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PhD thesis

Root microbiomes as controls of P use efficiency in woody crops

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Abbreviations:

DCP: di-calcium phosphate

G: Germany

HighP or HP: High phosphorus

K: potassium

L: Loden – *Salix* cultivar (SW 890129, *S. dasyclados*)

LowP or LP: Low phosphorus

Mg: magnesium

NoP or NP: No phosphorus

P: phosphorus

PCR: polymerase chain reaction

PSB: phosphorus solubilizing bacteria

PSF: phosphorus solubilizing fungi

PSM: phosphorus solubilizing microorganism

S: Sweden

SRC: Short rotation coppice

T: Tora – *Salix* cultivar (Svalöf-Weibull (SW) cultivar no. 910007, *S. schwerinii* × *S. viminalis*)

TCP: tri-calcium phosphate

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List of publications

1. List of publications that are a main part of the doctoral dissertation

Publication 1 - (P1)

Koczorski P., Furtado B., Hrynkiewicz K., Breezmann, M., Weih M. and Baum C., 2021. Site effects dominate the plant availability of nutrients under *Salix* species during the first cutting cycle. *Forests*, 12(9), p.1226.

https://doi.org/10.3390/f12091226

IF 2021 = 3.282; 5 Year IF: 3.292; punkty MEiN = 100; 2 citations

Publication 2 - (P2)

Koczorski P., Furtado B.U., Gołębiewski M., Hulisz P., Baum C., Weih M. and Hrynkiewicz K., 2021. The effects of host plant genotype and environmental conditions on fungal community composition and phosphorus solubilization in willow short rotation coppice. *Frontiers in Plant Science*, 12, p.647709.

https://doi.org/10.3389/fpls.2021.647709

IF 2021 = 6.627; 5 Year IF: 7.255; punkty MEiN = 100; 5 citations

Publication 3 - (P3):

Koczorski, P., Furtado B.U., Gołębiewski M., Hulisz P., Thiem D., Baum C., Weih M. and Hrynkiewicz K., 2022. Mixed growth of *Salix* species can promote phosphate-solubilizing bacteria in the roots and rhizosphere. *Frontiers in Microbiology*, *13*.

https://doi.org/10.3389/fmicb.2022.1006722

IF 2021 = 6.064; 5 Year IF: 6.843; punkty MEiN = 100

Publication 4 - (P4) (manuscript under revision)

Koczorski P., Furtado B.U., Baum C., Weih M., Ingvarsson P.K., Hulisz P. and Hrynkiewicz K., 2023. Large effect of P solubilizing bacteria on growth and gene expression of *Salix* spp. under stress conditions induced by reduced phosphorus levels. (manuscript under revision)

Summary of IF: 15.973

Summary of 5 Year IF: 17.390

Summary of MEiN points: 300

2

Abstract in Polish

2. Abstract in Polish

Fosfor jest jednym z kluczowych pierwiastków potrzebnych roślinie do prawidłowego wzrostu i rozwoju. Źródła P dostępne w przyrodzie są wyczerpywalne. W warunkach naturalnych P wymywany jest ze skał macierzystych i szybko tworzy trudno rozpuszczalne i niedostępne dla roślin kompleksy. Nowoczesna gospodarka rolna oparta na nawozach sztucznych nie jest wystarczająco wydajna. Sytuacja ta wymusza opracowanie alternatywnych technologii pozyskiwania fosforu, np. wspomagania mikrobiologicznego.

Mikroorganizmy ryzosferowe i endofityczne posiadają zdolności promujące roślin. Jedną z nich jest zdolność do mikrobiologicznej solubilizacji fosforu obecnego w glebie. Mikroorganizmy solubilizujące fosfor posiadają zdolność do uwalniania fosforu będącego częścią kompleksów z Al, Fe czy Ca, przekształcając go w formę, która jest dostępna dla roślin. Selekcja mikroorganizmów posiadających zdolność do zwiększania dostępności P w środowisku glebowym, zwłaszcza w połączeniu z innymi właściwościami stymulującymi wzrost roślin, np. synteza IAA, sideroforów, jest niezwykle ważnym kryterium doboru mikroorganizmów, które mogą znaleźć zastosowanie w uprawach roślin. Jest to szczególnie ważne zagadnienie w odniesieniu do popularnych w ostatnim czasie uprawach wieloletnich, np. wierzb uprawianych w systemie zagajników szybkiej rotacji (SRC), w których stosowanie nawozów jest ograniczone.

Dlatego też, celem pracy była ocena zróżnicowania oraz liczebności mikroorganizmów ryzosferowych i endofitycznych solubilizujących fosfor na dwóch stanowiskach SRC (prowadzonych w monokulturach i uprawach mieszanych) i zbadanie ich wpływu na wzrost i ekspresję genów dwóch gatunków wierzby.

Badania zaprezentowane w pracy doktorskiej prowadzono w SRC na stanowisku w Uppsali (Szwecja) i Rostoku (Niemcy), na których uprawiano dwie odmiany wierzby - Loden i Tora. Podczas realizacji doświadczeń wykorzystano klasyczne metody mikrobiologiczne umożliwiające izolację i selekcję mikroorganizmów solubilizujących fosfor (PSM) oraz metody molekularne umożliwiające identyfikację otrzymanych izolatów PSM (i) i ocenę zróżnicowania mikrobiomu (ii). W pracy wykorzystano również analizę transkryptomu wierzb poddanych inokulacji wyselekcjonowanymi PSM w warunkach niedoboru P w podłożu.

W efekcie przeprowadzonych badań wyselekcjonowano najbardziej efektywne bakterie solubilizujące fosfor: *Pantoea agglomerans* (B1) oraz *Paenibacillus* sp. (B2) i zastosowano je

w doświadczeniu donicowym w różnych warunkach dostępności fosforu (NP, LP, HP). Analiza mikrobiomu gleby ryzosferowej i endofitów korzeniowych wykazała, że uprawy mieszane wierzb promują wzrost różnorodności bakterii i grzybów, w tym tych solubilizujących fosfor, a wyższe zróżnicowanie mikroorganizmów jest obserwowane w ryzosferze. Wykazano, że aktywność kwaśnej fosfatazy na badanych stanowiskach SRC była trzykrotnie wyższa od fosfatazy zasadowej, co korelowało z wpływem pH stanowisk. Wśród najczęściej izolowanych grzybów solubilizujących fosfor dominowały szczepy *Penicillium* sp., a wśród bakterii *Pseudomonas* sp., *Bacillus* sp. i *Erwinia* sp. Badania mikrobiomu wykazały silny wpływ poziomu asocjacji z rośliną (mikroorganizmy ryzosferowe *vs.* endofity) na zróżnicowanie mikrobiomu wierzby. Na zróżnicowanie grzybów istotnie wpływały takie czynniki glebowe jak: całkowity węgiel organiczny oraz pH, zaś w przypadku bakterii: dostępny fosfor oraz całkowity azot. Inokulacja wierzb wyselekcjonowanymi bakteriami PSM przeprowadzona w doświadczeniu donicowym wykazała istotny wzrost parametrów wzrostu roślin w warunkach HP, jednak zależała od gatunku badanej wierzby (pędy gatunku Loden wykazały istotny przyrost biomasy, zaś Tora na długość). Analiza transkryptomu liści wierzb wykazała dla gatunku Tora wzrost, a dla Loden obniżenie ekspresji genów. Inokulacja wierzb bakteriami PSM znacząco wpłynęła na ekspresję genów związanych z fotosyntezą oraz syntezą skrobi (szczególnie dla gatunku Tora), w których to procesach fosfor pełni bardzo istotną rolę. W przypadku gatunku Loden obserwowano wzrost ekspresji genów związanych głównie z transportem jonów, regulacją transkrypcji oraz genami chromosomowymi.

Praca doktorska stanowi nowatorskie i kompleksowe podejście do badań nad analizą i selekcją mikroorganizmów PSM, stanowiąc punkt wyjścia dla potencjalnego wykorzystania mikroorganizmów PSM w celach komercyjnych.

3

Abstract in English

3. Abstract in English

Phosphorus (P) is one of the key elements needed by plants for proper growth and development. The plant availability of P in soils can be growth limiting, partly even with high total contents caused by rapid P fixation. Therefore, the P use efficiency should be promoted in arable soils rather than the P supply. Microbial P mobilization can substantially contribute to increasing the P use efficiency in soils.

Rhizosphere and endophytic microorganisms can have plant-promoting abilities. One of these is P mobilization, which is an important criterion for the selection of microorganisms for biofertilizers. This is a particularly important issue for perennial crops, such as willows (*Salix* spp.) grown in short rotation coppice (SRC) for biomass production.

Therefore, the aim of this study was to assess the diversity and abundance of P-solubilizing microorganisms in the rhizosphere and roots of *Salix* spp. in two SRC sites (grown in monoculture and mixed cropping) and to test if growth design, *Salix* spp. and the P solubilizing microbial strain inoculation can promote biomass production and P use efficiency.

The test sites of the present study are two SRCs in Uppsala (Sweden) and Rostock (Germany). The *Salix* genotypes tested were the cultivars 'Loden' (SW 890129, *S. dasyclados*) and 'Tora' (Svalöf-Weibull (SW) cultivar no. 910007, *S. schwerinii* × *S. viminalis*). Cultivation-dependent microbiological methods were used to isolate and select P solubilizing microorganisms (PSMs), and molecular methods were used to identify the PSM isolates obtained (i) and assess the diversity of the microbiome (ii). This study also utilized transcriptome analysis of willows inoculated with selected PSMs under P-deficient conditions in the substrate to control the plant physiological response to microbial inoculation.

The present study resulted in the selection of the two most effective P solubilizing bacterial strains (*Pantoea agglomerans* and *Paenibacillus* sp.). These strains were used for inoculation of *Salix* spp. at three levels of P availability in a pot experiment. Furthermore, it was revealed that mixed cropping of *Salix* spp. can increase bacterial and fungal diversity in general, as well as the diversity of P-solubilizing microorganisms. Among the most frequently isolated P solubilizing fungi, *Penicillium* spp. dominated, and among the P solubilizing bacteria, *Pseudomonas* sp., *Bacillus* sp. and *Erwinia* sp. dominated. The microbial diversity decreased significantly from the bulk soil to the rhizosphere and from the rhizosphere to root endophytes. Fungal diversity was significantly controlled by the chemical soil properties total

organic carbon content and pH, while bacterial diversity was significantly controlled by bioavailable P concentration and total nitrogen content in the soil. Inoculation of willows with PSM significantly increased plant growth under high P availability in the soil. However, this effect differed and was *Salix* genotype specific. The cv. Loden significantly increased biomass production, whereas cv. Tora significantly increased only the shoot lengths. Transcriptomics revealed an overall upregulation of photosynthesis and starch formation genes in the Tora species and a downregulation of ion transport, transcriptional regulation and chromosomal genes in the Loden species after inoculation with PSM.

This dissertation represents a novel and comprehensive approach to research on the selection and use of PSMs and on the promotion of PSMs by planting design, providing a starting point for the potential commercial use of PSMs and their promotion from the soil pool by site management.

4

Introduction

4. Introduction

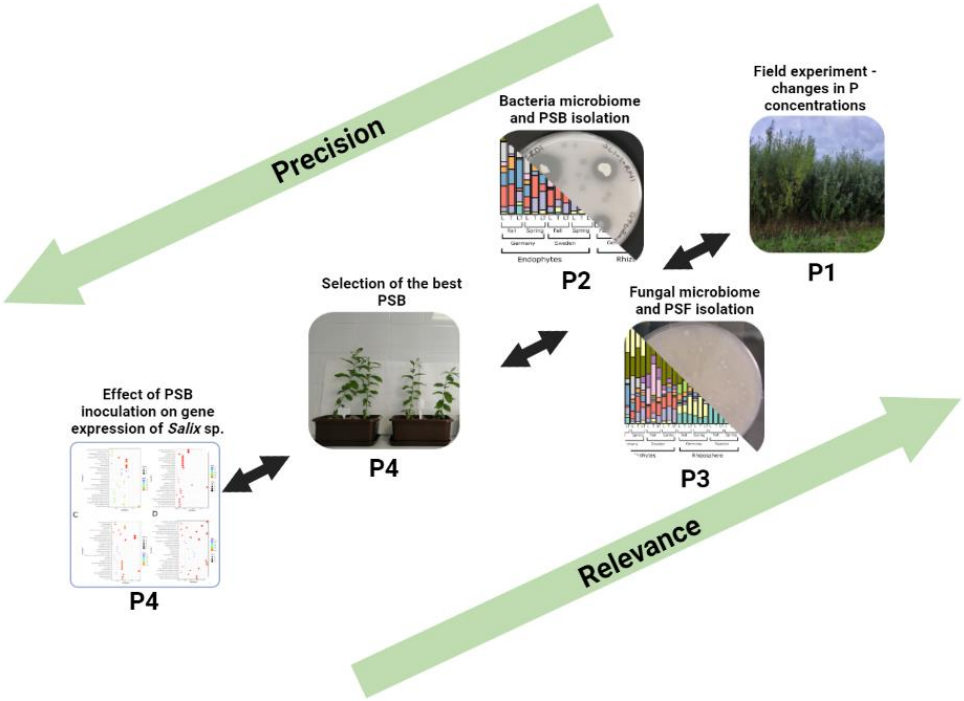

Figure 1 Diagram showing the most important findings and summary of the results from 4 publications that are a part of this dissertation.

Short-rotation coppices (SRCs) with fast-growing tree species are very popular in Europe because of the range of economic and ecological benefits that are derived from their cultivation. Trees grown in these coppices are primarily a source of biomass for energy production, which contributes to reducing fossil fuel consumption and deforestation (Berhongaray et al., 2017). SRCs have a positive impact on water management, protecting groundwater resources, increasing water retention and improving water quality at the same time (Dimitriou et al., 2021). Another important aspect of this type of SRC is its positive impact on the biodiversity of the soil environment compared to current agricultural practices, which focus on planting monocultures to obtain the highest possible biomass yield (Kahle et al. 2005). The most commonly selected tree species for this type of crop are those with fast growth rates, low nutrient requirements, tolerance to a wide range of pH values and adaptability to climatic conditions (Caslin and Teagasc, 2010; Langeveld et al., 2012; Amichev et al. 2014). In Europe, various willow (*Salix*) and poplar (*Populus*) species are most commonly used, as these are naturally occurring species that are part of floodplain forests composed of softwoods (Tullus et al., 2013; Pleguezuelo et al., 2015). Poplar is typical of western and central European

floodplains, whereas willow is more tolerant of low temperatures and can grow further north and in continental climates in eastern Europe (Hughes, 2003).

Willows are trees or shrubs comprising almost 350-500 species worldwide, meeting all the previously mentioned criteria for selecting species for cultivation in SRCs (Dickmann and Kuzovkina, 2014). They are additionally characterized by their ability to survive in conditions of extreme pollution and are often used for phytoremediation of such sites (Ruttens et al., 2011; Padoan et al., 2019). The productivity of willow coppice crops can be increased by establishing them on fertile land or by applying organic fertilizers such as manure and digested sewage sludge (Dimitriou et al., 2011). However, this involves the risk of leaching nitrates and phosphates. Willows are able to reduce these risks by a high uptake of nutrients (Mirc et al., 2005). The effective use of P is mainly based on its association with microorganisms in the roots and rhizosphere (Richardson and Simpson, 2011).

To date, SRCs have mainly been planted in monocultures; however, mixed cropping has become more common over the past 20 years because of ecological advantages revealed by ongoing research activities (Hoeber et al., 2018; Rödl, 2019). The increased diversity of trees in SRCs and their effects on growth and P mobilization in the soil and roots of willows in mixed cropping systems have been studied in detail in this work. According to my research, mixed cropping increases the diversity not only of P-solubilizing fungi and bacteria but also of the total microbiome of the rhizosphere and roots of willows (Koczorski et al., 2021; Koczorski et al., 2022). A reason for higher biodiversity in general and microbial diversity in mixed cropping may be the increased competition between both plants and microorganisms (Weih et al., 2019).

P sources, unlike nitrogen, are nonrenewable and intensive agriculture is based largely on mineral P fertilizers. For these reasons, global phosphate deposits decrease, and P recycling and more effective P use in agriculture are urgently needed (Gilbert, 2009). The low use efficiency of this type of fertilizer is a serious problem, as only a small portion of P will be assimilated by the plant, and the remainder will often be fixed in the soil (Filippelli, 2008; Singh and Satyanarayana, 2011). In addition to P fixation, P loss by leaching is problematic since it can lead to eutrophication and eventually complete its cycle on the seabed where there is no easy way to recover it (Khan et al., 2018).

Soils, especially if affected by roots, such as in the rhizosphere, host a large pool of microorganisms capable of promoting plant growth and development. Some of them can also penetrate plant roots and, as endophytes, provide the plant with essential metabolites (Hardoim

et al., 2008). Thus, the contribution of P solubilizing microorganisms (PSMs) in facilitating plant access to P supply is very important (Billah et al., 2019). There is a growing pool of literature describing plant growth promotion by PSMs (Kalayu, 2019; Prabhu et al., 2019; Divjot et al., 2021; Rawat et al., 2021). PSMs can be used as biofertilizers, which can help to reduce the need for fertilizer application and the accumulation of toxic elements in arable soil caused by geogenic cadmium and uranium contamination in mineral phosphates (Khan et al., 2009; Alori et al., 2017). Among fungi, the most commonly described species capable of P solubilization are *Aspergillus* spp., *Penicillium* spp., *Trichoderma viride*, *Arthrobotrys oligospora*, *Cephalosporium* sp., and *Cladosporium* sp. (Khan et al., 2010; Patil et al., 2012; Sharma et al., 2012; Ram et al., 2015; Li et al., 2016; Alori et al., 2017). The most commonly described and used P solubilizing bacteria (PSB) are *Pseudomonas* sp., *Bacillus* sp. and *Streptomyces* sp. (Wani et al., 2005; Chen et al., 2006; Ahemad and Khan, 2011; Kaur et al., 2011; Grafe et al., 2018; Rathinasabapathi et al., 2018; Ahmad et al., 2019; Wang et al., 2020). It is worth mentioning that new PSMs are still being identified and characterized, and the publications that are part of this work have contributed to this expanding effort (Koczorski et al., 2021; Koczorski et al., 2022). These publications describe lesser-known and sometimes undescribed PSMs such as *Talaromyces* sp., *Alternaria* sp., and *Juxiphoma* sp. among the fungi as well as *Mitsuaria* sp. *Ralstonia* sp. and *Lelliottia* sp. in the case of bacteria. Notably, the role of the abovementioned microorganisms is not limited to phosphorus solubilization. Fungi of the genera *Asperigllus* and *Penicillium* and bacteria of the genera *Pseudomonas* and *Bacillus* possess a number of additional plant growth-promoting properties, such as the ability to stimulate the synthesis of plant hormones, e.g., gibberellins (Khan et al., 2011; Preston et al., 2004; Abdel-Motaal et al., 2020), which stimulates the plant defence system by changing secondary metabolism (Hossain et al., 2007), etc.

In light of this research, it is important to gain a deeper understanding of the interaction of bacteria and fungi with plants to develop effective biofertilizers containing P-solubilizing microorganisms. To date, researchers have focused on studying the impact of P deficiency on plants directly and to a much smaller extent on the interactive effects of plants and microorganisms (Ren et al., 2018; Mo et al., 2019; Wang et al. 2019, Zhang et al. 2019; Sun et al. 2016). As established, the typical response of a plant to P deficiency is to trigger the transcription of genes involved in the synthesis of auxins, abscisic acid, jasmonic acid, salicylic acid or ethylene (Sun et al. 2016). The plant inoculation process itself, in the case of fungi, can affect the activity of genes related to auxin synthesis and responses to N or P deficiency

(Ludwig-Müller et al., 2015). Fungi can also stimulate the transcription of genes involved in phosphatase synthesis or genes related to P transport in plant tissues (Ray et al., 2021). In contrast, little is known about bacteria and their effects on the plant transcriptome regarding P cycling, and the only studies on this topic that I was able to find were those by Soni et al. (2021). By examining the transcriptome of a tobacco plant inoculated with the bacterium *Paenibacillus polymyxa*, the authors found that, in addition to its plant growth-promoting properties, it also activated a number of genes responsible for P transport (*pstA*, *pstB*, *pstC*, *pstS*, *phnD* or *phnE*). The above information indicates that there is a gap in our current knowledge of plant–microbe interactions under P deficiency stress. In my PhD thesis, I have investigated the effect of plant inoculation with P-solubilizing bacteria under varying conditions of phosphorus availability by combining two hitherto separately studied topics. This thesis therefore presents detailed and novel results on the topic of PSMs, which are available as a described collection for subsequent production of commercial bioinoculants.

5

Aims of study

5. Aims of the study

The main aim of this study was to reveal site effects and management options for the improved use of microbial P mobilization in biomass production with fast-growing tree species.

Objectives:

1. Determine the influence of factors such as environmental conditions, planting design (mono- *vs.* mixed cultures), and plant species on microbial diversity and activity in soils. **(Publication 1)**

2. Assessment of the impact of soil properties, planting design and the level of plant association effect on P solubilizing microorganisms to determine their role and contribution to the background of the entire *Salix* microbiome. **(P2, P3)**

3. Examine the effect of microbial inoculation on the expression of genes involved in P cycling in *Salix* under P deficiency. **(P4)**

6

Research hypotheses

6. Research hypotheses

1. Planting design with mixed *Salix* species may increase soil nutrient availability (P, K, Mg) and soil enzymatic P mobilization by phosphatases, thus preventing nutrient loss in the initial cutting cycle by increasing plant diversity. **(P1)**
2. The level of plant association (direct: endophytes *vs.* indirect: rhizosphere contact with plant tissue) may be the main factor determining the diversity of the P solubilizing microbiome, with little or no effect of factors such as the planting design, site and seasonal changes. **(P2 and P3)**
3. Microbial inoculation of *Salix* grown at different levels of P availability improves plant growth by plant physiological changes in gene expression. **(P4)**

7

Methodology of research

7. Research methodology

Table 1. Summary of techniques used during the research work

Technique	Scope of research	P 1	P 2	P 3	P 4
Selection of P solubilizing soil microorganisms	• Performed on three selective media: two containing tri-calcium phosphate (NBRIP, PVK) and one with di-phosphate (modified Pikovskaya). Composition of all media used is described in P 2.		✓	✓	✓
Identification of microorganism involved in the soil P cycling	• DNA isolation from bacteria was performed using Bacterial & Yeast Genomic DNA Purification Kit (EurX, Poland) • DNA isolation from fungi was performed using Plant & Fungi DNA Purification Kit (EurX, Poland) • Isolated bacterial DNA was amplified by PCR method using 27F and 1492R primers (described in P-3) while isolated fungal DNA was amplified using ITS1 and ITS4 primers (described in P 2) • Gel electrophoresis was performed on 2% agarose gel containing X and SimplySafe stain (EurX, Poland) to confirm presence of both DNA and RNA in samples (described in P 2)		✓	✓	✓
Biochemical and chemical soil analyses	• Acid and alkaline phosphatase activity (p-nitrophenyl colorimetric method) was performed to assess biocatalysis of P mobilization (P 1).	✓			✓

	• Total organic carbon (TOC), total nitrogen (TN), carbon to nitrogen ration (C:N), bioavailable phosphorus in soil and P uptake • Into the plant (Molybdenium blue for soil samples and inductively coupled plasma (ICP) for plant samples) and soil pH was measured in P-2 to provide more details on soil properties present on both test sites. • P content in leaves and rhizosphere soil was assessed using ICP method combine with soil fractionation method (P 4)				
Total DNA isolation and libraries purification for microbiome analysis	• Samples were taken from rhizosphere soil and roots of willows from two test sites and two seasons. Fungal ITS amplicon libraries (P 2) were generated in two-step PCR, as described by Thiem et al. (2018) using fungal primers (uITS1 and uITS2) then with M13 and M13R primers with P5/P7 adapters and barcodes (different MID sequences for each sample). For bacteria (P 3) different primers were used during first stem of PCR, namely: u357f and u786. • Libraries were purified twice with Agencourt AMPure XP (Beckman Coulter, USA) according to the manufacturer's protocol. The quality of the pooled libraries was assessed		✓	✓	

	on a Bioanalyzer chip (Agilent, USA) and they were quantified with KAPA Library Quantification Kit for the Illumina Platform using LightCycler 480 (Roche, Switzerland) according to the manufacturers' protocols.				
Total RNA isolation and sequencing	• RNA isolation using Chomczynski method described in details in P 4 • Sequencing of total RNA was done using the Illumina platform (SBS)				✓
Spectrophotometric analyses	• Performed to assess the quantity and quality of isolated DNA or RNA using a Nanodrop 2000 (Thermo Fisher, USA)		✓	✓	✓
Software used	**Scope of research**	**P1**	**P2**	**P3**	**P4**
Statisctica 13.3.721 (Statsoft)	• Used to perform all comparison between variants. ANOVA and Two-way ANOVA was used in combination with Tukey's or Kruskal-Wallice post hoc test depending on data normality (tested with Shaphiro Wilk test). Equality of variance was tested using Levene's test.	✓	✓	✓	✓
Sequencher 5.4.6	• Used to prepare contigs and correct errors from forward and reverse reads acquired from sequencing.		✓	✓	
RStudio	• Used to prepare figures with ggplot2, tidyverse, forcats and plotly packages.		✓	✓	✓

	• Additionally, a list of R packages used in microbiome analysis is presented in P 2. • Heatmap in P 4 was generated using ggplot2 package				
Inkscape 1.0	• Software was used to refine and group figures prepared in R.		✓	✓	✓
Transcriptome analysis performed by Novogene company	• Mapping was done using hisat2 software • Assembly was performed using Stringtie software • Quantification was conducted using featureCounts software • Differential analysis was performed using DESeq2 and edgeR software with following parameters: |log2(FoldChange)| >= 1 & padj <= 0.05 • Enrichment analysis was performed using clusterProfiler software				✓

8
Publications

8. Publications

forests

MDPI

Article

Site-Effects Dominate the Plant Availability of Nutrients under Salix Species during the First Cutting Cycle

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Abstract: Fast-growing willows (*Salix* spp.) provide alternative sources of renewable energy generation, but need an adequate nutrient availability in the soil for high biomass production. In general, species mixtures can be more nutrient-efficient than pure cultures, but this is scarcely known for *Salix* spp. Therefore, this study evaluates the nutrient availability and P mobilization under two willow species, *Salix dasyclados* var. 'Loden' and *S. schwerinii* × *viminalis* var. 'Tora', grown as pure and mixed cultures at non-fertilized former arable sites in Germany (Stagnic Cambisol) and Sweden (Vertic Cambisol). The plant availability of potassium (K), magnesium (Mg) and phosphorus (P) and soil phosphatase activities in the topsoil were measured in spring of the year of planting (initial) and under 4 years-old stocks (one year after the first 3-year cutting cycle). The initial plant availability of the nutrients significantly differed between the sites and the two sampling dates at both sites. The plant availability of K and Mg was optimal to high at both sites and sampling dates, but rather low for P (after 4 years $\leq$5 mg P 100 g^{-1} soil). The plant-available P and K content in soil significantly decreased within the 4 years of willow growth at both sites. The acid and alkaline phosphatase activity in the soil of the German site (Rostock) was significantly lower after 4 years of willow growth, but differed not significantly between the two sampling dates at the Swedish site (Uppsala). Higher activity of acid phosphatase compared to alkaline phosphatase was recorded in the soils at both test sites based on the site-specific soil pH (<7). The slight decrease of plant availability of P after 4 years of *Salix* growth in pure culture differed not significantly between the different species. Mixed growth did not decrease the plant availability of P within this period, although no significant difference in the biomass production of pure and mixed growth was observed. This was valid at both sites, and therefore, seems independent of the site-specific differences in soil and climate conditions. The general validity of the assumptions should be tested also for other species mixtures and soil conditions in the future before site-adapted growth designs can be recommended in biomass production of *Salix*.

Keywords: short rotation coppice; phosphatase activity; nutrient content; growth stages; biomass; willow; *Salix*

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1. Introduction

Short rotation coppices (SRCs) with poplar (*Populus*) and willows (*Salix*) species can be established on many types of land, including marginal lands that are unsuitable for agriculture [1,2]. SRCs offer a promising contribution to fuel wood supply, providing an alternative to fossil fuels and other nonrenewable resources [3]. The harvest from SRCs are usually used as feedstock in combined heat and power plants for energy generation [4,5]. Moreover, SRC plantations have ecological benefits compared to annual crops. They improve the soil water retention, enhance biodiversity in comparison to agricultural

monocultures, improve water quality, protect ground water, prevent soil erosion and it is a low-input agricultural practice, thus implying low greenhouse gas emissions due to limited applications of chemicals [6–10] SRCs have been investigated intensively for the last 20 years (reviewed by Rödl [11]). SRC are managed using agricultural techniques, including high-density plantings and a regular cutting every 2 to 6 years without replanting [12]. The choice of tree species for SRC is generally confined to fast-growing tree species, such as those from the genera *Populus* and *Salix* [13]. In Europe, poplar and willow (*Populus* and *Salix*) belong to the natural vegetation of the softwood floodplain forest [14,15]. Poplar is typical for Western and Central European floodplains, where willow is more tolerant to low temperatures and can grow further north and in continental climates in the east of Europe [16].

Willows are deciduous trees or shrubs and comprise 330–500 species around the world [17]. They adapt in cool climates and high altitudes or on wet soils [18]. Willows require sufficient moisture supply during site establishment, while in later plantation stages, they can adapt to dry environments with heat and drought stress [17]. Studies have proven that willows have high tolerance to marginal or contaminated soils [6,8,19]. Willow SRCs are gaining increasing interest, because of their efficient and sustainable land use in combination with a growing demand for biofuel resources [20]. In Sweden, willow coppices are often used for phytoremediation where wastewaters or sewage sludge is applied to plantations in order to reduce pollutants or excess nutrients in the water [21].

Previous studies have reported that the productivity in SRCs is determined mainly by the soil fertility [22], soil pH (usually 5–7.5, but willow and poplar are tolerant to pH outside this range) [23], climatic conditions, nutrient and water availability [24], plant species and plantation density [25]. The need for fertilizers in SRCs is small compared to conventional agricultural crops [26]. SRC yields can be maximized by establishing them at fertile soils or by applying organic fertilizers, such as slurry, digested sewage sludge, manure from biogas plants [27]. However, most of these products contain high levels of nitrogen and phosphorus (P), which is risky for the leaching of nitrate and phosphate. Many studies have shown that willows and poplars have high evapotranspiration rates and are able to uptake large amounts of nutrients present in waste, thus allowing significant wastewater disposal over the growing season [28]. Furthermore, some microorganisms, mainly bacteria, are capable of converting phosphates (through solubilization and mineralization processes), and in turn supplying P to plants [29]. Soil enzymes such as acid phosphatases and alkaline phosphatases aid in enzymatically mineralizing P hydrolytically [29]. These enzymes improve the P supply to plants and strengthen the activity of many beneficial microorganisms in the adjacent soil [29]. Additionally, studies have shown that phosphatases are involved in plant growth promotion, activity against plant pathogens, waste remediation and metal recovery [30–33].

The cultivation area of SRCs is expected to increase in many European countries, such as Sweden [27], Germany [34], Ireland [35] and England [25]. As the land under SRC cultivation increases, information on the potential impact of SRC on soil quality and nutrient use efficiency is needed. Early decreases in the nutrient availability under *Salix purpurea* (cv. Hotel) within the first cutting cycle were described from a Canadian site by Ens et al. [36].

Thus far, pure cultures of one *Salix* species are the common praxis, but mixed growth was tested during the last years [37]. Species mixtures can be more efficient in the nutrient mobilization [38], since they combine e.g., different microbial communities in the rhizosphere [39]. However, the impact of mixed growth on the nutrient availability was scarcely tested for *Salix* spp. thus far. We hypothesize that species mixtures of *Salix* can have a higher nutrient mobilization and soil nutrient availability than pure stands by their higher microbial diversity, and thereby, activity in the rhizosphere.

