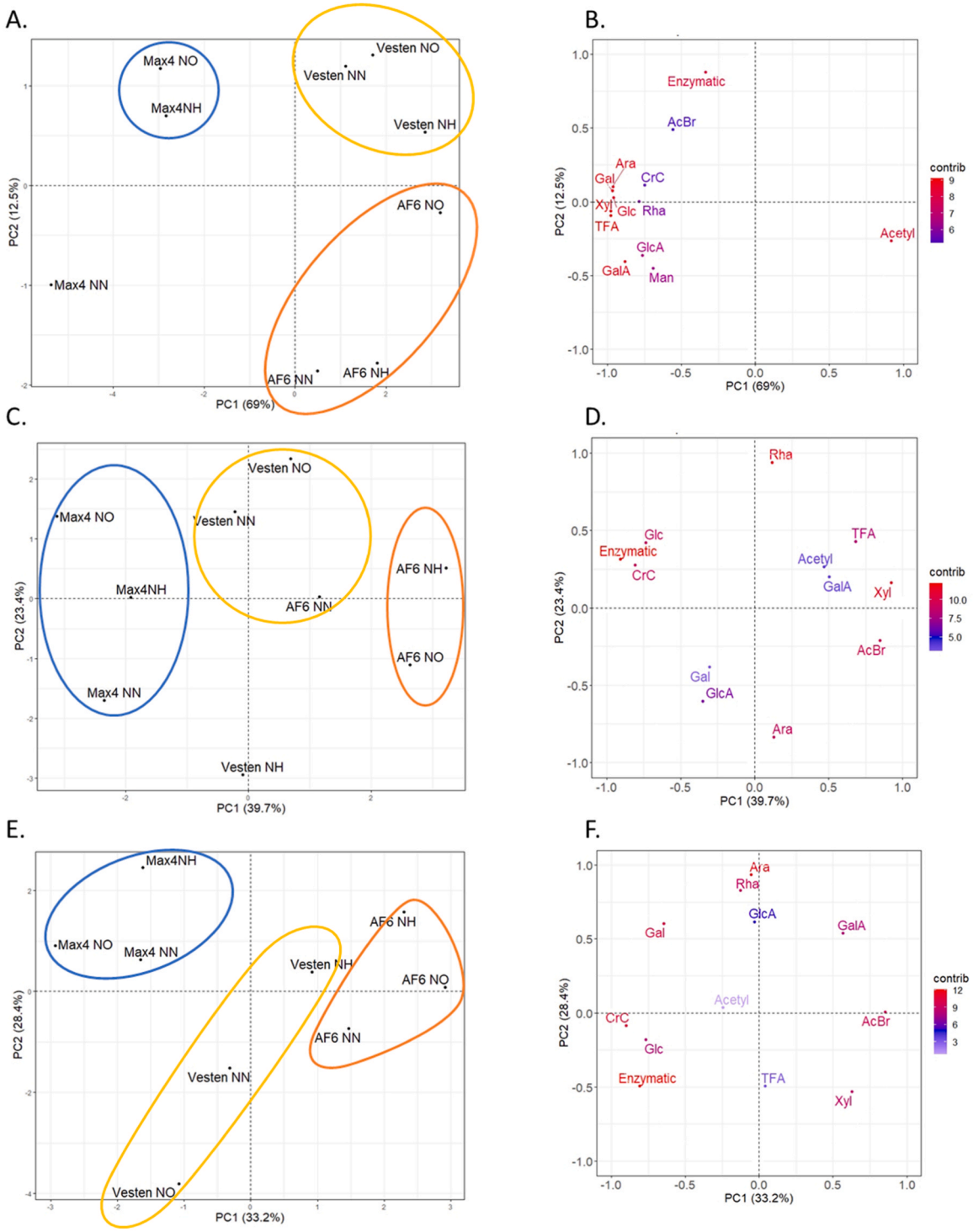

Fig. 2. Principal Component Analysis (PCA) of cell wall parameters from 3 genotypes of poplar fertilized with 3 different nitrogen forms. Loading plots and Scores plot on the first two components in unprocessed biomass (A/B) and pulp from low (C/D) and high (E/F) OrganoCat severities. Color gradient indicated percentage of contribution from parameters from each PC, red-greater percentage, lilac-lower percentage. 11 parameters were used for unprocessed biomass and 12 parameters for OrganoCat pulp, based on normalized data from mean values. AF6 NH, NN, NO n= 9; Max4 NH n=12, Max4 NN n=13, Max4 NO n=11; Vesten NH, NN n=9, Vesten NO n=7.

variance in the data. PC1 was predominantly composed of xylose and total sugar content (TFA fraction). In contrast, enzymatic saccharification was a key factor in PC2.

