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Received: 16 September 2022 | Revised: 10 January 2023 | Accepted: 13 February 2023

DOI: 10.1111/pce.14570

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

Root exudates and rhizosphere microbiomes jointly determine temporal shifts in plant-soil feedbacks

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Funding information
Nederlandse Organisatie voor Wetenschappelijk Onderzoek; Deutsche Forschungsgemeinschaft

Abstract

Plants influence numerous soil biotic factors that can alter the performance of later growing plants—defined as plant-soil feedback (PSF). Here, we investigate whether PSF effects are linked with the temporal changes in root exudate diversity and the rhizosphere microbiome of two common grassland species (*Holcus lanatus* and *Jacobaea vulgaris*). Both plant species were grown separately establishing conspecific and heterospecific soils. In the feedback phase, we determined plant biomass, measured root exudate composition, and characterised rhizosphere microbial communities weekly (eight time points). Over time, we found a strong negative conspecific PSF on *J. vulgaris* in its early growth phase which changed into a neutral PSF, whereas *H. lanatus* exhibited a more persistent negative PSF. Root exudate diversity increased considerably over time for both plant species. Rhizosphere microbial communities were distinct in conspecific and heterospecific soils and showed strong temporal patterns. Bacterial communities converged over time. Using path models, PSF effects could be linked to the temporal dynamics of root exudate diversity, whereby shifts in rhizosphere microbial diversity contributed to temporal variation in PSF to a lesser extent. Our results highlight the importance of root exudates and rhizosphere microbial communities in driving temporal changes in the strength of PSF effects.

KEYWORDS
ecometabolomics, illumina sequencing, soil bacteria and fungi, structural equation modelling

1 | INTRODUCTION

Plants have the capacity to influence their local abiotic and biotic soil environment (Bennett & Klironomos, 2019). These plant-driven changes in soil properties can influence the growth of conspecific or heterospecific plants that subsequently grow in the same soil—a process known as plant-soil feedback (PSF) (van der Putten et al., 2013). Beside abiotic factors, such as temperature, moisture and nutrient availability, the composition of soil microbial communities plays an important role in determining whether the PSF will be positive or negative (Bennett & Klironomos, 2019; Kaisermann et al., 2017). The accumulation of beneficial microorganisms, such as mycorrhizal fungi, typically leads to positive PSFs whereas the accumulation of pathogens commonly suppresses plant growth resulting in negative PSFs. Typically, plant–soil interactions exhibit short- and long-term temporal dynamics, and thus the direction and magnitude of PSFs can strongly depend on time (Bezemer et al., 2018; Dudenhöffer et al., 2018; Thakur et al., 2021; Zhao et al., 2021). To better understand temporal PSF dynamics, it is vital to simultaneously monitor the changes in plant performance and soil characteristics over time. Here, we focus on two key interdependent factors which are likely to drive temporal shifts in PSF: alterations of root exudation profiles and shifts in rhizosphere microbial communities. To this end, we examine the importance of these two factors in driving temporal shifts in PSF effects during the growth of two plant species grown in conspecific and heterospecific soils.

The rhizosphere (the area surrounding plant roots in soil; Philippot et al., 2013) is a hotspot of dynamic transformation of nutrients and contains complex populations of microorganisms (Ling et al., 2022; Philippot et al., 2013). Plant roots and soil microorganisms directly interact through root exudates, which typically comprise of primary metabolites such as sugars, amino acids, and organic acids, as well as a diverse set of secondary metabolites (Oburger & Jones, 2018; Rovira, 1969; van Dam & Bouwmeester, 2016). Besides being a source of carbon and nitrogen, root exudates have tremendous effects on soil microorganisms and the rhizosphere processes, such as nutrient mobilisation. They are involved in the establishment of beneficial symbioses, and the production of signalling and defence compounds (Baetz & Martinoia, 2014; Oburger et al., 2014; van Dam & Bouwmeester, 2016). The composition and diversity of root exudates substantially differ among plant species and among individuals of the same species. Even within a single plant individual, exudate composition may vary among various plant developmental stages (Haichar et al., 2014; Zhalnina et al., 2018). However, current knowledge of variation in root exudates is mainly based on a handful of crop species like *Avena barbata* (Zhalnina et al., 2018), *Zea mays* (Hu et al., 2018), and *Triticum aestivum* (Oburger et al., 2014) as well as from the model species *Arabidopsis thaliana* (Chaparro et al., 2013) and lately from tree species (Weinhold et al., 2022). Only recently, studies have begun focusing on root exudates of common European grassland species confirming strong species identity effects (Delory et al., 2021; Dietz et al., 2019; Herz et al., 2018; Steinauer et al., 2016). Moreover, we still know little about how species-specific differences in root exudate composition and their diversity change over time when plants are exposed to different microbial communities in the soil, and how these changes affect plant-microbial interactions in the soil and ultimately PSF effects.