Therefore, the main aims of this study were: (i) to evaluate the impact of growth of *Salix* species/varieties in pure and mixed cultures on the nutrient availability in the soil at

two test sites with different soil and climate conditions; and (ii) to analyze the effects of mixed vs. pure growth on soil enzymatic P mobilization by phosphatases.

2. Material and Methods

2.1. Study Sites and Soil Sampling

The SRCs selected for this study are among two of the three experimental field sites of the ECOLINK-*Salix* project. The goal of this project is to investigate the relationship between genotype diversity, genotype identity, productivity and ecosystem function [37]. The two SRC sites are located in Uppsala in Central Sweden (59°49′ N 17°39′ E) and Rostock in Northern Germany (54°02′ N 12°05′ E). These two field sites with different climatic and soil conditions were selected to test whether the effects of *Salix* species and mixture are common or limited to defined conditions only. The dominating soil type at the site in Uppsala is a Vertic Cambisol and was previously arable farmland. The area of 4147 m^2 is divided into 45 plots (92.16 m^2 in size). In 2014, 6480 trees of four different species/varieties were planted on this site. The species/variety pool of the trial in Uppsala includes four different *Salix* varieties partly belonging to different species [37], of which the pure and mixed culture for two of them was selected for the present study: *Salix dasyclados* var. 'Loden' (acronym L) and *S. schwerinii* × *viminalis* var. 'Tora' (T), based on their significant physiological differences [37]. The mean annual precipitation sum between March and October of 2014 to 2016 was 374 mm and the mean annual temperature between March and October of 2014 to 2016 was 11.0 °C in Uppsala. Samples from the trial in Uppsala were collected in an early stage (initial plantation year, 2014) and late plantation stage (after the first cutting cycle, 2018) of the plantation.

The site in Northern Germany near Rostock was previously used as arable farmland and is established on a Stagnic Cambisol. The area of 829 m^2 is smaller than in Uppsala, due to space and funding restrictions, which strongly compromised the trial size [37]. However, the plot size remained the same as in Uppsala, resulting in nine plots in Rostock. As a consequence of the smaller size of the trial area, the number of trees planted in 2014 was reduced to 1296, comprising only two species/varieties of *Salix* [Tora (T) and Loden (L)] instead of four [39,40]. The mean annual precipitation sum between March and October of 2014 to 2016 was lower in Rostock than Uppsala, with 281 mm, whereas the mean annual temperature between March and October of 2014 to 2016 was higher at 13.3 °C. Samples from the trial in Rostock were collected in an early stage (initial plantation year, 2014) and late plantation stage (after the first cutting cycle, 2018) of the plantation.

2.2. Planting Design and Sample Collection

In preparation for the experiment, both sites were treated with Roundup (glyphosate, 4 L ha^{-1}) in order to kill any existing weeds in the trial areas, which were subsequently cultivated with a rotavator prior to planting [37]. The planting of the 18 cm long stem cuttings was carried out manually [37]. All the cuttings were obtained from the same stock and were soaked in water for two days before being planted in such a way that the tips of the cuttings were flush with the surface [37]. In the beginning, the trial sites were weeded by hand; later, the weeds were controlled by mowing between the rows of plants when necessary [37]. No additional nutrient fertilizers were applied [37].

The planting set up on both the sites was a randomized block design with three replicates (blocks). The blocks in Uppsala have 15 plots each (i.e., four species/varieties and three replicates), whereas the blocks in Rostock consist of three plots [40]. The four (Uppsala) or two (Rostock) species/varieties of *Salix* were planted in every possible combination. Thus, some plots were planted with only one variety (e.g., L or T) pure cultures, some with mixtures of two varieties (e.g., LT) and, in Uppsala, even plots with three or four varieties were planted [37].

The patterns in which the cuttings were planted differed according to the number of species/varieties in the plots: if there were two species/varieties, they were planted in a checker board pattern; if there were three or four species/varieties, their planting positions

were randomized, although with the single restriction that no two individuals of the same species/variety should be planted directly next to each other in one row [37]. Twelve rows of twelve plants were fit into the 9.6 m × 9.6 m plots with every other row being set off [37]. This led to a hexagonal planting pattern with 0.8 m between every plant.

The selected two *Salix* species/varieties display contrasting characteristics (Weih and Nordh, 2002). For example, *S. schwerinii* × *viminalis* var. 'Tora' (T) is generally high-performing but less stress resistant, while *S. dasyclados* var. 'Loden' (L) is sturdier and more stress-tolerant.

Soil samples were taken with a soil corer (3 cm diameter) down to 10 cm soil depth with five replicates per plot in spring 2014 and 2018. The early plantation stage was defined as the year of establishment of the short rotation coppice (initial, 2014). The late plantation stage was defined as the year after the first cutting cycle (after four years of growth, 2018).

This soil depth was selected, since the highest fine root density of *Salix* spp. was revealed in this range [41], and therefore, the highest soil ecological impacts were assumed at this depth. For the soil chemical analyses, soil was sieved <2 mm. Soil phosphatases were measured in fresh wet soil. All other soil chemical properties were measured in air-dried soil.

2.3. Biochemical Analyses of Soil

The activity of acid and alkaline phosphatases in the soil were determined colorimetrically according to [42]. The enzyme activities were expressed as µg p nitrophenol (pNP) g^{-1} soil h^{-1} released from the pre-given substrate solution (p-nitrophenyl-phosphate) within one hour of incubation in modified universal buffer with pH 6.5 (for acid phosphatases) and pH 11 (for alkaline phosphatases) in April 2014 and April 2018.

2.4. Chemical Analyses of Soil

The total carbon (TC) and total nitrogen (TN) concentrations of soil samples were determined by dry combustion using a VARIO EL analyzer (Vario EL Fa. Foss Heraeus, Hanau, Germany). The concentration of SOC was valued by deducting the separately determined inorganic C (dissolution with HCl and volumetric CO_2-determination) from the concentration of TC.

The soil pH was measured in a 0.01 M $CaCl_2$ solution using a soil:solution ratio of w/v 1/2.5. Double lactate-extractable P (P_{dl}), Mg (Mg_{dl}) and K (K_{dl}) were considered to be the plant-available P fractions (e.g., [43]) and were determined by extracting P, Mg and K from 12 g soil with 150 mL lactate solution ($C_6H_{10}CaO_6$ * H_2O + 10 N HCl) according to [44]. Concentrations of P, Mg and K were determined with inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 8300, Perkin Elmer, Waltham, MA, USA) at wavelengths of 214.914 nm, 285.213 nm and 766.490 nm, respectively.

2.5. Biomass Measurements

During the late winter of 2016/2017, i.e., three growing seasons after planting, here representing the "late plantation stage", all individual shoots within a central measurement area of 8.0 m × 3.2 m of each plot were cut at 0.1 m above ground and weighed in fresh condition (fresh weight). A stratified sample of 30 shoots per species/variety was done among the plants situated outside the central measurement area of all pure culture plots to determine the relationships between fresh and dry weights of shoots separately for all species/varieties. The dry weights (biomasses) of the stratified sample shoots were determined after oven-drying at 70 °C for 96 h, and the species/variety specific regressions between the fresh and dry weights of the stratified samples were used to estimate the biomasses of all individual shoots sampled within the central measurement area of all plots [37].

2.6. Statistical Analyses

The effect of the site, the growth design and their interactions on the soil properties were analyzed by two-way ANOVA using the software PAST [45]. Statistical analysis was performed using the Statistica software package (version 13.0, StatSoft, Tulsa). Principal Component Analysis (PCA) was performed using R package. The samples used in the PCA were attributes (mean values) measured in two test sites (Rostock and Uppsala), two plantation stages (early and late) and three species identity and culture conditions ('Loden' pure culture, 'Tora' pure culture and a mixture of 'Loden' and 'Tora'). The attributes analyzed in the present study were: alkaline phosphatase activity, acidic phosphatase activity, willow biomass and plant-available K, Mg and P content in the soil.

3. Results

3.1. Plant-Available Nutrient Contents (K, Mg and P) in the Soil

The initial plant-available concentrations of K and Mg in the soil differed significantly between the two test sites and between the early and late plantation stage per test site (Table 1). The plant-available concentration of P was low and at the same level at both test sites (Figure 1). The plant-available concentrations of P and K in soil significantly decreased with the progressing willow growth (initial vs. 4 years of growth) at the test site Rostock (Figure 1 and Table 1).

Table 1. Nutrient contents (K, Mg and P) in mg/100 g soil in in sampling sites (a) Rostock and (b) Uppsala initally and after 4 years for each species identity and cultivation condition (Loden, Tora and their mixture). The biomass of the willow species was measured after harvest (kg dry matter per plot).

(a) Rostock							
Species	**Initial**			**After 4 Years**			**Biomass (First Harvest)**
	K (mg/100 g)	Mg (mg/100 g)	P (mg/100 g)	K (mg/100 g)	Mg (mg/100 g)	P (mg/100 g)	(kg dry matter/plot)
Loden [L]	13.4 ± 6.1	25.2 ± 9.5	6.8 ± 1.6 ^{A}	10 ± 2.2	20.4 ± 3.8	4.4 ± 0.01 ^{B}	16.14 ± 4.94
Tora [T]	32.9 ± 10.7 ^{A}	27.5 ± 5.9	5.8 ± 0.9 ^{A}	10.7 ± 3 ^{B}	22.0 ± 0.3	4 ± 0.1 ^{B}	30.71 ± 11.94
Loden, Tora [LT]	23.5 ± 14.3	21.4 ± 9.4	5.2 ± 1.9	10.2 ± 3.6	21.1 ± 4.3	4.4 ± 1.1	19.57 ± 5.21
(b) Uppsala							
Species	**Initial**			**After 4 Years**			**Biomass (First Harvest)**
	K (mg/100 g)	Mg (mg/100 g)	P (mg/100 g)	K (mg/100 g)	Mg (mg/100 g)	P (mg/100 g)	(kg dry matter/plot)
Loden [L]	17.6 ± 3.3	25.9 ± 1.6 ^{B}	5.1 ± 0.3 ^{A}	22.2 ± 2 *	32.1 ± 1.8 ^{A}*	4.3 ± 0.2 ^{B}	11.19 ± 5.14
Tora [T]	16.6 ± 2	27.7 ± 3.3	4.5 ± 0.6	19.9 ± 3.6 *	28.8 ± 3.3 *	3.6 ± 0.4	13.67 ± 2.89
Loden, Tora [LT]	17.8 ± 4.1	30.8 ± 12.3	6.0 ± 2.4	23.6 ± 4.6 *	27.9 ± 6.9	5.0 ± 1.8	13.76 ± 4.74

Soil properties were compared by site, growth stages (early and late plantation stage) and species (plots: L—Loden, T—Tora and LT—mixture). Values are means ± SDs (n = 3). The significant differences with $p < 0.05$ are marked by the following symbols: *—differences between sites, small letters—differences between species within one site, capital letters—differences between growth stages within one site.

Conversely, the amount of plant-available Mg in Uppsala soils increased from early to late plantation stages mainly under Loden (Figure 1). The plant-available concentration of K at the test site Uppsala was significantly higher than at the test site Rostock (see Tables 1 and 2). Likewise, the plant-available concentration of Mg in soil was higher in pure culture (Loden and Tora) in Uppsala in comparison to Rostock after 4 years of growth.

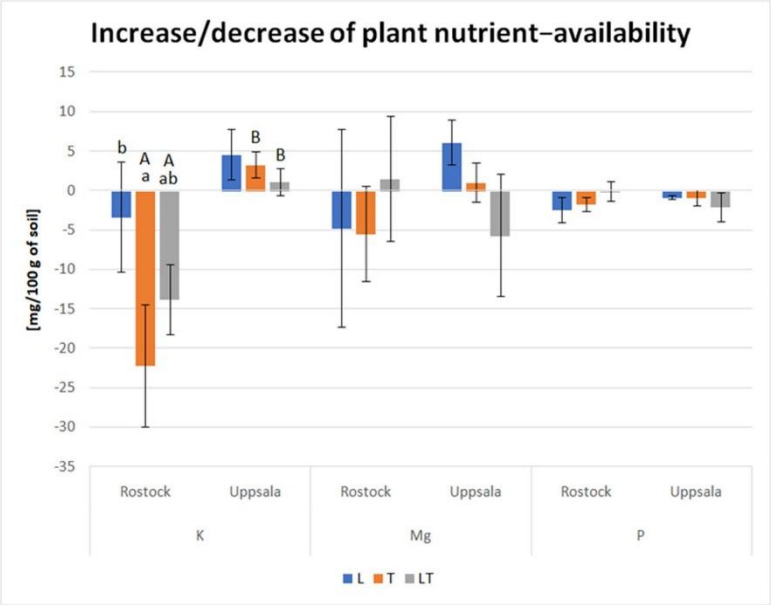

Figure 1. Nutrient levels increase/decrease between the years 2014 and 2018 in the soil at the two sites (Rostock and Uppsala). Small letters represent significant differences between the *Salix* species or the growth design on the same sampling site and capital letters represent the significant difference between sampling sites.

Table 2. Results of the two-way analysis of variance (ANOVA) on the effect of site, the growth design (with different host plant diversity; pure vs. mixture) and their interactions (site × growth design) on soil properties under *Salix* in spring 2014 (initial) and spring 2018 (after 4 years of growth).

Parameter		Site Initial 4 Years		Growth Design Initial 4 Years		Site × Growth Design Initial 4 Years	
Plant-available P content	p	0.304	0.950	0.603	0.249	0.335	0.590
	F	1.15	0.004	0.53	1.56	1.20	0.335
Plant-available Mg content	p	0.092	<0.001	0.612	0.747	0.101	0.495
	F	3.36	20.48	0.51	0.29	2.79	0.74
Plant-available K content	p	0.020	<0.001	0.053	0.702	0.039	0.536
	F	7.15	606.01	3.79	3.94	4.28	7.13
Alkaline phosphatase activity	p	0.378	<0.001	0.928	0.065	0.344	0.184
	F	0.84	95.30	0.07	3.45	1.17	1.95
Acid phosphatase activity	p	0.200	<0.001	0.016	0.513	0.147	0.297
	F	1.84	198.20	5.92	0.71	2.26	1.34

3.2. *Acid and Alkaline Phosphatase Activity in the Soil*

The site and the growth design affected the activities of acid and alkaline phosphatases significantly (Table 2). The alkaline phosphatase activity in the soil Rostock was significantly higher at the early plantation stages, and a significance between species (mainly in Loden) was observed (Figure 2). In the late plantation stage, the soils showed very low activity, specifically in the soil under Loden and under the mixture. Initially, under Loden, significantly higher alkaline phosphatase activity in the soil was revealed at the test site Rostock (Figure 2), while after 4 years under Tora and Loden, significantly higher activities were measured than under the mixture at the test site Uppsala.

The activity of acid phosphatases significantly increased in the early plantation stages of willow species than in the late plantation stages in Rostock (Figure 2). The highest activity in soil was observed in plots with the mixed culture plots (LT) in comparison to the plots with monocultures Tora and Loden. No significant differences were observed among the monocultures and mixed culture plots in the early nor in the late plantation stages in Uppsala. Only plots with Loden displayed a statistical significance, with the highest activity in the late plantation stage. Overall, the acid phosphatase activity was the lowest at the late plantation stages of species in Rostock. On average, the activity in the early plantation stage was approximately 320 µg p-nitrophenol g^{-1} DM h^{-1}, whereas in the late plantation stage, it lowered to an average of approximately 130 µg p-nitrophenol g^{-1} DM h^{-1}. A significantly high phosphatase activity was seen in the late plantation stages of willow species in Uppsala. Similarly, this activity was the lowest in Rostock.

Overall, a significantly higher acid phosphatase production was recorded at both the investigated sites compared to alkaline phosphatase production (Figure 2).

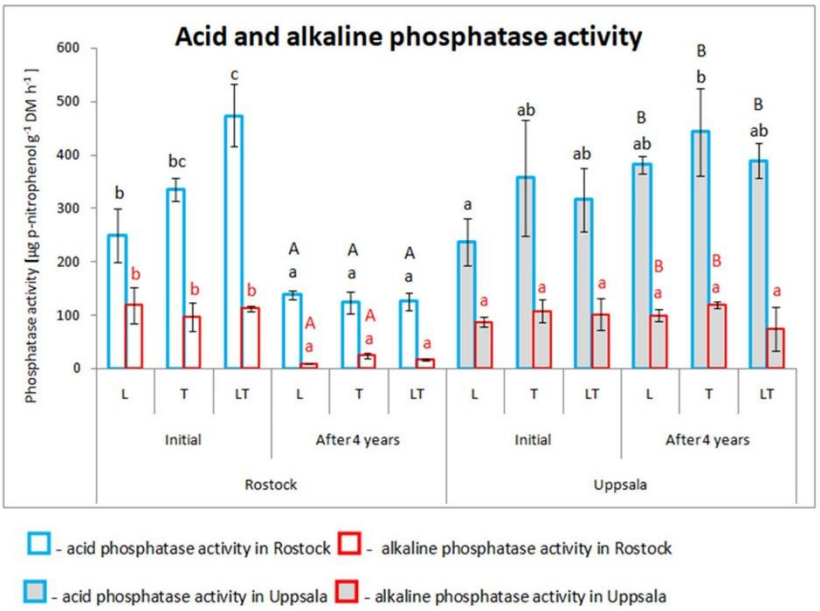

Figure 2. The alkaline phosphatase and acid phosphatase activity [µg p-nitrophenol g^{-1} DM h^{-1}] in soils obtained from Rostock and Uppsala. The data present comparisons between two sites, Rostock (on the left site) and Uppsala (on the right), between growth stages (initial and after 4 years) and species (plots: L—Loden, T—Tora and LT—mixture). Values are means ± SDs (n = 3). The significant differences with $p < 0.05$ are marked by the following symbols: small letters—differences between species within one site, capital letters—differences between initial and after four years. DM—dry matter.

Regardless of the tested parameters, approximately 55% of the total variance was explained by the first two components in the PCA analysis (Figure 3). The PCA analysis revealed that the samples were differentiated mainly based on the test sites, i.e., Rostock and Uppsala. A positive tendency towards the increase in willow biomass production was observed for Rostock samples with higher acid and alkaline phosphatase activity.

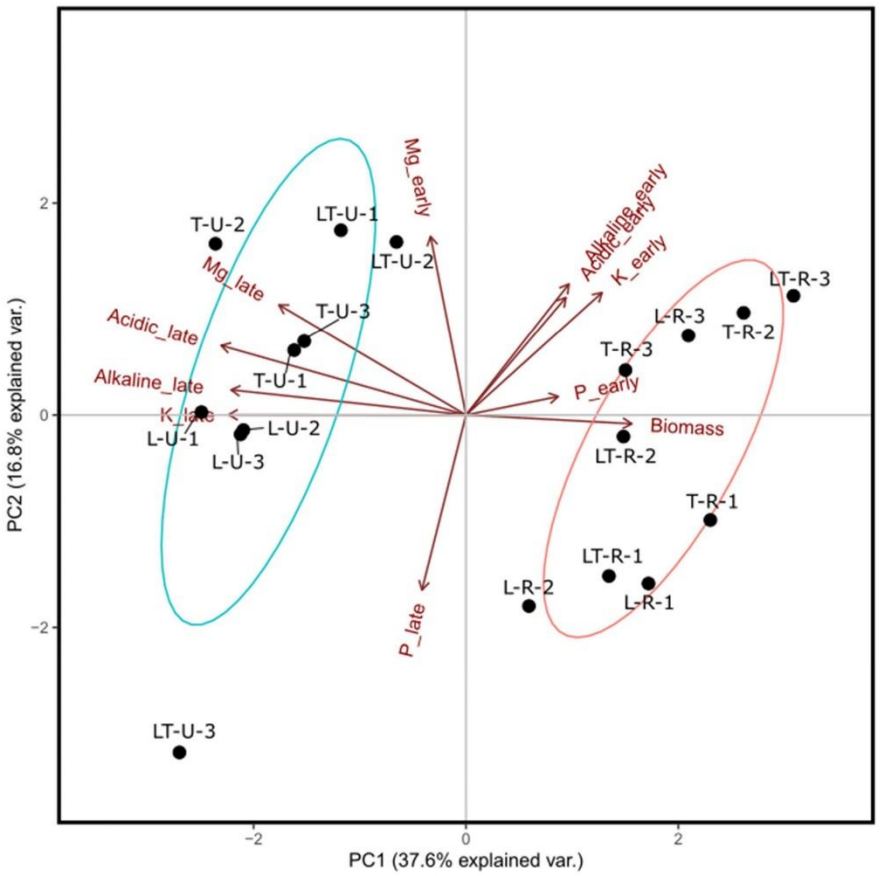

Figure 3. Grouping of samples and corresponding variable component loadings according to the Principal Component Analysis (PCA). Samples are attributes (mean values of the attributes indicated by arrows) measured in two test sites (Rostock—R and Uppsala—U), two plantation stages (initial (early) and after 4 years (late)) and three species identity and culture conditions (L—Loden pure culture, T—Tora pure culture and LT—mixture of Loden and Tora). Attributes were soil phosphatase activity (Alkaline and Acid), willow biomass and soil nutrient content (K, Mg and P in early and late plantation stages). PC1 explained 37.6% of total variance; PC2 explained 18.8% of total variance.

4. Discussion

Short rotation coppices (SRCs) generate crops used in renewable energy generation in Europe. The success of SRC establishment can be assessed by studying the adaptability of crops by monitoring their growth, climate and site conditions. Factors such as climate, soil nutrient availability, plant species and growth design may significantly influence plant nutrient cycling and overall biomass production. This study investigated the Ecolink *SALIX* SRC plantations located in Germany (Rostock) and Sweden (Uppsala). Both test sites were maintained in a similar way and planted with same two *Salix* species and their mixture. We analyzed the effect of the growth design on the plant availability and hydrolytic mobilization of P at the year of planting and after 4 years of growth, including the first harvest. The test cultivars Loden and Tora were selected for our experiment because they are both phenotypically and genotypically very distinct *Salix* species. Loden

is rather slow-growing but more stress-tolerant, while Tora is generally high-performing and less stress-tolerant [37].

The initial concentrations of plant-available nutrients (Mg, K and P) at both test sites in the present study were high compared to the recommended level for arable crops [44] for Mg and K, but below the recommended level (10–18 mg P/100 g soil) for an optimal P supply. The plant-available concentration of Mg in the soils of Rostock and Uppsala was even higher than the recommended value of >19, i.e., to 32 mg/100 g soil for an optimal plant supply [44]. Moreover, the soil of the test site Rostock displayed high initial levels of plant-available K concentrations (see Table 1).

Overall, the biomass production at the test site Rostock was higher than at the test site Uppsala; however, no significant differences were observed between species and pure vs. mixed culture plots [37]. The biomass production was not correlated with the plant-availability of one of the tested nutrients (Mg, K, P); however, a correlation between the phosphatase activities and the biomass production was indicated (see Figure 3).

In agreement with Ens et al. [36], we measured significantly decreased plant-available P concentrations in the soil under the pure stands after the first cutting period, and a significant site effect on the P cycling (phosphatase activities, see Table 2) was observed. However, significant differences were observed for soil nutrient concentrations (mainly P and K) between the initial stage early and after four years of growth at both test sites (see Table 1). The acid and alkaline phosphatase activities decreased strongly from the initial level to the 4-year growth stage, which might be caused by the former grassland vegetation with a higher fine root density [46].

A general effect of the species and the growth design on the phosphatase activity under *Salix* is in agreement with the results of the mycorrhizosphere observation by Baum et al. [47]. However, only two *Salix* species were investigated in the present study due to the limitation of available plant variants at the test site Rostock in Germany (only Loden and Tora were present). Increased number of plant species and a higher amount of diversity in the mixtures (three or more species) might have increased the validity of the present information. Furthermore, a joint impact of the P and N supply might be assumed [36] and was not investigated in the present study.

The initial nutrient surplus at the arable test sites in Sweden and Germany agrees with results of former investigations of SRC [48] and underlines no need for fertilization in the first cutting period at such sites. This is because formerly arable farmland was usually regularly fertilized, which often results in high nutrient contents [49]. The nutrient concentrations in the soil changed significantly within the first four years (see Table 1). The cultivar Loden, which was included in the present study, is frequently reported with great potential in nutrient acquisition from soil, especially nitrogen and increased biomass production when paired with other *Salix* species [40]. The analysis of the P content in soil of both the sampling sites showed a significant decrease from early plantation to late plantation stage, which might suggest that Loden is efficient in P uptake. Since both experimental sites were not fertilized after willows were planted, most of the P present is in organic or low soluble form, which is not easily accessible to plants. P depletion in Uppsala and Rostock was also paired with a significant increase and decrease of acid phosphatase activity, respectively (see Figures 1 and 2). This inconsistency might suggest that acid phosphatase activity is not strongly connected with P supply in soil, but may be connected with other factors. Study performed by Criquet et al. [50] revealed that increased leaf litter moisture is positively correlated with acid phosphatase activity. Additionally, experiments performed in three forest ecosystems in China showed that increased precipitation during the dry season had a positive effect on enzyme activity [51]. The cultivar Tora showed slightly fewer prominent differences from the initial nutrient availability to the level after the first cutting cycle; only in Rostock site it differed in both P and Mg between these two sampling dates. Although P depletion was not as severe as for Loden, the plant-available Mg content decreased by almost two-fold compared to the initial content.

Of note, differences were observed in willow biomass production at the two test sites, which may be due to the varying nutrient concentrations in soils. Most of the plant-available nutrient concentrations (Mg and P) were decreased in the soil within the first cutting period, although the level of K varied at both the test site and growth stages. The PCA analysis revealed that biomass was positively correlated with P and K during the early plantation stage and negatively with late plantation stage concentration of P and K (Figure 3). The correlation was prominent, although we observed K depletion in one experimental site and an increase in the other site. Willows are known to efficiently uptake organic P when paired with ectomycorrhizal fungi [52]. This suggests that the presence of these two compounds during early plantation stages is key to reaching higher biomass production efficiency in later plantation stages. Additionally, the K content was in direct correlation with alkaline and acid phosphatase activity in the early and late plantation stages, respectively, thus indicating that changes in phosphatase activity are not bound to soil P concentration but to other, more complex sets of factors. High correlation of plant-available K with phosphatase activity is probably connected with its important role as a co-factor of many enzymes. Tabaldi et al. [53] investigated the effect of various metals on *Cucumis sativus* L., e.g., Zn, K and Na. As a result, they observed increased acid phosphatase activity in higher presence of K ions.

Acid phosphatase activity was about three-fold higher than alkaline phosphatase. According to measurements performed in our previous study, pH on both sampling site is around 6 (measurements done at 2018 and 2019) [39], which promotes the activity of acid phosphatases. pH in which acid phosphatase is active is between 4.5–6, whereas for alkaline, this is 8–11 [54]. Additionally, the pH value in SRCs is known to drop slowly with time, which further promotes the activity of acid over alkaline phosphatase [55]. Another very important factor in acid phosphatase activity is the presence of arbuscular and ectomycorrhizal fungi. Baum et al. [47] pointed out the impact of both mycorrhiza types on various factors, including acid phosphatase activity. Loden was mostly colonized by ectomycorrhizal fungi and showed higher acid phosphatase activity in pure cultures, while Tora was colonized by arbuscular mycorrhizal fungi with slightly lower activity. Additionally, they reported that mixed growth of *Salix* possessed higher phosphatase activity than monocultures [47]. The abovementioned factors contribute to increased P mobilization and were in agreement with the insignificant decrease of the plant-available P concentrations in the soil under mixed growth of *Salix* species within the first cutting cycle in the present study.

5. Conclusions

The site impacts are the main controls of the changes in the concentrations of plant-available nutrients under *Salix*. The changes of the plant availability of P within one cutting cycle are generally low, independently of the site and growth design. Mixed growth of *Salix* species promotes the activity of alkaline phosphatases in P-deficient soil conditions. The impact of the growth design on the nutrient cycling differs significantly and site-specifically in the direction and amplitude. The future challenge will be to select a site-specific optimized growth design.

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The Effects of Host Plant Genotype and Environmental Conditions on Fungal Community Composition and Phosphorus Solubilization in Willow Short Rotation Coppice

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Phosphorus (P) is an essential plant nutrient. Low availability of P in soil is mainly caused by high content of Fe_2O_3 in the clay fraction that binds to P making it unavailable. Beneficial microbes, such as P solubilizing microorganisms can increase the available P in soil and improve plant growth and productivity. In this study, we evaluated the effects of environmental conditions (climate, soil parameters), plant genotype, and level of plant association (rhizosphere or endophytic root organism) on the abundance and diversity of phosphorus solubilizing microorganisms in a *Salix* production system. We hypothesized that a lower number of endophytic fungi may possess the ability to solubilize P compared to the number of rhizosphere fungi with the same ability. We also expect that the plant genotype and the experimental site with its environmental conditions will influence fungal diversity. Two *Salix* genotypes grown in pure and mixed cultures were investigated for their fungal microbiome community and diversity in the rhizosphere and endosphere during two growing seasons. We found that the rhizosphere fungal community was more diverse. A general dominance of Ascomycota (*Dothideomycetes*) and Basidiomycota (*Tremellomycetes*) was observed. The classes *Agaricomycetes* and *Pezizomycetes* were more frequent in the endosphere, while *Tremellomycetes* and *Mortierellomycetes* were more abundant in the rhizosphere. Plot-specific soil properties (pH, total organic carbon, and nitrogen) significantly influenced the fungal community structure. Among the culturable fungal diversities, 10 strains of phosphate solubilizing fungi (PSFs) from roots and 12 strains from rhizosphere soil were identified using selective media supplemented with di-calcium and tri-calcium phosphates. The fungal density and the number of PSF were much higher in the rhizosphere than in the endosphere. *Penicillium* was the dominant genus of PSF isolated from both sites; other less frequent genera of PSFs

were *Alternaria, Cladosporium,* and *Clonostachys*. Overall the main factors controlling the fungal communities (endophytic vs. rhizosphere fungi) were the soil properties and level of plant association, while no significant influence of growing season was observed. Differences between *Salix* genotypes were observed for culturable fungal diversity, while in metagenomic data analysis, only the class *Dothideomycetes* showed a significant effect from the plant genotype.

Keywords: diversity, fungal endophytes, phosphate solubilization, rhizosphere fungi, short rotation cropping, willow

INTRODUCTION

Phosphorus (P) is an essential plant nutrient provided by a non-renewable resource (Filippelli, 2008; Shen et al., 2011). Fluoroapatite is the main and non-renevable resource from which fertilizers are produced (Filippelli, 2008). Moreover, P compounds released during weathering are usually complex and are not immediately bio-available (Filippelli, 2008; Singh and Satyanarayana, 2011). Plants and microorganisms release extracellular phosphatases from roots and recover orthophosphate ions from mineralizing phosphor-organic compounds (Richardson and Simpson, 2011). Furthermore, P solubilization is a microbial-driven process of hydrolysis of organic and inorganic P compounds to simpler compounds that can be utilized by plants (Richardson and Simpson, 2011).

Phosphorus solubilizing microorganisms can increase the availability of P for plants from the soil P pool, reducing the need for P fertilization. More P solubilizing bacteria have been reported than P solubilizing fungi (PSFs), but the latter are more effective, as PSFs do not lose the ability to solubilize P after subculture (Zhang et al., 2011). There are reports of various PSF species, such as *Aspergillus* spp., *Penicillium* spp., *Trichoderma viride*, *Arthrobotrys oligospora*, *Cephalosporium* sp., or *Cladosporium* sp. (Khan et al., 2010; Patil et al., 2012; Sharma et al., 2012; Ram et al., 2015; Li et al., 2016; Alori et al., 2017). In certain cases, these fungi may form symbioses with plants, develop mycelial networks, produce plant growth-promoting metabolites, and increase plant P and nitrogen uptake from the soil, e.g., by increasing root surface area (Baum et al., 2009). Chatli et al. (2008) isolated PSFs belonging to the genera *Aspergillus* and *Penicillium* from the rhizosphere of willow (*Salix alba* L.) growing in the *trans*-Himalayan region. Few experiments have demonstrated that PSFs isolated from other host plants could be used to increase yields of tomato, maize, and wheat (Khan and Khan, 2002; Reyes et al., 2002; Sharma et al., 2012; Ram et al., 2015).

