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RESEARCH REPORT

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Optimized utilization of *Salix*—Perspectives for the genetic improvement toward sustainable biofuel value chains

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Abstract

Bioenergy will be one of the most important renewable energy sources in the conversion from fossil fuels to bio-based products. Short rotation coppice *Salix* could be a key player in this conversion since *Salix* has rapid growth, positive energy balance, easy to manage cultivation system with vegetative propagation of plant material and multiple harvests from the same plantation. The aim of the present paper is to provide an overview of the main challenges and key issues in willow genetic improvement toward sustainable biofuel value chains. Primarily based on results from the research project "Optimized Utilization of *Salix*" (OPTUS), the influence of *Salix* wood quality on the potential for biofuel use is discussed, followed by issues related to the conversion of *Salix* biomass into liquid and gaseous transportation fuels. Thereafter, the studies address genotypic influence on soil carbon sequestration in *Salix* plantations, as well as on soil carbon dynamics and climate change impacts. Finally, the opportunities for plant breeding are discussed using willow as a resource for sustainable biofuel production. Substantial phenotypic and genotypic variation was reported for different wood quality traits important in biological (i.e., enzymatic and anaerobic) and thermochemical conversion processes, which is a prerequisite for plant breeding. Furthermore, different *Salix* genotypes can affect soil carbon sequestration variably, and life cycle assessment illustrates that these differences can result in different climate mitigation potential depending on genotype. Thus, the potential of *Salix* plantations for sustainable biomass production and its conversion into biofuels is shown. Large genetic variation in various wood and biomass traits, important for different conversion processes and carbon sequestration, provides opportunities to enhance the sustainability of the production system via plant breeding. This includes new breeding targets in addition to traditional targets for high yield to improve biomass quality and carbon sequestration potential.

KEYWORDS
bioethanol, biofuels, biogas, biomass recalcitrance, carbon sequestration, LCA, plant breeding, *Salix*, tension wood

1 | INTRODUCTION

The transformation into a bio-based economy requires a high and sustainable production of woody biomass to reduce the need for fossil fuels. One of the key challenges for this transition is to find additional sources of biomass, with high yield potential, low conversion and production costs, and an energy-efficient and sustainable value chain. Here, short rotation coppice (SRC) willows (genus *Salix*) represent a natural resource to produce woody biomass with enormous potential as feedstock for bioenergy, both for heating and different biorefinery conversion processes generating biofuels (Horn et al., 2011; Sassner, Galbe, et al., 2008; Sassner, Martensson, et al., 2008; Serapiglia et al., 2013) and additional products for use in the chemical industry (Krzyżaniak et al., 2014). In addition, *Salix* can grow on marginal or less favorable land, thereby reducing land-use competition between food and energy crops (Karp et al., 2011). Moreover, the SRC system allows repeated harvesting every 2–5 years, enabling more resource-efficient biomass production compared to annual crops (Boehmel et al., 2008). Since the lifetime of a plantation (rotation time) may be more than 20 years, multiple harvests can be made using the same rootstock (Karp & Shield, 2008; Weih, 2004). In addition to its role as a bioenergy source, *Salix* also provides agriculture and society with several ecosystem services, such as carbon storage, absorption of nutrients and heavy metals from the soil, reduction on the effect of floods and provides an increased biodiversity that benefits wildlife and pollinating insects (Weih et al., 2020). Since the late 1980s and until today, willow biomass has been used almost exclusively commercially as biofuel in the form of wood chips for direct combustion in heat and power plants (Kuzovkina et al., 2008). However, there is also a growing interest of using this biomass as raw material for conversion into other biofuels such as biogas and bioethanol (Jarunglumlert & Prommuak, 2021; Phitsuwan et al., 2013), a development that likely requires genetic improvements of additional breeding targets compared to using willow biomass solely for direct combustion. To date, *Salix* breeding programs have produced varieties for the European as well as the international market (Karp et al., 2011; Smart & Cameron, 2008) and varieties with increased yield and resistance toward different pests, especially rust fungi (*Melampsora epitea*) are now available (Åhman et al., 1994; Karp et al., 2011). To date, no breeding program has been developed to address properties important for use of *Salix* in the whole bioconversion process. Breeding targets of interest include biomass quality traits affecting biomass conversion efficiencies, and production system properties such as soil carbon accumulation, influencing the ecological sustainability of this biofuel feedstock (Weih et al., 2020).

The high genetic diversity in *Salix*, together with its low level of domestication, the short generation time, and the ability for vegetative propagation, makes *Salix* an excellent woody biomass crop with possibilities for improvement via plant breeding. *Salix*, together with *Populus*, is a member of the Salicaceae family. The *Salix* genus consists of hundreds of species distributed worldwide, with most species in the northern hemisphere in arctic and temperate regions, although *Salix* species exist in both subtropical and tropical regions exist (Kuzovkina et al., 2008). There is great phenotypic variation within the genus ranging from small arctic plants to large trees. Many species within the subgenus *Vetrix*, consisting of bushes and smaller trees like *Salix viminalis*, *Salix schwerinii*, *Salix dasyclados* and *Salix purpurea*, are used today for cultivation in SRC systems in Europe, United States and Canada (Kuzovkina et al., 2008). Valuable genomic resources also exist for the breeding of *Salix*, as several sequenced genomes are now publicly available, for example, for *S. purpurea* (Zhou et al., 2020) and for *S. viminalis* (Almeida et al., 2020). In addition, biparental populations and association mapping populations are available for many species together with a large number of molecular markers (Berlin et al., 2017; Carlson et al., 2019; Hallingbäck et al., 2019; Hanley & Karp, 2014; Ohlsson et al., 2019). All these resources facilitate development toward selection using genomic tools.

Development and breeding of *Salix* for lignocellulose production requires understanding of the composition (i.e., structure, chemistry) of *Salix* wood, and knowledge about the genetic variation in the relevant wood traits that will provide improved lignocellulose disintegration and subsequent saccharification. Composition of the major components has been studied in different *Salix* species and clones (Fabio et al., 2017; Ray et al., 2012;

Sandak & Sandak, 2011; Serapiglia et al., 2008, 2012, 2013). Typical reported composition of the major biomass components in *Salix* biomass is in the approximate ranges of 40%–45% cellulose, 15%–30% hemicellulose, and 20%–25% lignin, with large variation in reported values (Weih et al., 2020). These studies demonstrate the great potential for breeding, and for producing new *Salix* varieties with targeted chemical compositions for alternative applications other than heat production. How much of the demonstrated variation is due to genetic causes, however, requires further analysis to realize the full potential of *Salix*.

Breeding of *Salix* for sustainable biomass production should also consider the characteristics of the biomass crop and the entire production system in terms of ecological sustainability, which is challenging to accommodate in breeding programs. This is because plant breeding is strictly trait-based while ecological sustainability is evaluated at the production system or even larger scale, which requires linking plant traits to production system characteristics (Weih et al., 2014). In this context, both above- and belowground perspectives are important to consider, since the interaction with soil ecological factors, in particular root and soil microbiology, may differ depending on the *Salix* species and variety (Baum et al., 2020; Weih et al., 2019). The interactions between biomass crop characteristics (species, variety) and its abiotic and biotic environment strongly affect the environmental impact of a production system, and environmental impact can be evaluated using life cycle assessment (LCA). In a breeding context, LCA and related methods can be used to investigate the environmental impact of various biomass value chains based on the use of different *Salix* species and varieties, which links plant breeding to ecological sustainability and thereby further adds to the total range of possibilities for breeding (Ericsson et al., 2014).

