

Pronounced lignin variation in a decades-old ex vitro collection of *Cereus hildmannianus* somaclones

Silvana Alves Rosa

Universidade Estadual de Maringá (UEM)

Felipe Francisco Santos Dias

Universidade Estadual de Maringá (UEM)

Diego Eduardo Romero Gonzaga

Universidade Estadual de Maringá (UEM)

Maria de Fátima Pires da Silva Machado

Universidade Estadual de Maringá (UEM)

Claudete Aparecida Mangolin

mangolimca@gmail.com

Universidade Estadual de Maringá (UEM)

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Abstract

Long-term collections derived from callus-mediated plant regeneration offer an opportunity to characterize biochemical heterogeneity decades after establishment under ex vitro conditions. We quantified lignin content in 17 individual somaclones from a historical collection originally described as *Cereus peruvianus* and referred to here as *C. hildmannianus*. The plants were regenerated through a callus phase, maintained ex vitro for several decades, and previously characterized for morphological and molecular variation. Lignin was determined in dried stem material using the acetyl-bromide method; repeated analytical determinations were used to assess measurement precision. Mean lignin content ranged from 28.61 mg g⁻¹ PCIP in SC15 to 62.46 mg g⁻¹ PCIP in SC29, representing a 2.18-fold difference between the observed extremes. The collection therefore exhibits substantial contemporary heterogeneity in a cell-wall biochemical trait, complementing its previously documented morphological and molecular diversity. These findings do not establish whether lignin differences arose during tissue culture or persisted unchanged over subsequent decades. The contrasting individuals provide a basis for future investigations of cell-wall composition and biomass processing.

Key Message

A decades-old collection of callus-derived somaclones exhibits 2.18-fold variation in lignin content, revealing biochemical heterogeneity in plants previously characterized for morphological and molecular diversity.

1. Introduction

Cactaceae comprise a diverse group of succulent plants with remarkable morphological and physiological adaptations to environments characterized by water limitation, high temperatures, and other abiotic constraints. These characteristics have renewed interest in cacti as plant genetic resources in the context of climate change, crop diversification, and the development of more resilient production systems (Khan et al. 2024). Beyond their ecological importance, several cactus species provide fruits, cladodes, mucilage, fibers, and phytochemicals with nutritional, functional, and technological applications. Nevertheless, much of this diversity remains underutilized when compared with a small number of commercially established cactus crops (Araújo et al. 2021; Khan et al. 2024).

Among South American columnar cacti, *Cereus hildmannianus* K. Schum. has attracted interest because of the chemical diversity of its tissues and its potential for food, pharmaceutical, biotechnological, and biomass-related applications. Its cladodes contain structurally and functionally relevant polysaccharides and other metabolites, while different fractions of the plant have been investigated as sources of compounds of potential technological value (Araújo et al. 2021). More recently, the polysaccharide composition of the cell wall of *C. hildmannianus* has also been examined from the perspective of biomass utilization, reinforcing the interest in this cactus as a non-conventional plant resource (Corrêa et al. 2022).

The historical somaclonal collection investigated in this study was originally reported under the name *Cereus peruvianus* Mill. (Oliveira et al. 1995; Mangolin et al. 1997). Subsequent research on related in vitro-propagated material has employed the name *Cereus hildmannianus* K. Schum. (Corrêa et al. 2022). Because the taxonomic identity of the present collection has not been independently reassessed, we retain the historical identification when referring to the original studies and use *C. hildmannianus* provisionally for the material examined here, following the nomenclature adopted in the more recent literature. This usage does not imply a formal taxonomic redetermination or establish synonymy between the two names.

A particularly valuable resource associated with this material is a collection of plants regenerated through callus culture and maintained under ex vitro conditions for several decades. Earlier studies documented considerable phenotypic heterogeneity among regenerated plants. Morphological and isozyme differences were evident in young plants (Mangolin et al. 1997), and subsequent studies reported further somaclonal variation (Ruvolo-Takasusuki et al. 2006; Mangolin et al. 2009). Microsatellite analysis also documented molecular diversity among somaclones with typical and atypical morphologies (Martin et al. 2018). Together, these findings make the collection a valuable system for examining phenotypic and molecular diversity long after regeneration and field establishment.

The biochemical dimension of this long-term somaclonal diversity has received less attention than its morphological and molecular components. Cell-wall traits are particularly relevant because their contemporary variation can be documented even when genetic, epigenetic, developmental, and environmental contributions cannot be separated by a single sampling event. Previous work on cell-wall polysaccharides in *C. hildmannianus* reported differences between propagated and conventionally cultivated material (Corrêa et al. 2022). However, lignin content has not been characterized across individual plants in this historical collection.