The European Union directive (April 2009) on the promotion of the use of energy from renewable sources (Directive 2009/28/EC) states that 20% of the total energy in Europe should be generated from renewable sources[1]. Generally modern agriculture is focused mostly on reaching high yields by the use of best-performing plant species grown in pure culture. Such pure cultures are often easier in maintenance than mixed cultures, but pure cultures have been shown to be more vulnerable to pests and diseases in many cases. Thus, the introduction of mixed genotypes plantations could limit the losses due to pests and diseases while significantly enhancing biodiversity (Hoeber et al., 2018; Schweier et al., 2019). This may improve the sustainability of biomass production on SRCs but our knowledge in this area is still limited. Additionally selection of tree species for SRC is critical and depends on the local climate and soil conditions. SRCs are generally confined to fast growing tree species, mainly from the genera *Populus*, *Salix*, *Eucalyptus*, and *Robinia* (Navarro et al., 2016). *Salix* species are fast growing trees that possess high economic value because of their high potential to contribute to renewable energy generation in Europe (Sevel et al., 2012; Weih et al., 2021). This woody crop can be planted on soils that are less suitable for farming of food crops and can be fertilized with sewage sludge, wastewater, or ashes which contain high amounts of nitrogen and phosphorus (Dimitriou and Aronsson, 2011). Many species of *Salix* can control P uptake and metabolism, although the corresponding mechanisms are still largely unknown (Rennenberg and Herschbach, 2013).

Rhizosphere and endophytic fungi play important roles in plant growth and development. Their ecology and function is different and depends on various parameters (Hrynkiewicz and Baum, 2012). The level of plant association may be affected by soil properties, climate, weather conditions, and host plant genotype. Likewise, understanding how the willow plantations in the form of monoculture and mixed genotypes could affect the overall microbial diversity is key information. This study bridges the gap by providing knowledge on the rhizosphere and endophytic fungal diversity in willow SRCs. Hence, the main aim of this research was to investigate the diversity of rhizosphere and endophytic fungi from two willow species, *S. dasyclados* (cultivar "Loden") and *S. schwerinii* × *S. viminalis* ("Tora"), as well as their mixture at two sites located in Germany and Sweden. The two species selected for the study are phenotypically different, and "Tora" is known for its high productivity compared to "Loden" (Hoeber et al., 2017). The mixture of two host species may result in increased diversity of fungi compared to growing the same species in pure culture. The two investigated experimental sites represent ECOLINK-Salix within a global tree diversity network (Verheyen et al., 2016). The two selected sites are similar in terms of planting time, design, and management (e.g., fertilization, timing of shoot harvests) but vary by local climate and soil conditions. This might provide more information about importance of climate and soil nutrient content on willow microbiome. We

[1] https://ec.europa.eu/eurostat/web/energy/data/shares

evaluated the effect of climate, soil conditions, level of plant association, and planting design on the abundance and diversity of PSFs by applying culture-dependent and culture-independent (metagenomic) methods. Using culture-dependent techniques is critical as it allows application of this study for future research in using these strains as inoculants in plants. Moreover, culture-independent methods give more insight into the culturable and unculturable fungal diversity present in the two experimental sites. In addition, to assess the potential impact of PSFs on the P supply of *Salix* in fall, we tested the P solubilization efficiency of fungal isolates. We hypothesized that a lower number of endophytic fungi may possess the ability to solubilize P compared to the number of rhizosphere fungi with the same ability. Second, the site-specific climate and soil conditions and the *Salix* species genotypes may determine the abundance and diversity of total fungi and PSFs.

MATERIALS AND METHODS

Site Description and Sampling

The two experimental sites have been well-established SRCs since spring 2014 (Hoeber et al., 2018). The first experimental site is located in Uppsala, Sweden (59°49′13.4″N, 17°38′25.2″E), and the second is located in Rostock, Germany (54°03′41.0″N, 12°04′54.7″E). Both sites are former arable fields. The experimental site in Uppsala is located on fine-textured mineral soils, mainly Vertic Cambisols (according to IUSS Working Group WRB, 2015). The experimental site Rostock is dominated by Stagnic Cambisols developed from loamy sands.

The study sites vary by local climate and soil conditions. According to the Köppen–Geiger classification, the climate in Uppsala is boreal (Dfb: snow, fully humid with warm summers; Kottek et al., 2006). The average annual rainfall is 551 mm, and the average annual air temperature is 5.8°C (1970–2000; http://www.worldclim.org/current). Winters are usually not as cold as in other cities at similar latitudes due to the influence of the Gulf Stream. Rostock is situated on the Baltic coast in a warm temperate climate (Cfb: fully humid with warm summers). The average air temperature of Rostock is higher than that of Uppsala (8.4°C), but the rainfall sum is similar (600 mm).

The meteorological data obtained from https://www.worldweatheronline.com/showed similar trends in the 2018–2019 period for both studied localities. In general, these years were warmer than average (2009–2019). However, lower than average monthly temperatures were recorded in February (Rostock and Uppsala) and March (Rostock) 2018. The interannual variability in the monthly distribution of rainfall was high. The 2018 year was significantly drier and the 2019 year was much wetter than average (2009–2019; **Supplementary Table 1** and **Supplementary Figure 1**). Two *Salix* genotypes, "Loden" (*S. dasyclados*) [L] and "Tora" (*S. schwerinii* × *S. viminalis)* [T], were cultivated as pure cultures and mixtures [LT] at the two sites. The L genotype is characterized by shorter shoots and a larger leaf area than the T genotype (Hoeber et al., 2018).

Willow roots and soils were sampled from the two experimental sites during two seasons: Fall 2018 (Sweden – October 23rd, Germany – October 27th) and spring 2019 (Sweden – May 15th, Germany – May 18th). Both experimental sites were organized into three blocks, and the density of plants was 15,600 ha^{-1}. Blocks were divided into plots randomly planted with different *Salix* genotypes (**Figure 1**) and their mixtures in all possible combinations. Treatments L, T, and LT were selected for investigation since they were present at both experimental sites (**Figure 1**). There were three replicates per treatment (nine plots per experimental site, in total), and each plot was 9.6 m × 9.6 m.

Three samples per plot (81 from Sweden and 81 from Germany per season, 324 in total) were taken by digging root samples with adhering soil (15 cm × 15 cm × 15 cm) at a distance of 6 m from each other for microbiological analysis. The organic litter layer (up to 5 cm thick) was removed and topsoil (0–25 cm) was sampled. Samples were carefully transferred to collection bags and covered with a thin layer of soil to prevent drying. Collected samples were immediately transported to the laboratory and used for analyses.

Soil Physicochemical Analysis

Air-dried soil samples were passed through a 2 mm sieve. The total organic carbon (TOC) and total nitrogen (TN) contents were measured after dry combustion using a CHNS Vario Macro Cube elemental analyzer. Available phosphorus (P_{av}) in 1% citric acid (van Reeuwijk, 2002) was determined by a spectrophotometric method using spectrophotometer UV–Vis Rayleigh UV-1601 (van Reeuwijk, 2002), and the pH (in H_2O and 1M KCl) at 1:2.5 soil to solution ratio was determined by the potentiometric method using an Elmetron CP-105 pH-meter. **Table 1** shows differences in soil parameters on both sampling sites and two growing seasons.

Total Cultivable Fungal Density

Samples from fall 2018 were processed for cultivable fungal density and phosphate solubilizationability. The willow roots were carefully separated from the adhering rhizosphere soil. One and a half grams of roots were surface sterilized with 60% alcohol (3 min) by vigorous shaking and then washed three times in sterile 2% NaCl solution. The roots were washed in sterile 5% H_2O_2 solution (10 min) and rinsed three times in sterile 2% NaCl solution. The last wash of 2% NaCl was plated on R2A medium (BD Difco, United States; sterilization control). The surface sterilized roots (1 g) were homogenized using a sterile mortar and pestle under sterile conditions and then transferred to Falcon tubes containing 9 ml of sterile 0.5% NaCl solution. For the rhizosphere soil samples (from uppermost mineral part of soil affected by the plant roots), 1 g was transferred to Falcon tubes with 9 ml of sterile distilled water, and dilutions were performed.

For the total fungal density, potato dextrose agar (PDA) medium (BD Difco, United States) supplemented with tetracycline at 100 mg/l was used. Serial dilutions of 10^{-2} and 10^{-3} of the root samples and 10^{-3} and 10^{-4} of the rhizosphere soil were selected for spread plating. Plates were kept at 24°C, and the total number of fungal colonies on PDA medium was counted after day 7 and presented as colony forming units (c.f.u).

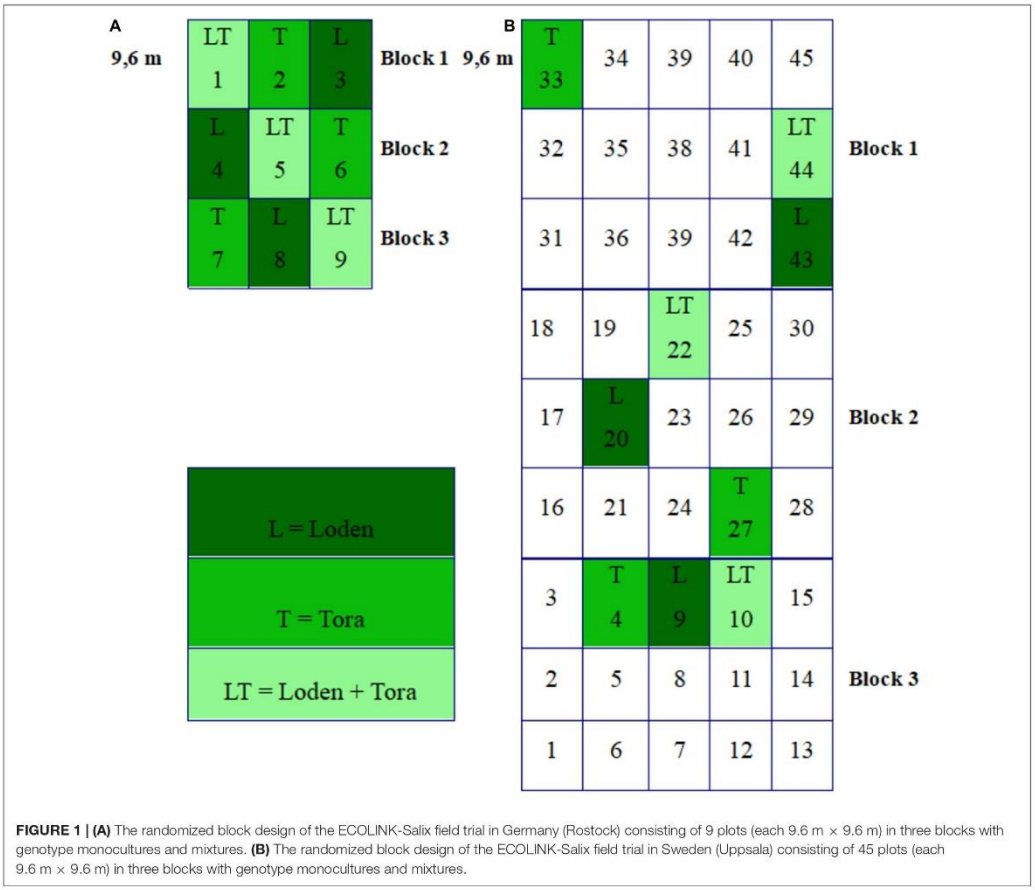

FIGURE 1 | (A) The randomized block design of the ECOLINK-Salix field trial in Germany (Rostock) consisting of 9 plots (each 9.6 m × 9.6 m) in three blocks with genotype monocultures and mixtures. **(B)** The randomized block design of the ECOLINK-Salix field trial in Sweden (Uppsala) consisting of 45 plots (each 9.6 m × 9.6 m) in three blocks with genotype monocultures and mixtures.

Screening PSFs From Roots and Rhizosphere Soil

The isolation and selection of PSFs were performed on three selective media (NBRIP, PVK; Nautiyal, 1999, and DCP; modified Pikovskaya, 1948; **Supplementary Table 2**) containing either tri- (NVRIP, PVK) or diphosphates (DCP). In total, 108 plates per medium were used to evaluate P solubilizing and non-PSF.

The serial dilutions from the roots and the rhizosphere soil (100 μl each) were spread plated on the three selective media. For NBRIP and PVK media, 10^{-5}–10^{-6} dilutions were used for both types of samples. For DCP medium, 10^{-3}–10^{-4} dilutions were used for the roots, and 10^{-4}–10^{-5} dilutions were used for the rhizosphere soil samples. Three technical replicates were prepared for each sample. Plates were kept at 24°C for 7 days and observed for fungal halos indicated phosphorous solubilization. Calculations were performed to determine the number of PSF and the total number of fungi growing on each of the three media. All PSF isolates unique to each plot were subjected to molecular identification. The fungal density was determined by the $\log_{10}$ [c.f.u. (g f.w. soil)$^{-1}$ or (g f.w. roots)$^{-1}$] values for both the total fungal count and those screened with selective media.

Molecular Identification of Fungal Strains

Twenty-two selected fungal isolates (12 from Germany and 10 from Sweden) were cultivated on fresh PDA medium (BD Difco, United States). Fungal DNA was isolated from fresh mycelium using the Plant and Fungi DNA Purification Kit (Eurx, Poland). The concentration of DNA was measured using a UV–Vis spectrophotometer (Thermo Scientific NanoDrop2000, United States). The ITS region was amplified using ITS1 (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) primers (Martin and Rygiewicz, 2005; Manter and Vivanco, 2007). PCR clean-up was carried out using a PCR/DNA Clean-Up Purification Kit

TABLE 1 | Characteristics of rhizosphere soils.

Site	Season	Genotype	TOC (%)	TN (%)	C:N	P_{av} (mg·kg^{-1})	pH(H_2O)	pH(KCl)
Sweden	Fall	L	1.69 ± 0.24*	0.16 ± 0.02*	10.3 ± 0.5b	101 ± 12.2b	5.7 ± 0.2b	5.3 ± 0.1
		T	1.59 ± 0.21*	0.16 ± 0.01*	10.2 ± 0.7	94.2 ± 13.6*	5.7 ± 0.1b	5.2 ± 0.2
		LT	1.65 ± 0.2*	0.16 ± 0.02*	10.4 ± 0.6	105 ± 28.1b*	5.7 ± 0.1	5.1 ± 0.1
	Spring	L	1.89 ± 0.29*	0.17 ± 0.02*	11.1 ± 0.5Aa	75.7 ± 20.1Ba*	6.0 ± 0.2Ba	5.2 ± 0.2
		T	1.72 ± 0.22*	0.17 ± 0.02*	10.2 ± 0.6B	93.6 ± 14.7A*	6.2 ± 0.2Ba	5.2 ± 0.1
		LT	1.63 ± 0.18	0.16 ± 0.02*	10.3 ± 0.5B	63.1 ± 19.8Ba	5.7 ± 0.2A	5.2 ± 0.1
Germany	Fall	L	1.21 ± 0.17	0.11 ± 0.02	11.3 ± 0.7*	101 ± 22.0Aa	6.0 ± 0.4*	5.8 ± 0.4*
		T	1.13 ± 0.13b	0.1 ± 0.01b	11.3 ± 0.7*	57.0 ± 7.3B	6.1 ± 0.3*	6.0 ± 0.3*
		LT	1.22 ± 0.21b	0.1 ± 0.01b	11.7 ± 1.0*	55.0 ± 12.3B	6.0 ± 0.3b*	5.7 ± 0.4*
	Spring	L	1.24 ± 0.2	0.11 ± 0.01	11.3 ± 0.9	50.5 ± 9.5b	6.2 ± 0.3*	6.1 ± 0.3*
		T	1.4 ± 0.24a	0.11 ± 0.01a	12.5 ± 1.3*	54.8 ± 11.3	6.3 ± 0.2*	6.2 ± 0.2*
		LT	1.49 ± 0.19a	0.12 ± 0.01a	12.4 ± 0.8*	57.3 ± 11.2	6.2 ± 0.2a*	6.1 ± 0.3*

*At each sampling site (Sweden and Germany), three plots per three blocks for each variant of the experiment (Loden, Tora, and mixture of both genotypes) were sampled. In total, 108 root and soil samples from both samplings were collected. Soil properties were compared by site, season, and genotype (plots: L-Loden, T-Tora, and LT-mixture). Values are means ± SDs (n = 9). The significant differences with p < 0.05 are marked by the following symbols: * – differences between sites in the same season, small letters – differences between seasons within one site, and capital letters – differences between genotypes on the same site and season.*

(EurX, Poland). The presence of ITS sequences was confirmed on a 1% agarose gel (1X TBE buffer) with the addition of Simply Safe dye (EurX, Poland). Samples were sent for sequencing to the Institute of Biochemistry and Biophysics PAS[2]. Contigs were assembled using Sequencher 5.4.6 software and compared with the NCBI database using BLASTn[3] to find sequences that showed the highest similarity to the assembled contigs. The DNA sequence generated for this study were deposited in the NCBI GenBank under the following accession numbers: MW342736–MW342757 (as shown in **Supplementary Table 3**). The phylogenetic tree was constructed using the NJ method in MEGA 7, and 1,000 bootstrap replicates were used to assess branching support (Tamura et al., 2013; Kumar et al., 2016). The *p*-distance method was calculated (Saitou and Nei, 1987). The phylogenetic tree was visualized using Interactive Tree of Life (iTOL) v3 (Letunic and Bork, 2016).

Metagenomic Analysis

Total DNA was extracted from 50 mg of lyophilized willow roots and rhizosphere soil samples using Plant and Fungi DNA Purification Kit (EURx, Poland) according to the manufacturer's protocol. Three biological replicates were prepared for each plot. The amount of isolated DNA was quantified fluorometrically (InvitrogeneQubit 2.0, United States) and the quality was assessed spectrophotometrically (Thermo Scientific NanoDrop 2000, United States) and the preparations were diluted to 1 ng/μl.

Fungal ITS amplicon libraries were generated in two-step PCR, as described by Thiem et al. (2018) using fungal primers (uITS1 and uITS2) then with M13 and M13R primers with P5/P7 adapters and barcodes (different MID sequences for each sample). Libraries were purified twice with Agencourt AMPure XP (Beckman Coulter) according to the manufacturer's protocol. The quality of the pooled libraries was assessed on a Bioanalyzer chip (Agilent) and they were quantified with KAPA Library Quantification Kit for Illumina Platform using LightCycler 480 (Roche) according to the manufacturers' protocols. The final pool was diluted to 4 nM, denaturated, mixed with 5% of PhiX control library and sequenced with the use of 2 × 300 cycles kit v.3 on a MiSeq machine (Illumina).

The resulting read pairs were denoised with dada2 (Callahan et al., 2016) and the fungal sequences were processed with ITSx (Bengtsson-Palme et al., 2013), and all fungal ITS1 sequences were used in the downstream analyzes. The reads were de-replicated and OTUs were constructed using vsearch (Rognes et al., 2016) at 0.03 dissimilarity level, then singletons as well as doubletons (OTUs consisting of one or two sequences only) were removed. The sequences were classified with naive Bayesian classifier (minimum 80% bootstrap support was required; Wang et al., 2003) using ITS1 extracted from UNITE v.7 (fungi), and the non-fungal sequences were removed. The final data were sub-sampled to 300 (fungi) sequences per sample 20 times, sequences names were mangled to reflect the iteration, the sets were pooled, de-replicated, and OTUs were constructed as described earlier. OTU tables were then averaged over the 20 subsamples and the entries were rounded to the nearest integer with a Perl script to yield the final tables.

Data Analysis

Statistical analysis for screening of PSFs was performed using the Statistica software package (version 13.0, StatSoft) based on three replicates for each sample variant. For total density of culturable strains (**Figure 2**) and PSF screening (**Figure 3**) nine replicates (three samples from each of three plots) for each genotype present on site were used. Normality was tested using Shapiro–Wilk test and homogeneity of variance using Levene's test. Samples that were outside of two standard deviations range from mean were removed. The one-way analysis of variance (ANOVA) was used to determine whether there are any statistically significant differences between the means of total fungi count for each genotype on PDA and selective media and PSF count for

[2]http://oligo.ibb.waw.pl/

[3]www.ncbi.nlm.nih.gov/BLAST

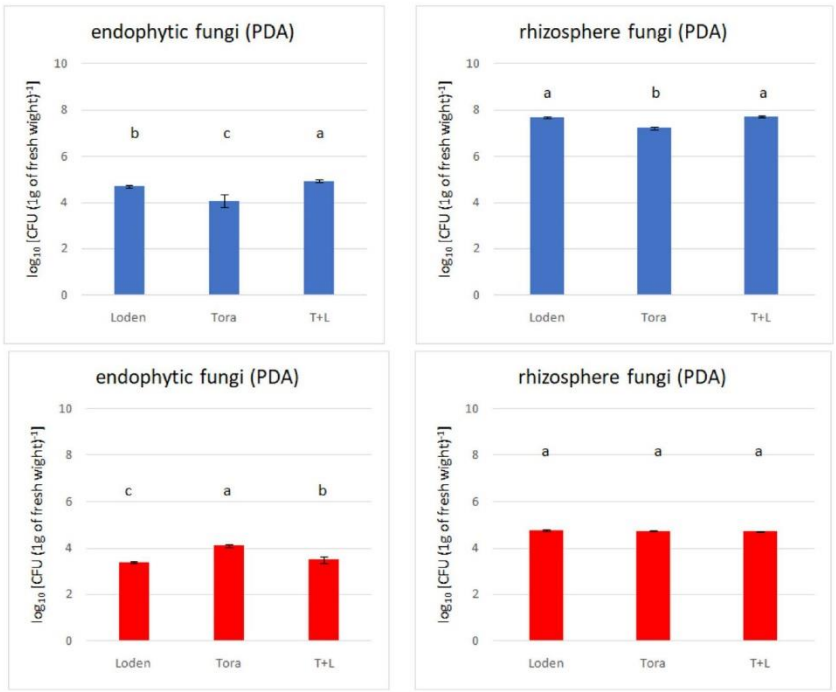

FIGURE 2 | Total fungal count (endophytes and rhizosphere) from two experimental sites (Sweden and Germany) in fall 2018. The upper panels (blue bars) indicate the total fungal counts for Swedish samples; the lower panels (red bars) indicate the total fungal counts for Germany samples.

genotypes for each experimental site. Upper case letters represent significant differences for PSF count for genotypes for each experimental site (**Figures 2**, **3**).

For metagenomic data analysis, Bray–Curtis distance matrices based on Wisconsin double-standardized OTU tables were calculated with vegdist in R. Non-metric multidimensional scaling (NMDS) and canonical correspondence analysis (CCA) analyzes were performed within R with vegan's metaMDS and cca functions. In case of CCA, forward selection procedure implemented in ordistep was used for stepwise model building. Significance of differences between sample clusters was assessed with ANOSIM and PERMANOVA in vegan's anosim and adonis functions, respectively. *P*-values < 0.05 was considered significant. Variance partitioning was performed with the varpart function. Significance of differences in means (number of observed OTUs, Shannon's H′, Shannon's E, taxa, and functional groups distribution) was assessed with ANOVA with *post hoc* Tukey's HSD analysis, unless assumptions of normality of data and/or homogeneity of variance were violated, in which case robust ANOVA implemented in raov of the Rfit package was used to check for general *p*-value. All figures were plotted with standard R graphic functions.

RESULTS

Soil Properties and Climatic Conditions at the Two Experimental Sites

As shown in **Table 1**, in Uppsala the rhizosphere soil parameters TOC, TN, C:N was higher while P_{av}, pH(H_2O), pH(KCl) was lower. ANOVA analysis revealed that the soil properties differed significantly between the experimental sites (**Table 1**). Soil samples from Sweden had higher contents of TOC, TN, and P_{av}, while soil samples from Germany were characterized by higher C:N ratios and pH values. Soil parameters at both experimental sites differed slightly in spring. Significant differences between genotypes were observed for the TOC (T and LT in Germany), pH (all plots in Sweden) and P_{av} content, which were higher in fall (L and LT plots in Sweden and L plots in Germany; **Table 1**).

Identification of Dominant PSFs and Total Fungal Density in Willow Genotypes and Mixtures

The overall density of cultivable fungi ranged from 3.38 to 4.94 log10 [measured as colony-forming units: c.f.u. (g d.w. roots)$^{-1}$]

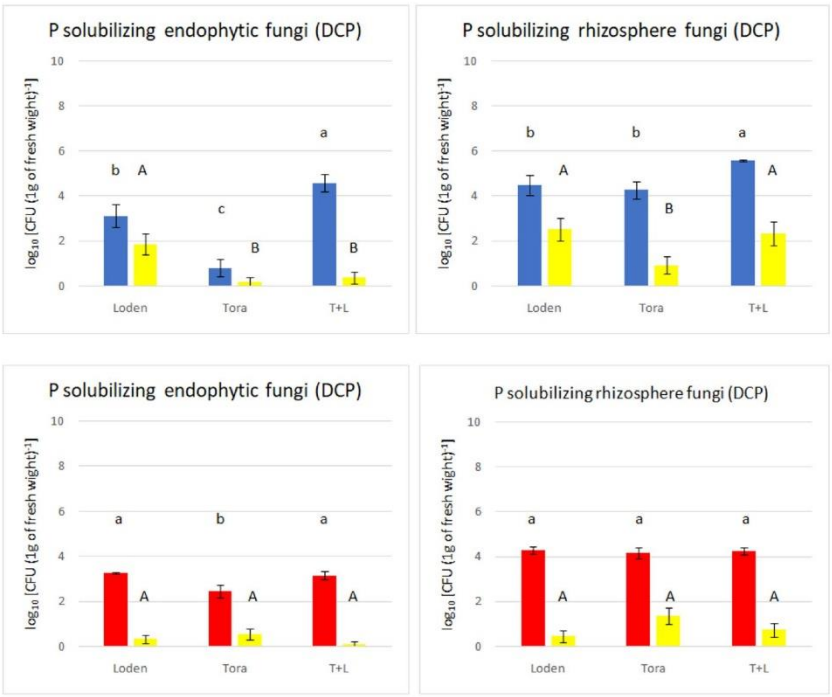

FIGURE 3 | Total non-P solubilizing and P solubilizing fungi count (endophytes and rhizosphere) from two experimental sites (Sweden and Germany) in fall 2018. The upper panels (blue bars) indicate the total fungal counts from selective media for Swedish samples; the lower panels (red bars) indicate the total fungal counts from selective media for Germany samples. The yellow bars indicate the number of phosphate solubilizing fungi found among the non-P solubilizing fungi.

for endophytes from both experimental sites. In the rhizosphere soil, the total fungal count ranged from 4.71 to 7.72 log10 [c.f.u. (g d.w. soil)$^{-1}$]. Significant differences among the analyzed willow genotypes were observed for endophytic fungal density (**Figure 2**), but this was not observed for rhizosphere fungi. The endophytic fungal density was lower than that of rhizosphere fungi. The difference in fungal density between the endophytes and rhizosphere soil was significantly greater in Swedish samples than German ones. Generally, the highest fungal density was recorded in LT samples from Sweden and T samples from Germany.

The medium supplemented with triphosphate (PVK) showed nofungal growth, and therefore, it was excluded from the analysis. The total number of culturable endophytic fungi ranged from 0.80 to 4.57 log10 c.f.u g^{-1}, among which PSF ranged from 0.12 to 1.86 log10 c.f.u. g^{-1} but showed no significant differences with the exception of Loden (only in Sweden). The endophytic fungal density was lower than that of rhizosphere fungi, ranging from 3.38 to 4.94 log10 c.f.u. g^{-1}, whereas rhizosphere fungi ranged from 4.75 to 7.72 log10 c.f.u. g^{-1}. The LT genotype showed the highest non-PSF density at both experimental sites (**Figure 3**). The endophytic fungal density was lowest for the Tora genotype at both experimental sites. The phosphate solubilizing ability of fungi from the Swedish site (found mostly in rhizosphere soil) was higher than that in fungi from the German site.

In total, 22 fungal strains were isolated from roots (10 strains) and rhizosphere soil (12 strains; **Figure 4**). *Clonostachys* and *Penicillium* were the dominant genera at the German site (30% for each), while at the Swedish site, *Penicillium* alone was the dominant genus (70%; **Figure 4**). Most of the strains found at the German site were isolated from the rhizosphere soil of T, in contrast to the Swedish site, where the majority was endophytic isolates from the L genotype. *Penicillium* was the only fungal genus occurring at both sites. The German site showed higher diversity in the rhizosphere, with five different fungal genera (*Penicillium*, *Clonostachys*, *Alternaria*, *Gibellulopsis*, and *Cladosporium*). For the Swedish site, the highest diversity was obtained for endophytes of the L genotype with three different species (*Penicillium*, *Talaromyces*, and *Juxtiphoma*). In total, 54% of the identified strains were isolated from T, 31% from L, and 13% from LT samples.

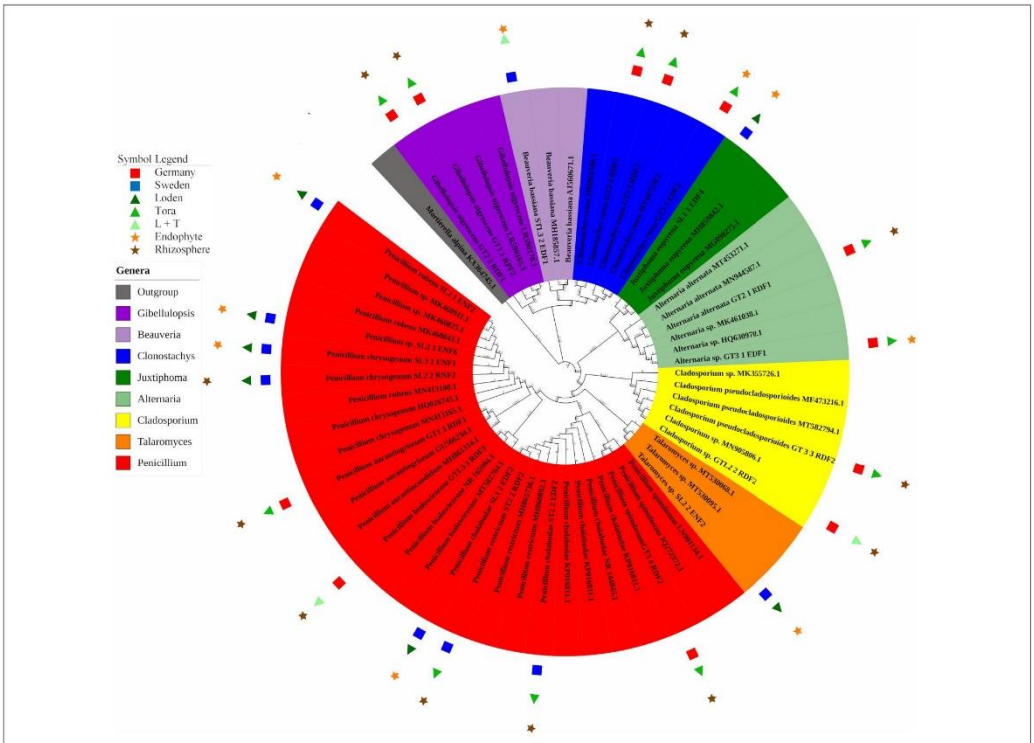

FIGURE 4 | Phylogenetic analysis of culturable fungi isolated from three willow genotypes classified at the genus level. For details of isolates with their GenBank accession numbers, see **Supplementary Table 3**. Reference sequences with closest BLAST match were used (strains without symbols) to construct the phylogenetic tree. The 22 fungal strains with PSF activity are marked with their respective symbols (see legend on the left).

The endophytes comprised 54% of the total number of identified fungi.

Experimental Site and Level of Plant Association Shaped the Community Structure in Willow SRCs

The alpha diversity of the fungal community was not influenced by genotype but by site or the level of fungi association with the plant (rhizosphere vs. endosphere; **Figure 5**). Diversity (H′), species richness (observed OTU number) and evenness (E) were higher in the rhizosphere than in the endosphere. Endophytic communities at the German site were more diverse and even harbored more OTUs than those at the Swedish site; however, there were no differences between seasons. At the Swedish site, alpha diversity was higher in spring than in fall. There were no differences between variants in rhizospheric communities.