As discussed above, genetic variation in important wood traits as well as the whole SRC system of *Salix* is important to consider for further development of new plant material for sustainable biomass value chains and these aspects were studied in the project "Optimized utilisation of *Salix*" (OPTUS; https://www.slu.se/en/Collaborative-Centres-and-Projects/optus/). This paper provides an overview of the main challenges and key issues in genetic improvement of willows for sustainable biofuel value chains, illustrating the different scales, approaches, and methodologies needed to address breeding aspects for different parts of the biomass value chain. Various field sites were used as project resources to collect biomass samples from Table 1 and the workflow between the different parts of the OPTUS project

TABLE 1 Plant material and field experiments referred to in the paper

Plant material (number)	Description of plant material	Field experiment	Reference	Results from OPTUS presented in section
Association mapping population (1)	A collection of ca. 300 genetically distinct accessions of *Salix viminalis* from Europe	Pustnäs, Uppsala (59°48′N, 17°39′E). Vegetative propagated accessions planted in a completely randomized block design, six blocks	Berlin et al. (2014); Hallingbäck et al. (2019); Ohlsson et al. (2019)	2, 3, 6
Ecological characterization (2)	Six commercial varieties: *S. viminalis* (*S. viminalis* × *S. schwerinii*), *S. dasyclados* (*S. burjatica* × *S. dasyclados*)	Pustnäs, Uppsala (59°48′N, 17°39′E). Two fertilization treatments, four blocks for each variety and treatment	Weih and Nordh (2005)	2, 3, 4, 5
Biogas experiment (3)	Six clones/varieties; *S. viminalis*, (*S. viminalis* × *S. schwerinii*)	Ultuna, Uppsala (58°81′N, 17°66′E). Different ages of shoots, split plot design with three blocks	Ohlsson et al. (2020)	2, 3
Commercial variety (4)	Clone "Tora"; (*S. viminalis* × *S. schwerinii*)	Material taken from plantations		3

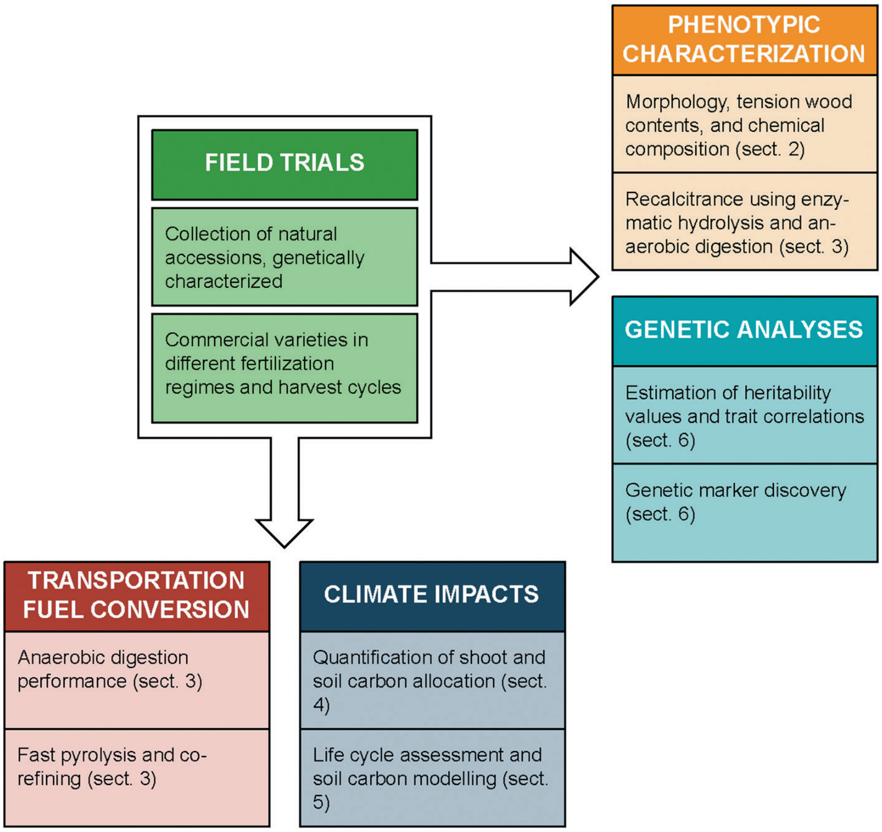

FIGURE 1 The different parts and their interactions in the OPTUS program. Sect = sections

(Figure 1) motivated the structure of this paper: Starting with the influence of wood quality on the potential for biofuel use and related issue of converting the biomass into liquid and gaseous transportation fuels, the paper addresses breeding aspects related to soil carbon sequestration and climate change mitigation, before a section on breeding potential integrates much of the information presented in the previous sections.

2 | INFLUENCE OF *SALIX* WOOD QUALITY ON THE POTENTIAL FOR BIOFUEL USE

One of the major controlling factors for using any lignocellulose as a resource for biofuels is its native recalcitrance, which is normally considered dependent on both its inherent gross chemistry (e.g., cellulose/lignin ratio) and accessibility of cellulose for enzymatic depolymerization (Himmel et al., 2007). Recalcitrance is influenced by the overall wood anatomy, which reflects variations in the spatial distribution of the cellular structure at tissue level, as well as the major wood polymers at ultrastructure/supramolecular levels (Zhao et al., 2012). Therefore, attractive *Salix* varieties should have high cellulose/lignin ratio with highly accessible cellulose in addition to physiological traits of fast growth and high yield (Gao et al., 2021a, 2021b).

2.1 | Physical and chemical characteristics of *Salix* clones

Over one thousand (i.e., 1100) individuals from a population (ca. 300) of genetically distinct *Salix* clones (Table 1, subset of 1) and several commercial varieties (i.e., Tora, Björn, Olof, Jorr; Table 1, subset of 2,3) have been analyzed for traits interesting for biofuels using a systematic approach (Gao et al., 2021a, 2021b). A number of complementary physical, chemical, histochemical, image and fiber analysis and microscopy approaches were employed allowing for comparisons of stem density, wood anatomy, and % area of tension wood (i.e., presence of reaction wood), cellular and spatial distribution of wood polymers, and fiber morphometric characteristics. The absolute dry density of the approximately 1100 debarked individual samples varied between 305 and 662 kg/m^3, while the density of the four commercial varieties was well within that range and varied between 457 and 520 kg/m^3. To understand if the observed differences in density reflected variations in cellular structure, anatomy, and chemistry, in-depth studies were conducted on a number of selected clones (i.e., 20 individual samples representing 19 clones). Wet chemical analysis of samples from the clones showed a variation in lignin of 5.6% ranging from 16% to 22% of the dry weight with acid insoluble lignin (i.e., kraft lignin) representing between 85% and 92% of total lignin in debarked samples. A greater variation was noted in total sugar (i.e., total carbohydrates) that varied by approximately 16% (ca. 61% and 77%) with glucose (i.e., cellulose) representing the major sugar of the biomass, with lesser amounts of xylose > mannose and >galactose. Using Pearson r ($p < 0.05$), moderate and strong positive correlations were obtained between % tension wood area, density, and total sugars, while the correlation with lignin was negative. From the analyses, clone samples showing high glucose content (i.e., cellulose) also had pronounced tension wood, while clones with highest lignin levels tended to have lower % tension wood present (Gao et al., 2021a). Histochemical studies on *Salix* stem cross sections confirmed the lack of lignin in the G-layers of tension wood fibers and varied spatial distribution of S- and G-lignin in fibers, vessels, and middle lamellae regions.