Lignin is a structurally complex phenolic polymer of plant secondary cell walls and a major determinant of the properties and processing behavior of lignocellulosic biomass. Its abundance and structural characteristics contribute to biomass recalcitrance and influence the accessibility of cell-wall polysaccharides during biomass deconstruction. At the same time, lignin is increasingly regarded not merely as an obstacle to biomass processing but as a renewable aromatic resource with potential applications in fuels, chemicals, polymers, and advanced materials (Brienza et al. 2024; Dixon et al. 2024). This dual role makes variation in lignin content relevant both to biomass deconstruction and to integrated biorefinery strategies.

In this context, lignin provides a useful biochemical trait with which to ask whether the diversity already documented in this decades-old somaclonal collection is also expressed at the level of cell-wall composition. Contrasting lignin phenotypes would broaden the biological characterization of the collection and, secondarily, identify materials for subsequent functional studies. Plants at the lower end of the lignin distribution could be tested for pretreatment efficiency and enzymatic saccharification,

whereas lignin-rich materials could be examined for lignin recovery and valorization. These applications are prospective and cannot be inferred from lignin concentration alone.

Here, we characterized lignin content in 17 long-established *C. hildmannianus* somaclones from this historical collection. Our objective was to determine whether its previously documented morphological and molecular heterogeneity is accompanied by contemporary differences in lignin content after decades of ex vitro maintenance. We also identified individuals with contrasting lignin contents for future cell-wall and biomass-processing studies. These measurements cannot determine whether the differences originated during in vitro regeneration or remained stable over time.

2. Materials and methods

2.1. Origin and long-term establishment of the somaclonal collection

The plant material belongs to a historical somaclonal collection originally described as *Cereus peruvianus* Mill. and provisionally referred to here as *C. hildmannianus* K. Schum., as explained in the Introduction. The regeneration program began in the 1980s with seedlings obtained by in vitro germination of seeds collected from a single wild mother plant. Cladode segments from these seedlings were used as explants for callus induction and subsequent plant regeneration (Oliveira et al. 1995).

Callus induction and regeneration were performed on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg et al. 1968), agar (0.8%), sucrose (3.0%), 4.0 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin at concentrations of 4.0, 6.0, or 8.0 mg L⁻¹ (Oliveira et al. 1995). Following regeneration in 1987, the plants were acclimatized under controlled laboratory conditions, transferred to pots for ex vitro development, and subsequently established in the Experimental Botanical Garden of the State University of Maringá (UEM), Maringá, Paraná, Brazil (approximately 554.9 m altitude; 23°25' S, 51°25' W), where they have been maintained under field conditions for several decades.

The somaclones share a maternal origin, chronological age, and cultivation history. However, because the original seedlings developed from sexually produced seeds, the regenerated plants should not be regarded as genetically identical. Earlier studies documented morphological, isozyme, and molecular variation in the collection (Mangolin et al. 1997; Ruvolo-Takasusuki et al. 2006; Mangolin et al. 2009; Martin et al. 2018).

2.2. Plant material, sampling and sample preparation

Seventeen individual somaclones from the historical collection were analyzed: SC8, SC9, SC10, SC11, SC12, SC14, SC15, SC17, SC29, SC32, SC55, SC56, SC56.1, SCC, SCE, SCG1, and SCI. Each identifier denotes one independently maintained plant and therefore one biological unit for descriptive comparisons. All 17 plants were of the same chronological age, originated from seeds collected from

the same mother plant, and had been maintained in the same experimental garden for several decades. This shared history limits differences in age, geographic origin, and cultivation history, although genotypic, developmental, and local environmental differences cannot be excluded.

Stem (cladode) material was collected from all 17 somaclones during the summer of 2023 at the Experimental Botanical Garden of UEM. Approximately 500 g of cladode material was collected separately from each plant. Spines were removed, and the material was washed with distilled water and cut into smaller pieces. Samples were dried in a forced-air oven at 55°C for 72 h, ground in a ball mill, and stored as powder in glass containers at room temperature until analysis.