The alpha diversity analysis did not show significant differences between genotypes. Overall, Shannon's diversity (H′), Shannon's evenness, and observed OTUs revealed significant differences between the endophytes and rhizosphere fungal diversity regardless of the experimental site and seasons. The endophytic diversity at the two experimental sites in Germany and Sweden was significantly different from that of the rhizosphere fungi. A significant effect of seasonality was observed only for endophytes from Sweden (**Figure 5**). The number of observed OTUs for rhizosphere fungal diversity showed a greater tendency than that of the endophytes, and this difference was prominent between the experimental sites in Germany and Sweden during the fall.

The NMDS analysis revealed that the fungal communities clustered according to the experimental sites, but this was not observed for seasons and genotypes (**Figure 6**). The grouping was significant for roots (PERMANOVA, $F = 0.5050$, df = 1, and $P = 0.0001$) and for soil (PERMANOVA, $F = 0.1830$, df = 1, and $P = 0.0001$). The differences in variance were not significant for roots (PERMDISP, $P = 0.579$) or soil (PERMDISP, $P = 0.2911$). The distance between the fungal communities in the two experimental sites was significantly larger for the rhizosphere soil than for roots.

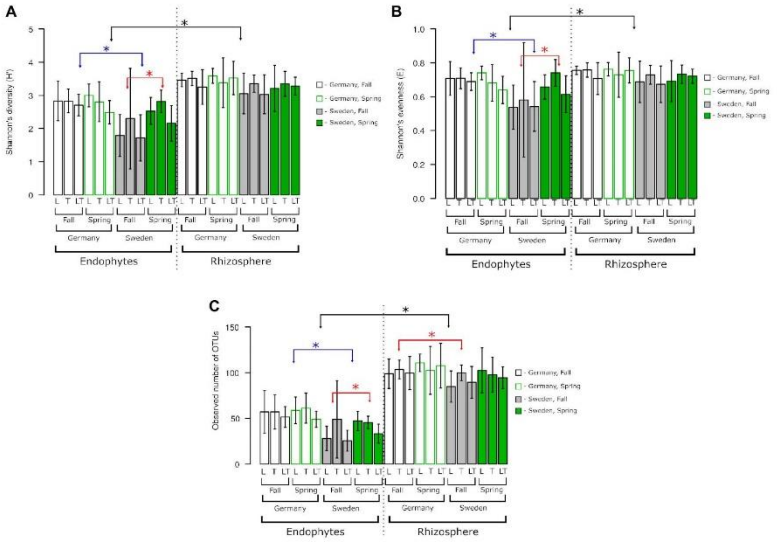

FIGURE 5 | Fungal species richness, diversity, and evenness across two experimental sites, rhizosphere soil and roots. OTUs constructed at 0.03 dissimilarity for fungal sequences. **(A)** Shannon's (H'), **(B)** Shannon's (E), **(C)** observed number of OTUs. Robust ANOVA with Tukey's *post hoc* analysis was used to assess the significance of differences between experimental sites, rhizosphere soil, and roots. Colors denote the following: black – significant difference between endophytes/rhizosphere. *Indicates significant difference between variants indicated by arrows.

The CCA showed that the fungal communities were significantly different between the two experimental sites only (**Figures 6B,D**). In the rhizosphere soil, total organic C and total N were the two environmental variables shaping fungal diversity at the Swedish experimental site (**Figure 6**). TN explained 7.5% of variance whereas TOC explained 2.4%. In contrast, the fungal communities in the roots of the German experimental site were mostly influenced by pH(H_2O), whereas at the Swedish experimental site, the most influential factor was TN (**Figure 6**). TN explained 2.5% of the variance, while pH(H_2O) explained 1.5%.

In conjunction with the culturable fungal diversity, the fungal libraries from the rhizosphere soil and endophytic community showed significant dominance of the phylum Ascomycota, followed by Basidiomycota and Mortierellomycota (**Supplementary Figure 2**).

At the class level, the fungal community was dominated by *Dothideomycetes* and *Leotiomycetes*, with significant differences seen for both endophytes and rhizosphere soil fungi. The above two classes were significantly different based on both the experimental sites and seasons as well (**Figure 7**). *Agaricomycetes* and *Pezizomycetes* showed high abundance and were exclusive to endophytes only. All classes except *Agaricomycetes* displayed significant differences between the two seasons. The rhizosphere soil fungal community was dominated by *Tremellomycetes* and *Mortierellomycetes*; the former showed significance among the two sites and seasons, while the latter showed only seasonal effects. *Dothideomycetes* was the only class exhibiting differences among genotypes.

At the family level, *Piskurozymaceae* showed greater fungal diversity among the rhizospheric fungi than the endophytes (**Figure 7**). *Helotiaceae* and *Phaeosphaeriaceae* were dominant and significantly represented in the Swedish site. *Tuberaceae* and *Herpotrichiellaceae* reads were found mostly in German samples, but the former did not show seasonal changes. Among the fungal endophytic communities, the effect of season was not observed for *Tuberaceae* and *Thelephoraceae*. In the rhizosphere fungal community, both sites were dominated by *Piskurozymaceae* and *Mortierellaceae,* mainly at the Swedish site. At the German site, the family *Plectosphaerellaceae* displayed a significant seasonal effect.

The endophytes *Paraphoma* and *Exophiala* were the most frequently occurring genera in both the experimental sites and seasons. The genus *Exophiala* was present in greater numbers at the German site than at the Swedish site; in contrast, the *Tetracladium* genus was present mostly at the Swedish site. On the other hand, the rhizosphere fungal libraries were dominated by reads of the genera *Solicoccozyma* and *Mortierella* mainly at the Swedish site. The rhizospheric fungi were significantly different in the two seasons and were more prominent at the Swedish site than at the German site.

At the species level, the endophytic fungal community consisted mostly of *Tetracladium* sp., *Paraphoma rhaphiolepidis* and *Exophiala salmonis,* whereas most of the identified fungi

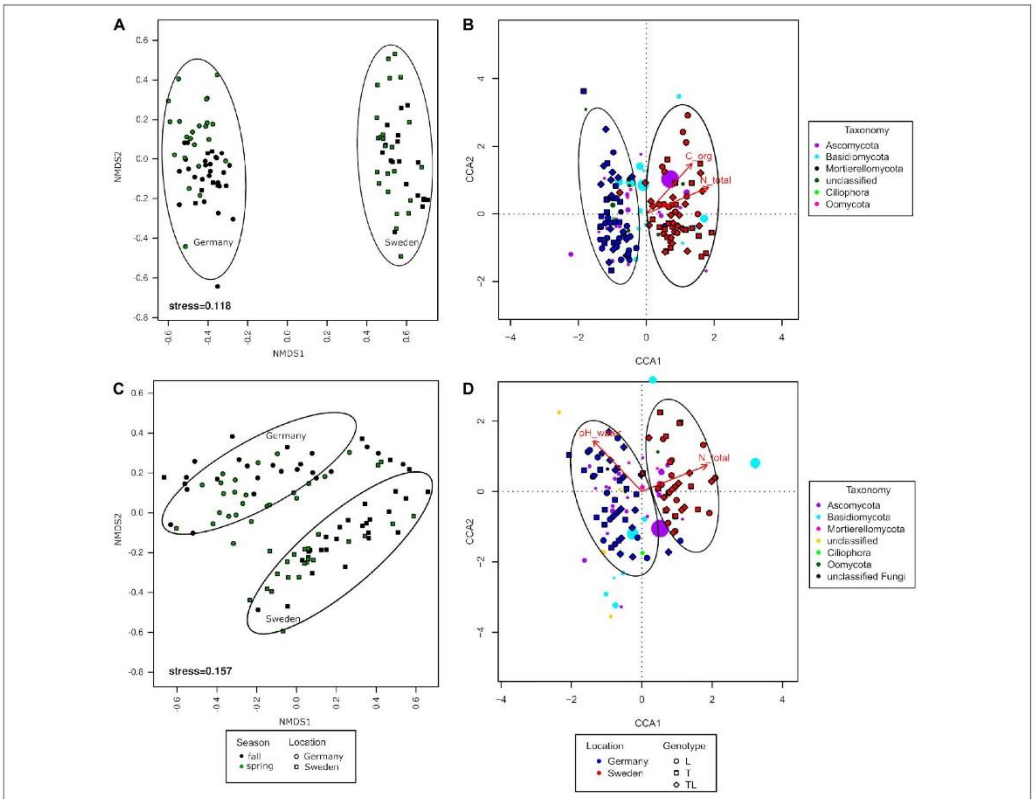

FIGURE 6 | Analysis of log-transformed and Wisconsin double-standardized Bray-Curtis dissimilarity matrix for rhizosphere soil and root fungal communities associated with *Salix* genotypes L (Loden), T (Tora), and LT (mixture) between two seasons (fall, spring) and two sampling sites (Germany and Sweden). **(A,C)** NMDS (non-metric multidimensional scaling analysis); **(B,D)** CCA (canonical correspondence analysis).

from the rhizosphere soil belonged to *Salicoccozyma terrea* (with the highest occurrence at the Swedish site). Based on the experimental sites, the rhizosphere fungi at the German site showed significantly more fungal reads belonging to *E. salmonis*. *E. salmonis* was the only species to show significant differences between the endophytic and rhizospheric communities at the German site.

DISCUSSION

This study is first in describing the PSF in woody SRC and comparing the fungal diversity and community structure between sites, seasons, and genotypes. The beta diversity revealed that experimental site drives the fungal community structure. Abundance as well as alpha and beta-diversity of fungal community were mainly driven by TOC, TN, and pH. We found *Penicillium* to be the dominant genus of PSF in the group of isolated fungal strains, while this genus was not detected in the metagenomic analysis. This may be due to the presence of other abundant fungal genera that may mask its presence. The level of plant association (endophytic or rhizosphere fungi) was the main factor driving fungal diversity and community structure (number of observed OTUs was greater for rhizosphere fungi than endophytic fungi). Differences for seasons and genotypes were present but were not particularly prominent.

Physico-Chemical Soil Properties and Climate Distinct at the Two Experimental Sites

To date, only a few studies have characterized the fungi associated with woody crops from different geographic locations, e.g., *S. alba* in India (Chatli et al., 2008), *Salix viminalis* in the United Kingdom (Barnes et al., 2018), *S. viminalis* and *S.* × *dasyclados* in Germany (Baum et al., 2006), and *S. viminalis*

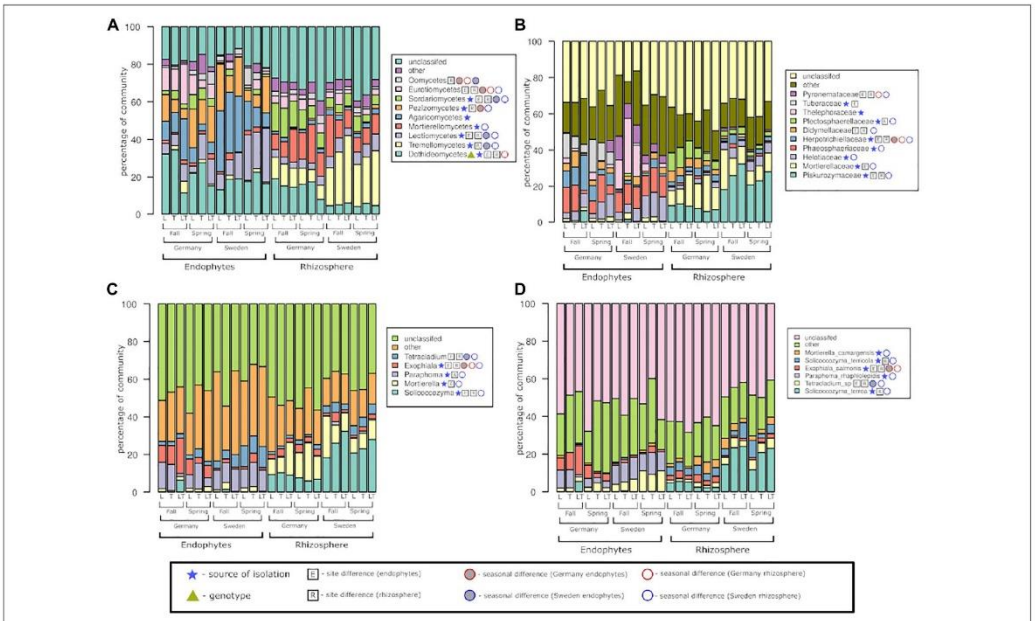

FIGURE 7 | Fungal community structure at the level of class **(A)**, family **(B)**, genus **(C)**, and species **(D)** among rhizosphere soil and roots, two experimental sites, and two seasons.

and *S. schwerinii* in Sweden (Hrynkiewicz and Baum, 2012). The soils in the investigated sites can be described as fertile and biologically active, with optimal properties for willow cultivation (Guidi et al., 2013). Soils at the Swedish site contained a higher content of TOC, TN, and P_{av} than soils at the German site, which likely resulted from primary soil properties related to their different pedogenesis (Vertic and Stagnic Cambisols) such as texture and nutrient abundance. However, the studied soils were moderately to slightly acidic, which undoubtedly favors the bioavailability of P (Yue et al., 2000; Richardson et al., 2009; Yadav et al., 2012). TN is frequently found to be a critical factor for fungal diversity (Allison et al., 2007; Lauber et al., 2008; Zhou et al., 2016). The available P in soil may influence fungal communities in the rhizosphere to some extent (He et al., 2016; Rosenstock et al., 2016; Williams et al., 2017). In our case, the Swedish site had significantly higher P_{av}, but we observed no significant influence of this variable. This confirms N-limited rather than P-limited conditions, as is common at most arable sites (Williams et al., 2017).

A statistically significant short-term seasonal increase in TOC was recorded at the experimental site in Germany, but a similar trend was also observed at the experimental site in Sweden. This can be attributed to the leaf and root litter inputs in combination with no-till management. However, research by Hoeber et al. (2020) showed that climatic factors may significantly determine the rate of decomposition of leaf litter. These authors found that the decomposition rates in Germany were 43% faster than those in Sweden. The fine roots of willows can also be an important source of soil organic matter (Kahle et al., 2007). A slight decrease in P_{av} concentrations in spring suggested nutrient depletion. No effects of nutrient depletion on the yields were observed in Sweden. These results were supported by the findings of (Kahle et al., 2007).

Dominating PSF Identity and Total Fungal Density in Willows

Among the culturable diversities, the number of PSF was much higher in the rhizosphere fungi than in the endophytic fungi. This supports our hypothesis that lower number of endophytic fungi may possess ability to solubilize P than rhizosphere fungi. We found *Penicillium* to be the dominant genus of PSF isolates from the SRCs; other, less frequent isolates were of the genera *Alternaria, Cladosporium,* and *Clonostachys*. Similarly, Chatli et al. (2008) reported members of *Penicillium* to be among the dominant strains isolated from woody crop species. These strains were also abundant in the rhizosphere of *Salix* spp. in Lithuania (Repečkienė et al., 2009). The genus *Clonostachys* (isolated from the rhizosphere and endosphere) was specific to the German site, whereas *Juxtiphoma*, *Talaromyces,* and *Beauveria* were specific to the Swedish site. Apart from PSFs, we isolated non-PSF that were able to grow on selective media (NBRIP, PVK DCP). These

fungi probably require low P concentrations to support growth. At the Swedish site, the fungal density in the three genotypes was found to range as follows: LT > Loden > Tora, in contrast to the German site, where it ranged as follows: Loden > LT > Tora. This effect might be based on the varying site-specific environmental conditions and their interactions with the genotypes.

Penicillium is commonly found as a PSF taxon (Chatli et al., 2008; Patil et al., 2012; Sharma et al., 2012). *Penicillium bilaiae* is even sold by NovoZymes as a bioinoculant enabling soil P mobilization. Levels of plant growth-promoting effects by *Penicillium* species were associated with increased uptake of P into shoots (Qiao et al., 2019). Although P solubilization ability is common in the genus *Penicillium*, various species and strains differ in their capacity to mobilize P due to differences in the secretion of organic acids, phosphatases, and phytase or in the operation of other P solubilizing mechanisms. The genus *Clonostachys* (isolated both from the endosphere and rhizosphere) was previously reported as an endophyte in *Salix* species growing in SRCs (Hosseini-Nasabnia et al., 2016). *Clonostachys rosea* is reported to be a mycoparasite in *Theobroma gileri* and as a biocontrol agent against *Phytophthora palmivora* and *Moniliophthora roreri* (ten Hoopen et al., 2003). To date, reports on the P solubilizing abilities of *Alternaria* and *Cladosporium* are scarce. There is not much information about PSF isolates from other woody plants. Schmidt et al. (2018) investigated endophytic community of poplar, species that is also commonly grown as SRCs but did not found any PSFs. Mishra et al. (2014) reported presence of several phosphorus solubilizing fungi in banana tree including *Fusarium* sp., *Trichoderma* sp. and present in this study *Penicillium* sp. In forest environment *Aspergillus* sp., *Cladosporium* sp., *Curvularia* sp. and several *Penicillium* species are frequently reported to possess high potential in P solubilization (Manoharachary and Nagaraju, 2017). There is several studies that use *Penicillium* species as bioinoculants that improve P availability for various plants (Omar, 1997; Ram et al., 2015; Yin et al., 2015; Li et al., 2016).

Multiple Factors Influencing Alpha Diversity and Fungal Community Structure in Willow SRCs

The rhizosphere fungal community was more diverse in culture independent (metabarcoding) analyses, suggesting strong selective pressure in the interior of willow roots. Similar observations are common both for bacterial (e.g., Kielak et al., 2008; Bulgarelli et al., 2012) and fungal (e.g., Hrynkiewicz et al., 2012; Yergeau et al., 2015; Thiem et al., 2018; Furtado et al., 2019) communities associated with various plants. The reason for this phenomenon could be that roots may act as a filter or selection barrier for fungal species present in the rhizosphere soil, which can result in a lower number of endophytes (Garbeva et al., 2004; Lauber et al., 2008). This might be especially valid for woody roots such as those of *Salix* spp.

The classes *Agaricomycetes* and *Pezizomycetes* were more frequent in the endosphere, while *Tremellomycetes* and *Mortierellomycetes* were more abundant in the rhizosphere. These facts could be explained by the former two classes comprising mostly ectomycorrhizal fungi (Li et al., 2018), whereas the latter contains mostly saprophytic organisms (Francioli et al., 2020). Yergeau et al. (2015) reported the dominance of the class *Dothideomycetes* in willows, and the same was observed in our study. Members of this class are mainly endophytes and epiphytes and can be lichenized or lichenicolous fungi (Schoch et al., 2009). Moreover, *Dothideomycetes* was the only fungal class whose abundance differed between genotypes. *Agaricomycetes* was the only class whose abundance did not differ between seasons, which was caused by large differences within variants (i.e., high standard deviation). Similarly, reports by Shakya et al. (2013) from poplar showed no seasonal effect for rhizosphere fungi at any of their investigated sites.

At species level fungal community was mostly build from six species. For endophytes *Tetracladium* sp., *P. rhaphiolepidis* and *E. salmonis* were the most frequent. All three species are known grass endophytes while *E. salmonis* was additionally reported as an animal pathogen (Maciá-Vicente et al., 2016; Ricks and Koide, 2019; Gomzhina et al., 2020). Besides being pathogenic in animals it was previously isolated from the roots of poplar, which is another commonly grown tree species in SRCs (Maciá-Vicente et al., 2016). Most of the fungal reads from the rhizosphere soil belonged to *S. terrea,* but no information is available on this fungal species. The other *Solicoccozyma* species found in this study, *S. terricola* is a well known psychrotolerant yeast used in lipid production (Filippucci et al., 2016; Stosiek et al., 2019; Tasselli et al., 2019). Lastly, *Mortierella camargensis* isolated from grassland soils showed ability to produce arachidonic acid and lipase activity (Botha et al., 1999; Miklós et al., 2012).

The absence of the PSFs cultured from our samples in amplicon libraries is probably due to technical reasons. First, universal primers used to generate libraries might be biased against particular sequence variants (SVs), which together with SV scarcity in samples might result in excluding them from libraries.

Unconstrained ordination revealed that the level of community association with plants (rhizosphere vs. endosphere) and experimental sites were the two most important factors grouping the samples. This is expected, as (i) the difference between rhizosphere and endophytic communities is frequently observed (Thiem et al., 2018) and (ii) the sites differed in both climatic and soil conditions. Indeed, soil environmental parameters (TOC, TN, and pH) significantly influenced the fungal community structure. A significant influence of pH was unexpected, as it is usually not a limiting factor for fungi. Out of four factors we hypothesized would influence fungal communities in willow, level of community association with the plant, experimental site location and season turned out to exert a significant impact on fungi, while the effect of tree genotype was not as prominent. This fact can be explained by (i) the spatial distribution of fungal mycelium in soil, i.e., mycelium is able to freely grow out of particular field boundaries, effectively canceling actual differences, and (ii) genetic differences between analyzed genotypes may cause little effect on the soil fungal microbiome.

CONCLUSION

The level of fungal community association with the plant (rhizosphere vs. root endophytes) is the most important factor shaping its diversity. The site, season, and planting design have a lower impact. The fungal diversity at the same level of plant association was mainly driven by soil properties such as TN, TOC, and pH. Among the culturable fungal diversities, *Penicillium* was dominant and commonly isolated genus as a P solubilizing taxon from both SRCs, while others less frequently isolated were the genera *Alternaria, Cladosporium,* and *Clonostachys*. Apart from PSFs, we isolated non-PSF that may require less P to support their growth. In general, a lower number of endophytic fungal strains possessed the ability to solubilize P compared to the number of rhizosphere fungal strains with this ability. The rhizosphere fungal community was generally more diverse than that in the endosphere at both willow SRC sites. This might suggest selective pressure on willow roots and emphasize the uniqueness of the fungal community. Fungal libraries of rhizosphere soil and endophytic communities showed significant dominance of the phyla Ascomycota followed by Basidiomycota and Mortierellomycota. The genus *Exophiala* was present in greater numbers at the German site, while the genus *Tetracladium* was present mostly at the Swedish site.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject, PRJNA716888; https://www.ncbi.nlm.nih.gov/genbank/, MW342736–MW342757.

AUTHOR CONTRIBUTIONS

PK participated in all analyses and wrote the first version of the manuscript. BF participated in preparation of manuscript. MG designed the bioinformatics pipeline, performed bioinformatics analyses, and participated in the preparation of the manuscript. PK and BF analyzed the results and the statistical output. PK, CB, and MW performed sampling at the locations, selected plant genotypes for the experiments, and provided input to the manuscript. PH did soil analyzes and participated in the preparation of the manuscript. KH designed and managed the field and lab experiments and participated in the preparation of the manuscript. All authors revised the manuscript and approve of its publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.647709/full#supplementary-material

Supplementary Figure 1 | Average temperatures and average rainfalls in Rostock (Germany) and Uppsala (Sweden) in 2018 and 2019.

Supplementary Figure 2 | Fungal community structure at the phylum level among rhizosphere soil and root samples, two experimental sites, and two seasons.

Supplementary Table 1 | Meteorological data from two experimental sites in Sweden (SE) and Germany (GER). The maximal monthly temperature (max temp), minimal monthly temperature (min temp), average monthly temperature (avg temp), precipitation (rain), and monthly sunshine hours (sun) in 2018–2019. Data source: https://www.worldweatheronline.com.

Supplementary Table 2 | Selective media (NBRIP, PVK, and DCP) used for the selection of phosphate solubilizing fungi.

Supplementary Table 3 | Identified fungi with accession numbers. S – Sweden, G – Germany, L – Loden, T – Tora, LT – mixture, E – endophyte, and R – rhizosphere fungi.

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Mixed growth of Salix species can promote phosphate-solubilizing bacteria in the roots and rhizosphere

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Phosphorus (P) is an essential plant nutrient that can limit plant growth due to low availability in the soil. P-solubilizing bacteria in the roots and rhizosphere increase the P use efficiency of plants. This study addressed the impact of plant species, the level of plant association with bacteria (rhizosphere or root endophyte) and environmental factors (e.g., seasons, soil properties) on the abundance and diversity of P-solubilizing bacteria in short-rotation coppices (SRC) of willows (*Salix* spp.) for biomass production. Two willow species (*S. dasyclados cv.* Loden and *S. schwerinii* × *S. viminalis cv.* Tora) grown in mono-and mixed culture plots were examined for the abundance and diversity of bacteria in the root endosphere and rhizosphere during two seasons (fall and spring) in central Sweden and northern Germany. Soil properties, such as pH and available P and N, had a significant effect on the structure of the bacterial community. Microbiome analysis and culture-based methods revealed a higher diversity of rhizospheric bacteria than endophytic bacteria. The P-solubilizing bacterial isolates belonged mainly to Proteobacteria (85%), Actinobacteria (6%) and Firmicutes (9%). *Pseudomonas* was the most frequently isolated cultivable bacterial genus from both the root endosphere and the rhizosphere. The remaining cultivable bacterial isolates belonged to the phyla *Actinobacteria* and *Firmicutes*. In conclusion, site-specific soil conditions and the level of plant association with bacteria were the main factors shaping the bacterial communities in the willow SRCs. In particular, the concentration of available P along with the total nitrogen in the soil controlled the total bacterial diversity in willow SRCs. A lower number of endophytic and rhizospheric bacteria was observed in Loden willow species compared to that of Tora and the mix of the two, indicating that mixed growth of *Salix* species promotes P-solubilizing bacterial diversity and abundance. Therefore, a mixed plant design was presented as a management option to increase the P availability for *Salix* in SRCs. This design should be tested for further species mixtures.

KEYWORDS

diversity, bacterial endophytes, rhizosphere bacteria, phosphate solubilization, short-rotation cropping, willow diversity, willow

Introduction

Most of the research on plant-bacterial interactions, specifically on woody plant species, focuses either on the general bacterial community diversity or on the role of bacteria in plant interactions, i.e., the bacterial properties that directly affect host plant growth such as plant growth-promoting metabolites, synthesis of phytohormones, and N_2 binding (Basu et al., 2021). Few studies have addressed bacterial diversity based on their level of association (root endosphere or rhizosphere), and their properties or specific function in woody plants have been scarcely investigated (e.g., Thiem et al., 2018). Meanwhile, in the pool of endophytic and rhizospheric bacteria, there may be those whose direct or indirect influence on plant development is underestimated and not sufficiently investigated to date.

One of the most important contributions of bacteria to plant host growth is their role in facilitating the plant host's access to available forms of phosphorus (P) (Billah et al., 2019). Several reviews describe the important role of P-solubilizing microorganisms in enhancing overall plant (shoot) growth (Kalayu, 2019; Prabhu et al., 2019; Divjot et al., 2021; Rawat et al., 2021). It is frequently reported that the use of P-solubilizing bacteria (PSB) as bioinoculants is cost-effective and a sustainable alternative to chemical fertilizers, as the excessive use of the latter contributes to lowering the quality of groundwater and soil, as well as the accumulation of toxic elements such as selenium (Se) or arsenic (As) in the soil (Khan et al., 2009; Alori et al., 2017). Experimental studies on the role of P-solubilizing bacteria in plants have mostly used well-known strains of bacteria belonging mainly to *Pseudomonas* sp., *Bacillus* sp. or *Streptomyces* sp. (Wani et al., 2005; Chen et al., 2006; Ahemad and Khan, 2011; Kaur et al., 2011; Grafe et al., 2018; Rathinasabapathi et al., 2018; Ahmad et al., 2019; Wang et al., 2020;). Additionally, screening studies exploring new bacterial strains with P-solubilizing abilities are carried out relatively often (e.g., El Habil-Addas et al., 2017; Rahman et al., 2017; Boubekri et al., 2021; Chen et al., 2021). However, only in some cases are studies on P-solubilizing bacteria conducted on larger scales that take into account (i) the selection and differentiation of strains that are able to degrade easily and sparingly soluble P compounds (belonging to the groups diphosphates and triphosphates, respectively), (ii) the compatibility of specific groups of P-solubilizing bacteria with specific plant species and specific environmental conditions and cultivation systems (e.g., monoculture or mixed cultivation), and (iii) the application potential of these microorganisms. High-throughput methods such as metagenomic analysis, which can provide the background of the entire community of bacteria of a given environment, are also rarely targeted by the previously mentioned research (Sarikhani et al., 2019; Teng et al., 2019; Chawngthu et al., 2020; Rfaki et al., 2020). Meanwhile, the evaluation of the microbiological background of the microorganisms coexisting with P-solubilizing microbes is extremely important because the composition of the microbiological background can be used as a guide for selecting the right bacteria that will be compatible not only with a specific plant species but also with its microbiome.

Our work responds to the above deficiencies and presents not only the screening of cultivable P-solubilizing bacteria (e.g., di-and triphosphates) but also a metagenomic analysis of the rhizosphere and root endosphere, which provides a proper background of the bacterial community. The research was carried out in experimental trials including single-species and mixed-species plots of two willow varieties belonging to different species (Loden (*S. dasyclados*) [L] and Tora (*S. schwerinii* × *S. viminalis*) [T]), which were grown in two locations with different soil and climatic conditions (Germany and Sweden). This study focused on bacteria and complements our previous results on P-solubilizing fungi in the same experimental trials (Koczorski et al., 2021). The main objective was to assess the influence of soil properties, seasons, the level of plant association with bacteria (root endophyte and rhizosphere), and the host plant cultivation system (single-species vs. mixed-species) on the diversity of P-solubilizing bacteria and to determine their role and contribution to the background of the entire willow microbiome. The application of culturable and nonculturable research techniques allowed us to evaluate the culturable PSB in the context of a complete community of bacteria, as well as to determine the influence of soil properties and seasons on changes in community structure. We hypothesized that (i) bacteria with the ability to solubilize P would be more abundant in the rhizosphere than the root endosphere of the tested plants and (ii) the plant species and the test site conditions would significantly affect the bacterial diversity and bacterial community.

Materials and methods

Sample collection and processing

The test sites used in this study are two experimental trials of willow short-rotation coppices (SRCs) located in Uppsala (Sweden: 59.820375, 17.640334) and Rostock (Germany: 54.061391, 12.081857). Both sites were previously used as arable sites, and they differ in the soil type. The soil at site S is characterized as a Vertic

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Cambisol type (moderate amount of clay), while at site G, the soil type is described as a Stagnic Cambisol (sandy loam dominance) (IUSS Working Group WRB, 2015). The experiment was established in 2014 as part of the ECOLINK-Salix to investigate the impact of willow diversity on growth, nutrient use and ecosystem functioning (Hoeber et al., 2018). A detailed description of the experimental trials is found elsewhere (Hoeber et al., 2018; Koczorski et al., 2021; Figure 1). In our investigation, we only used the data from two willow genotypes belonging to two different species, Loden (*S. dasyclados*) and Tora (*S. schwerinii* × *S. viminalis*), and compared them between the two test sites (German and Swedish). Tora is characterized by a higher growth rate and smaller total leaf area than Loden (Hoeber et al., 2018). At each test site, three blocks (replicates) were established, each consisting of three plots with willows planted in single-species or mixed-species cultures. The size of each plot was 9.6 × 9.6 m, and the tree planting density was 15,600 plants per ha (Hoeber et al., 2018). Samples for analysis were collected at both sites in two seasons: fall (October) 2018 and spring (May) 2019. During each season, samples from each experimental site were collected from three plots in three replicates: Loden monoculture, Tora monoculture and mixed cultivation of both (Loden and Tora). In total, 162 samples (81 from Sweden and 81 from Germany) of willow roots and adjacent soils (15 × 15 × 15 cm) sampled at equal intervals of 6 m from each other were collected. The collected samples were immediately transported to the laboratory in Poland (Department of

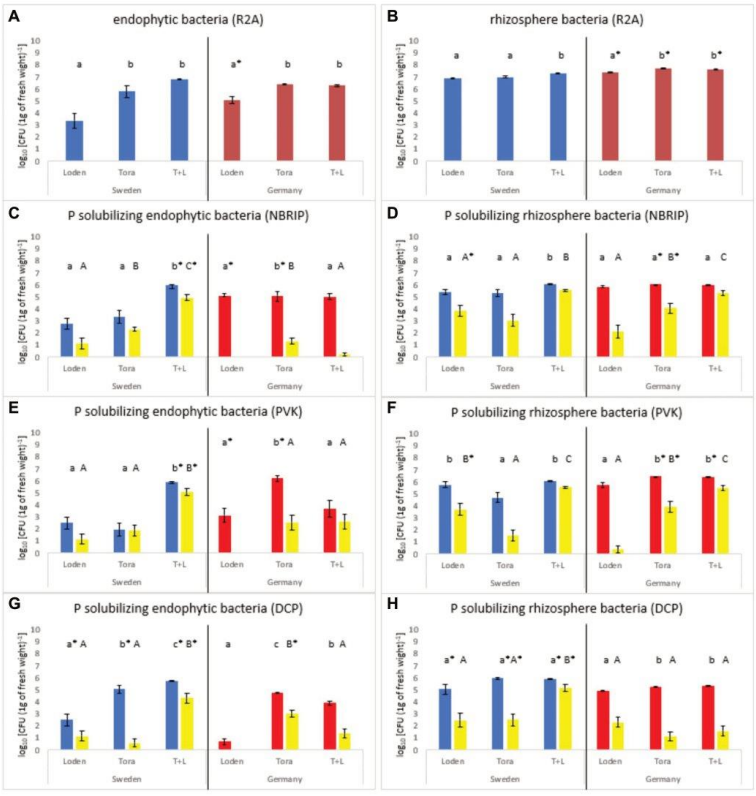

FIGURE 1
Total **(A,B)** and P-solubilizing bacteria **(C–H)** abundance on different media. Samples were taken in fall 2018 from two test sites (Sweden and Germany). **(A,B)** present the total bacterial count on plates with R2A medium for two levels of plant associations (rhizosphere and endophytic bacteria). Blue bars represent samples from Sweden and red from Germany. **(C–H)** show the abundance of bacteria present on selective media plates (blue and red bars) and the abundance of P-solubilizing bacteria (yellow bars). Small and capital letters indicate significant differences between the two willow species used (Tora=*S. schwerinii x viminalis*, Loden=*S. dasyclados*) (small letters for total bacteria count; capital letters for P-solubilizing bacteria). *Indicates differences between sites.