2.2 | Morphometric analyses of *Salix* clones

The combined physical and chemical evaluation indicated considerable variability among the *Salix* clones and commercial varieties at macro-, micro-, and ultrastructural levels (e.g., stem length and diameter, density, growth ring thickness, size of pith, presence of tension wood, and G-layers). Since the majority of the characteristics relate to the inherent stem cellular structure including the universal presence of tension wood, a morphometric approach was developed to characterize the cellular elements. This involved using an optical fiber analyzer (OFA) to quantify cellular features (e.g., fiber length and width) of 1000s (minimum 100,000 particles) of macerated wood cells providing statistical results of different classes and distribution of length weighted (LW) fiber length or width versus proportion of total fiber length or width in per mille (‰). In addition, analyses of vessel frequency in *Salix* samples were conducted as this property varies greatly between tissue types in wood, especially in tension wood (Clair et al., 2006; Jourez et al., 2001). Using OFA to analyze macerated cells from different *Salix* clones, a cumulative normal distribution function and Gaussian fit was applied to describe the fiber length distribution, and the stem diameter was found to be significantly and strongly correlated to the LW fiber length's distribution curve (Gao et al., 2021a). This strong relationship indicates that tension wood in *Salix* clones can be distinguished by fiber length (i.e., which is longer than normal or opposite wood fibers) when stems of similar diameter and density are compared (Gao et al., 2021a). Similarly, differences in vessel frequency and length may be used as a secondary character to distinguish *Salix* tension wood from opposite wood.

2.3 | Importance of the presence of tension wood

Microscopy analyses of stem cross sections showed the presence of tension wood was universal in all the *Salix* clones and commercial varieties examined. Tension wood was characterized by the development of various multilateral and unilateral bands in the first and second annual growth rings of the 2-year-old stems examined (Gao et al., 2021b), an observation consistent with previous studies (Berthod et al., 2015; Brereton et al., 2015). Microscopy and histochemical analyses for lignin showed tension wood was typically composed of characteristic G-fibers with cellulose-rich and lignin-free G-layers forming a modified fiber secondary wall structure composed of S1, S2, and G-layer (Gao et al., 2021b). The G-layer varied greatly in thickness between early and latewood. Image analysis of % tension wood and histochemical analysis of sections along the stems of several commercial varieties (Tora, Björn, Jorr, Loden) confirmed the presence (e.g., from ca. 18% to 45% area of cross section in variety Tora) of tension wood (Gao et al., 2021a). Quantifying the volume presence of tension wood is difficult and currently only an approach based on X-ray microtomography has been evaluated (Brereton et al., 2015). A novel enzymatic method was therefore developed where stem cross sections (ca. 30 μm) after determining % tension wood were subjected to hydrolysis by a commercial cellulase preparation and the development of D-glucose determined (Gao et al., 2021a, 2021b, 2022). The open fibers (average length of *Salix* fibers is ca. 0.5 mm) allows ingress of the enzyme into the fiber cell lumina and interface regions between the G-layer and S2 layer. Since the G-layer is non-lignified and non-recalcitrant, hydrolysis of the G-layer occurs in contrast to the other lignified cell walls and middle lamella regions (i.e., fiber and vessel secondary cell walls). G-layer cellulose in tension wood fiber cross sections was readily hydrolyzed by cellulase and produced approximately 44 and 38 kg/m^3 D-glucose for *Salix* varieties Björn and Tora, with D-glucose release increasing with stem height. By correlating stem tension wood volume with average cellulose content reported for *Salix* in the literature (i.e., 45%), it was estimated that G-layer cellulose contributed between 16% and 20% of the total cellulose yield in the two *Salix* varieties studied. The result further emphasized the significant contribution that tension wood and G-layer cellulose may have on the total cellulose yield of *Salix* grown under field situations, a result consistent with previous studies (Berthod et al., 2015; Brereton et al., 2012;

Sawada et al., 2018). Therefore, tension wood represents an important trait for consideration in breeding studies with respect to optimizing biofuel production from this coppice plant.

The viability of using *Salix* stem cross sections directly for gross chemical analysis of targeted stem areas instead of milling entire stems and using powder for comparing clones was further developed in the present study (Gao et al., 2022). By using serial *Salix* stem sections and applying sequential analyses of total sugars (i.e., HPLC) and lignin (soluble/insoluble) together with cellulose saccharification and D-glucose determination, the relative amounts of the major wood polymers could not only be determined but also the D-glucose derived from tension wood (i.e., G-layer) and that from the wood cell walls (i.e., structural) be distinguished. This methodology provides an estimation of the presence and relative importance of tension wood allowing for a direct comparison between clones (Gao et al., 2022). Using the stem section approach, we calculated the contribution of tension wood G-glucose (i.e., that derived only from the gelatinous layer of tension fibers) as a percentage of dry matter content for four commercial *Salix* varieties (Tora, Björn, Jorr, and Loden) as between approximately 7.0% and 12.0% (Gao et al., 2022).

3 | CONVERSION OF *SALIX* BIOMASS INTO LIQUID AND GASEOUS TRANSPORTATION FUELS

Transforming lignocellulose biomass like *Salix* into energy dense transportation fuels offers several advantages over using the biomass directly for heat and power generation. Fuels such as ethanol, biomethane, and pyrolysis oil are considerably more energy dense than the corresponding *Salix* biomass and have a broader range of applications. Biomass can be refined into such fuels using several biological and thermochemical routes (Cherubini et al., 2009). The overall economics of conversion processes depend on several factors, including specific production aspects, for example, reactant concentrations and pentose utilization (Sassner, Galbe, et al., 2008; Sassner, Martensson, et al., 2008), cultivation and fertilization regimes (Gouker et al., 2021; Stolarski et al., 2017), and biomass qualities including both biomass yield and composition (Happs et al., 2020).

Biological conversion systems suitable for *Salix* biomass can be broadly classified as enzymatic hydrolysis (EH) or anaerobic digestion (AD) processes. The former system uses cellulolytic enzyme cocktails for breaking down the biomass into sugars, which can then be further processed into fuels (e.g., ethanol) by microorganisms such as brewer's yeast (*Saccharomyces cerevisiae*) (Chundawat et al., 2011). In contrast, AD systems employ undefined mixed microbial cultures that are responsible for both disintegrating and converting the biomass, often into methane-rich biogas (Jarunglumlert & Prommuak, 2021; Monlau et al., 2013). A major impediment to biological conversion of lignocellulose biomass is its inherent resistance to deconstruction, a property commonly referred as biomass recalcitrance (see Section 2). Thus, most biorefineries employ some kind of pretreatment technology to disintegrate the lignocellulose structures responsible for recalcitrance (Zhu et al., 2010). However, reducing biomass recalcitrance via plant genetic improvement may be necessary in order to lessen pretreatment requirements to a degree compatible with economic requirements for biorefineries (Himmel et al., 2007).