Upon processing the biomass (Fig. 2C and Fig. 2E), the clustering pattern changed, particularly between OrganoCat severities. Notably, at low severity (Fig. 2C), Vesten NH emerged as an outlier and AF6 NH and NO showed similarities based on this characterization. 63.1 % of variance can be attributed to PC1 and PC2, with the largest contribution from enzymatic saccharification, xylose content, and CrC in PC1, whereas PC2 was mainly influenced by arabinose and rhamnose content (Fig. 2D). At high severity (Fig. 2E), poplars are grouped by genotype in this analysis, similar to the initial unprocessed poplar. In addition, Max4 showed little difference in all nitrogen fertilization after OrganoCat processing, indicating that fertilization has little effect on cell wall

structure and recalcitrance in this hybrid. A total of 61.6 % could be attributed to PC1 and PC2, similar to that for low severity. Enzymatic saccharification and CrC remained the major contributors to PC1, whereas arabinose and rhamnose were the major contributors to PC2 (Fig. 2F). Nevertheless, the score plots for low and high OrganoCat severities (Fig. 2D and Fig. 2F, respectively) showed a greater scattering of lignocellulosic parameters than the unprocessed biomass (Fig. 2B), suggesting that the relationship between parameters and components after OrganoCat processing is more intricate and not as straightforward as in the case of unprocessed biomass.

The principal component analyses revealed that CrC and enzymatic saccharification are important discriminating factors in this setup. Subsequently, these two parameters were examined more closely, as shown in the following graph (Fig. 3).

A depicts the CrC content, grouped by processing conditions and fertilization and color-coded-coded by genotype. Following the OrganoCat fractionation process, the CrC content exhibited a gradual increase, fluctuating between 45.7 % and 51.5 % at low severity, and between 64.3 % and 71.2 % at high severity. OrganoCat processing disintegrates hemicelluloses and removes lignin, the cellulose remains in the pulp. Poplar generally performed as other biomasses under similar severities (Martinez Diaz et al., 2023; Schrey et al., 2023). As for the genotypes, Max4 showed the highest CrC content and AF6 had the lowest levels under both OrganoCat severities. Higher variation was noted in unprocessed poplars, but this diminished after biomass was subjected to OrganoCat processing, suggesting that the produced pulp exhibits less compositional differences than the initial untreated biomass when comparing different genetic backgrounds and nutritional variations on the final product. The results of enzymatic saccharification are shown in Fig. 3B. At low severity, poplars treated with NH showed a