Microbiology, Nicolaus Copernicus University) and analyzed as described below. The samples collected in fall 2018 were used to determine the total number of bacteria and the total number of P-solubilizing bacteria (PSB), for which a collection was also established. The roots were carefully separated from adherent soil and subjected to a surface sterilization process as described previously (Koczorski et al., 2021). In the first step of surface sterilization, 60% alcohol was used (3 min), and then the roots were rinsed 3 times in a sterile 2% NaCl solution. In the second step, 5% H_2O_2 solution (10 min) was used for sterilization, and roots were again rinsed 3x in sterile 2% NaCl solution. The solutions from the last rinse were used to perform sterilization control on R2A medium (Difco, United States).

Soil analysis

In this study, soil data obtained in previous studies were used (Koczorski et al., 2021; Supplementary Table S1). Soil samples were sieved through a 2-mm-mesh screen after air-drying for the following analyses: total organic carbon (TOC) and total nitrogen (TN) contents were measured after dry combustion using a CHNS Vario Macro Cube elemental analyzer. Available phosphorus (P_{av}) in 1% citric acid (Van Reeuwijk, 2002) was determined by a spectrophotometric method using a UV–Vis Rayleigh UV-1601 spectrophotometer (Van Reeuwijk, 2002), and the pH at a 1:2.5 soil to water ratio was determined by the potentiometric method using an Elmetron CP-105 pH meter.

Isolation of endophytic and rhizospheric total culturable bacteria and screening for P-solubilizing bacteria

Sterile roots (1 g) were homogenized in a sterile mortar and transferred to 9 ml of a sterile 0.5% NaCl solution. The processed rhizosphere soil samples (1 g) were transferred to 9 ml of a sterile 0.5% NaCl solution. Serial dilutions (10^{-1} – 10^{-7}) were prepared and used for inoculations (spread plating technique) on R2A media with the addition of 40 mg/μl nystatin (for roots: 10^{-3} and 10^{-4}, for rhizosphere soil: 10^{-4} and 10^{-5}) in 3 replicates for each variant and dilution (108 Petri plates, in total). The plates were incubated for 7 days at 24°C, and then total bacterial counts were determined and presented as colony-forming units (cfu).

Three selective media were used for the isolation of phosphate (P)-solubilizing bacteria: NBRIP, PVK containing tri-phosphates (Nautiyal, 1999) and DCP containing di-phosphates (modified by Pikovskaya, 1948) (composition of media presented as Supplementary materials in Koczorski et al., 2021).[1] The inoculations were made from the same serial dilutions (mentioned above) that were used to determine the total number of bacteria. For NBRIP and PVK media, dilutions of 10^{-6} and 10^{-7} were used (roots and rhizosphere soil), and for DCP media, dilutions of 10^{-4}– 10^{-5} (roots) and 10^{-5} – 10^{-6} (rhizosphere soil) were used. All analyses were performed in three technical replications (324 plates in total, 108 plates for each of the selective media). After 7 days of cultivation at 24°C, colonies with visible halo zones were noted. The presence of halo zones indicated the P-solubilizing ability of the isolated strains and allowed for the selection of positive strains for further analysis.

In the experiment, the total number of P-solubilizing bacteria and the total number of bacteria grown on the tested media were determined to assess the potential of the investigated sites and zones (rhizospheric and endophytic) for colonization by potential P-solubilizing bacteria. The bacterial isolates with the ability to solubilize P on selective media were selected and transferred to R2A medium for further identification using molecular methods.

Identification of culturable P-solubilizing bacterial strains

A Bacterial and Yeast Genomic DNA Purification Kit (EurX, Poland) was used to isolate bacterial DNA. The isolated DNA was quantified using a UV–Vis spectrophotometer (NanoDrop 2000, United States). Bacteria were identified based on the 16S rRNA region using primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5'-TACGGYTACCTTGTTACGACT-3′; Frank et al., 2008). The PCR products were purified using the PCR/DNA Clean-Up Purification Kit (EurX). A 1% agarose gel (1 x TBE buffer) with the addition of Simply Safe (EurX) dye was used to confirm the presence of PCR products after purification. The length of the PCR products was determined based on the 100 bp ladder (Perfect 100 bp DNA Ladder, EurX). The genetic material was sequenced at the Institute of Biochemistry and Biophysics.[2] Sequencher 5.4.6 software was used to prepare contigs, and the obtained sequences were compared with those in the NCBI database using BLASTn, which is available on the National Center for Biotechnology Information (NCBI) servers.[3] Mega X software (Kumar et al., 2018) was used for phylogenetic analysis according to the procedure described by Furtado et al. (2019). Reference sequences were obtained from NCBI, and phylogenetic analysis was performed using the neighbour-joining method. Evolutionary distances were determined with the use of the p-distance method (Saitou and Nei, 1987). The phylogenetic tree was visualized with Interactive Tree of Life (iTOL) v3 (Letunic and Bork, 2016).

Statistical analysis

Statistical analyses were performed using Statistica software (version 13.0, StatSoft). Mean values and standard deviations were

1 https://www.frontiersin.org/articles/10.3389/fpls.2021.647709/full

2 http://oligo.ibb.waw.pl/

3 http://www.ncbi.nlm.nih.gov/BLAST

calculated. The computed data from both the isolation of total culturable bacteria and screening for culturable P-solubilizing bacteria are presented as Colony-Forming Unit (CFU)/g dry weight.

Assessing nonculturable bacterial diversity

Total DNA (from the roots and rhizosphere) was isolated according to Koczorski et al. (2021). DNA was isolated from 50 mg of lyophilized roots (for endophytic diversity) and 50 mg of air-dried rhizosphere soil, and each sample was prepared in 3 replicates. Root samples were homogenized with plastic beads prior to isolation. The spectrophotometric (NanoDrop 2000, United States) and fluorometric (Qubit 2.0) methods were used to determine the concentration of DNA in the samples. DNA prepared in this way was used to create libraries in a two-step PCR and purified using Agencourt AMPure XP (Beckman Coulter). The quality and quantity of libraries were assessed using the Bioanalyzer chip (Agilent) and the KAPA Library Quantification Kit for the Illumina Platform – LightCycler 480 (Roche). The complete process of DNA isolation and library preparation was described in our previous work (Thiem et al., 2018), and the method of statistical analysis was described in the publication by Koczorski et al. (2021).

Results

Culturable bacteria and their phosphate solubilization activity at two sites

The number of culturable bacteria ranged between 3.5 and 6.8 cfu/g of dry roots for endophytes and 6.89–7.89 cfu/g of dry soil for the rhizosphere. In general, the tests showed that there was a significant difference in endophytic bacterial abundance between the tested variants of the experiment (mono and mixed cultures). In the case of the willow species, a lower number of endophytic and rhizospheric bacteria were observed in Loden than in both Tora and the mix of both (Loden and Tora; Figure 1). The difference between P-solubilizing bacterial abundance in the Loden species and the mixed variant was an almost twofold lower, indicating the importance of Tora species presence. The NBRIP and PVK selective media showed very similar trends for Loden, where the frequency of occurrence of isolates with phosphate solubilization was lower than in the other tested variants (single-species Tora and the species mix). At the Swedish site, the mixed cultures showed the highest number of PSB both in the case of endophytic and rhizospheric bacteria, while at the German site, the number of PSB was the highest in Tora for endophytic and the mixed culture for the rhizospheric bacteria (Figure 1). Loden showed a lower number of PSBs associated with the plant (both endophytic and rhizospheric bacteria). Overall, the NBRIP medium selected more PSBs from the Swedish site, while in the PVK medium, the PSB abundance was higher in the German site. The total abundance of PSB on the DCP medium was lower than that on the NBRIP and PVK media. The number of PSBs on the DCP medium was lowest for Loden at both experimental sites and both levels of association with the plant (endophytes and rhizosphere). For endophytes in the Swedish site, the mixed cultures showed the highest number of PSB, while for the German site, it was Tora. Similarly, for the rhizosphere, the PSB was the highest for the mixed cultures in the Swedish site, while no significant differences were observed in the German site.

Identification of culturable phosphate-solubilizing bacteria

During the experiment, 88 different bacterial strains with the ability to solubilize P were isolated. Among this bacterial collection, 61 were isolated from tri-phosphate-containing media (26 from NBRIP, 35 from PVK) and 27 from di-phosphate (DCP) media. A total of 41 different endophytic and 47 rhizospheric strains were isolated. Almost 85% of the isolated bacteria (74 strains) belonged to *Proteobacteria*, 6 belonged to *Actinobacteria* (6%) and 8 belonged to *Firmicutes* (9%). The dominant taxa among *Proteobacteria* included species of *Pseudomonas* with 12 strains (13%), *Erwinia* with 17 (19%) and *Rahnella* with 10 (11%). *Actinobacteria* was dominated by the genus *Curtobacterium*, with 3 identified strains (50%), and *Firmicutes* was dominated by 6 strains from the *Bacillus* (67%) genus. A total of 51 strains with the ability to solubilize P at the German site and 37 at the Swedish site were obtained. At the German site, more rhizospheric than endophytic PSBs were isolated, while at the Swedish site, we observed the reverse trend. The most frequently isolated PSB at the Swedish site were bacteria belonging to the genera *Pseudomonas* and *Rahnella*, both found in the endosphere and the rhizosphere. The genus *Erwinia* was characteristic of the German site for both levels of association with the plant (endosphere and rhizosphere). The highest number of strains belonging to *Actinobacteria* were isolated from the German site. Almost 50% of the strains were isolated from the mixed culture (LT; Figure 2).

Microbiome analysis

The bacterial alpha diversity was mainly determined by the level of association with the plant (endosphere and rhizosphere), whereas in the case of endophytes, it was additionally determined by the experimental sites (S, G; Figures 3A–C). A significant difference between the rhizospheric and endophytic bacterial diversity was found in the Shannon's H′ and the number of OTUs observed, wherein the bacterial population in the rhizosphere soil was more diverse (Figure 3A). In the case of endophytic bacteria at site G, significant differences were observed between the cultivable variants and the seasons. This is due to the high values

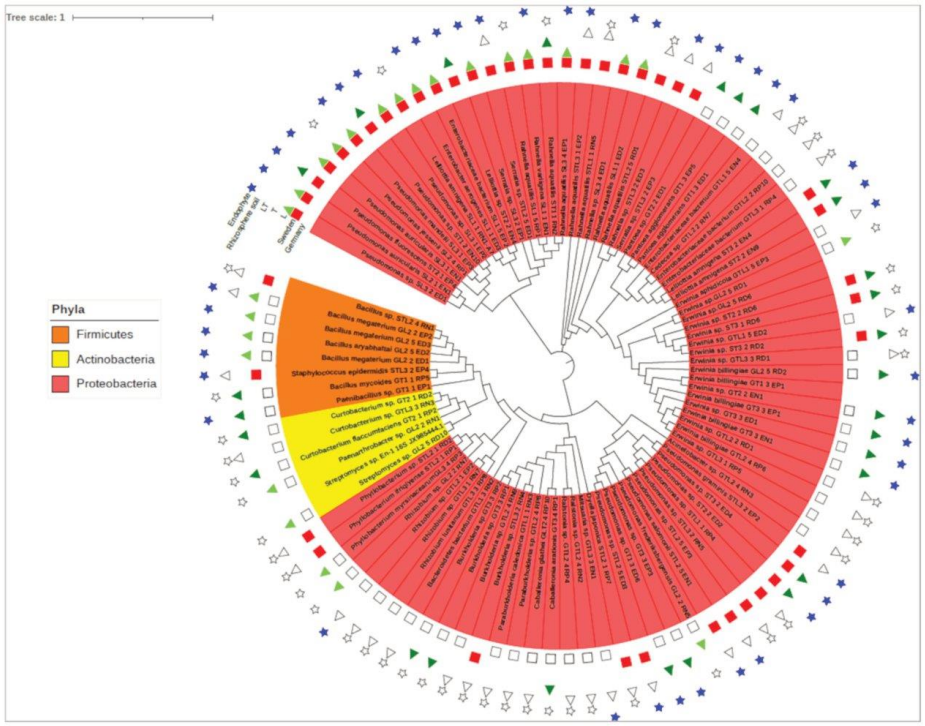

FIGURE 2
Phylogenetic analysis of culturable bacteria isolated from two willow species and their mixture classified at the phyla level. For details of isolates with their GenBank accession numbers, see Supplementary Table S1B. Reference sequences with the closest BLAST match were used (strains without symbols) to construct the phylogenetic tree.

obtained for the samples from the Tora mono-and mixed cultures, which differed significantly from those of the other variants (Figure 3A). The analysis of the Shannon evenness index showed a significant difference between the endophytic communities from the German and Swedish sites (Figure 3B). No significant differences were observed in other variants of the experiment (Figure 3B). The number of observed OTUs showed a significant difference between endophytes and the rhizosphere, as well as between the sites within the endophyte variant (Figure 3C). Overall, the most prominent difference in the diversity of the bacterial microbiome was observed for endophytes and is related to the differences between the studied sites (Swedish and German) Shannon's H′, Shannon's E and the number of observed OTUs.

NMDS and CCA analysis

In this study, soil data obtained in previous studies were used (Koczorski et al., 2021; Supplementary Table S1). According to the results of NMDS analysis, the bacterial communities in the rhizosphere (Figure 4A) and endosphere (Figure 4C) were clustered based on sites (S and G). A PERMANOVA test confirmed that the grouping of rhizosphere and endosphere variants was statistically significant (rhizosphere soil: $F = 20.241$ $r^2 = 0.180$ $p = 0.0001$ roots: $F = 44.85$ $R^2 = 0.099$ $p = 0.0001$). Grouping of bacterial communities by season was not observed. The samples from the German site were more scattered, especially from the roots, indicating higher variability at this test site. The separation between sites was much more prominent in the case of the rhizosphere, which suggests that these communities show greater differentiation than the endophytic communities. NMDS ordination showed clear division of samples from the German site according to willow species, with Loden grouped with Sweden samples, while Tora and mixed variants remained grouped together (Figure 4C). The samples from Tora and the mixed cultures were separated from the Loden samples that were grouped with the samples from the Swedish site. The CCA confirmed the separation of samples from the rhizosphere by site. In Figure 4B, the total nitrogen (TN) and partially available phosphorus (P_{av}) were the key factors responsible for the

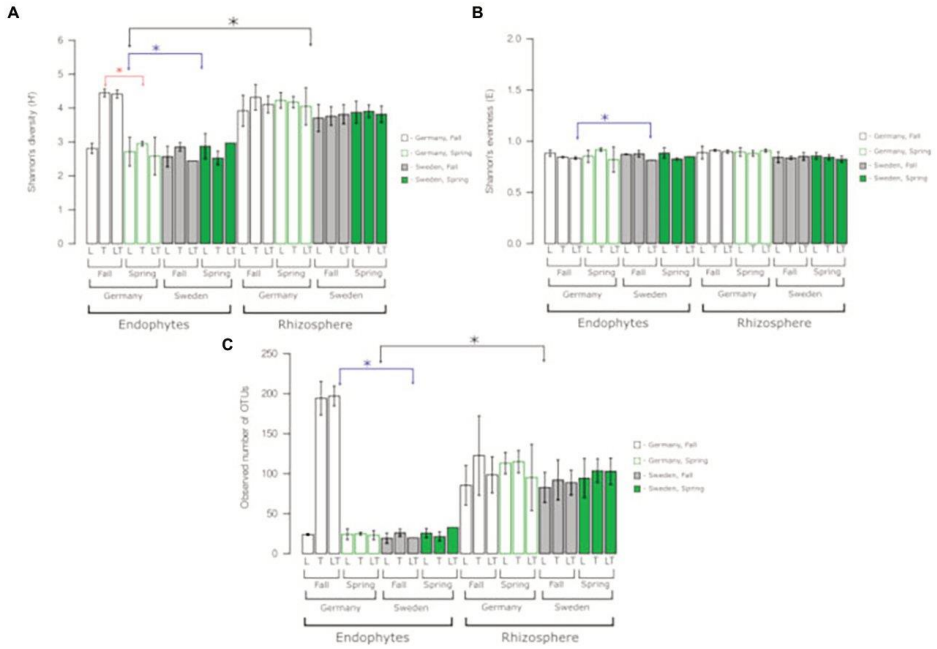

FIGURE 3
Bacterial species richness, diversity, and evenness across two experimental sites, rhizosphere soil and roots. OTUs were constructed at 0.03 dissimilarity for bacterial sequences. **(A)** Shannon's (H), **(B)** Shannon's (E), **(C)** observed number of OTUs. Robust ANOVA with Tukey's post-hoc analysis was used to assess the significance of differences between experimental sites, rhizosphere soil, and roots. Colours denote the following: black – significant difference between endophytes/rhizosphere, blue – significant differences between sites, red-significant difference between seasons. *Indicates a significant difference between variants indicated by arrows.

separation of samples with a positive correlation for site S. The rhizosphere samples were more scattered and formed 3 separate groups. The first group correlated with P_{av} and consisted mainly of samples from the Tora and mixed cultures (TL) from Sweden. The second group showed a high correlation with pH (pH_water) and was composed of the samples belonging to Tora and mixed cultures (TL) from Germany. The remaining samples that formed the third group showed a negative correlation with all the previously mentioned factors and with organic carbon (TOC). CCA analysis revealed that for soil samples, the phylum *Firmicutes* showed a positive correlation with P_{av}, while *Proteobacteria* correlated with TN. For root samples, there was no clear correlation with any of the bacterial strains.

Bacterial diversity at different taxonomic levels

As with the identification of isolated bacteria, most of the reads obtained in the microbiome analysis belonged to the phyla *Actinobacteria* and *Proteobacteria*, especially in the communities of endophytic bacteria from Sweden (Figure 5A). At the class level, significant differences were also observed between the level of bacterial association and the location of the test site. Additionally, some seasonal differences were observed, but only for samples from site G. At the class level, no differences between the two willow species were observed (Figure 5A). At the order level (Figure 5B), we observed a high number of different taxa across all samples. Most of the samples showed significant differences in the level of plant association (endophyte, rhizosphere) and site (Figure 5B). Bacterial community analysis showed that similar to the class level (Figure 5A), a small number of samples showed differences between seasons. However, in the case of the samples from Sweden, differences between the willow species were observed. Figures 5C,D show that this trend is also valid for the remaining taxonomic levels (family and genus). It was also shown that the lower we are at the taxonomic level, the higher the number of reads belonging to the 'rare' category (number of reads is less than a predetermined threshold).

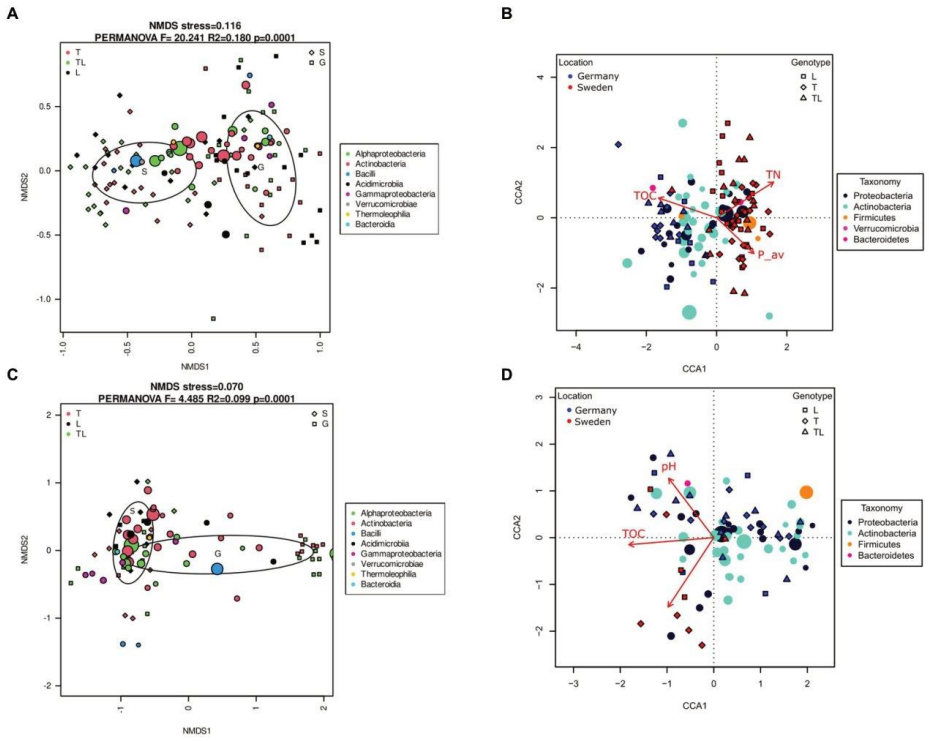

FIGURE 4
Analysis of log-transformed and Wisconsin double-standardized Bray–Curtis dissimilarity matrix for rhizosphere soil and root bacterial communities associated with *Salix* species L (Loden=*S. dasyclados*), T (Tora=*S. schwerinii x viminalis*), and LT (mixture) between two seasons (fall, spring) and two sampling sites (Germany and Sweden). **(A,C)** NMDS (nonmetric multidimensional scaling analysis); **(B,D)** CCA (canonical correspondence analysis); **A,B** plots – Soil; **C,D** – Root samples.

Discussion

The aim of this work was to gain insight into bacterial diversity and to identify P-solubilizing bacteria in the rhizosphere and endosphere of single-species and two-species plots of short-rotation willow coppices. Moreover, this is also an extension of a previous investigation that aimed at isolating and identifying P-solubilizing microorganisms (fungi) from the same experimental trials and comparing them with the results of the microbiome analysis (season comparison included; Koczorski et al., 2021). In the present work, we expected that the increased diversity of host plant species grown in the species mixture may favor a greater diversity of microorganisms, including phosphate-solubilizing microorganisms. Consequently, biomass production may be enhanced (Chen et al., 2021). To verify this, we used a classical microbiological approach combined with NGS analysis. The results presenting the number of P-solubilizing bacteria revealed that there was a significantly higher number of bacteria in the rhizosphere than in the endosphere. It could be that only selected bacterial strains can penetrate the root's intercellular space and form endophytes. Most plant species are known to produce substances that either attract or inhibit microbial root entry (Jacoby et al., 2017). As such, the roots can act as a filter through which only selected bacteria can penetrate and form a well-functioning symbiosis with the host plant (Taulé et al., 2021). Moreover, bacteria that form endophytes are known to possess specific properties or functions that help to survive in plant tissues and establish this symbiosis (Papik et al., 2020). Furthermore, the results from the culturable diversity showed the lowest number of P-solubilizing bacteria for Loden species (*S. dasyclados*) grown in monoculture and the highest number in the case of the species mixture (at both sites and levels of association of microorganisms with the plant: endo-and rhizosphere). Therefore, we can expect that an increased number of plant species in a cultivation system

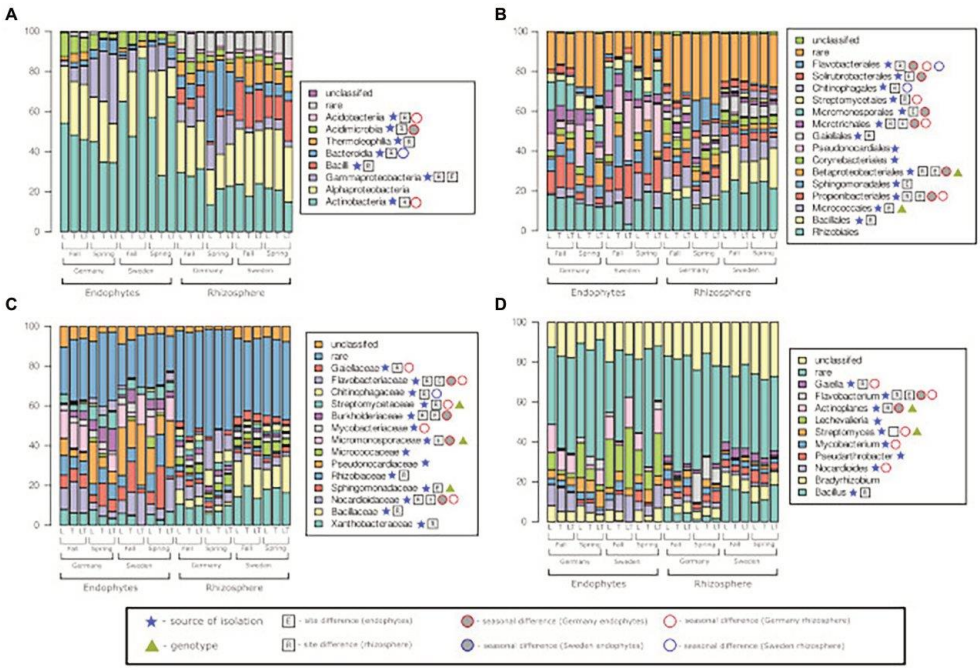

FIGURE 5
Bacterial community structure at the level of class **(A)**, order **(B)**, family **(C)**, and genus **(D)**, among rhizosphere soil and roots, two test sites and two seasons.

will promote microbial diversity and function. This suggests that both species identity and the cultivation of mixed species at the same site can have positive effects on the number of P-solubilizing bacteria. The mixing effect may be due to the higher competition of microorganisms for nutrients in mixed cultures, which promotes the diversity of rhizospheric and endophytic microorganisms (Weih et al., 2019). A similar conclusion was made by Schweier et al. (2019), where mixed cultures of willow species and genotypes produced higher biomass and positively influenced the diversity of microorganisms at the site.

The soil analysis presented in our earlier publication showed a much higher level of P at the Swedish site than at the German site (Koczorski et al., 2021). This may suggest that the increased P availability in the soil has a positive effect on the abundance of PSBs (rhizosphere and endophytes). Our experiment showed the presence of bacteria that were able to grow on media without available P but at the same time did not show any visible signs of P solubilization (halo zones). Since the bacteria were grown on the media for 7 days, it is possible that their cells had some reservoir of P that allowed them to grow. Liu et al. (2015) made similar observations using media supplemented with the same P source as we did, and they stated that lack of clear signs of P solubilization might also be a result of their very low activity. The most frequently isolated PSB in our experiment were *Pseudomonas*, *Erwinia* and *Bacillus*. These genera are well known and frequently isolated as PSBs, especially *Pseudomonas* (Yu et al., 2011; Sarker et al., 2014) and *Bacillus* (Liu et al., 2015; Chawngthu et al., 2020). These strains have found applications as biofertilizers for the cultivation of wheat (Liu et al., 2019), maize (Viruel et al., 2014), rice (Gomez-Ramirez and Uribe-Velez, 2021) and fruit trees, e.g., apple trees (Kurek et al., 2013). *Erwinia* sp. is often mentioned in the literature as a plant pathogen; however, the *Salix* trees grown at the two test sites did not show any symptoms of infection during sampling. Few researchers have shown the potential of *Erwinia* sp. as a plant growth-promoting bacterium and confirmed its presence in underground and aboveground parts of plants, e.g., almond trees (Guzmán et al., 2021), apple trees, pear trees (Rezzonico et al., 2016) and barley (Li et al., 2021). This suggests that willow trees are a potential source of both well-known and completely new P-solubilizing microorganisms. Our work also provides insight into P-solubilizing bacteria that can potentially be used as a part of specific willow SRC biofertilizers.

The Shannon diversity index (H′) and the number of observed OTUs confirmed that the bacterial diversity of the rhizosphere was higher than that of the endosphere. The same trend was observed by Tardif et al. (2016), where alpha diversity was also

higher in the rhizospheric than endophytic bacteria. Additionally, we observed a very large difference between the root endophytes derived from site G of the single-species Tora plots and the mixed-species plots, which showed much higher diversity than the rhizosphere samples. This is in agreement with the results obtained from the culturable bacterial abundance assessment on R2A medium, where Loden showed significantly lower numbers of bacterial isolates than Tora and the mixed plots. This result suggests that there is a strong connection between host plant species and microbial community composition in the rhizosphere and the endosphere.

In our previous publication that describes the abundance and diversity of fungi, we observed differences in alpha diversity for sites and levels of association with the plant, while no differences between host plant species were seen (Koczorski et al., 2021). In comparison to the fungal diversity, the bacterial diversity analysed using Shannon's index (H′) was higher than that of fungi with 4.5 (bacteria) and 3 (fungi) for rhizosphere soil and 3 (bacteria) and 2.5 (fungi) for endophytes. The NMDS analysis showed a clear division of samples according to the test sites, except for endophytic bacteria at site G, where a statistically significant separation between genotypes was observed. The separation of samples by the two sites in the NMDS analysis for fungi was more distinct, and the samples showed a greater concentration (Koczorski et al., 2021). This suggests that fungal communities are more dependent on soil and weather conditions or nutrient content, while bacteria are mostly dependent on pH and soil structure (Lauber et al., 2008; Furtado et al., 2019).

The results of the CCA analysis showed that among the assessed variables, total nitrogen and available P were the main factors driving bacterial community diversity, while for fungi, total nitrogen and organic carbon were the main factors driving community diversity (Koczorski et al., 2021). The high similarity of samples from the Tora monoculture and the mixed cultures at site G with soil pH suggests that the increase in bacterial diversity could be caused by the higher pH of the soil at this site. The significant effect of pH on bacterial diversity was analyzed by Tripathi et al. (2018), and their results indicate that any deviation from neutral pH significantly affects the structure of soil microorganisms. Furthermore, the research conducted by Kuzovkina et al. (2018) showed a significant positive effect of higher soil pH on the growth of willow, which may also increase the diversity of microorganisms by promoting P-solubilizing microorganism abundance. Although acidification is a strong indicator of P solubilization, we did not observe a positive correlation between those two factors in our analyses. The optimal pH for P solubilization appears to range from 5.5 to 7.5 (Alori et al., 2017), and at both of our sites, the pH was in the reported range. This indicates that there are other factors that can influence P solubilization efficiency.