Biomass recalcitrance is commonly determined using enzymatic assays that quantify the sugar released after pretreatment and enzymatic saccharification (Decker et al., 2018). In order to generate data for genetic analyses, sugar release was evaluated in *Salix* samples taken from 2-year-old shoots in four blocks of a field experiment established with approximately 300 vegetative propagated natural accessions of *S. viminalis* (Table 1, subset of 1) (Ohlsson et al., 2019). The analyses showed clear phenotypic variation for all traits studied and part of this variation also had genetic causes, implying possibilities for breeding, and the implications for breeding are discussed in Section 6.

Since AD and EH systems use distinct enzymes for lignocellulose hydrolysis (i.e., fungal vs bacterial) (Schwarz, 2001), and AD cultures have a broader substrate range, the pretreatment requirements may be less stringent for AD systems. Thus, traditional biomass recalcitrance assays may not fully reflect recalcitrance under AD. To investigate correlations between AD and EH recalcitrance in *Salix*, 94 clones (Table 1, subset of 1) were evaluated using both sugar release and biomethanation potential (BMP; an assay for estimation of methane productivity and AD convertibility) assays, omitting any non-mechanical pretreatment step for the BMP assay (Ohlsson, Harman-Ware, et al., 2020). In the BMP assay, non-pretreated *Salix* clones exhibited a relatively low variation in total yield of methane per unit biomass (Ohlsson, Harman-Ware, et al., 2020). However, samples with low recalcitrance in the EH sugar release assay showed higher initial rates of methane production, indicating faster hydrolysis. This suggests that low-recalcitrance *Salix* biomass can provide economic benefits through both EH and AD processes, although by different mechanisms: in EH systems, reduced recalcitrance translates to larger amounts of product per unit biomass or lower requirements for enzyme and pretreatment inputs, while in AD systems, it translates into higher outputs per unit time due to a faster turnover rate (Ohlsson, Harman-Ware, et al., 2020).

The performance of pretreated (steam explosion) *Salix* biomass in AD systems has previously been evaluated (Estevez et al., 2012; Sassner, Galbe, et al., 2008; Sassner, Martensson, et al., 2008). *Salix* is well suited for small-scale conversion plants located close to the cultivation site, such as farm-operated AD plants, where pretreatment other than mechanical (i.e., size reduction) may not be economically feasible. However, increasing the retention time of the process could allow for a more thorough deconstruction of recalcitrant material. Therefore, a serial digester AD process was evaluated using *Salix* and animal manure in a co-digestion system (Ohlsson, Rönnberg-Wästljung, et al., 2020). By using serial digesters, the effective retention time could be doubled. The resulting conversion yields closely mirrored those from steam-pretreated biomass, implying that serial digestion could be an option for using *Salix* as a substrate in small-scale AD plants. Moreover, a comparison of six commercial clones with different harvest frequencies (Table 1, subset of 3), the clone choice and coppicing frequency affected BMPs, although differences in biomass yields were approximately an order of magnitude greater. In effect, the land area required for supplying a typical farm-scale digester with biomass differed by a factor 3.7 between the highest and lowest biomass yielding clone/harvest frequency combinations, highlighting the importance of using high-yielding clones for AD (Ohlsson, Rönnberg-Wästljung, et al., 2020).

Thermochemical conversion by gasification, pyrolysis, and hydrothermal liquefaction also allows conversion of *Salix* biomass into transportation fuels. One attractive and cost-efficient thermochemical route for the rapid introduction of large-scale production of biofuels into the transport sector is to convert biomass into a liquid pyrolysis oil via fast pyrolysis followed by co-processing with fossil oil using existing refinery installations (Talmadge et al., 2013). In fast pyrolysis, biomass is thermally decomposed in an inert atmosphere producing pyrolysis oil, biochar, and non-condensable gas. However, when the aim is to produce transportation fuels, the highly oxygenated pyrolysis oil requires further refining (Talmadge et al., 2013).

To demonstrate how *Salix* can be transformed into gasoline and diesel range products, *Salix* biomass was liquefied using fast pyrolysis, followed by co-refining with other fossil oils using two different upgrading routes, either hydroprocessing or catalytic cracking (Johansson et al., 2022). The commercial *Salix* clone Tora (Table 1) was first liquefied to pyrolysis oil in a cyclone fast pyrolysis pilot plant. Subsequently, the oil was co-refined with fossil oil in a continuous slurry hydrocracking pilot and fluidized catalytic cracking laboratory units, respectively. Results showed that *Salix* could be significantly deoxygenated and converted to liquid gasoline and diesel range products via both co-refining routes. In the hydroprocessing route, the biogenic carbon from the *Salix* biomass resulted in significantly higher yields of hydrocarbon products (26 wt% vs 11 wt%), although high pressure hydrogen is required for this process. In contrast, in the catalytic cracking route, a significant part of the biogenic carbon ended up as coke on the catalyst (16 wt%). The choice of routes is therefore highly dependent on the available amount of bio-oil and refining infrastructure available (Johansson et al., 2022).

4 | GENOTYPE INFLUENCE ON SOIL CARBON SEQUESTRATION IN *SALIX* PLANTATIONS

Climate regulation is an important ecosystem service and increasing soil carbon (C) sequestration plays an important role in the mitigation and adaptation to climate change. In this context, genetic variations between *Salix* genotypes in C sequestration capacity could be used to enhance the sustainability of willow production systems. This is because *Salix* plantations are known to enhance soil C sequestration (Borzêcka-Walker et al., 2008; Gregory et al., 2018; Pacaldo et al., 2014; Rytter, 2012) through net transfer of C from the atmosphere to belowground where it is stored in the form of organic matter (Lal, 2008). In general, plants can directly affect soil C sequestration through the chemical composition of their inputs to soil in the form of plant (e.g., root and leaf) litter, rhizodeposits, and root-associated fungi including mycorrhizas. Different species and genotypes of *Salix* vary considerably in their biomass allocation to roots and leaves (Cunniff et al., 2015; Weih & Nordh, 2005; Weih & Nordh, 2002), their root associations with mycorrhiza, and fungi affecting soil C accumulation (Baum et al., 2009; Rooney et al., 2009), as well as leaf litter quality affecting soil C cycling and accumulation (Hoeber et al., 2020). Exploring the potential of various *Salix* genotypes may be a promising strategy for increasing C sequestration, because there is sufficient variation in the relevant traits (e.g., root allocation, mycorrhiza colonization, leaf litter quantity and quality).