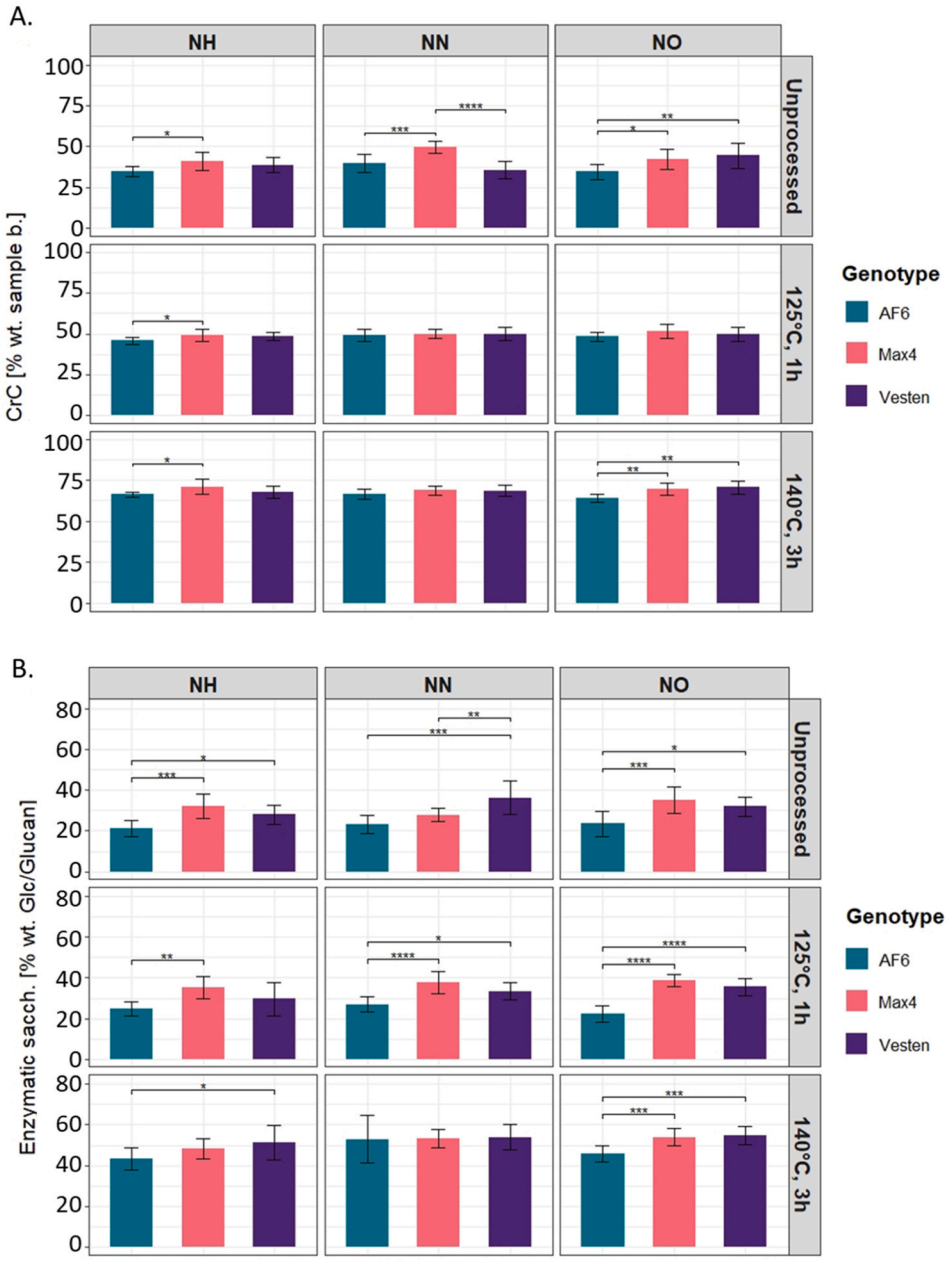

Fig. 3. Composition of poplars from 3 genotypes under 3 different N fertilization in unprocessed biomass and pulp under low (125°C, 1 h) and high (140°C, 3 h) OrganoCat severities based on A. Crystalline cellulose content, in units of relative percentages and B. Enzymatic saccharification, in units of percentage of glucose released per glucan, mg/mg. NH: ammonium (NH4+), NN: ammonium nitrate (NH4NO3), NO: nitrate (NO3-). 2-way ANOVA and Tukey HSD test (p-value *0.05, **0.01,***0.001). AF6 NH, NN, NO n= 9; Max4 NH n=12, Max4 NN n=13, Max4 NO n=11; Vesten NH, NN n=9, Vesten NO n=7.

slight increase in enzymatic yield, ranging now from 27.0 % to 37.8 % Glc/glucan. In fertilization with NH and NO, the trend among genotypes remained largely unchanged, but in NN fertilization, an increase in yield was observed for Max4. Notably, this genotype appeared to be more prone to glucose release as enzymatic saccharification increased in all fertilization. At high severity, all poplars demonstrated a considerable increment in enzymatic yield, oscillating between 64.0 % to 71.2 % Glc/glucan. The trend remained the same between NH and NO fertilization, having AF6 at the lowest rate.