For the bacterial community, the dominance of *Actinobacteria* and *Alphaproteobacteria* in the soil environment and willow roots was also reported by Tardif et al. (2016) and Yergeau et al. (2015). However, it should be mentioned that these studies were conducted in contaminated areas that are usually dominated by *Proteobacteria*. In the abovementioned studies, as in our work, there was a difference in the bacterial community between the endo-and rhizosphere, which was evident in the significant differences in the abundances of *Actinobacteria*, *Alphaproteobacteria* and *Firmicutes*. Bacteria belonging to 10 different genera were observed. Among them, we found possible P solubilizers. *Bacillus* is a genus reportedly comprising many P-solubilizing organisms. They are also known for their antifungal properties and IAA and ACC deaminase synthesis (Cherif-Silini et al., 2016). Another efficient P-solubilizing genus of bacteria found in willows is *Streptomyces*. They are also known for their antifungal properties (Cao et al., 2004; Khamna et al., 2009; Jog et al., 2014). Other, not as explored, genera of P solubilizers found in our experiment are *Gaiella* and *Nocardioides* (Albuquerque et al., 2011). *Nocardioides* was also reported to possess the ability to degrade casein and Tweens 20, 40 and 80 (Roh et al., 2020). Additionally, in Figure 5D, it can be observed that bacteria from the genus *Bradyrhizobium* do not differ significantly between the variants, which suggests that they belong to the group of microorganisms characteristic of willow regardless of the tested variants of the experiment. Other genera found in our study were also reported and are briefly described in Supplementary Table S1A in the supplementary materials. The collection of isolated and characterized P-solubilizing bacteria will be used in our future experiments to select the most efficient P-solubilizing bacteria in short-rotation coppices of willow.

Conclusion

The level of plant association (direct: endophytes vs. indirect: rhizosphere contact with plant tissue) is the most important factor shaping the bacterial community in willow short-rotation coppices, with dominance of Actinobacteria in the endosphere and Gammaproteobacteria and Bacilli in the rhizosphere. Cultivation of a mixed willow species system increases the abundance of P-solubilizing bacteria, which can ultimately minimize the problem of low P availability in agricultural soils and lower the need for fertilizer application.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject, PRJNA716888; https://www.ncbi.nlm.nih.gov/genbank/, OP102593- OP102680.

Author contributions

PK participated in all analyses and wrote the first version of the manuscript. BF participated in preparation of manuscript. MG

designed the bioinformatics pipeline, performed bioinformatics analyses, and participated in the preparation of the manuscript. DT participated in manuscript preparations and total DNA isolations and prepared libraries required for sequencing. PK and BF analyzed the results and the statistical output. PK, CB, and MW performed sampling at the locations, selected plant genotypes for the experiments, and provided input to the manuscript. PH did soil analyses and participated in the preparation of the manuscript. KH designed and managed the field and lab experiments and participated in the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1006722/full#supplementary-material

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Large effect of phosphorus-solubilizing bacteria on the growth and gene expression of *Salix* spp. at low phosphorus levels

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Abstract

Phosphorus (P) is one of the most important nutrients required for plant growth and development. However, owing to its low availability in the soil, phosphorus is also one of the most difficult elements for plants to acquire. Phosphorus released into the soil from bedrock quickly becomes unavailable to plants, forming poorly soluble complexes. Phosphorus-solubilizing bacteria (PSB) can solubilize unavailable phosphorus-containing compounds into forms in which phosphorus is readily available, thus promoting plant growth. In this study, two willow species, *Salix dasyclados* cv. Loden and *Salix schwerinii* × *Salix viminalis* cv. Tora, were inoculated with two selected bacterial strains, Pantoea agglomerans and Paenibacillus spp., to evaluate the plant growth parameters and changes in gene expression in the presence of different concentrations of tricalcium phosphate: 0 mM (NP), 1 mM (LP), and 2 mM (HP). Inoculation with PSB increased root, shoot and leaf biomass, and for the HP treatment, significant changes in growth patterns were observed. However, the growth responses to plant treatments tested depended on the willow species. Analysis of the leaf transcriptomes of the phosphate-solubilizing bacterium-inoculated plants showed a large variation in gene expression between the two willow species. For the Tora willow species, upregulation of genes was observed, particularly for those involved in pathways related to photosynthesis, and this effect was strongly influenced by bacterial phosphorus solubilization. The Loden willow species was characterized by a general downregulation of genes involved in pathway activity that included ion transport, transcription regulation and chromosomes. The results obtained in this study provide an improved understanding of the dynamics of *Salix* growth and gene expression under the influence of PSB, contributing to an increase in yield and phosphorus-use efficiency.

Introduction

Phosphorus is a key element required for proper plant development, and its deficiency can lead to disrupted shoot growth, delayed plant maturation, reduced plant resistance to pathogens or reduced leaf area and number (Prabhu et al., 2019). Furthermore, phosphorus in the soil is nonrenewable, and research in recent years confirms that the demand for this element is continually increasing (Bindraban et al., 2020). Modern artificial fertilizers contain phosphorus most often in the form of rock phosphate or superphosphate, the sources of which are limited worldwide, and their increasing price is becoming a serious problem for farmers wishing to use this type of fertilizer. In the soil, phosphorus compounds unavailable to plants can exist in two forms, namely, inorganic form and organic form, and only mineralization and solubilization processes can result in these phosphorus forms becoming available to plants (Richardson and Simpson, 2011). Soil phosphorus availability is impacted by pH (Penn and Camberato, 2019). In general, two phosphorus solubility maxima are observed at approximately pH levels of 4.5 and 6.5, which are associated with the lowest degree of P fixation by Ca, Al, and Fe minerals (Barrow, 2017; Penn and Camberato, 2019). In acidic soils, the dominant forms of inorganic phosphorus are aluminium ($AlPO_4$-$2H_2O$) or iron ($FePO_4$-$2H_2O$) compounds (Kalayu, 2019; Zhang et al., 2019). In alkaline soils, low-soluble and indirectly plant-available calcium compounds (dicalcium phosphate (DCP), tricalcium phosphate (TCP), etc.) are most often found (Bashan et al., 2021). In these form, phosphorus can be leached from soils and ends up in groundwater and lakes until it reaches the bottom of the oceans where it is unrecoverable (Bashan et al., 2021).

Phosphorus-solubilizing microorganisms (PSMs) can play a key role in the conversion of phosphorus compounds into bioavailable forms for plant uptake. Interest in this particular group of microorganisms and in the possibilities for their practical use in agriculture and forestry has been particularly important in recent years due to climate change, soil contamination by excessive use of fertilizers, drought and overexploitation of agricultural land (Tian et al. 2021). Knowledge of PSMs is progressively advancing by scientists from all over the world. A key action that would allow full use of PSMs in practice is the selection of strains with the highest efficiency, i.e., the ability to solubilize phosphorus compounds. PSMs can enhance phosphorus solubilization directly, e.g., through proton efflux, synthesis of acid and alkaline phosphatases or chelation of iron ions (*via* siderophores) (Tian et al. 2021). PSMs can also indirectly improve the ability of plants to take up nutrients (including phosphorus) through

phytostimulation (synthesis of indole-acetic acid (IAA) or ACC deaminase) or the production of hydrolytic enzymes (Richardson and Simpson 2021). Microorganisms in this group can promote the development of the plant root system and increased uptake efficiency of other key nutrients needed by plants (Bargaz et al. 2021). For this reason, PSMs are used as key components of engineered biofertilizers in modern agriculture, for example, in wheat, maize, rice or fruit trees such as apple trees (Kurek et al., 2013; Viruel et al., 2014; Liu et al. 2019; Kour et al., 2020).

We chose two *Salix* species that are commonly grown as short-rotation coppices in Europe as a renewable energy source as model plant species. The two *Salix* species, Loden and Tora, are characterized by substantial morphological differences. Most of the biomass of Loden willow species is allocated to the leaves, while that of Tora is mainly allocated to the shoots (Hoeber et al., 2018). In general, *Salix* species are also considered P efficient, suggesting that microorganisms associated with these plant species help promote their ability to solubilize and maintain stable P levels in the soil, making them the perfect model plant species for both investigation of microbial diversity and phosphorus-focused research.

Currently, in terms of phosphorus, most attention is given to understanding plant responses to phosphorus deficiency alone, without no consideration of the influence of microorganisms on this process (Ren et al., 2018; Mo et al., 2019; Wang et al. 2019, Zhang et al. 2019; Sun et al. 2021). Analysing the transcriptome of a plant inoculated with PSMs can help provide answers on the extent to which PSMs affect or influence their plant host. Under phosphorus-deficiency stress, plants activate gene expression cascades responsible for the synthesis of auxins, abscisic acid, jasmonic acid, salicylic acid and ethylene (Sun et al. 2016). Researchers have investigated the interactions of plants with arbuscular fungi and the effects of these interactions on the plant transcriptome (Liu et al. 2020; Ray et al. 2021). Fungi are responsible for activating genes responsible for auxin synthesis and responses to nitrogen or phosphorus deficiency (Ludwig-Müller et al., 2015). In terms of phosphorus metabolism, fungi are capable of activating genes responsible for the synthesis of phosphatases or genes related to the transport of phosphorus in plant tissues (Ray et al. 2021). However, there is a large gap in knowledge, as little attention has been given to the effect of bacteria on plants under phosphorus-deficient conditions. Soni (2021) and his team examined the effect of the bacterium *Paenibacillus polymyxa* on the tobacco transcriptome. The researchers observed that in addition to activating genes responsible for promoting plant growth, bacteria can influence the transcription of many genes responsible for phosphorus transport (pstA, pstB, pstC, pstS, phnD

or phnE) within the plant. However, this was the only publication we could find on the subject. A more in-depth understanding of the effects of bacteria on the plant transcriptome will allow a more accurate determination of their positive effects on plants, resulting in more effective bioinoculants in the future.

This study aimed to assess the effect of inoculation with phosphorus-solubilizing bacterial strains on the growth and gene expression dynamics in the leaves of two *Salix* species grown under three different phosphate concentrations. Therefore, our research involved screening from a pool of 64 phosphorus-solubilizing bacterial strains obtained in our previous study (Koczorski et al. (2022)) and selecting strains with the highest efficiency for solubilization of TCP and DCP for in vitro experiments. Second, evaluation of their potential to promote plant growth under conditions of phosphorus deficiency in the media was not only determined based on plant growth parameters but also characterized by the level of gene expression changes during plant inoculation with the phosphorus-solubilizing bacterial strains. We hypothesize that phosphorus-solubilizing bacterial strains not only can solubilize phosphate in the substrate but also can regulate phosphorus-related pathways in plants as well as stimulate plant growth and development. We speculate that at the initial stages of *Salix* growth, the effect of phosphorus-solubilizing bacteria (PSB) will be noticeable at the transcriptomic level in the leaf tissue.

Materials and Methods

Phosphorus-solubilizing bacterial strain collection

The phosphorus-solubilizing bacterial strains used in this experiment are part of a collection reported in our previous study (Koczorski et al., 2021). The bacteria were isolated (autumn 2018) from the roots and rhizosphere soil of two willow species: 'Tora' (Svalöf-Weibull (SW species No. 910007, *S. schwerinii* × *S. viminalis*) and 'Loden' (SW 890129, *S. dasyclados*). These willow trees were grown at two test sites established in 2014 as part of the ECOLINK project in Uppsala (Sweden) and Rostock (Germany) (Hoeber et al., 2018). The selection of PSB was performed using three selection media containing calcium triphosphate (National Botanical Research Institute's phosphate growth medium (NBRIP) and Pikovskaya (PVK)) or calcium diphosphate (DCP) as phosphorus sources (Koczorski et al., 2021). The bacteria that showed the ability to solubilize phosphorus (presence of halo zones) were classified into the PSB group and identified based on their 16S rRNA sequences (OP102593-OP102680) (Koczorski et al., 2021).

Selection of phosphorus-solubilizing bacterial strains with the highest phosphorus solubilization efficiency

In the present study, a total of 64 bacterial strains from the PSB collection belonging to the species *Pseudomonas*, *Bacillus*, *Erwinia*, *Serratia*, *Paenibacillus* or *Burkholderia* (Koczorski et al 2022) were screened. Solid NBRIP and DCP media were used to select the phosphorus-solubilizing bacterial strains with the highest efficiency for phosphorus solubilization (the composition of the media is given in Koczorski et al., 2021). The bacteria were spot inoculated onto media (4 strains per Petri plate), and there were three replicates. The bacteria were cultured in the dark at 28°C, and the diameter of the halo zone was measured after 1, 5 and 10 days of culture. The results obtained were statistically analysed; the 10 most efficient phosphorus-solubilizing bacterial strains were selected and used for further analysis. The 10 selected bacteria did not include strains that were pathogenic to humans and plants, despite their high activity (data verified based on published literature).

The ten phosphorus-solubilizing bacterial strains selected in the previous step were tested in liquid NBRIP media. NBRIP medium was chosen since plants cannot efficiently solubilize TCP on their own (Ticconiand Abel, 2004). In this part of the experiment, three different P concentrations were used, namely, 0 M (NP), 1.0 M (0.3129 g/L TCP) (LP) and 2.0

M (0.6259 g/L TCP) (HP), and noninoculated media with the same variants were maintained as controls. The concentration of available P in the liquid NBRIP medium for all 10 strains was determined using the spectrophotometric molybdenum blue method (30 samples in total). Based on a statistical analysis, the 2 most effective phosphorus-solubilizing bacterial strains, *Paenibacillus* spp. and *Pantoea agglomerans*, were selected and used to inoculate the plants in the pot experiment.

Inoculation of two willow species with PSB in conjunction with varying phosphorous contents

Pots were divided into two compartments using membranes and filled with sterile sand. Loden and Tora willow cuttings with two or more nodes were inserted into the pots such that there were two cuttings per pot. To minimize the effect of phosphorus sorption in the soil, quartz sand was used as a substrate. The pot experiment was designed to include three phosphorus concentrations and two willow species for inoculation treatments (control (Ctr), bacterium 1 (*Pantoea agglomerans*) and bacterium 2 (*Paenibacillus* spp.)). The temperature in the room was set to 22°C with a photoperiod comprising 10 hours of light and 14 hours of darkness. After the cuttings acclimated for 7 days, with the exception of the control plants, the pots were inoculated with bacteria. The plants were watered thrice a week with a fertilizer solution with one of three different P concentrations (NP, LP, HP) for 1 month. We aimed to reach the final P concentrations (0.3129 g/L for LP and 0.6259 g/L for HP) in the first week of the experiment. After the plants were cultivated for 5 weeks, we harvested them and measured the length of their roots and shoots, along with measuring the fresh weights of their roots, shoots and leaves. In addition, the total P content in leaves at the end of the experiment was examined via inductively coupled plasma–optical emission spectrometry (ICP–OES). ~~The available P in soil substrate was measured using the~~ spectrophotometric molybdenum blue method after sample extraction with 1% citric acid solution (Van Reeuwijk, 2002) for all the analysed variants in the experiment (180 samples in total). A portion of the leaves obtained at harvest was flash frozen in liquid nitrogen and stored at -80°C.

Statistical analysis

The plant growth parameters (leaf weight, shoot weight, root weight, shoot length, root length) and phosphorus concentrations in leaves and soil were analysed using STATISTICA v.13.3.721 (StatSoft, Poland). As the results obtained were not normally distributed, the

Kruskal–Wallis test was used to determine significant differences. Dunn's post hoc test was performed to determine differences between inoculation variants, different phosphorus concentrations and the willow genotypes used. The data were analysed using 3-way ANOVA, with soil P concentration (3 levels), *Salix* species (2 levels) and bacterial inoculation (2 levels) included as factors, and their interactions were also evaluated. The response variables included all the growth parameters and P concentrations in the soil and plant leaves.

RNA isolation

Total RNA was extracted from 100 mg of leaf tissue and crushed in sterile mortars filled with liquid nitrogen. RNA extraction was performed according to the protocol of Chomczynski and Sacchi (2006), with slight modifications. A total of 1000 µl of TRIzol reagent (Life Technologies, Poland) was added to the crushed leaf material, which was then incubated for 5 minutes at room temperature. Then, 300 µl of chloroform was added, after which the material was incubated for 15 minutes and then centrifuged at 12000 rpm for 15 minutes. A mixture of 0.8 M sodium chloride, 1.2 M sodium citrate and 200 µl of isopropanol was added to the upper layer of the supernatant, after which the mixture was centrifuged. The resulting pellet was washed twice with 75% ethanol. The RNA pellet was then resuspended in RNAse-free water (50 µl) and stored at -80°C. The RNA samples were outsourced to the company Novogene (Great Britain) for library preparation and transcriptome analysis.

Transcriptome analysis

The reference genome of *Salix viminalis* was acquired from Almeida et al. (2020). The total RNA was sequenced using the Illumina platform in conjunction with P5 (P5-AATGATACGGCGACCACCGAGA (5'-3') and P5'-TTACTATGCCGCTGGTGGCTCT (3'-5') and P7 adapters (CGTATGCCGTCTTCTGCTTG-P7' (5'-3') and CATACGGCAGAAGACGAAC-P7 (3'-5')). After the removal of reads that were contaminated with adapter sequences, that included unknown nucleotides that constituted more than 10% of either read (N > 10%) and that were of low quality (base quality < 5) and constituted more than 50% of the sequence, the genome of *Salix viminalis* retrieved from the European Nucleotide Archive (ENA) was used as a reference for mapping using HISAT2 (cite the publication of this reference). Assembly of the sequences was performed using StringTie software with transcripts of class code type 'u'. Quantification was performed using featureCounts, and a differential analysis was performed using DESeq2 and edgeR with the

following parameters: |log2(fold-change)| >= 1 &padj<= 0.05. Finally, enrichment analysis was performed using clusterProfiler with a padj< 0.05.

Results

Selection of bacterial strains with the highest efficiency of P solubilization

The solubilization indices obtained from the experiment (given in the range from 0 to 1.6) are presented in the form of a heatmap where for each experimental variant (growth medium: DCP and NBRIP, strain: 64 in total and time of cultivation: 1, 5 and 10 days), the values are indicated based on colour differences (from blue to yellow) (Figure 1 A-B). The values in Figure 1 are sorted according to the P solubilization efficiency measured on the NBRIP medium after 1 day of bacterial cultivation as the main criterion for the selection of microorganisms for further studies.

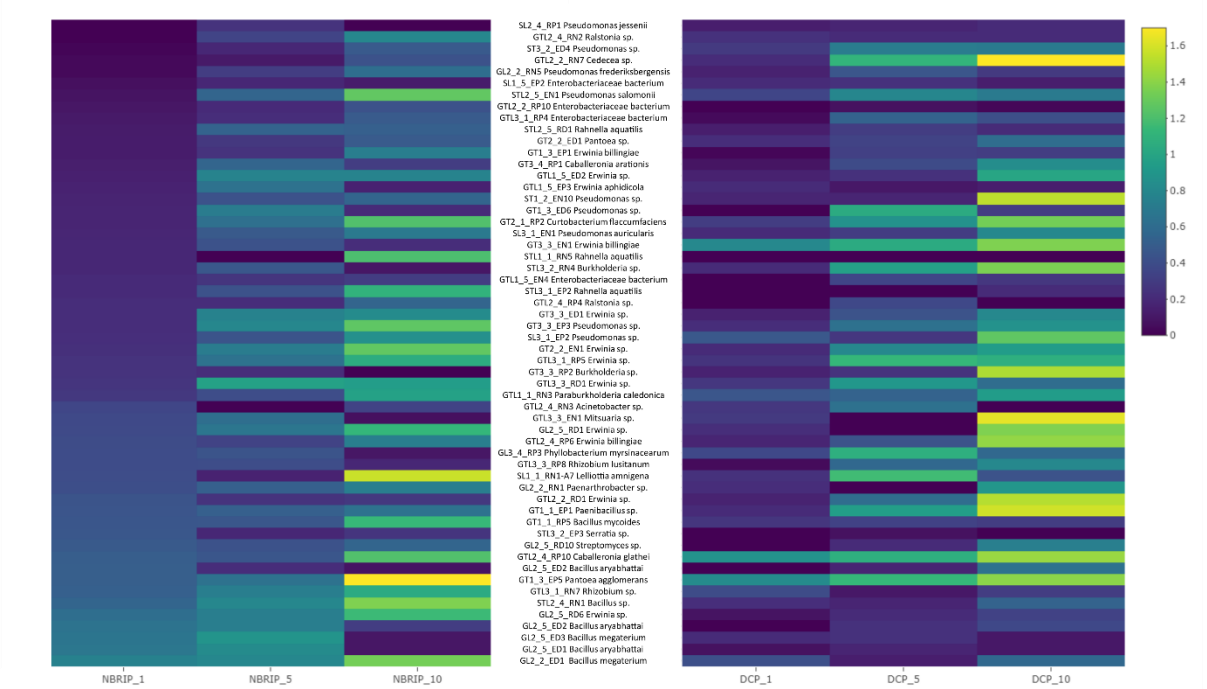

Figure 1 Heatmap of bacteria isolates ordered according to their P solubilization efficiency after 1 day of growth (from down to top). Activity was measured using solubilization index (from halo zone diameter we subtracted colony diameter). Measurements were taken after day 1, 5 and 10 from two selective media DCP and TCP. Blue colour indicates low solubilization index, while yellow high solubilization index.

Of the 64 strains tested, 15 with the strongest ability to solubilize P on DCP and NBRIP solid media were selected. In this group, we observed a predominance of species of the genus

Bacillus (7 out of 15: *Bacillus megaterium*, *Bacillus aryabhattai*, *Bacillus* spp. and *Bacillus mycoides*); *Erwinia* (2 out of 15: *Erwinia* spp.); and single strains of *Rhizobium* spp., *Streptomyces* spp., *Pantoea agglomerans*, *Paenibacillus* spp., *Caballeronia glathei* and *Serratia* spp. The strains with the highest activity on NBRIP media after 10 days of incubation belonged to *Bacillus megaterium*, *Erwinia* spp., *Bacillus* spp. and *Bacillus aryabhattai*. The second DCP medium showed a slightly different distribution of strains in terms of their activity. Among the 15 strains previously selected (based on the NBRIP medium), only four displayed high activity for P solubilization of DCP: *Caballeronia glathei*, *Bacillus aryabhattai*, *Pantoea agglomerans* and *Paenibacillus* spp. Notably, the strains with the highest activity on the DCP medium included *Pseudomonas frederiksbergensis*, *Mitsuaria* spp., *Paenibacillus* spp. and *Pseudomonas* spp. Among the group of strains, *Bacillus megaterium*, *Bacillus aryabhattai*, *Erwinia* spp. and *Caballeronia glathei* were excluded from further study, as they were previously reported to be potential plant pathogens (Abdel-Monaim et al., 2012, Narsing Rao et al., 2019, Dobritsa et al., 2019, Parcey et al. 2022). A more detailed quantitative determination of bacterial P solubilization efficiency in liquid media (NBRIP) with different P contents showed that *Rhizobium* spp., *Pantoea agglomerans*, and *Paenibacillus* spp. were most efficient at solubilizing P compounds (Figure 2). Among the strains with significantly lower activity were *Streptomyces* spp., *Bacillus megaterium*, *Bacillus aryabhattai*, *Acinetobacter* spp. and *Pseudomonas* spp., while the *Mitsuaria* spp. and *Rhizobium lusitanum* strains displayed the lowest activity (Figure 2). These last two strains were excluded from subsequent stages of the study. *Rhizobium* spp. emerged as members among the most active strains; however, this species is not a typical endophyte and was excluded from further stages of the study.

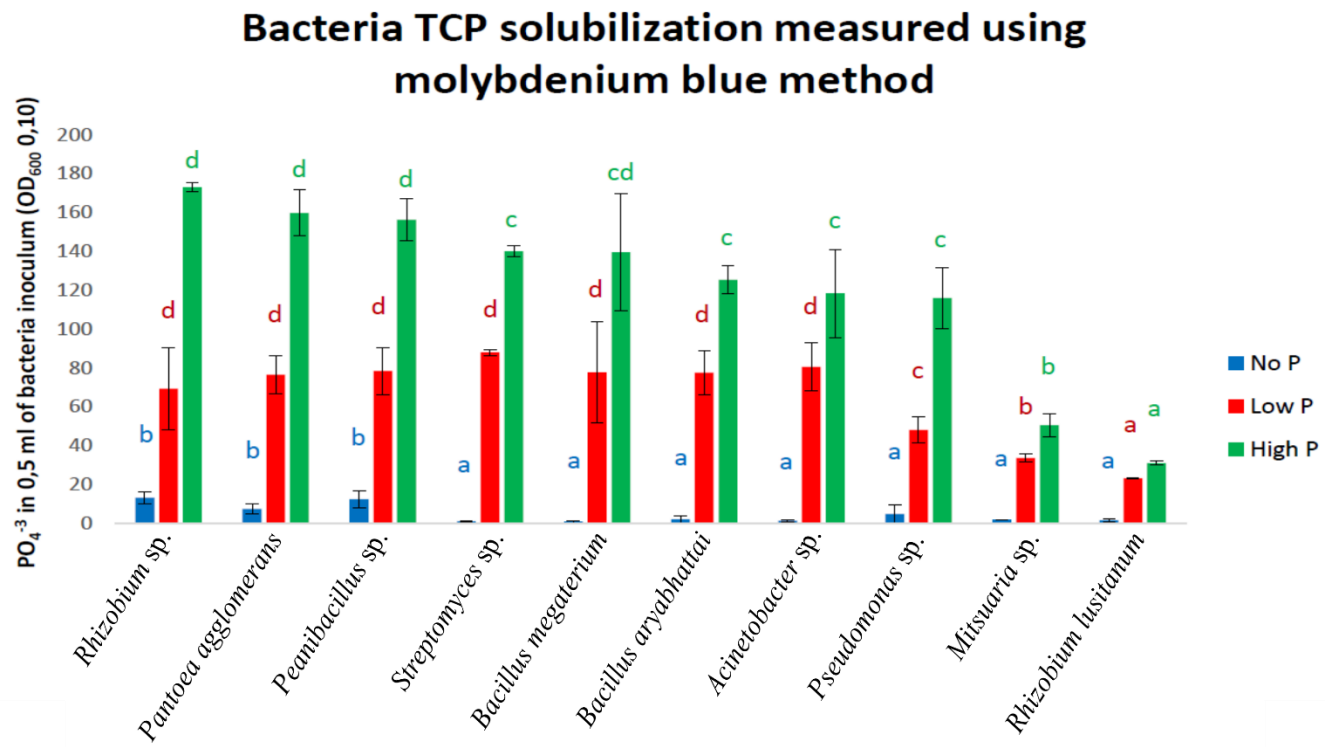

Figure 2 P solubilization level represented by presence of PO_4^{-3} in 0,5 ml of bacteria inoculum (OD_{600} 0.10) in liquid TCP medium. Media were supplemented with 3 different P concentrations: NoP – blue, LowP – red, HighP – green. Letters indicate significant differences between bacteria for each P variant.

Bacterial contribution to plant growth promotion under phosphorus-limited conditions

All experimental factors (3 P concentrations, 2 willow species and 2 bacterial inoculations) showed statistically significant effects on most plant growth parameters (Table 1; Supplementary Figure 1,2). A slightly better plant growth stimulation effect was observed for *Paenibacillus* spp. than for the other species (B2). Significant differences were also observed in response to different concentrations of P in the soil substrate. The positive or negative effects of the bacteria were dependent on the parameters tested, and the direction of change varied between the willow species and the bacterial variants (e.g., significant inoculation x species interactions listed in Table 1). High P concentrations in the soil significantly increased root fresh weight and length, while a decrease in leaf fresh weight was observed (Table 1). Soil P effects on plant growth patterns varied between the two willow species (e.g., the significant species x factor interaction effects in Table 1). Changes in soil P concentration did not affect the P content in the leaves (Supplementary Figure 3). Only B1 showed a significant decrease

in leaf P content for the LP and HP treatments compared to the NP treatment for both Loden and Tora (Supplementary Figure 3A-B).

	Root length			Shoot length			Wet weight of roots			Wet weight of leaf			Wet weight of shoot		
	MS effect	F	p-level	MS effect	F	p-level	MS effect	F	p-level	MS effect	F	p-level	MS effect	F	p-level
(1) Inoculation	145.42*	20.198*	0.00000*	1891.82*	99.578*	0.00000*	1.78164*	139.884*	0.00000*	30.4661*	130.063*	0.00000*	6.0201*	44.970*	0.00000*
(2) P concentration	92.89*	12.902*	0.00001*	81.30*	4.280*	0.01677*	2.27967*	178.986*	0.00000*	7.1424*	30.491*	0.00000*	1.7321*	12.939*	0.00001*
(3) Species	1068.67*	148.435*	0.00000*	264.36*	13.915*	0.00033*	2.41824*	189.865*	0.00000*	0.1916	0.818	0.368185	3.1836*	23.781*	0.00001*
(1) x (2)	163.31*	22.684*	0.00000*	280.32*	14.755*	0.00000*	0.17112*	13.435*	0.00000*	2.4937*	10.646*	0.00000*	1.0162*	7.591*	0.00003*
(1) x (3)	193.21*	26.836*	0.00000*	38.16	2.009	0.140142	0.31205*	24.500*	0.00000*	0.1297	0.554	0.576823	1.9774*	14.771*	0.00000*
(2) x (3)	154.13*	21.408*	0.00000*	315.16*	16.589*	0.00000*	1.43592*	112.740*	0.00000*	4.9392*	21.086*	0.00000*	0.0498	0.372	0.690559
(1) x (2) x (3)	122.62*	17.032*	0.00000*	357.90*	18.839*	0.00000*	0.56060*	44.015*	0.00000*	1.8046*	7.704*	0.00002*	1.1084*	8.280*	0.00001*
Error	7.20			19.00			0.01274			0.2342			0.1339		
Tukey test for unequal sample sizes:															
(1) Inoculation	Ctr	11.62 A		Ctr	21.16 A		Ctr	0.38 A		Ctr	1.12 A		Ctr	1.07 A	
	B1	12.38 A		B1	31.41 B		B1	0.58 B		B1	2.74 B		B1	1.55 B	
	B2	15.42 B		B2	35.16 C		B2	0.83 C		B2	2.69 B		B2	1.89 C	
(2) P concentration	N P	11.29 A		No P	30.62 B		No P	0.36 A		No P	2.67 C		No P	1.41 A	
	LP	14.19 B		Low P	27.64 A		Low P	0.57 B		Low P	2.08 B		Low P	1.35 A	
	HP	13.93 B		High P	29.47 AB		High P	0.86 C		High P	1.80 A		High P	1.76 B	
(3) Species	Loden	9.99 A		Loden	27.68 A		Loden	0.45 A		Loden	2.23 A		Loden	1.68 B	
	Tora	16.28 B		Tora	30.81 B		Tora	0.75 B		Tora	2.14 A		Tora	1.33 A	

Table 1 ANOVA results for the effects of bacterial Inoculation (B1, B2), soil P concentration (NP, LP, HP) and willow plant Species (Loden, Tora) on various plant growth traits. * indicate significant difference.