To investigate the differential effects of *Salix* genotypes on C accumulation above- and belowground, a field study was carried out in Central Sweden. In this study, considerable variation in shoot C accumulation (35–122 $\mathrm{Mg\,ha^{-1}}$) and soil C accumulation (6–17 $\mathrm{Mg\,ha^{-1}}$) was observed among six different *Salix* genotypes grown with and without nutrient fertilization for 17 years (Table 1, subset of 2) (Baum et al., 2020). Notably, the effect of fertilization on C accumulation varied considerably between the genotypes (genotype × environment interaction); that is, the fertilization changed shoot C

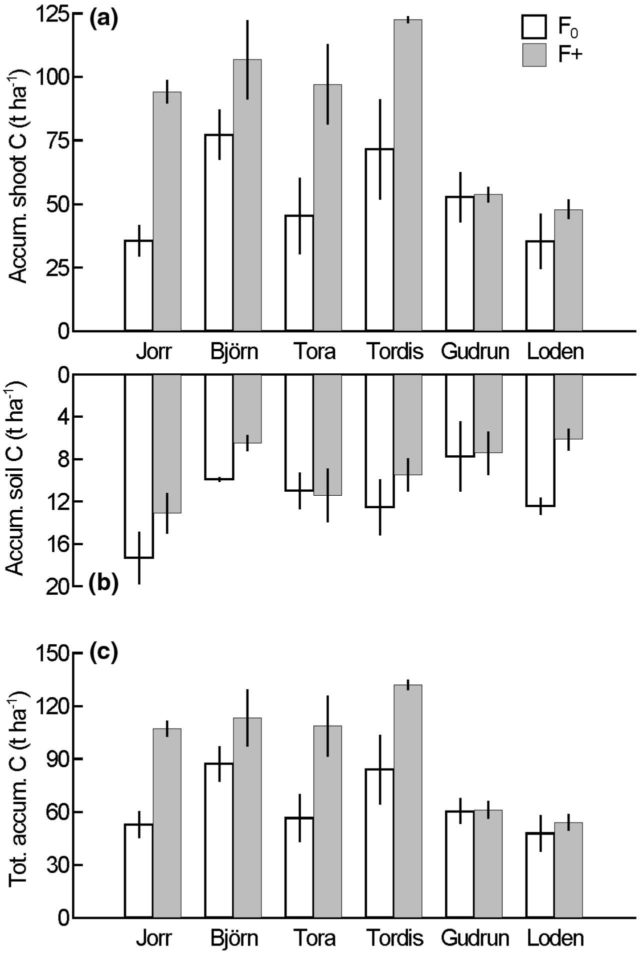

FIGURE 2 Changes in C stocks in soils (0–10 cm depth) and total accumulated shoot C biomass of six willow genotypes grown with and without nutrient (NPK) fertilization (F_0 vs $F+$) for 17 years in central Sweden (mean ± standard error); shoot harvests were done according to a 3-year cutting cycle. Total shoot C was determined assuming 50.5% C in the biomass (Stolarski, 2008). The initial soil C stock in 0–10 cm soil depth in 2001 was 14 ± 0.6 Mg/ha. All data are from Baum et al. (2020).

accumulation by a factor between 1.0 and 2.2, and soil C accumulation by a factor between 0.5 and 1.0 (Figure 2). At a first glance, the results indicate a large breeding potential for maximizing soil C accumulation under plantations of *Salix*. However, the mechanisms at plant level leading to these differences are often complex, involving genetic, physiological, and ecological mechanisms (including their interactions), and the identification of feasible breeding targets appears a formidable challenge. Theoretically, breeding for maximizing soil C accumulation could make use of the proposed links between soil C and aboveground biomass yield, because soil C estimation models commonly use allometric functions where belowground biomass inputs and soil C accumulation are assumed proportional to harvested yield (Taghizadeh-Toosi et al., 2016). However, our results indicate that aboveground shoot biomass allocation was poorly correlated with soil C accumulation (Figure 2), suggesting that independent data on biomass production and soil C accumulation are needed to assess the breeding potential for maximizing soil C accumulation under biomass plantations.

Apart from genotype, management of *Salix* plantations (including genotype × management interaction) may also affect soil C accumulation. Baum et al. (2020, Figure 2) highlight the importance of nutrient fertilization and genotype interactions. Planting design and cutting cycle length are further examples for adopting management practices that may also increase C sequestration in soils.

In summary, *Salix* genotype influences shoot and soil C accumulation, providing opportunities for adopting management strategies including breeding activities toward *Salix* germplasm with larger belowground C sequestration potential. However, as soil C sequestration is a complex process involving diverse organisms and ecological processes, only exploring the breeding process will not be sufficient, and further studies are required to disentangle processes within the complex soil–plant system that will contribute to larger C sequestration potential (Weih et al., 2014).

5 | INFLUENCE OF *SALIX* GENOTYPE VARIATIONS ON SOIL CARBON DYNAMICS AND CLIMATE IMPACTS

The assessment of climate impacts from bioenergy systems is an important step to ensure that such systems are sustainable and lead to climate change mitigation, although the direct comparison of different biomass-to-bioenergy systems can be difficult due to the use of different methodologies, systems boundaries, and assumptions across different studies. From cultivation to final end use, the entire bioenergy chain involves many steps and inputs with environmental impacts arising from emissions caused or prevented, soil C dynamics, and possible land-use changes. Therefore, a systems analysis approach to break down the system and study the individual components and their interactions is highly beneficial. LCA has emerged as a popular and well-established tool for evaluation of environmental impact as it considers all the energy flows and emissions during the whole lifespan of the product or service (i.e., energy from different *Salix* varieties in this case) (Garrigues et al., 2012; Henryson et al., 2018). Although the complexity of biomass systems and their interaction with biodiversity, C dynamics, water availability, and other ecological functions leads to uncertainty and variability (McKone et al., 2011), our understanding of biomass systems and methodologies is evolving to increase accuracy of impact assessments in LCA.

The climate impact of six varieties of *Salix* grown under fertilized and unfertilized conditions (Table 1, subset of 2) was analyzed and compared using LCA methodology, using the data generated from a *Salix* field study at Pustnäs, Uppsala, Sweden (Table 1, subset of 2). The study system involved the steps from field site preparation to end use of the *Salix* biomass as biofuel for an incineration plant, with heat energy as the end product, described in Kalita et al. (2021). The system did not include the distribution of heat and transportation of ash. The study period was 50 years, with a coppice cycle of 3 years, and a new rotation established at 25 years. The *Salix* systems are compared to a reference scenario with equivalent heat generation from natural gas and green fallow as land use. Climate impacts were expressed in terms of two metrics—global warming potential (GWP_{100}) (Myhre et al., 2013) and time-dependent climate metric (ΔT_s) as defined in Ericsson et al. (2013). GWP_{100} expresses the climate impact as CO_2-equivalent emissions while ΔT_s expresses CO_2 as a change in temporal global mean surface temperature. The change in soil carbon at the cultivation site over the study period was calculated using a soil carbon model ICBM (Introductory Carbon Balance Model) developed by Andrén and Kätterer (1997) using the field data presented in Section 4 (Figure 2; Table 1).

The soil carbon modeling showed that all the *Salix* cases led to an increase in soil organic carbon (SOC) in the topsoil (0–20 cm) over a 50-year study period (Kalita et al., 2021) as shown in Figure 3. However, magnitude of SOC change was highly dependent on *Salix* variety and fertilization. Fertilization had a negative effect on SOC increase as the unfertilized treatment for each variety sequestered 1.2–3.3 times more carbon as SOC relative to the equivalent fertilized treatment. Overall, the low-yielding variety of Jorr was the best performing in terms of SOC increase potential. While fertilized Loden and high-yielding Björn showed the lowest potential for SOC increase being only marginally better than the fallow reference.