2.3. Preparation of the protein-free cell-wall fraction

The cell-wall fraction used for lignin determination was prepared following the washing procedure described by Moreira-Vilar et al. (2014). For each analytical determination, 0.08 g of dried, ground material was transferred to a microcentrifuge tube. Samples were subjected to sequential washing steps, each followed by centrifugation at $8,000 \times g$ for 5 min: five washes with 1.6 mL sodium phosphate buffer (50 mM, pH 7.0), five washes with 1.6 mL 1% (v/v) Triton® in phosphate buffer (50 mM, pH 7.0), ten washes with 1.6 mL 1 M NaCl in phosphate buffer (50 mM, pH 7.0), ten washes with 1.6 mL distilled water, and two washes with 1 mL acetone. The resulting pellet was dried in a forced-air oven at 60°C for 24 h. The dried material was designated the protein-free cell-wall fraction (PCIP) and was used for lignin determination.

2.4. Acetyl-bromide lignin determination

Total lignin was determined using the acetyl-bromide method adapted from Moreira-Vilar et al. (2014). An aliquot of 0.02 g of PCIP was transferred to a screw-cap glass tube and mixed with 0.5 mL of 25% (v/v) acetyl bromide in chilled acetic acid. The reaction mixture was incubated in a water bath at 70°C for 30 min and then rapidly cooled in an ice bath. Subsequently, 0.9 mL of 2 M NaOH, 0.1 mL of 7.5 M hydroxylamine-HCl and 3 mL of acetic acid were added. After centrifugation at $1,400 \times g$ for 5 min, the absorbance of the supernatant was measured at 280 nm using a Shimadzu UV-2450 spectrophotometer. Lignin content was quantified using a calibration curve prepared with commercial lignin (Aldrich, product no. 37,096 – 7), with the reported calibration range of 0–75 mg lignin g⁻¹ PCIP. Results were expressed as mg lignin g⁻¹ PCIP.

Four analytical determinations were available for 14 somaclones, whereas three were available for SC15, SC29, and SC32. These measurements were used to assess analytical precision and were not treated as independent biological replicates.

2.5. Data treatment and statistical analysis

For each somaclone, analytical determinations were summarized as the mean, standard deviation (SD), and coefficient of variation (CV). Each of the 17 somaclones represents a unique biological individual; replication within a somaclone was therefore not part of the study design. Repeated measurements assessed analytical precision rather than biological variability. Accordingly, we did not perform inferential

tests that would treat analytical determinations as independent biological observations. Variation among the 17 individuals was described using the range, median, mean, SD, and CV of somaclone means. Individuals were ranked by mean lignin content.

3. Results

3.1. Variation in lignin content among long-term established somaclones

Lignin content varied markedly across the 17 long-term established somaclones (Table 1; Fig. 1). Mean values ranged from 28.61 mg g⁻¹ PCIP in SC15 to 62.46 mg g⁻¹ PCIP in SC29, corresponding to an absolute range of 33.85 mg g⁻¹ and a 2.18-fold difference between the lowest and highest observed means. Across the 17 individual somaclones, the mean lignin content was 46.82 mg g⁻¹ PCIP, the median was 47.86 mg g⁻¹ PCIP, and the standard deviation among somaclone means was 9.85 mg g⁻¹ (CV = 21.0%).

The lowest mean lignin contents were observed in SC15 (28.61 mg g⁻¹), SCC (32.45 mg g⁻¹), SCI (35.28 mg g⁻¹), SC55 (35.92 mg g⁻¹), and SC32 (38.09 mg g⁻¹). The highest values occurred in SC29 (62.46 mg g⁻¹), SCG1 (58.86 mg g⁻¹), SCE (55.55 mg g⁻¹), SC12 (55.18 mg g⁻¹), and SC11 (53.95 mg g⁻¹). These rankings describe individual plants and do not imply statistically distinct groups.

Analytical precision varied among samples. The analytical CV was below 10% in 11 of the 17 somaclones and was highest in SC56 (22.45%), SC14 (18.03%), SC55 (15.94%), and SC17 (15.17%). SC9 had identical recorded analytical values and therefore an SD and CV of zero. The SD values in Table 1 and the error bars in Fig. 1 describe analytical variability, not population-level variation.

Table 1

Lignin content of 17 long-term established *C. hildmannianus* somaclones. Values are means of analytical determinations; n is the number of analytical determinations per individual. SD and CV quantify analytical variability, not biological replication.