Pretreated biomass from the poplars exhibited less variance in CrC, resulting in a nearly slim trend between genotypes in each fertilization. For example, Max4 exhibited the highest content in unprocessed biomass, while AF6 had the lowest. However, after processing, the genotypes show similar content levels. As for enzymatic saccharification, variation remains more evident under low severity but becomes less pronounced at higher severity. Nonetheless, at high severity for both parameters, variation is reduced in the dataset, which indicates that the impact of genetic and nutritional variations in poplar pulp is reduced, emphasizing the importance of processing severity over inherent variations.

3.2.2. Sugar analysis from OrganoCat processing in pulp and hydrolysate yield

To determine the potential relationships in the yield and lignocellulosic parameters of poplar pulp under both severities, Pearson correlations were calculated. Our findings revealed consistent trends in poplar based on hemicellulose composition (Fig. 4).

In the pulp, a positive correlation was observed between the total TFA sugar and xylose content at low severity (Fig. A). This correlation was expected because xylose is the main building block of xylan as the dominant polysaccharide of hemicellulose fraction. Consequently, it takes longer to completely hydrolyze it due to its higher concentration compared to that of other sugars. These other sugars did not significantly affect pulp composition, as indicated by the lack of strong correlations, as they had already undergone hydrolysis, and xylose is left disproportionally high. This finding supports the correlations shown in Fig. B, which had a greater influence on the total hydrolysate content (Sugar). Despite this, xylose and glucose maintained the strongest correlations as they were the main constituents of the biomass.

At high severity, more positive correlations were observed for the remaining sugars in the pulp (Fig. C), with xylose and glucose being the predominant sugars and exhibiting a stronger correlation with the total sugar content. Similarly, the total content was positively correlated with xylose and glucose levels in the hydrolysate (Fig. D). Overall, some minor sugars showed greater fluctuations in their correlations However, xylose plays a crucial role in the composition of sugars hydrolyzed during OrganoCat. Therefore, a more in-depth look is provided in Figure, depicting the xylose proportion in the unprocessed biomass and OrganoCat pulp to evaluate its processability after OrganoCat Fig. 5.