Possible explanations for these differences in SOC increase are variations in biomass growth and allocation between *Salix* varieties as well as growing conditions that have been reported in previous studies (Cunniff et al., 2015; Gregory et al., 2018). Studies on aboveground and belowground biomass components in *Salix* support the concept that fertilization can lead to lower levels of belowground biomass production (Heinsoo et al., 2009; Rytter, 2001, 2013). The SOC results questions the common assumption that fertilization contributes to greater aboveground growth and is indicative of a higher belowground biomass growth and consequently more SOC sequestration. However, the soil is not an infinite C sink, and overtime the SOC sequestration rate can be expected to be negatively affected by rising temperatures. The magnitude

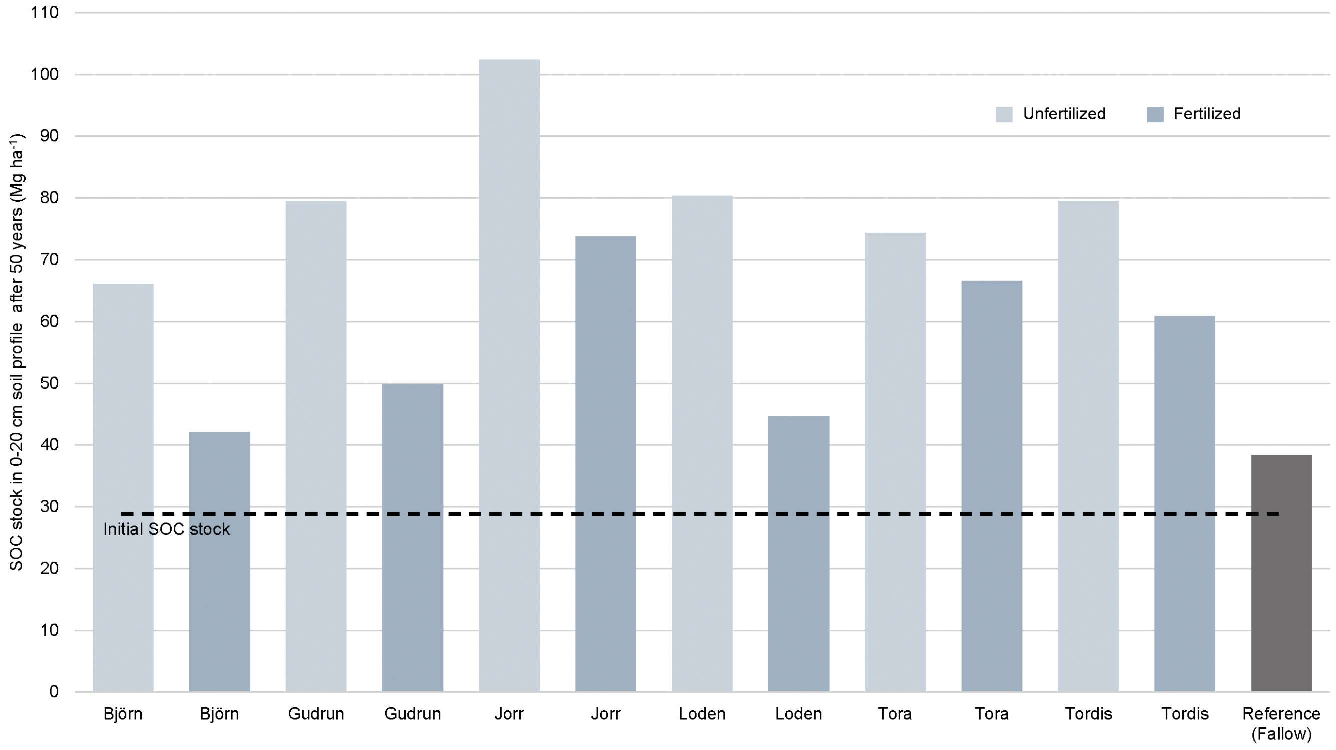

FIGURE 3 Soil organic carbon stocks under the different Salix plantations in the 0–20 cm soil layer after 50 years as calculated using ICBM soil carbon model (modified from Kalita et al., 2021).

and potential for SOC change are site specific being dependent on previous land use, as well as soil and climate conditions (Agostini et al., 2015; Rytter et al., 2015; Walter et al., 2015).

When including the effects of substituting fossil-based energy, all *Salix* systems had negative values for both GWP_{100} and ΔT_s, which means that they lead to a net removal of GHGs from the atmosphere and have a cooling effect on the Earth's climate over this study period (Figure 4). This indicates that cultivation of selected *Salix* varieties under the given set of conditions in central Sweden for bioenergy production to substitute fossil energy would have a cooling effect on the climate, and thus a potential to mitigate climate change. The climate impacts of the different *Salix* biomass-to-bioenergy systems after 50 years under the system conditions including substitution effects of the reference scenario are shown in Figure 4 with two functional units; namely, 1 ha of land and one MJ of heat (Kalita et al., 2021). Even when looking at the *Salix* systems alone, that is, without substitution effects, all except fertilized Björn lead to negative emissions over the lifecycle under the given system conditions.

From a land use perspective (i.e., per hectare), the biomass harvest yield was the governing factor for the climate impact results. Increased yield levels indicates more fossil energy replaced by *Salix*, which in turn leads to reduced emissions from fossil fuels. As a result, the high-yielding fertilized Tordis, Björn, Tora, and Jorr had the greatest cooling effect on the climate. Gudrun and Loden were the worst climate performers in this case, as fertilization did not significantly improve yields of these two varieties (Figure 4). However, a different scenario arises when investigated from the point of final energy output (per MJ_{heat}). Here, the potential to sequester carbon as SOC becomes the dominant factor in determining the climate impacts. The unfertilized varieties of Jorr and Loden offer the greatest potential for climate change mitigation per MJ of heat output (Figure 4) as they sequester the most CO_2 as SOC during the study period.

The results from Kalita et al.'s (2021) study show that *Salix* genotype influences the biomass yield, response to fertilization, SOC sequestration rate, and ultimately the climate impact. It is important to account for these genotype effects in systems studies and environmental impact assessments. Hence, selection of the optimal *Salix* variety and fertilization practice is very important to obtain the desired energy and climate change mitigation outcome from the biomass-to-bioenergy system. In the quest for high harvest yields, root biomass should not be ignored as they can sequester significant amount of SOC and greatly influence final climate impacts. Further studies on belowground biomass growth and persistence of soil carbon are necessary to improve accuracy of soil carbon modeling results. Development of *Salix* varieties that produce high shoot biomass yields along with great allocation of biomass and persistent C forms (roots and exudates) below ground would be optimal for climate change mitigation. There are apparent links between the biomass and C accumulation aspects discussed here and the wood quality aspects discussed in Section 2, although we have so far been unable to integrate these aspects. Future studies should integrate those aspects in a quantitative way in order to guide *Salix* breeding for climate change mitigation.

6 | BREEDING OF *SALIX* VARIETIES

Breeding of *Salix* with a main goal to increase yield has been conducted for decades in both Europe and the United States. Since *Salix* species have large phenotypic and genetic variations, and are very early in their domestication process, great progress has been accomplished in the development of new and higher yielding cultivars (Kuzovkina et al., 2008; Larson, 1998; Smart & Cameron, 2008). The practical *Salix* breeding programs have mainly focused on recurrent selection based on phenotypic measurements and crossing within and between species and considerable phenotypic variation remains unused in breeding populations. Recent focus on prebreeding research on *Salix* has been on understanding the genetic background of the phenotype and developing genomic tools for early selection of individuals, with potential of making breeding more efficient (Carlson et al., 2019; Hallingbäck et al., 2019; Hanley & Karp, 2014).