Transcriptome analysis of two willow species in response to inoculation and limited phosphorus concentrations

A transcriptome analysis of two willow species was performed to determine the effects of inoculation with the two selected PSB (B1 and B2) and different concentrations of P compounds (NP, LP and HP). The most distinctive difference was observed for plants inoculated with B2 of the Loden variety compared with the Ctr, where 2147 genes were downregulated and 2296 genes were upregulated. In addition, a similar trend was observed for both willow species in the HP and LP treatments: there was a greater number of genes that were upregulated compared to those in the NP treatment, the number of which ranged from 426 to 1920 genes (Supplementary Figure 4). No significant trend was observed in terms of downregulation of genes. The average numbers of all genes in Loden and Tora whose transcription patterns differed from those of the control variant were approximately 2500 and 1000, respectively. According to Venn diagrams for the Loden species, a pool of genes common to both the Ctr and B1 and B2 variants was lower in the NP (15009 genes) treatment compared to the LP (18099 genes) and HP (18054 genes) treatments (Figure 3A-C). In addition, a very low number of common genes between the B1 variants and the Ctr were observed in the LP treatment for the Loden (Figure 4B) and Tora (Figure 3E) variants, which were 355 and 430, respectively, the lowest values recorded. As shown in Figure 8, a Gene Ontology (GO) enrichment analysis was performed, and the 30 most differentially expressed genes associated

with *p* values under 0.05 are presented for three P concentrations and two inoculation variants (bacteria B1 and B2 are compared to the corresponding non inoculated Ctr). Analysing the effect of different P concentrations on the Loden willow species, we characterized the molecular functions in the NP treatments according to the presence of a large number of genes responsible for tetrapyrrole binding, haem binding, iron ion binding, GTP binding or ribonucleotide binding (Figure 4A-B). The LP treatment revealed a high number of genes responsible for the process of translation, and the HP treatment displayed a high number of genes associated with cytoskeleton formation and rearrangement (Figure 5A-B, Figure 6A-B). In the case of the inoculation effect, the Loden variants inoculated with B1 (rather than B2) demonstrated a high expression of genes involved in cell membrane structure, phosphatase activity and acid phosphatase activity (Figure 4, 5, 6). The variants inoculated with B2 were characterized by genes involved in hydrolase activity and pyrophosphatase activity and many genes related to cation and ion transport (Figure 4, 5, 6). For the LP treatment, significant changes in the molecular functions of plants inoculated with B1 and B2 were observed, although some similarities were detected. In response to this variant, a significant increase in the number of genes responsible for DNA binding and regulation of transcription and translation was observed (Figure 5A-B). However, enrichment of GO terms related to direct phosphorus solubilization was not observed. Similar to the NP treatment, a number of genes responsible for the biosynthesis and metabolism of peptides that make up cell membranes were noted. In the category of biological processes, the variants inoculated with B1 under LP revealed processes related to the stress response (especially oxidative stress) and the plant defence system (Figure 5A). In the HP treatment, a shift from transcription to translation and, in particular, to ribosome synthesis was observed for the genes (Figure 5A-B). Moreover, genes related to chromatin formation and the formation of ribonucleoprotein complexes were found. For the NP, LP and HP treatments, GO terms associated with cell membranes were detected among the differentially expressed genes. The plants inoculated with B2 for HP were also characterized by genes related to different types of hydrolases, which was not observed in response to B1.

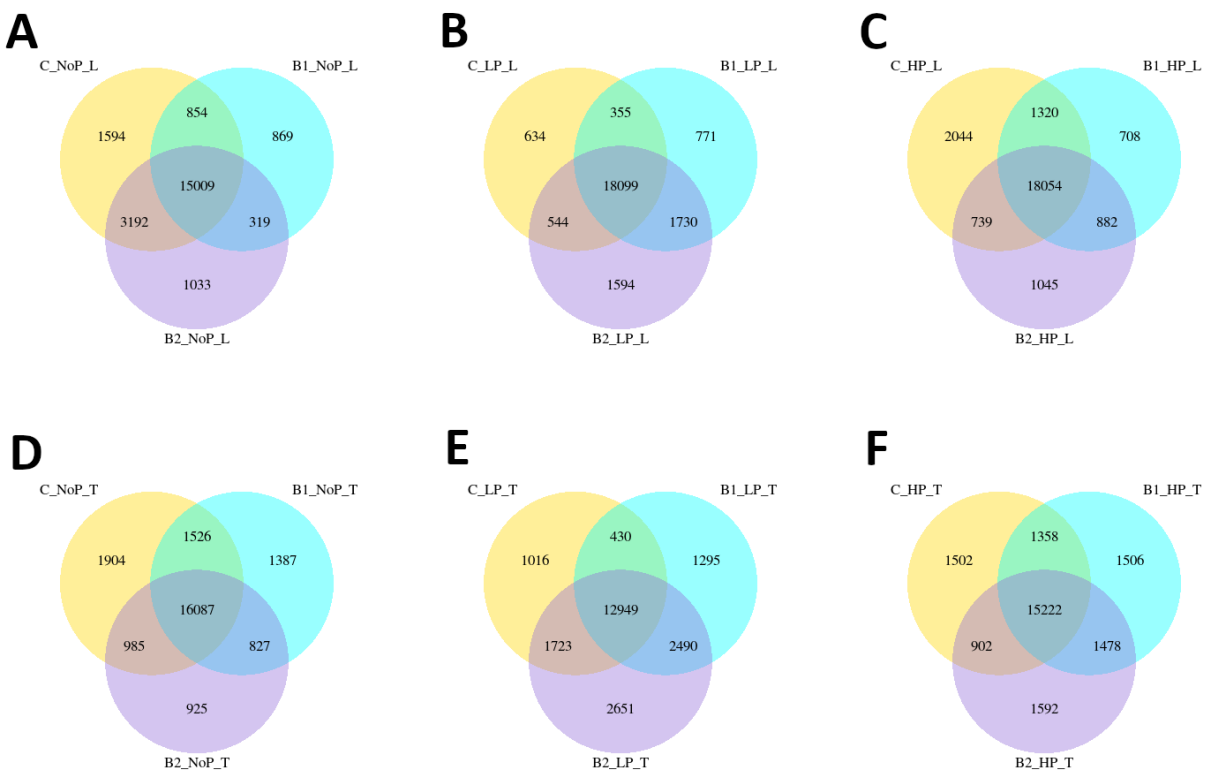

Figure 3 Venn diagrams representing common and unique genes expressed among inoculation variants. Loden – A,B and C; Tora – D,E andF;C – non inoculated control; B1 – Bacteria 1; B2 – Bacteria 2; NoP – No Phosphorus; LP – LowP; HP- HighP.

For the Tora willow species under NP, a high frequency of genes responsible for transcription, DNA binding and organic acid metabolism was observed (Figure 4C-D). Plants under the LP treatments were characterized by genes associated with transport and pollination, whereas under HP, a high frequency of genes associated with chloroplasts, thylakoids and photosystems I and II was observed (Figure 5C-D, Figure 6C-D). For the effect of inoculation, B1-inoculated plants showed molecular functions belonging to tetrapyrrole and haem-binding activity and biological processes related to pollen recognition and formation and intercellular communication (Figure 4C, 5C, 6C). The plant inoculated with B2 was mainly characterized as expressing genes involved in metabolic processes and the biosynthesis of carboxylic acids, oxyacids and organic acids (Figure 4D, 5D, 6D). The Tora willow species under LP showed a change in gene expression and significant differences between the B1 and B2 variants (Figure 5C-D). In the case of the B1 variant, a high number of genes responsible for the stimulus response even at the cellular level was noted. A large number of genes responsible for transport also was detected. B2 under LP elicited the expression of a large number of genes responsible for transcription (Figure 5D). The he expression of genes related to pollen production,

pollination and modification of various proteins was also evident, albeit with lower numbers. As with the LP treatment, little similarity was observed between B1 and B2 inoculation (with the exception of genes encoding transferases) (Figure 5C-D). Inoculation of plants with B1 resulted in high expression of genes responsible for transferase activity and coenzyme binding. A large number of genes related to the metabolic activity of various acids, glucan, lipids and carbohydrates also emerged. Inoculation with B2 increased the expression of genes involved in photosynthesis (thylakoid structure, photosystems I and II) and the synthesis of structural elements of plant cells. For each of the NP, LP and HP treatments, upregulation of a large number of genes responsible for the activity of different types of transferases was observed.

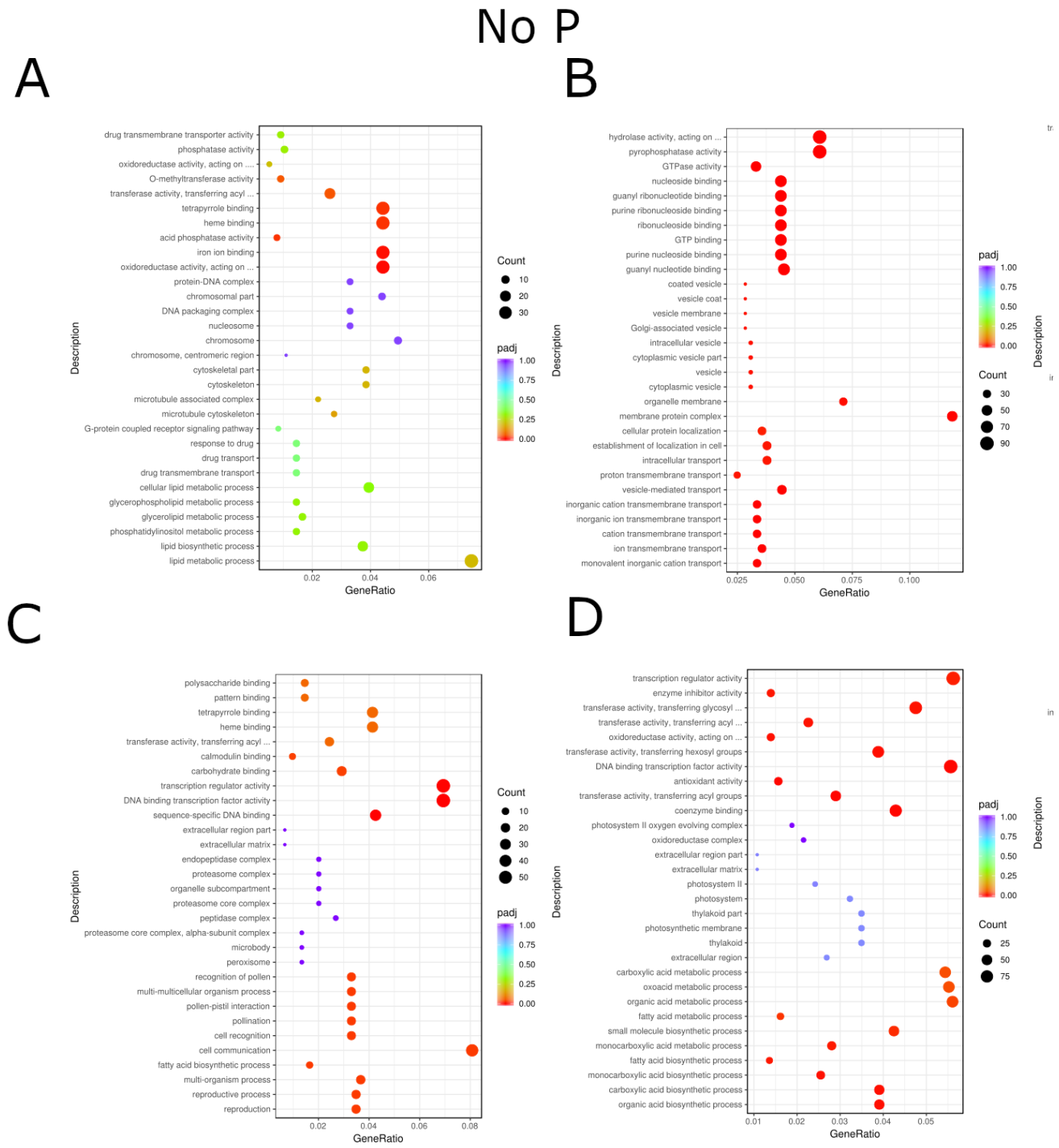

Figure 4 GO enrichment analysis scatter plots presenting the top 30 genes detected for the NP treatment of inoculation and P concentration for two willow species, Loden (A-B) and Tora (C-D). The results are presented in pairs (B1 and B2) and are compared to the respective controls.

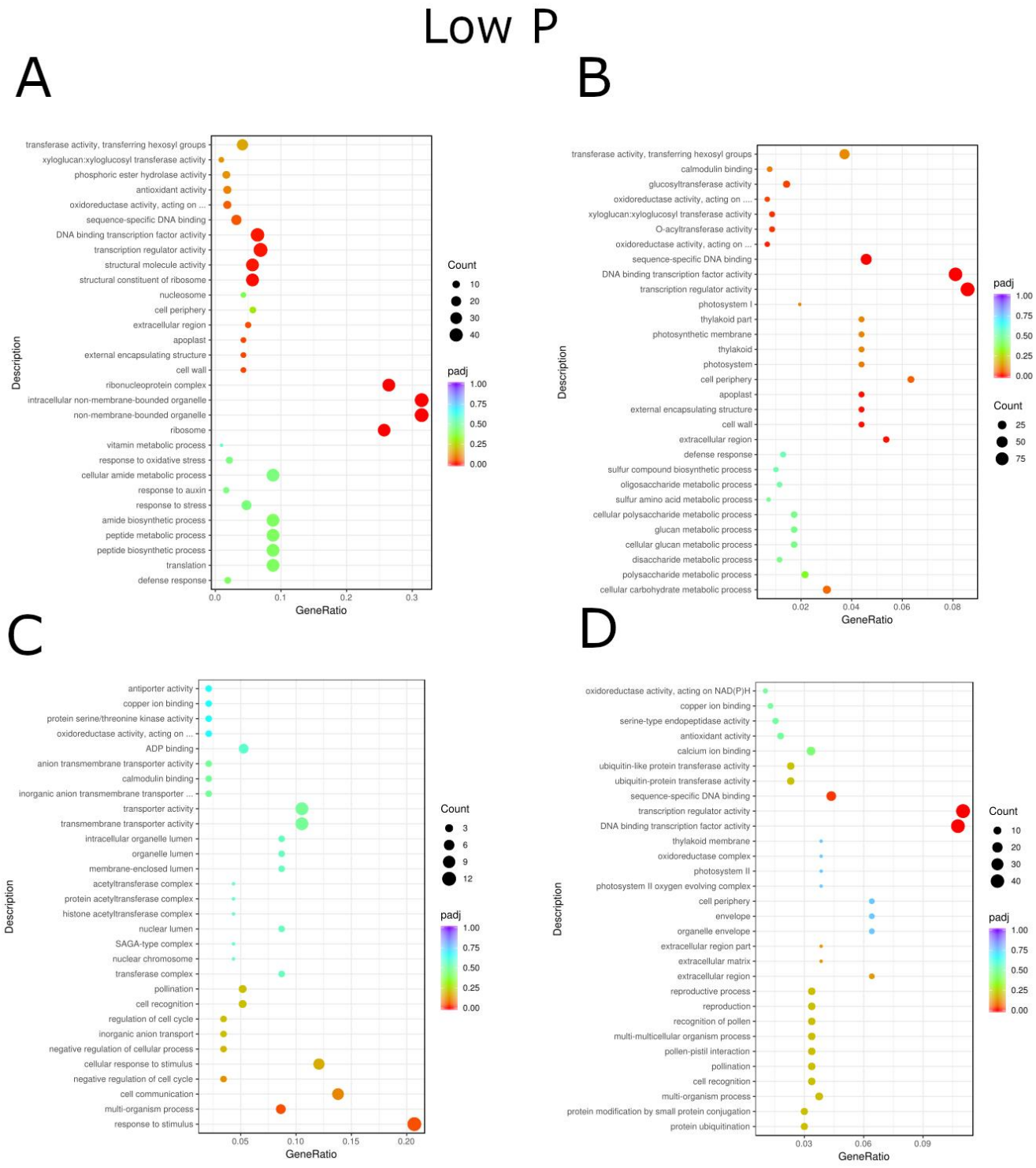

Figure 5 GO enrichment analysis scatter plots presenting the top 30 genes present for the LP treatment of inoculation and P concentration for two willow species, Loden (A-B) and Tora (C-D). The results are presented in pairs (B1 and B2) and are compared to the respective controls.

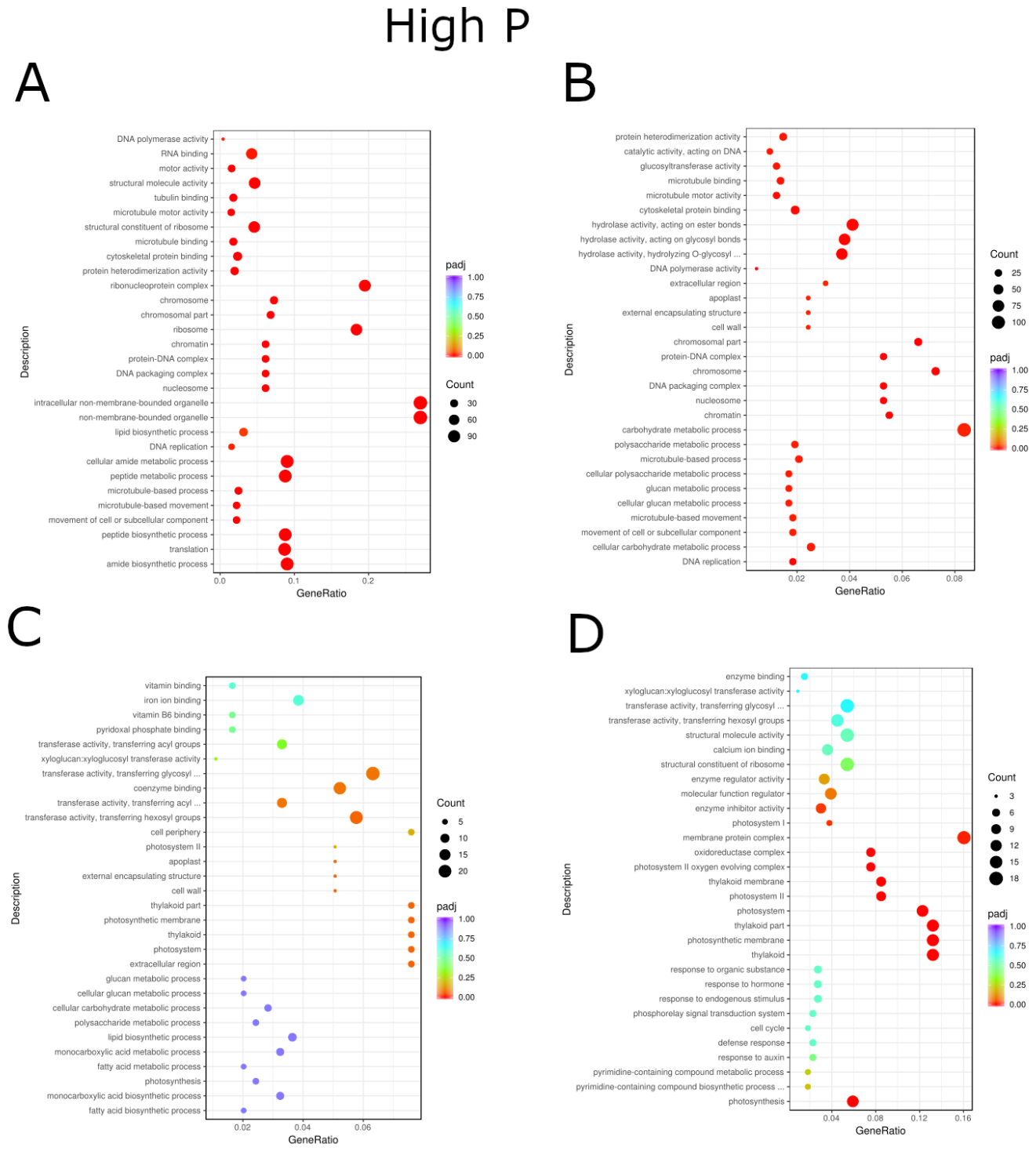

Figure 6 GO enrichment analysis scatter plots presenting the top 30 genes present for the HP treatment of inoculation and P concentration for two willow species, Loden (A-B) and Tora (C-D). The results are presented in pairs (B1 and B2) and are compared to the respective controls.

Gene expression regulation

Among the 10 most frequently up- or downregulated genes found in each experimental variant, a general trend based on the willow species/species was observed in which genes for the Loden willow species were downregulated and genes for the Tora willow species were upregulated regardless of the experimental conditions tested (bacterial inoculant B1 or B2 and P concentration). This was particularly evident for genes associated with biological processes (Figure 7) and molecular functions (Figure 9).

Analysis of GO biological processes (Figure 7) revealed the occurrence of 86 different up- or downregulated genes across all factors tested in the experiment. Among these genes, 43 were specific only to Loden, and 37 were specific to Tora. There were also 6 genes that were either up- or downregulated in both plant species tested. These genes were responsible for the cellular carbohydrate metabolic process, glucan metabolic process, glucan metabolic process, defence response, response to auxin, and lipid biosynthetic process. In response to both inoculation variants (B1 and B2), 13 common genes were observed, mainly those related to the metabolism and biosynthesis of fatty acids, glucan, and carboxylic acids. A strong effect of phosphorus (NP, LP, HP) was observed for fatty acid and monocarboxylic acid biosynthesis and metabolism, for which downregulation was observed in the NP treatment and upregulation in LP and HP treatments.

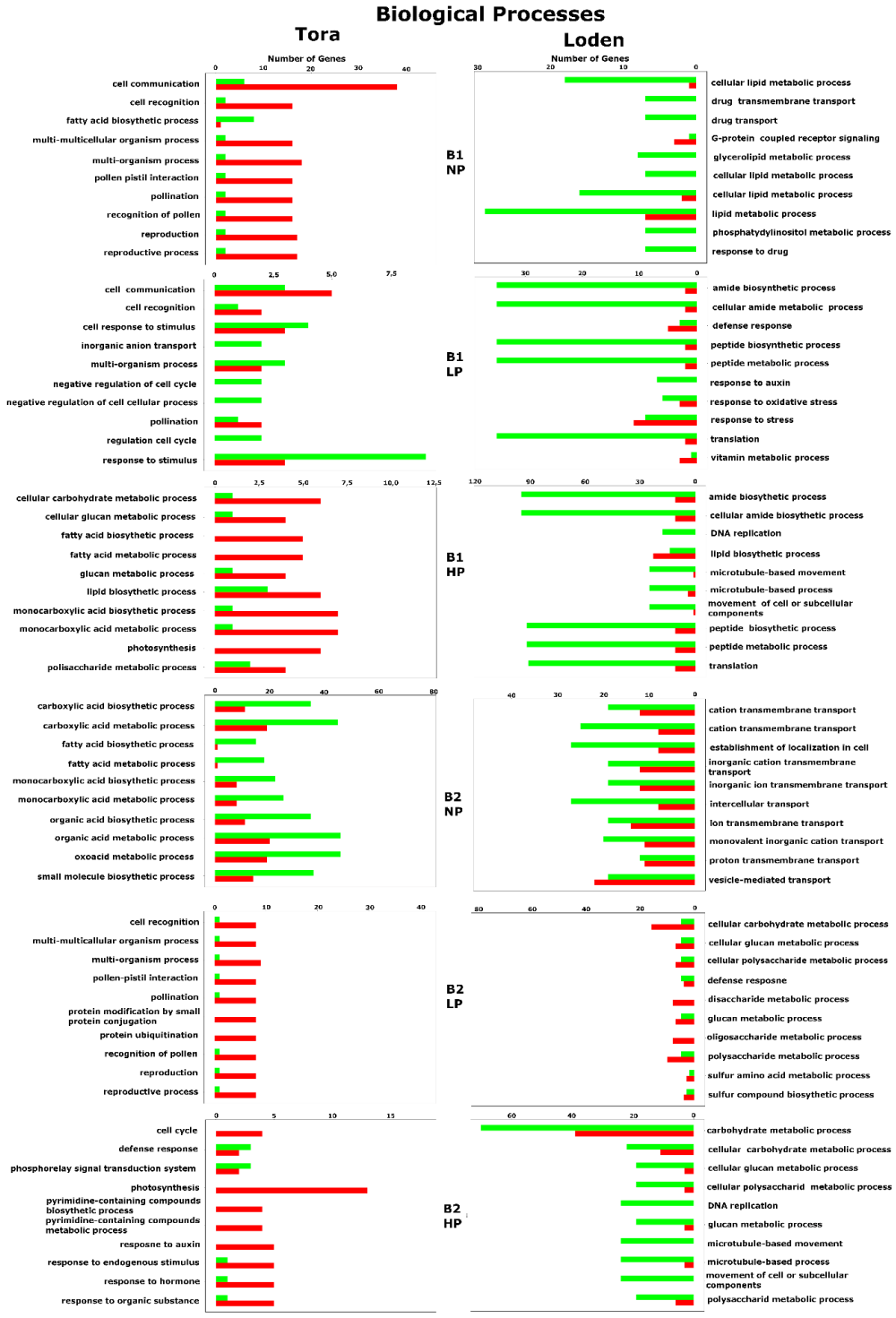

Figure 7 Biological process-associated gene expression regulation for all willow species, PSB and phosphorus concentrations. The red colour indicates upregulation of genes, while the green colour indicates downregulation.

In terms of the GO cellular components, 65 different genes were observed, of which 28 were specific only to Loden (mainly downregulation), and 27 were specific to Tora (mainly upregulation) (Figure 8). At 10, the pool of common genes for cellular components was larger than that for biological processes and included a large number of genes related to cellular elements involved in photosynthesis, i.e., thylakoids, photosystems I and II, the photosystem II oxygen-evolving complex and photosynthetic membranes. In response to different inoculants (B1 vs. B2), we observed the occurrence of 16 common genes, which were associated with terms related to chromosomes, the apoplast, DNA packaging or extracellular elements in addition to photosystem elements. For genes associated with cellular components, we also observed a strong effect of P on photosystems I and II, thylakoids, the photosystem II oxygen-evolving complex, and the oxidoreductase complex; moreover, the NP treatment downregulated the expression of these, in contrast to the LP and HP treatments, which upregulated the expression of these genes.

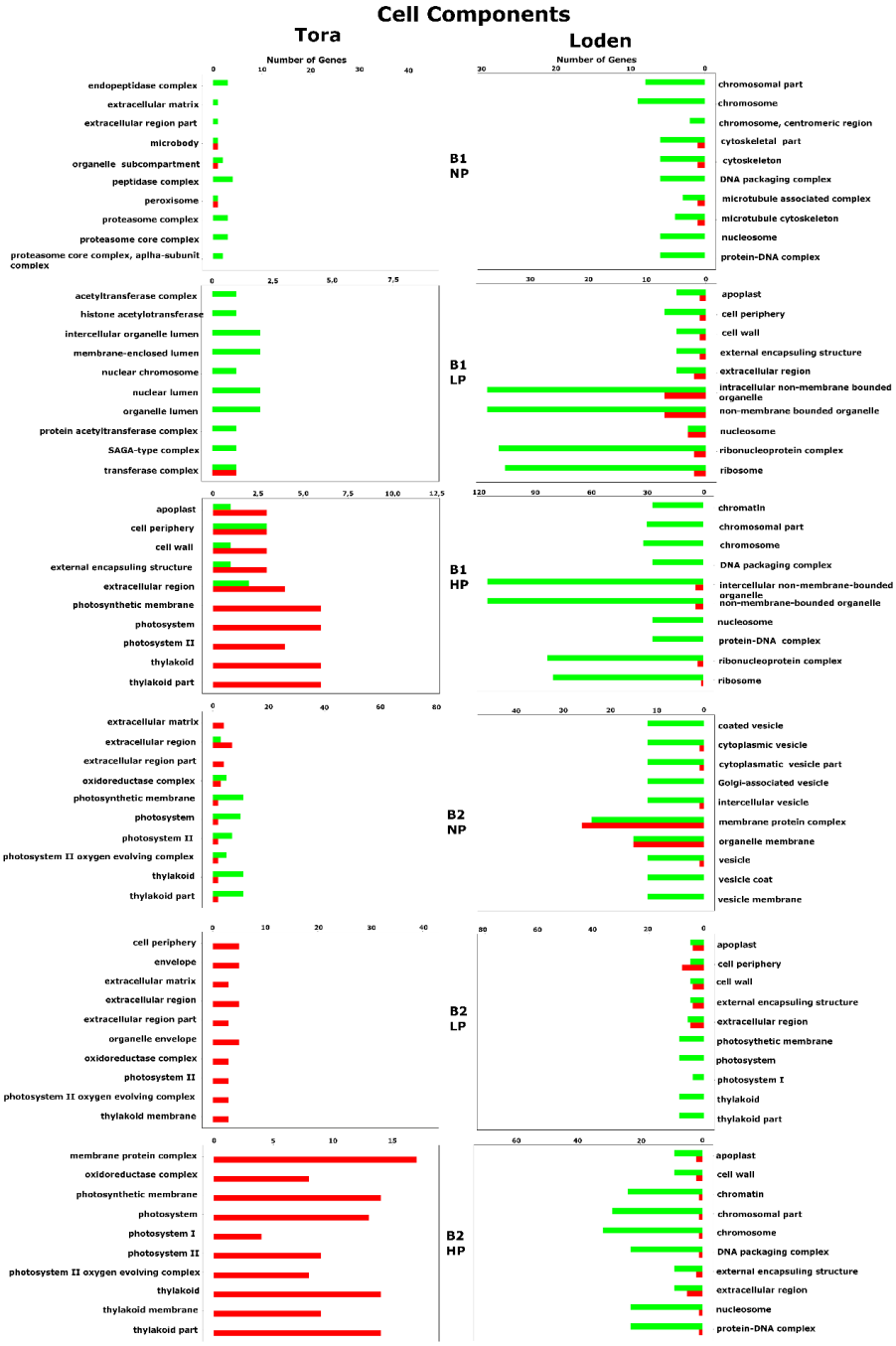

Figure 8 Cell component-associated gene expression regulation for all willow species, PSB and phosphorus concentrations. The red colour indicates upregulation of genes, while the green colour indicates downregulation.

For GO molecular functions, our analysis revealed the presence of 73 genes, among which 37 were expressed only in Loden, and 27 were expression in Tora (Figure 9). The pool of common genes comprised 11 genes and included those mainly responsible for calmodulin, haem and calcium binding, transferase activity, transcription and DNA-binding processes. We observed that the gene pool shared by Loden and Tora was characterized by the lack of an apparent division between downregulated and upregulated genes. The pool of common genes that responded to both B1 and B2 totalled 12. The abovementioned group of genes was joined by microtubule-binding activity, DNA-binding transcription factor activity and copper ion binding. In the case of molecular functions, the influence of P on gene regulation was observed only in the case of coenzyme binding, where the HP treatment elicited upregulation, which contrasted with the NP treatment, which caused those genes to be downregulated. Additionally, downregulation of two genes related to phosphatase activity was characteristic for the Loden B1 NP treatment combination.

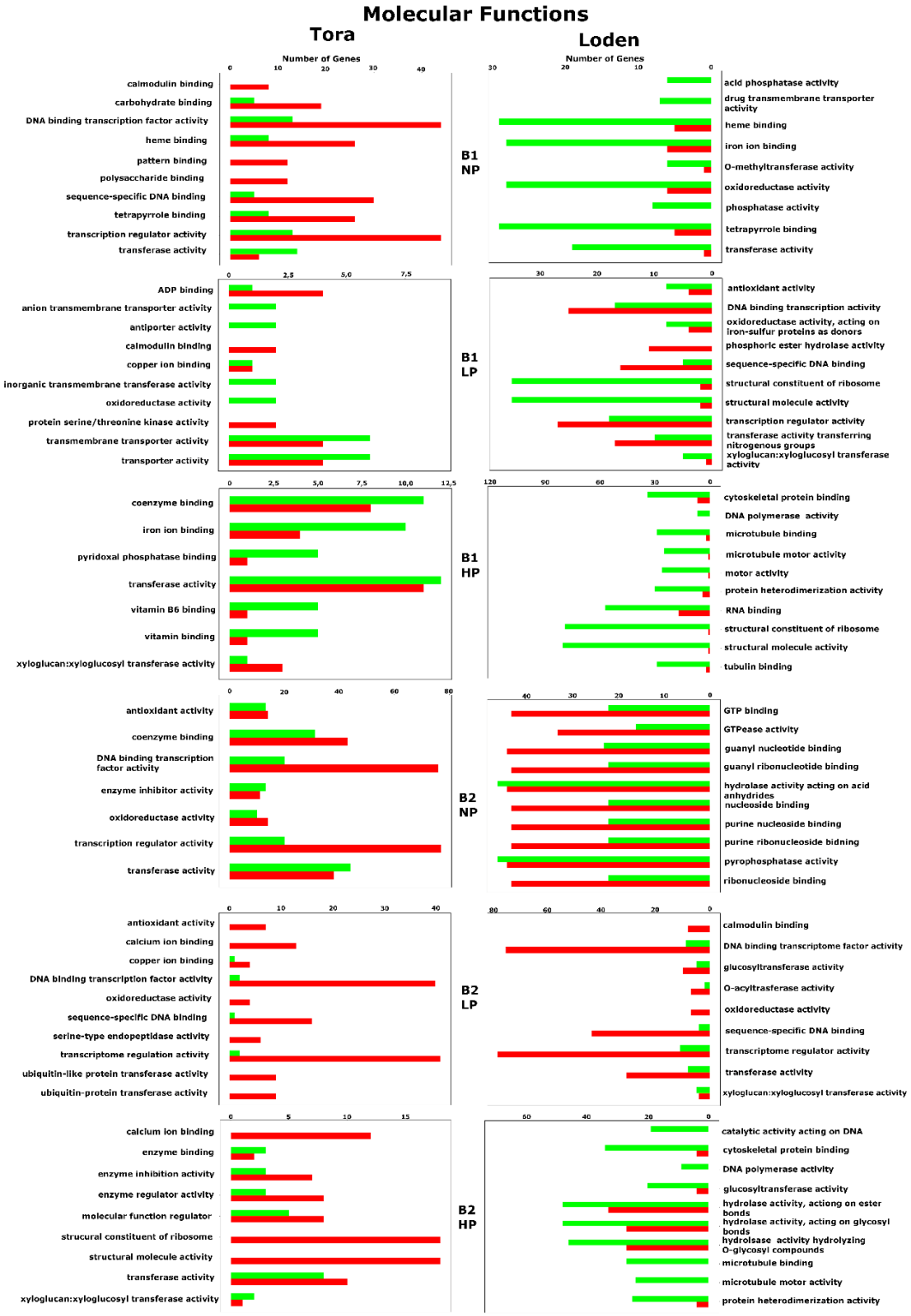

Figure 9 Molecular function-associated gene expression regulation for all willow species, PSB and phosphorus concentrations. The red colour indicates upregulation of genes, while the green colour indicates downregulation.