Somaclone	n	Lignin mean (mg g ⁻¹ PCIP)	Analytical SD	Analytical CV (%)
SC15	3	28.61	1.47	5.13
SCC	4	32.45	2.71	8.36
SCI	4	35.28	1.88	5.34
SC55	4	35.92	5.73	15.94
SC32	3	38.09	1.31	3.44
SC9	4	43.44	0.00	0.00
SC10	4	45.95	2.41	5.25
SC56.1	4	46.06	1.87	4.05
SC14	4	47.86	8.63	18.03
SC8	4	50.90	4.64	9.12
SC56	4	52.08	11.69	22.45
SC17	4	53.35	8.09	15.17
SC11	4	53.95	3.01	5.57
SC12	4	55.18	5.58	10.11
SCE	4	55.55	2.23	4.02
SCG1	4	58.86	6.12	10.40
SC29	3	62.46	5.13	8.21

3.2. Contrasting materials for subsequent biomass-oriented studies

The observed distribution identifies contrasting individuals for future experiments. SC15 had the lowest mean lignin content, followed by SCC, SCI, SC55, and SC32; SC29 had the highest, followed by SCG1, SCE, and SC12. These individuals provide a basis for subsequent studies of cell-wall composition, pretreatment response, enzymatic saccharification, and lignin recovery. The present measurements do not establish superior biomass-processing performance in either group.

4. Discussion

The main finding is that a decades-old somaclonal collection, derived from callus-mediated regeneration and previously characterized for morphological and molecular variation, also exhibits substantial contemporary differences in lignin content. Mean values ranged from 28.61 mg g⁻¹ PCIP in SC15 to 62.46 mg g⁻¹ PCIP in SC29, a 2.18-fold difference. Although analytical determinations cannot serve as biological replicates for comparisons among individual plants, the observed range establishes the presence of contrasting lignin phenotypes within the collection. The study therefore adds a cell-wall biochemical trait to its existing morphological and molecular characterization.

This finding is especially informative in the context of the collection's history. The plants were regenerated through a callus phase and have subsequently been maintained under ex vitro conditions for decades. Morphological and isozyme variation was already detected in regenerated *C. peruvianus* by Mangolin et al. (1997), who reported conspicuous morphological variants but found that morphological divergence was not simply associated with isozyme changes. Subsequent RAPD analysis found greater polymorphism among somaclones than among cultivated seed-derived plants, while molecular polymorphisms were not consistently associated with typical versus atypical shoot morphology (Gazoli et al. 2010). Microsatellite analysis later reinforced this complex picture: Martin et al. (2018) found high genetic variability within the somaclonal collection but only low molecular divergence between morphologically typical and atypical groups. The present lignin data add a further, independently measured phenotypic dimension to this long-term biological resource rather than defining another categorical grouping of the somaclones.

The present lignin heterogeneity should therefore be interpreted within this broader biological framework. Plant tissue culture, particularly regeneration involving a callus phase, can generate genetic and epigenetic variation through changes in DNA sequence, chromosome constitution, DNA methylation and chromatin state (Miguel and Marum 2011; Bidabadi and Jain 2020; Ferreira et al. 2023). Such variation may persist after regeneration and can occasionally provide useful phenotypes for selection. Nevertheless, the current experiment does not establish that the differences in lignin content were directly caused by tissue culture, nor does it demonstrate longitudinal stability of lignin accumulation throughout the lifetime of the plants. Developmental, anatomical and microenvironmental effects may also contribute to the observed values. The defensible conclusion is that pronounced lignin heterogeneity is currently expressed in a long-term established somaclonal collection whose morphological and molecular diversity has been independently documented.

A direct connection with cell-wall biology is provided by previous biochemical work with *C. hildmannianus*. Corrêa et al. (2022) characterized cell-wall-related polysaccharides in in vitro propagated plants and identified type I arabinogalactan and arabinoxylan in the somaclonal material; in comparison with plants cultivated in vivo, the regenerated material also showed marked differences in mucilage and starch accumulation. Those observations cannot be combined quantitatively with the present lignin data, and they should not be assumed to involve the same individual plants. They nevertheless provide independent evidence that regenerated *Cereus* material can express biochemical phenotypes involving carbohydrate fractions relevant to biomass composition. The present results extend this perspective by

showing substantial variation in lignin, a structurally distinct and technologically important component of secondary cell walls.

It is important, however, to distinguish variation in lignin content from variation in overall cell-wall architecture. The present measurements do not provide information on cellulose content, hemicellulose abundance, cellulose crystallinity, wall thickness, porosity, lignin monomer composition, or lignin-carbohydrate linkages. Consequently, SC15 cannot simply be described as having a reduced cell wall, nor can SC29 be assumed to possess a thicker or mechanically stronger wall. These plants are more appropriately regarded as contrasting cell-wall biochemical phenotypes with respect to lignin accumulation. This distinction is important because biomass recalcitrance emerges from interactions among several structural and chemical properties rather than from lignin concentration alone.