To study the genetic background of *Salix* traits, different strategies or a combination of strategies are applicable. An early attempt to identify the genes and genetic variants underlying phenotypic variation for traits connected to *Salix* wood and growth traits relevant for biofuel production was made by Brereton et al. (2010) who performed a quantitative trait locus (QTL) mapping study in a *Salix* hybrid population. More recently, using a biparental population of *S. viminalis* and QTL mapping, Pawar et al. (2018) identified regions in the genome connected to different FT-IR chemotypes (lignin, hemicellulose, cellulose) and saw large variation in methane production in a subset of the offspring population. Compared to QTL mapping studies, genomewide association studies (GWAS) allow for a more general view of the genetic background of complex traits since unrelated individuals from large populations are studied (Thavamanikumar et al., 2013). Due to the unrelatedness of the individuals in these populations, genetic markers associated with traits are likely to be located very close to the causative mutation or gene. In outcrossing species like *S. viminalis*, linkage disequilibrium

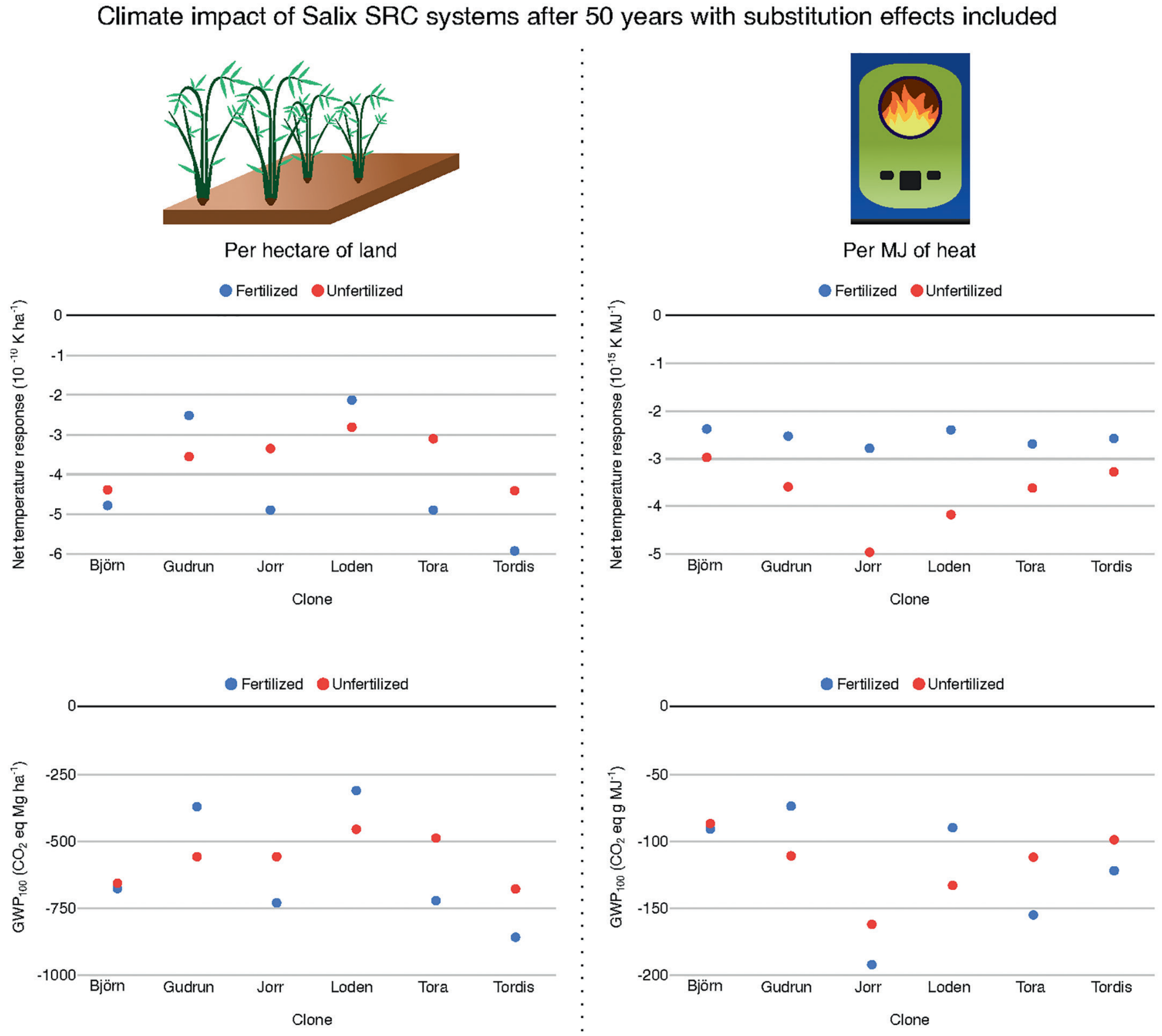

FIGURE 4 Climate impacts of Salix (SRC) systems after 50 years including substitution effects. The climate impacts are given in terms of GWP100 and ΔT_s per hectare of land and per MJ of heat (modified from Kalita et al., 2021).

(LD, i.e., the statistical correlation between neighboring genetic markers) is usually very low (Berlin et al., 2011). Consequently, large numbers of markers are required in GWAS to identify reliable marker–trait associations. Using a population of *S. viminalis* accessions collected from across Europe into Russia (Table 1, subset of 1), together with around 20,000 markers, Hallingbäck et al. (2019) identified markers connected to different growth traits. In the taxonomically related genus *Populus*, several studies of large populations of unrelated accessions have also demonstrated the utility of GWAS methods for identifying genes controlling various wood traits (Guerra et al., 2013; Muchero et al., 2015; Porth, Klápště, Skyba, Hannemann, et al., 2013; Porth, Klápště, Skyba, Lai, et al., 2013; Wegrzyn et al., 2010).

In the OPTUS project, one aim was to evaluate the possibilities for breeding by studying the genetic variation of traits important for biomass growth and recalcitrance as well as finding genetic markers for these traits. By investigating the genetic correlations between traits, another aim was to understand which traits are the most important for breeding. To study these aims, a large population of approximately 300 individuals (see above, Table 1, subset of 1) (Hallingbäck et al., 2019) was used to assess growth, wood traits, and sugar release after pretreatment, to obtain an overview of the variation in *S. viminalis* (see also Section 3; Ohlsson et al., 2019). Considerable phenotypic variation was found for all traits studied, and part of this variation also had genetic causes as shown in the moderate to high narrow sense heritabilities (Figure 5; Ohlsson