Discussion

In an era of climate change and a growing human population on Earth, there is a challenge in providing humanity with adequate food and energy resources, which are critical for such rapid population growth. The increased use of P fertilizers in agriculture worldwide is causing many undesirable environmental changes and depleting the planet's supply of this element (Filippelli, 2008; Alewell et al., 2020). The results of this experiment highlight the large effects of phosphorus provided by PSB in terms of altering growth patterns and gene expression, which manifested in the form of increased shoot growth for both willow species and general upregulation of genes for Tora and downregulation of them for Loden. The findings presented in this paper could constitute an important piece of the puzzle for developing effective bioinoculants containing PSB to increase the soil P use efficiency of plants.

Phosphate-solubilizing bacterium selection

From a pool of 64 PSB, two strains with the highest TCP-solubilization potential were selected (based on two screening stages carried out on solid and liquid TCP media) to investigate the effects of inoculation on the growth of willow seedlings under different P concentrations. The willow plants were grown in a substrate supplemented with TCP ($Ca_3(PO_4)_2$), which is not a readily available source of P, and the phosphate solubilization process carried out by the bacterium inoculants was the only source of available P, with the exceptions of strains *Bacillus aryabhattai* (GL2_5_ED2) and *Caballeronia glathei* (GTL2_4_RP10), which solubilized TCP and DCP equally well. *Bacillus* spp. are well-known and well-documented P-solubilizing microorganisms and have been tested many times for their use as potential biofertilizers of crop plants (Tahir et al., 2019; Ahmad et al., 2019; Miljaković et al., 2020; Mosela et al., 2022). In the present experiment, among the 15 most effective strains that solubilized phosphates, *Bacillus* spp. also were predominant (7 out of 15). This study found that *Rhizobium* sp. bacteria solubilized phosphate compounds most effectively, which highlights their great potential not only for nitrogen fixation but also as free-living soil bacteria that can solubilize P. Recent literature has confirmed the high efficiency of *Rhizobium* applied to crop plants, but there are no reports of the role of this species in tree growth (Afzal and Bano, 2008; Jaybhay et al. 2017; Verma et al., 2020; Mir et al., 2021; Shome et al., 2022).

In the pot experiment, strains *Pantoea agglomerans* (B1) and *Paenibacillus* sp. (B2) that effectively solubilized phosphates in both LP and HP media (analysed by the molybdenum

blue method) were selected. *Pantoea agglomerans* is described as a plant growth-promoting rhizobacterium (PGPR) capable of auxin biosynthesis, ACC deaminase production, ammonia production, P solubilization and increasing plant tolerance to salt stress (Majumdar et al, 2015; Cherif-Silini et al., 2019; Emami et al., 2021). Because willow trees do not constitute a food crop, the potential pathogenicity of willow to humans was not an issue (Mackiewicz et al., 2016). The second strain we selected was *Paenibacillus* sp., which is described in the literature as a bacterium capable of nitrogen fixation; P solubilization; stimulation of the plant defence system; and production of ammonia, HCN, IAA and siderophores (Kumari & Thakur, 2018; Hussain et al., 2020; Selim, 2022). Both bacterial species have been previously experimented with as bioinoculants of plant species such as barley (Canbolat et al., 2006), wheat (Hussain et al., 2020), sugarcane (Quecine et al., 2012), poplar (Vaitiekūnaitė et al., 2021) and palm trees (Saadouli al., 2021).

Effects of inoculation on willow growth

Inoculation with PSB and P treatments had significant effects on the growth patterns of the tested plants. However, it was difficult to determine whether the changes were caused by different soil P levels, bacterial inoculation, or a combination of both. Growth responses were different for Loden and Tora. For Loden, an increase in shoot thickness was found in response to higher soil P, while for Tora, the shoot length increased. In the NP treatment, the decrease in the leaf fresh weight could have been the result of P deficiency, because P deficiency has been shown to stimulate root allocation at the cost of leaf allocation (Ericsson, 1995). However, changes in P allocation to the roots or the leaves could also be related to changes in substrate pH, because increasing TCP concentrations may have increased the substrate pH. According to Walle et al. (2007), the optimum pH for willow growth is between 5 and 7.5, and the process of P solubilization by microorganisms is often associated with soil acidification. While alkaline phosphorus compounds are being formed, the pH becomes equilibrated by the process of microbial phosphate solubilization (Khan et al., 2014).

Effects of increasing P concentration and phosphate-solubilizing bacterium inoculation on willow gene expression

Plant transcriptome studies are becoming an integral part of all analyses aimed at determining the impact of biotic or abiotic stresses on various biological processes, cellular components or molecular functions. There are a growing number of publications examining the

transcriptomic responses of tree crops and the impact of factors such as drought (Pucholt et al., 2015; Jia et al., 2020; Xu et al., 2021), salinity (Yao et al., 2018; Zhou et al., 2020; Pang et al., 2022) and pest resistance (Wang et al. 2020). To date, however, there are no publications showing differences in gene expression under P-deficient conditions. Our results allowed us to determine the effects of phosphate-solubilizing bacterium inoculation with increasing concentrations of plant-available phosphate on the expression of genes related to the P stress response and P metabolism, mobilization and remobilization.

Our transcriptomic analysis showed high variability between the gene expression in the leaves of the two willow species tested (Loden vs. Tora), the inoculation variants (B1 and B2) and the P concentrations in the substrate (NP, LP, HP). The results showed that Loden exhibited approximately 1500 more differentially expressed genes than did the Tora willow species. As our previous study has shown, these species differ significantly in their morphology (Hoeber et al. 2017) and root-associated microbiome (Koczorski et al. 2021). As reported by Hoeber et al. (2017), Tora is characterized by highly efficient biomass production (tall shoots) but a smaller leaf area. Conversely, Loden is characterized by a large leaf area but relatively low amount of biomass production. Moreover, under NP conditions, Loden was shown to express a low number of genes in common between both the B1 and B2 variants, suggesting that inoculation significantly affects gene activity, specifically under these conditions. According to previous studies, one of the plant responses to inoculation is increased activity of genes responsible for DNA replication and cell division (Camilios-Neto et al. 2014). In addition, the activity of genes related to response to stimuli, signalling, regulation of biological processes and metabolic processes were observed (Sasha and Seal et al. 2015). In our analysis, similar gene activities in both the Loden and the Tora willow species, especially under LP conditions, were observed. Additionally, the expression of genes responsible for photosynthesis, transcription and translation in the LP and HP treatments was also observed. This was manifested by the activity of genes such as those involved in the glucan metabolic process, thylakoids, photosystems I and II, translation, DNA-binding translation factor activity, transcription regulation and sequence-specific DNA binding.

Phosphorus plays a very important role in the photosynthetic carbon reduction cycle, also known as the Calvin-Benson cycle, because the final product of this process is 3-phosphoglyceraldehyde (triose-P), which is then (already in the form of sucrose) transported to the cytosol of the cells or remains in the chloroplasts where it is converted into starch (Rychter

and Rao, 2005). Among the previously mentioned genes are also those responsible for glucan metabolic processes. Glucan phosphatase plays an important role in the transient metabolism of starch in leaves, as glucan phosphatase is the main storage material and consists mainly of glucose polymers. During the diurnal cycle of photosynthesis, starch is deposited during the day to be used up at night to provide sufficient metabolites needed for plant growth (Meekins et al. 2016). Our results suggest that the bacteria present in the substrate significantly improved the availability of starch and thus had a positive effect on plant growth. For the Tora willow species inoculated with B1, activity of genes responsible for pollen recognition and formation (NP and LP treatments) was observed. The direct effect of inoculation on pollen formation has not yet been investigated, but according to a study by Lankinen et al. (2000), phosphorus has a positive effect on the quantity and quality of pollen formed, while abiotic stresses such as salinity do not affect this process. Among the variants tested, only the Loden willow species inoculated with B1 under LP showed the expression of genes directly related to stress and defence mechanisms. Studies conducted on *Arabidopsis thaliana* leaves under P-deficiency conditions showed that these plants exhibit high expression of genes involved in phosphatase synthesis and genes that encode ribonucleases and sulfolipid biosynthesis proteins that replace P, contributing to P remobilization (Muller et al., 2007). Similarly, we detected genes involved in ribonuclease activity and phosphoric ester hydrolase activity, which may indicate P-deficiency stress. The genes indicated above (except for those encoding ribonucleases) were also expressed at lower numbers in response to the other treatment combinations in this study, which may be related to the rapid growth of the plants at an early stage of development. As a woody plant species, willows are known for their rapid growth, and as research has indicated, P remobilization and phospholipid replacement by sulfur and galactolipids is a strategy employed to increase photosynthetic efficiency (Lambers et al., 2012).

Gene expression regulation

The information obtained in this analysis largely overlaps with the results obtained from the GO enrichment analysis, providing additional information about genes that are upregulated and downregulated. The first and most prominent result of the analysis is the observed trend, in which the genes of the Loden willow species were downregulated and those of the Tora willow species were upregulated. As mentioned earlier, this may be a result of the difference in biomass allocation between the two willow species (Ericsson, 1995).

For the Tora willow species, a large effect of P on genes related to photosynthesis, thylakoids and starch metabolism in the form of upregulation of photosynthesis, photosystems I and II, thylakoid parts, carboxylic acid, carbohydrates, glucan metabolism and biosynthesis genes was found. All of the abovementioned genes are highly dependent on P because it is a component of ATP, NADPH, nucleic acids, sugar phosphates and phospholipids (Carstensen et al., 2018). The effect of phosphorus on carboxylic acids seems to be substantial due to the important role of these compounds as plant signalling molecules; also important are compounds like malate, which can be converted into NADH and NADPH, thus increasing the efficiency of photosynthesis (Mutz et al. 2013). Additionally, the presence of carboxylic acids has been confirmed in the apoplast, where activity related to the regulation of stomatal opening occurs (Mayer et al. 2010).

Among the downregulated genes for the Loden willow species, genes involved in lipid peptide metabolism and biosynthesis, nucleic acids, and ion and metabolite transfer were detected, while upregulation of genes involved in the defence mechanism and response to stress was also observed. Gene expression studies conducted on plants subjected to P-deficiency stress have indicated that the most common plant response to P deficiency is the downregulation of genes involved in ATP synthesis, translation, transport, carbohydrate synthesis and photosynthesis, which was observed for all the treatment combinations involving Loden (Bai et al. 2014; Chu et al. 2018). In addition, the upregulation of genes related to sulfur compounds was observed for the B2-inoculated Loden treatment combinations, indicating an attempt to replace P to increase phosphorus-use efficiency (Lambers et al., 2012). Notably, plants in the LP and HP treatments did not show signs of P deficiency, so the phenomena are related to species differences and biomass allocation rather than P itself.

Conclusions

Inoculation of *Salix* spp. with PSB increased soil phosphorus uptake and stimulated plant growth, which was manifested through changes in the growth patterns of both willow species and changes in the regulation of gene expression at the transcriptional level. Significant species differences were observed between Loden and Tora in their responses to the two inoculation variants and the different P concentrations in the substrates, the differences of which were evident, e.g., an increase in shoot thickness for Loden in response to higher soil P and a greater shoot length for Tora under the same treatment conditions. Transcriptomic analysis showed that phosphate-solubilizing bacterium inoculation of the Tora willow species significantly affected transcription in the leaves, affecting the upregulation of most genes, especially those related to photosynthesis, which are highly influenced by phosphorus. A general reduction in gene transcription levels was observed in Loden, especially for genes involved in ion transport, transcriptional regulation and chromosomes.

Data Availability Statement

The reference genome of *Salix viminalis* was acquired from Almeida et al. (2020). The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject, PRJNA967604.

Author Contributions

PK participation in all analyses and preparation of initial draft, data processing and preparation of figures and tables, statistical analysis, BF participation in all analyses and preparation of initial draft, review and editing of draft, CB pot experiment design, review and editing of draft, MW review and editing of draft, PI substantive support during transcriptome data interpretation, review and editing of draft PH soil analyses, review and editing of draft KH experiment design and supervision, review and editing of draft.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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9

Summary of results

9. Summary of results

The subject of the research described in this thesis was the soil microbial and ecological value of *Salix* spp. in short rotation coppices in Europe. According to the European Union Directive (of April 2009) on the promotion of the use of energy from renewable sources (Directive 2009/28/EC), 20% of all energy in Europe should be produced from renewable sources. Biomass production for energy generation using *Salix* as a fast-growing tree species is one of the promising ecological and efficient options. The first stage of the research was the analysis of two *Salix* field sites from different geographic origins within Europe. The first site was located in Uppsala (Sweden: 59.820375, 17.640334), and the second was located in Rostock (Germany: 54.061391, 12.081857). Both sites were created through the ECOLINK-*Salix* project, and the *Salix* genotypes were planted there identically. Two willow cultivars, 'Loden' (S. dasyclados) [L] and 'Tora' (S. *schwerinii* × S. *viminalis*) [T], were used in the study, which are characterized by great morphological and physiological differences. 'Loden' is characterized by a larger leaf area but less shoot growth, and 'Tora' has a smaller leaf area but reaches a larger size. This made it possible to test the influence of factors such as site conditions (soil and climate) and planting design (monoculture *vs.* mixed cropping) on the occurrence and diversity of the soil microbial diversity and the ratio of P solubilizing microorganisms (bacteria and fungi).

In the first publication **(P1)**, attention was focused on assessing the effect of growing *Salix* genotypes in monoculture and mixed culture on soil nutrient availability at two test sites with different soil and climatic conditions and analysing their effects on enzymatic P mobilization. Using data obtained from 2014 and 2018 (the first rotation cycle), a comparison was made between the plant availability of potassium, magnesium and phosphorus in the soil, and the activity of acid and alkaline phosphatase was assessed as a marker for biocatalysis in P mobilization. Mixed cultures, in contrast to monocultures, did not show a decrease in phosphorus availability after a period of 4 years, but this did not affect biomass production significantly. A higher activity of acid phosphatase compared to alkaline phosphatase was observed in soils at both test sites based on site-specific soil pH (<7). As the initial nutrient stocks at the two sites differed significantly, it was assessed that site interactions were the main regulators of changes in nutrient concentrations available to willow.

- The main factor influencing changes in nutrient availability (including phosphorus) in short-rotation willow crops is the site and its biodiversity, which, by promoting the diversity of the soil microbiome, contributes to the efficiency of P mobilization.

The next step **(P2 and P3)** was to isolate and evaluate the abundance of culturable microorganisms capable of solubilizing phosphorus and to put this in the context of the total microbial population in the rhizosphere willow roots (microbiome studies). At the selection stage, three selective media were used, two containing calcium phosphate (NBRIP and PVK) and calcium hydrogen phosphate (DCP). In this way, 22 fungal strains were isolated, among which fungi of the genus *Penicillium* predominated. The fungi were characterized by a high ability to solubilize calcium hydrogen phosphate, and their abundance was significantly higher in samples taken from mixed sites (cv. Loden and Tora). The selection also yielded 88 bacterial strains capable of solubilizing phosphorus, among which the genera *Pseudomonas*, *Bacillus* and *Erwinia* dominated. The bacteria solubilized calcium phosphate much better, showing relatively little activity on medium containing calcium hydrogen phosphate. The abundance of both bacteria and fungi was higher in the rhizosphere soil, and higher in roots at mixed sites than under monocultures. A higher abundance of P-solubilizing rhizosphere bacteria and fungi was also observed relative to endophytes in the mixtures. When the total bacterial and fungal community was examined, it was shown that the level of association with the plant (rhizosphere microorganisms *vs.* endophytic microorganisms) most significantly affected both the abundance and diversity of microorganisms. In the case of P-solubilizing fungi, *Penicillum* spp. dominated only in soil, not in the roots, but in the case of bacteria, the Alpha- and Gammaproteobacteria classes were maintained in soil and roots.

- The level of association with the plant (endophytes *vs.* rhizosphere microorganisms) is the main factor influencing the diversity of both PSMs (bacteria and fungi) and the total microbial diversity.

The final stage of the work **(P4)** involved setting up a pot experiment and starting to select bacterial strains with the highest P solubilization efficiency. From a collection of 64 P-solubilizing bacteria obtained at the beginning of the study, two strains were selected in two stages. The first stage of selection was carried out on solid NBRIP (tri-calcium phosphate) and DCP (di-calcium phosphate) medium and allowed the selection of 15 strains, which were then subjected to further selection in NBRIP liquid medium after potential pathogens were discarded. The two strains we selected were *Pantoea agglomerans* (B1) and *Paenibacillus* sp.

(B2). The pot experiment examined 3 factors (in each possible combination) affecting willow growth: cultivar (Loden, Tora), inoculation (control, B1, B2) and phosphorus concentration (NoP - 0 g/l, LowP - 0.3129 g/l and HighP 0.6259 g/l). The experiment was conducted for one month, and at the end, the following growth parameters were measured: wet weight of leaves, shoot, and roots; length of shoot and roots; and phosphorus concentration in soil and leaves. In addition, transcriptome analysis of leaves obtained from each variant of the experiment was performed. The analysis of growth parameters showed a large effect of phosphorus on plant growth that was manifested by a change in biomass allocation in shoots. Both willow species at high phosphorus had significantly increased shoot biomass, but in the case of Loden, the change was an increase in shoot thickness, and in the case of Tora, the change was in length. Transcriptome analysis showed large differences between Loden and Tora species, where common genes accounted for only ~15%. In addition, a trend was observed where genes of the Tora species underwent upregulation and Loden downregulation. The Tora species was characterized by high activity of genes responsible for photosynthesis and starch synthesis, both of which are strongly influenced by phosphorus. The Loden species showed a general downregulation of gene transcription, especially for ion transport, transcriptional regulation and chromosome genes. Inoculation seemed to have less of an effect than species but did affect the activity of genes such as fatty acids, glucan, carboxylic acid biosynthesis and metabolism and transcription-related processes. The greatest effect of phosphorus was seen primarily for the genes encoding photosystem I and II, thylakoids, photosystem II oxygen evolving complex, and oxidoreductase complex, which were upregulated for the HP viroid and downregulated for the NP viroid. Among the active genes were also those responsible for phosphatase synthesis but only for the Loden B1 NP variant.

- Phosphorus significantly alters biomass allocation by stimulating shoot growth in length for Tora and in width for Loden. In addition, it significantly affects gene transcription, causing upregulation of genes related to photosynthesis for Tora and downregulation of genes related to transcription or ion transport in Loden.

10

Discussion

10. Discussion

Mixed *Salix* plantations prevent nutrient loss

The first aim of the study was to examine the nutrient availability and phosphatase activity of soils and their changes depending on the design of *Salix* spp., i.e., pure cultures or mixed cultures. The samples were from 2014 and 2018, i.e., the first cutting cycle of *Salix* rotation systems. Soil analysis showed that Mg and K reserves were at optimal levels, but phosphorus was below the recommended amount (10-18 mg P/100 g soil) (Hoffmann, 1991). The high availability of nutrients in the soil at both sites is understandable, as these areas were previously used as agricultural land and thus were frequently fertilized (Lutter et al., 2016). The nutrient surplus at the German and Swedish sites, which were agricultural land in the past, is in agreement with previous studies (Kahle et al., 2010) and clearly indicates that there is no need to fertilize the coppice during the initial growth phase. Additionally, a positive correlation was observed between biomass and P and K in the early growth phase and a negative correlation with P and K in the later growth phase, indicating an important role for these two elements in the early growth phase of willows. Willows are known for their high phosphorus uptake capacity, especially in situations where they have a properly functioning symbiosis formed with ectomycorrhizal fungi (Rennenberg and Herschbach, 2013). The soil nutrient supply at both sites decreased over 4 years, especially for plants planted as monocultures, and similar results were also obtained for the willow species *Salix purpurea*, 'Hotel' (Ens et al., 2013). In addition, the Loden species used in this experiment is known for its ability to efficiently uptake nutrients, especially in combination with other willow species (Hoeber et al. 2017).

The activity of acid phosphatase was three times higher than that of alkaline phosphatase. Previous studies conducted at these sites reported a pH value of approximately 6 (Koczorski et al., 2021), which promotes the activity of this type of phosphatase. In addition, studies indicate a slow decrease in pH in short rotation coppices, which gradually increases acid phosphatase activity (Pellegrino et al., 2011). Phosphatase activity at the German site decreased significantly after 4 years, which may be related to the naturally occurring vegetation forming a dense network of fine roots (Lugli et al., 2019). No correlation of phosphatases with phosphorus was observed, while a positive correlation with potassium appeared. This indicates a probable lack of influence of phosphorus on phosphatase activity and suggests that the activity

of these enzymes may be controlled by a number of other, more complex factors. The high correlation of phosphatases with K may be related to its important role as a cofactor for many enzymes. Studies conducted on *Cucumis sativus* L. indicated increased activity of acid phosphatases in the presence of K ions (Tabaldi et al., 2007). Increased acid phosphatase activity may also be related to the interaction of arbuscular and ectomycorrhizal fungi. As established by Baum et al. (2018), these fungi play an important role in promoting acid phosphatase activity in Loden and Tora species and indicate increased activity in mixed cultures.

The level of plant association as the main factor shaping fungal and bacterial diversity in willow short-rotation coppices

The next stage of the work was to investigate the influence of soil properties, planting system and level of association with the plant on phosphorus-solubilizing bacteria and fungi and to establish their role and contribution compared to that of the willow microbiome. A selection of fungi (P2) and bacteria (P3) on solid media was performed, resulting in the isolation of 22 fungal and 88 bacterial strains with the ability to solubilize phosphorus. Among the most frequently isolated fungal strains at both test sites examined in this study was the genus *Penicillium*. This fungus is often used in research on P solubilizing fungi, and *Penicillium bilaiae* is marketed by NovoZymes as a bioinoculant to increase phosphorus mobilization (Chatli et al., 2008; Patil et al., 2012; Sharma et al., 2012). For the test site in Germany, the fungal genus most frequently isolated was *Clonostachys*. It was isolated from both the rhizosphere and roots of willow. In the literature, this fungal species is described as one of the endophytes of willow (Hosseini-Nasabnia et al., 2016). Among the isolated P solubilizing bacteria, the genera *Pseudomonas*, *Erwinia* and *Bacillus* predominated. All the aforementioned genera are known for their ability to solubilize phosphorus and other plant growth-promoting properties and are frequently used in research, especially *Pseudomonas* (Yu et al., 2011; Sarker et al., 2014) and Bacillus (Liu et al., 2015; Chawngthu et al., 2020). These strains have positive effects on crops such as wheat (Liu et al., 2019), maize (Viruel et al., 2014), rice (Gomez-Ramirez and Uribe-Velez, 2021) and some trees (Kurek et al., 2013). Another phosphorus solubilizing bacterial strain I frequently isolated was *Erwinia* sp. In the literature, this bacterium is often described as a plant pathogen, but we did not observe any visible symptoms of infection in willows. *Erwinia* sp. are quite rare in the literature but are reported to have the ability to promote plant growth, and their presence has been confirmed in the underground and

aboveground parts of trees such as almond (Guzmán et al., 2021), apple and pear (Rezzonico et al., 2016) and in agricultural crops such as barley (Li et al., 2021). Among the microorganisms isolated, rhizosphere bacterial and fungal strains are much more common than endophytic strains. Plants are known to synthesize substances capable of attracting or inhibiting microbial entry by acting as a filter through which only selected microorganisms can enter (Jacoby et al., 2017; Taulé et al., 2021). In addition, we observed a much higher abundance of P solubilizing microorganisms isolated from mixed crops and the lowest for the Loden species. This indicates that both species and planting system (mixed cropping) have an effect on bacterial and fungal biodiversity. This effect may be related to increased competition between both plants and microorganisms, consequently leading to increased biodiversity (Weih et al., 2019).

The data obtained from the microbiome survey indicated a dominance of *Dothideomycetes*, which is consistent with other studies conducted on willows (Yergeau et al., 2015). *Dothideomycetes* was also the only fungal class that showed differences between willow species. For bacteria, dominance of the genera *Actinobacteria* and *Alphaproteobacteria* was observed, both in the soil environment and in willow roots. Similar results, but from contaminated sites, were obtained by Tardif et al. (2016) and Yergeau et al. (2015). Microbiome studies again showed large, significant differences between the levels of association with the plant. This indicates the strong selective pressure that the plant exerts on rhizosphere microorganisms, and higher diversity in this environment is observed for both fungi (e.g., Hrynkiewicz et al., 2012; Yergeau et al., 2015; Thiem et al., 2018; Furtado et al., 2019) and bacteria (e.g., Kielak et al., 2008; Bulgarelli et al., 2012). In the case of fungi, the classes *Agaricomycetes* and *Pezizomycetes* occurred more frequently as endophytes, and *Tremellomycetes* and *Mortierellomycetes* occurred more frequently as rhizosphere fungi. Among the first two classes, ectomycorrhizal fungi are mainly found, and saprophytic fungi are found in the second group, which explains the results we obtained (Li et al., 2018; Francioli et al., 2020). In the case of bacteria, as mentioned above, the differences between association levels were not due to changes in bacterial diversity but rather to changes in bacterial abundance. Studies of soil parameters have shown that fungal communities are mainly dependent on climatic and soil conditions and nutrient content. Furthermore, bacterial communities are dependent on pH and soil structure (Lauber et al., 2008; Furtado et al., 2019).

Phosphorus solubilizing bacteria alter the growth patterns and gene expression of willows

The final stage of this work (P4) was to investigate the dynamics of gene expression and the effect of inoculation on two *Salix* species under phosphorus-deficient conditions. In the first part of the study, two strains with the highest phosphorus solubilization efficiency were selected from a group of 64 P solubilizing bacteria to carry out a pot experiment on two willow species (Loden and Tora). The first was a strain belonging to the species *Pantoea agglomerans*, which has been described in the literature as a plant growth-promoting rhizobacterium capable of synthesizing ACC deaminase, auxins, and ammonia and stimulating plant salt stress tolerance (Majumdar et al., 2015; Cherif-Silini et al., 2019; Emami et al., 2021). As willow is not a food crop, its pathogenicity was not an issue (Mackiewicz et al., 2016). The second P solubilizing bacterial strain we selected was *Paenibacillus* sp., capable of stimulating the plant defence system and synthesizing ammonia, HCN, IAA and siderophores (Kumari & Thakur, 2018; Hussain et al., 2020; Samain, 2022). Both bacterial species have been used as bioinoculants of plants such as barley (Canbolat et al., 2006), wheat (Hussain et al., 2020), sugarcane (Quecine et al., 2012), poplar (Vaitiekūnaitė et al., 2021) and palm trees (Saadouli al., 2021). The study showed a large positive effect of phosphorus-solubilizing bacteria on plant growth parameters. For the high phosphorus (HP) variant of the experiment, the plants additionally showed changes in the root:shoot ratio in both willow species (Loden and Tora). The change in biomass allocation observed between the variant without access to phosphorus and the one with high soil P content was manifested by an increase in shoot biomass in the HP variant. The phenomenon of a change in biomass allocation was noted by Ericsson (1995), where increased phosphorus concentration resulted in an increase in the root-to-shoot length ratio of the plants tested. Investigations of the willow leaf transcriptome showed a large difference in the number of active genes, with the average number of active genes being 1,500 higher for Loden. The two willow species used show a large difference in both morphology (Hoeber et al. 2017) and the root microbiome (Koczorski et al. 2021). The study by Hoeber et al. (2017) shows that Tora has a high biomass production efficiency and smaller leaf area, while Loden has a larger leaf area and smaller biomass. Further analysis of the leaf transcriptome revealed the presence of GO terms such as DNA replication, cell division, response to stimulus, signalling, and regulation of biological and metabolic processes. The activity of these genes in the literature is often linked to the plant's response to inoculation, and their occurrence was observed for both Loden and Tora under low phosphorus (LP) availability conditions

(Camilios-Neto et al. 2014; Sasha and Seal, 2015). Additionally, a high number of GO terms related to photosynthesis was observed for the LP and HP variants. This was associated with the expression of photosystem I and II, thylakoids and glucan metabolism process genes. Phosphorus is very important in the photosynthetic carbon reduction cycle called the Calvin-Benson cycle, the final product of which is 3-phosphoglyceraldehyde (triose-P), and glucan phosphatase plays an important role in transient starch metabolism in leaves (Meekins et al. 2016). Of all the experimental variants, only Loden inoculated with strain B1 showed expression of genes directly related to stress. Studies in *Arabidopsis thaliana* under P-deficient conditions showed high activity of phosphatase synthesis genes, ribonucleases and sulfolipid biosynthesis genes that replace P, contributing to P remobilization (Muller et al., 2007). Willows are species characterized by a very fast growth rate, which may explain some of the symptoms of phosphorus deficiency, and attempts to replace phosphorus by sulfur and galactolipids may be an attempt to increase phosphorus use efficiency (Lambers et al., 2012). The study observed large differences between the upregulation and downregulation of genes, where the Tora species was characterized by overall upregulation and Loden by downregulation of genes. For the Tora species, a large P effect was found on genes related to photosynthesis, thylakoids and starch metabolism in the form of upregulation of photosynthesis, photosystem I and II, thylakoid part, carboxylic acid, carbohydrates, glucan metabolism and biosynthesis. All the abovementioned genes are strongly associated with phosphorus because it is a component of compounds such as ATP, NADPH, nucleic acids, sugar phosphates or phospholipids (Carstensen et al., 2018). Loden showed downregulation of lipid peptide metabolism and biosynthesis, nucleic acid, ion and metabolite transfer genes. As indicated in the literature, some of these genes are associated with the phosphorus deficiency stress response, and these genes were expressed in all phosphorus variants (Bai et al. 2014; Chu et al. 2018). Notably, the LP and HP variants for Loden showed no visible signs of deficiency of this element.

11

Final conclusions

11. Final conclusions

1. Mixed culture willow planting systems, in contrast to monocultures, increase enzymatic P mobilization by promoting the diversity of microorganisms in both the rhizosphere and root interior of *Salix* species.
2. The level of plant association is the main factor driving both fungal and bacterial diversity, signifying the importance of species-specific selection of P solubilizing microorganisms for potential biofertilizers.
3. Phosphorus solubilizing bacteria can significantly promote *Salix* growth and change gene expression by regulating P cycling-related genes.

12

Future outlooks

12. Future outlooks

- Application of the P solubilizing microorganisms isolated in the present study to other tree species and arable crops to compare their compatibility and efficiency in interaction with other host plants.
- Further analysis of plant physiological traits, which are affected by microbial colonization of plants, was performed to describe the potential of biofertilizers to secure crop yield.
- Extension of the selection scheme for P solubilization efficiency to a larger number of microorganisms, including mixtures of bacteria and fungi. This will allow the inclusion of synergistic or antagonistic interactions between microorganisms.
- Extending the characterization of P solubilizing microorganisms to establish their other properties related to promoting plant growth and development.
- Development of biofertilizers to increase the P use efficiency from the soil pool in crop production and decrease the need for P fertilization and P loss into ground and surface waters.

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13. References

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