The magnitude of the variation nevertheless has clear experimental relevance for biomass utilization. Lignin contributes to lignocellulosic recalcitrance by limiting accessibility to structural polysaccharides and through interactions that can reduce the efficiency of enzymatic deconstruction; at the same time, cellulose crystallinity, hemicellulose, porosity and other structural features also influence processing behavior (Brienza et al. 2024). Accordingly, the relatively low-lignin somaclones identified here, particularly SC15 and, secondarily, SCC, SCI, SC55 and SC32, constitute rational candidates for testing whether lower lignin content is associated with improved accessibility of structural carbohydrates. This is a testable hypothesis rather than a demonstrated technological advantage. Direct measurements of cellulose and hemicellulose, followed by standardized pretreatment and enzymatic saccharification assays, would be required to determine whether these low-lignin phenotypes are indeed less recalcitrant.

Evidence from other Cactaceae provides a useful proof of principle. Cladodes of *Opuntia ficus-indica* have been evaluated as lignocellulosic feedstock for ethanol production, with enzymatic hydrolysates successfully fermented by *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* (Kuloyo et al. 2014). More directly, alkaline hydrogen peroxide pretreatment of *O. ficus-indica* cladodes reduced the lignin fraction and was accompanied by a substantial increase in biogas production (Belay et al. 2021). These studies do not demonstrate that low-lignin *Cereus* somaclones will behave similarly, because species, biomass composition, analytical procedures and conversion technologies differ. They do, however, show that lignin modification can have functional consequences for cactus cladode conversion and provide a biologically relevant rationale for testing the contrasting *Cereus* phenotypes identified here.

An equally important implication emerges at the opposite end of the lignin distribution. Contemporary biorefinery concepts increasingly regard lignin not only as a barrier to carbohydrate utilization but also as a renewable aromatic resource whose effective recovery and conversion can improve the value obtained from lignocellulosic biomass. Lignin can serve as a precursor for fuels, aromatic chemicals, polymers and functional materials, and integrated plant-biology and biorefining strategies increasingly seek to optimize both lignin amount and structure for downstream valorization (Brienza et al. 2024; Dixon et al. 2024). From this perspective, SC29, SCG1, SCE and SC12 represent a second, conceptually different set

of candidate materials. Their comparatively high lignin contents make them appropriate starting points for future studies of lignin yield, extractability, structural composition and downstream valorization.

This dual interpretation avoids treating high lignin as intrinsically undesirable and low lignin as intrinsically advantageous. Instead, the collection contains contrasting biomass phenotypes that may be useful for different conversion strategies: low-lignin materials can be tested for reduced recalcitrance and enhanced polysaccharide accessibility, whereas high-lignin materials can be investigated for lignin recovery and lignin-derived products. The immediate value of the present study is therefore not the demonstration of an industrially superior phenotype, but the identification of biologically contrasting materials that permit these hypotheses to be tested experimentally.

Taken together, these findings extend the characterization of this historical *C. hildmannianus* collection. Earlier studies documented morphological, isozyme, and DNA-marker variation, whereas the present measurements reveal a broad range of lignin contents decades after ex vitro establishment. They do not establish a shared mechanism underlying these traits or demonstrate that lignin content has remained stable since regeneration. Rather, they identify contemporary biochemical heterogeneity in a collection with independently documented morphological and molecular diversity.

Future work should compare individuals with contrasting lignin contents for cellulose and hemicellulose composition, lignin structure, pretreatment response, and enzymatic saccharification. SC15 and SC29 provide useful extremes, while including additional individuals across the observed range would avoid reliance on a single pair. Such experiments could test whether differences in lignin content translate into differences in biomass processing. The primary contribution of the present study remains the characterization of lignin heterogeneity in this historical collection.

5. Conclusion

The historical *C. hildmannianus* collection, derived from callus-mediated regeneration and maintained ex vitro for decades, currently exhibits substantial variation in lignin content, including a 2.18-fold difference between the lowest- and highest-lignin individuals. This finding extends the collection's previously documented morphological and molecular variation to a cell-wall biochemical trait. It does not establish that the lignin differences were caused by in vitro regeneration or remained unchanged during subsequent decades.

The contrasting individuals provide material for future functional studies. Somaclones with relatively low lignin content, particularly SC15, can be evaluated for polysaccharide accessibility and biomass recalcitrance, whereas high-lignin individuals such as SC29 may be useful for studies of lignin recovery and composition. These possibilities require direct experimental testing, including measurements of other cell-wall components and biomass-processing performance.