Figure shows the proportion of xylose based on the total sugar

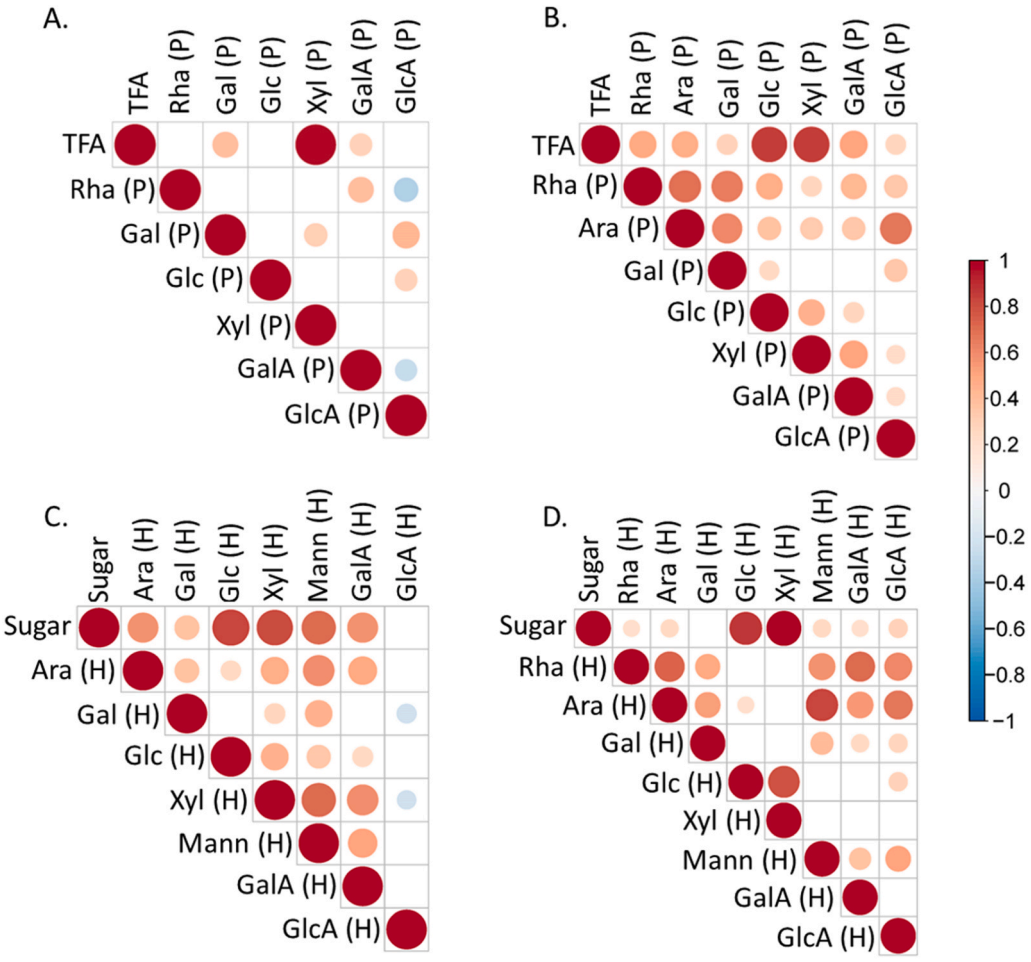

Fig. 4. Pearson correlations of sugar composition in pulp and hydrolysate after (A/B) low and (C/D) high OrganoCat severities. Positive correlations are shown in red and negative correlations in blue. The diameter of the circle represents the magnitude of correlation ($p < 0.5$). TFA: total sugar content from TFA hydrolysis; Rha: rhamnose; Ara: arabinose; Gal: galactose; Glc: glucose; Xyl: xylose; Man: mannose; GalA: galacturonic acid; GlcA: glucuronic acid in pulp (P) or in hydrolysate (H). Sugar: total sugar content in hydrolysate phase.