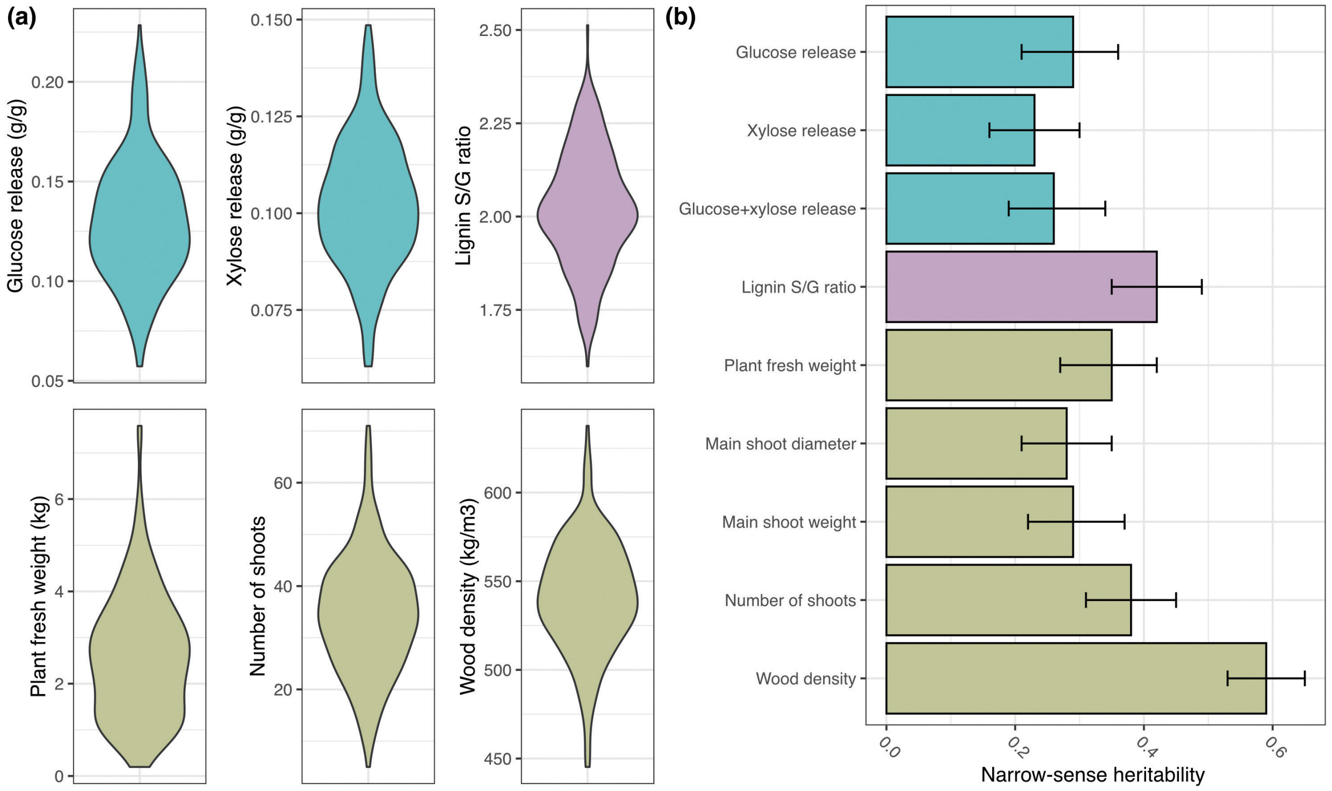

FIGURE 5 Phenotypic variation for selected traits illustrated with violin plots (a) and narrow sense heritabilities for different growth and wood traits (b), 95% CI is indicated for each heritability value (modified from Ohlsson et al., 2019).

et al., 2019). In particular, wood density and lignin S/G ratio showed high narrow sense heritability values and are interesting traits to improve through breeding. The heritability values for sugar release (0.23–0.29) are in the same range if somewhat lower than in other studies of woody crops (Fahrenkrog et al., 2016; Guerra et al., 2016; Harman-Ware et al., 2022; Porth, Klápště, Skyba, Hannemann, et al., 2013; Porth, Klápště, Skyba, Lai, et al., 2013).

Additional findings included the high significant additive genetic correlations between sugar release and different growth traits (e.g., correlation between combined release and main shoot weight and with main shoot diameter were 0.65 and 0.64, respectively) indicating that growth traits could be used as a proxy for sugar release during selection in breeding (Ohlsson et al., 2019). In *Populus trichocarpa*, it was shown that sugar content is an important trait for the financial viability of biorefineries only after a certain biomass yield has been attained. This suggests that biomass yield may still be more important than biomass recalcitrance as a target for biorefinery-targeted breeding (Happs et al., 2020). The low genetic correlations between density and sugar release indicate traits that may be selected independently (Ohlsson et al., 2019).

The GWAS made with this material and the approximately 20,000 markers developed from the genotyping by sequencing showed few suggestive marker associations. Among these, one marker was related to glucose release and gave an effect of 27% increase in glucose release in individuals having the homozygote genotype of the rare allele (Ohlsson et al., 2019). Further studies to confirm this association and to study variation in genes close to this marker would be interesting.

Breeding of low-recalcitrance *Salix* varieties therefore seems feasible since traits of importance have a high phenotypic and genotypic variability. The polygenic nature of most of these traits (i.e., sugar release, growth traits) makes it difficult to identify specific markers of high significance interesting for selection programs. However, since several *Salix* genomes are now available (Almeida et al., 2020; Zhou et al., 2020) and the cost for resequencing individuals has been significantly reduced, genomic selection seems to be a realistic alternative selection method. With this method, the variation in the whole genome is considered and can be used to estimate genomic breeding values taking into consideration the effects of all genes influencing the traits.

7 | CONCLUSIONS

Compared to other biomass crops, SRC *Salix* represents an interesting alternative to increase biomass production that could be used for conversion to transportation biofuels. Knowledge of the chemical composition and the wood anatomical micro/macromolecular structure of the wood is important for the conversion efficiency into biofuels. For the commercial willows and the *S. viminalis* population addressed in this work, great variation was found in several wood traits including wood density, S/G ratio, and recalcitrance (sugar release after pretreatment and EH). Heritabilities between 0.23 and 0.59 indicate an appropriate genetic background for the different traits, which is necessary for further genetic improvements by plant breeding. Strong correlations between the amount of tension wood and sugar accessibility (glucose) were shown while the development of a novel method using *Salix* stem cross sections and chemical analyses demonstrated the possibility to estimate the relative importance of tension wood for the comparison of different plant materials.

Biological conversion systems suitable for *Salix* biomass include EH and AD processes, with low-recalcitrance *Salix* biomass providing economic benefits through both processes, although using different mechanisms. Using serial digestion in AD plants could increase the conversion yield and be of interest for small-scale AD plants. When comparing *Salix* genotypes regarding conversion yield on area bases, the high-yielding genotypes produced most biomass, indicating the importance of high biomass production for high conversion yields. In terms of thermochemical conversion, two different conversion routes after fast pyrolysis were investigated, and both hydroprocessing and catalytic cracking of *Salix* biomass represent interesting alternatives for producing gasoline and diesel range products.

An important aspect in the sustainability evaluation of *Salix* biomass systems is the potential for carbon sequestration and the climatic impact of *Salix* plantations. Appropriate genotype selection is important for the overall climatic impact, since biomass yield and allocation to above- and below-ground plant systems, the growth response to fertilization, and ultimately the carbon sequestration potential vary, depending on the *Salix* genotype. The preferred *Salix* plant material to select and breed should have high biomass production both above- and below ground, although results show that aboveground (shoot) growth is not necessarily correlated with belowground (root) growth and carbon accumulation in willows. In addition, complex interactions with cultivation regimes and other ecological processes affecting belowground carbon sequestration must be considered.

To conclude, the different aspects investigated in the OPTUS project show a high potential for using *Salix* biomass for conversion into transportation biofuels. Large genetic variation in various wood and biomass traits important for different conversion processes and carbon sequestration indicates great opportunities to enhance the sustainability of the production system via plant breeding, for example, by adding new breeding targets to the traditional targets for high yield to improve biomass quality and carbon sequestration potential.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Genotypic data for the study population (Table 1, subset of 1) generated by the genotyping-by-sequencing (GBS) process are publically available at the Zenodo repository (http://doi.org/10.5281/zenodo.2607520). All other data are available on request.

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