Overall, the results identify a distinct range of lignin phenotypes within a decades-old somaclonal collection and establish a basis for further studies of cell-wall biology and biomass utilization.

Declarations

Competing interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Ethics approval

Not applicable.

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Author Contribution

S.A.R., F.F.S.D., D.E.R.G., M.F.P.S.M. and C.A.M. contributed to the study conception and design. Material preparation, data collection and analysis were performed by S.A.R. The statistical analyses were performed by F.F.S.D. The first draft of the manuscript was written by C.A.M. All authors contributed to the formal analysis and critically revised the manuscript.

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Data availability

The underlying analytical data supporting the findings of this study will be made available by the corresponding author upon reasonable request.

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Figures

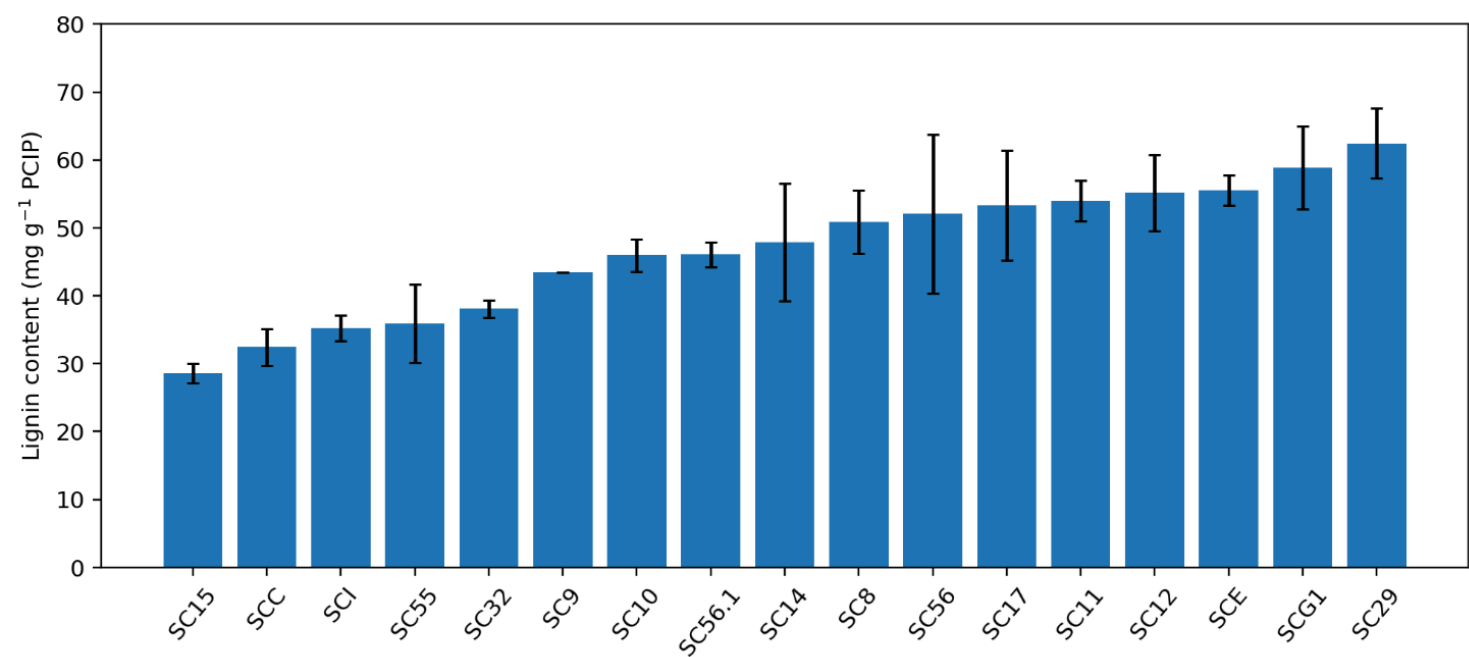


Figure 1

Lignin content in 17 long-term established *C. hildmannianus* somaclones, arranged in ascending order of mean lignin content. Bars represent mean lignin content (mg g⁻¹ PCIP), and error bars indicate the standard deviation of analytical determinations (n = 4, except for SC15, SC29 and SC32, for which n = 3). Error bars reflect analytical variability and do not represent biological replication. Each somaclone corresponds to a single biological individual. Numerical SD values are provided in Table 1