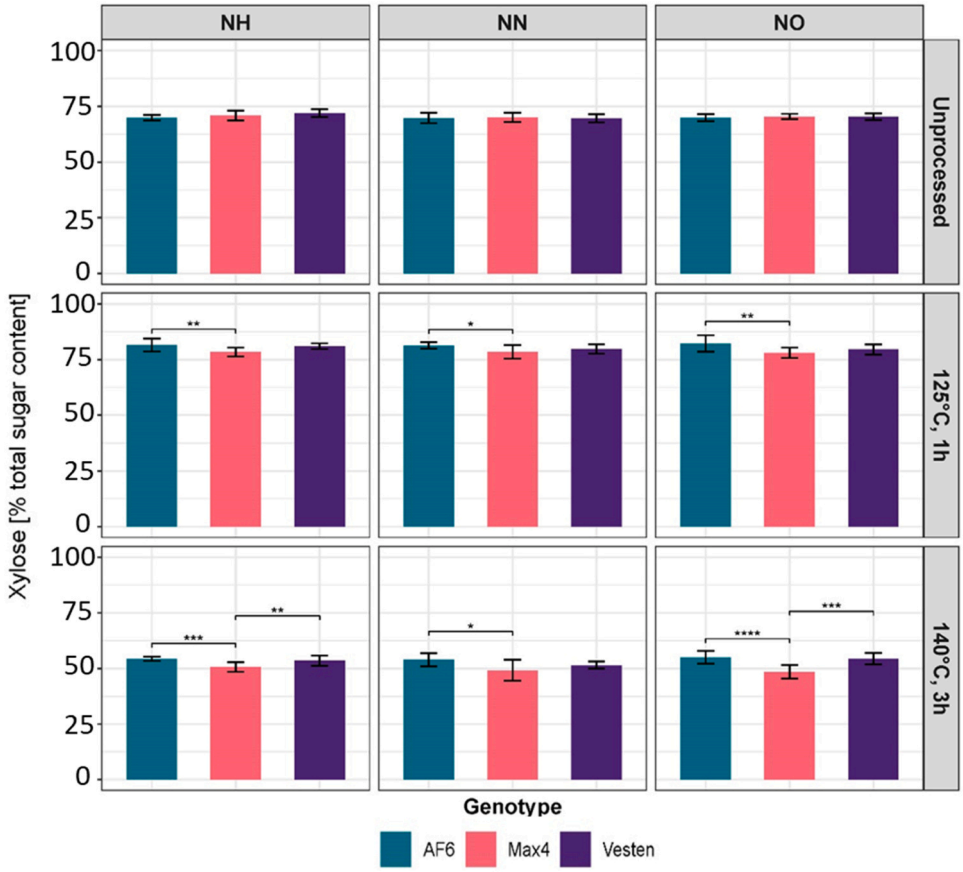

Fig. 5. Xylose content in the TFA-fraction of poplars from 3 genotypes under 3 different N fertilization in unprocessed biomass and in pulp at low (125°C, 1 h) and high (140°C, 3 h) OrganoCat severities. In units of percentage of xylose in total sugar content. NH: ammonium (NH_4^+), NN: ammonium nitrate (NH_4NO_3), NO: nitrate (NO_3^-). 2-way ANOVA and Tukey HSD test (p-value *0.05,**0.01,***0.001). AF6 NH, NN, NO n= 9; Max4 NH n=12, Max4 NN n=13, Max4 NO n=11; Vesten NH, NN n=9, Vesten NO n=7. Detailed Proportion of all sugars in the untreated biomass (Table B), the pulps at low and high severity (Table D) as well as the OrganoCat hydrolysate (Table C) can be found in the supporting information.

composition of the poplar biomass or pulp. Xylose compromised approximately 70 % of sugars in the TFA-fraction of the unprocessed biomass. A similar percentage was obtained for all genotypes and fertilization. After OrganoCat processing at low severity, in the pulp, 78–82 % of TFA-fraction consisted of xylose. Overall, there was an approximately 10 % increase in xylose in the TFA-fraction compared to unprocessed biomass. This was because all minor sugars were hydrolyzed and transferred to the hydrolysate, whereas xylose remained unhydrolyzed, which led to an increase in the proportion of xylose in the pulp. At high severity, the percentage of xylose was between 48 % and 55 %, suggesting that xylan underwent hydrolysis and was removed from the pulp, as was also the case for du Pasquier et al. (2024), at their higher severities in dilute acid pretreatment. Despite this, it remains one of the primary sugars present in the pulp, along with glucose, although at significantly lower levels (approximately 1–5 % relative weight).

The general trend observed in the xylose proportion in the pulp, based on the genotype following OrganoCat processing, was consistent at both severities. Specifically, Max4 showed a slightly lower proportion of xylose than AF6 at high severity. It seems that in Max4, xylan is more likely to be hydrolyzed than in AF6. This could be to the fact that xylose proportion in Max4 (15 %) is higher than in AF6 (12 %) and Vesten (12 %). But generally, after processing with OrganoCat at low severity, xylose levels remained largely unchanged in the pulp. Only at severe conditions, there was a marked reduction of xylose present in the pulp; and the proportion of xylose to other sugars in the pulp was reduced (Supplementary file).

4. Conclusions

This study investigated the impact of plant genotype, N fertilization and process severity on the recalcitrance of lignocellulose from young poplars grown at controlled greenhouse conditions towards OrganoCat processing. The genotype influences most of the parameters associated with the cell wall, indicating its predominant role in shaping the composition of the biomass. This influence is particularly pronounced in unprocessed biomass, where there is a notable interaction between genotype and N fertilization, especially in hemicellulose sugars derived from the TFA fraction. However, the chosen high process severity can diminish the overall compositional variation in OrganoCat-processed poplar pulps. Lignin and pulp yield exhibit minimal variation in response to genotype and fertilization individually, but their interaction becomes more pronounced at high OrganoCat severities.

The outcomes indicate that the consequences of higher OrganoCat processing are more pronounced than the initial variation in cell wall traits for the poplar genotypes when subjected to varying N fertilization. The results indicate that OrganoCat processing significantly reduces the impact of genetic and nutritional variations in this poplar set. This is evidenced by the observed decrease in variation following fractionation process.

CRediT authorship contribution statement

Holger Klose: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Conceptualization.

Philipp M Grande: Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Jimena Martines Diaz:** Writing – original draft, Visualization, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2024.119820.

Data Availability

Data will be made available on request.

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