Changes in root exudation patterns are known to affect the composition and relative abundance of microbial communities in the rhizosphere (Philippot et al., 2013; Zhao et al., 2021). Due to the continuous developmental changes of the plant individual, soil microbial communities underlie strong temporal dynamics (Hannula et al., 2019). The few studies that have examined the temporal variability in soil microbial communities indicate that their composition can vary at the scale of days (Zhang et al., 2011), months (Hannula et al., 2019; Lauber et al., 2013), and seasons (Mellado-Vázquez et al., 2019). Thus, it is likely that a seedling, a juvenile, an adult, or a senescing plant shape their soil microbial community differentially. Consequently, a single plant individual may get exposed to different soil microbial communities with different functional roles over its life history. A succeeding plant—independent of being of the same or of a different species—is often exposed to the soil microbial community that was left behind by the plant that grew previously in the soil. Measuring how fast the succeeding plant-individual is capable to either adapt or re-shape the soil microbial community to its own benefit, is key to predict the strength and temporal dynamics of PSFs. For instance, seedlings and juvenile plants are considered to be more sensitive to the soil microbial legacy of the previous plant than adult plants (Elger et al., 2009; Hannula et al., 2021). The exact involvement of root exudate composition in shaping the soil microbial community over time is also poorly understood. Thus, it is of great importance to simultaneously study the effects of temporal changes during plant growth on root exudate dynamics along with soil microbial dynamics.

Here, we experimentally examine whether temporal shifts in PSF are linked with temporal changes in root exudate composition and the rhizosphere microbial community of two common grassland species representing two different plant functional groups, the grass *Holcus lanatus* and the forb *Jacobaea vulgaris*. These species were chosen because previous studies have shown that both species grow worse in soil of the other species than in own soil (Bezemer et al., 2018, 2006; van de Voorde et al., 2012). We investigate root exudate and microbial dynamics in soils by studying their composition and diversity during the growth of these two plants. Previous studies have shown that both species exhibit negative conspecific PSFs (Bezemer et al., 2018, 2006; van de Voorde et al., 2012). During the conditioning phase, we grew both plant species separately in pots establishing conspecific ('home soil') and heterospecific ('away soil') soil for both plant species (Figure 1). Hereafter, in the feedback phase, we grew individual plants from the seedling stage in soil conditioned by conspecifics or heterospecifics in a full-factorial design for 10 weeks. From Week 3 onwards, we destructively harvested a subset of plants weekly to determine plant biomass. We expected (1) negative conspecific PSF effects on plant growth in both plant species in early plant growth stages due to a higher

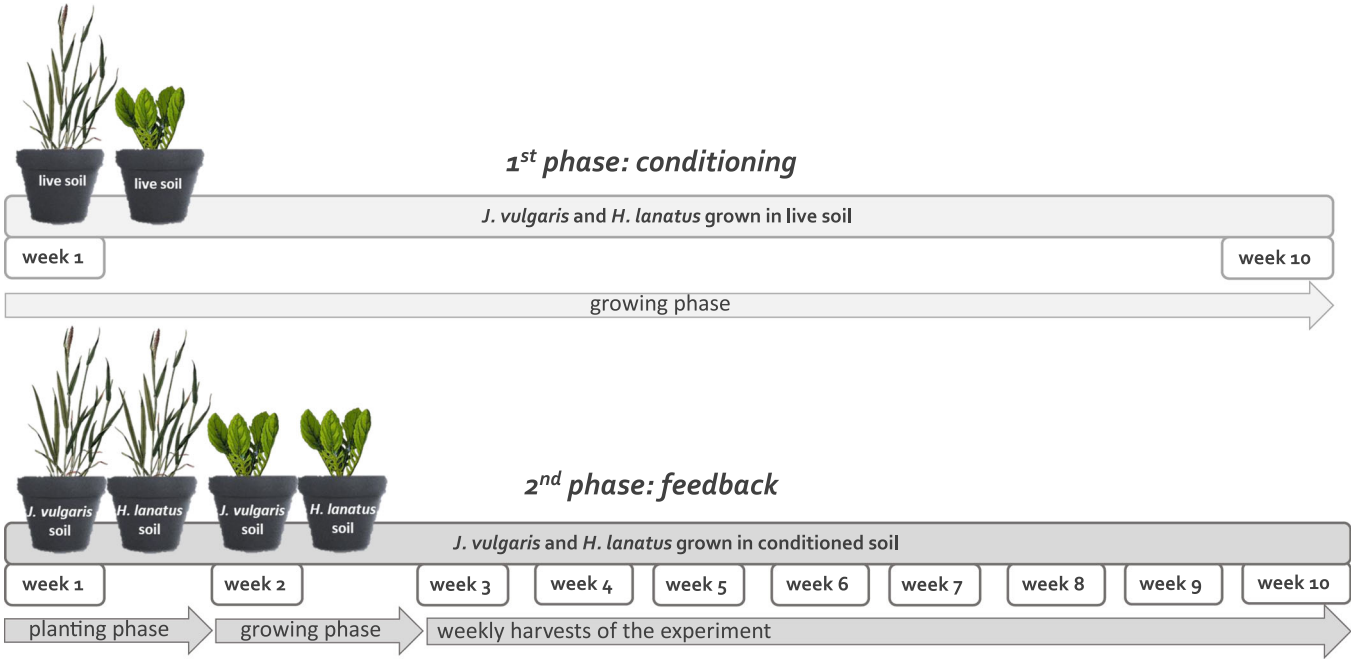


FIGURE 1 Experimental design of both conditioning and feedback phase and timeline of weekly harvests.

susceptibility of young plants to soil pathogens (Hersh et al., 2012). Furthermore, we expected (2) a shift in the strength and direction of PSF effects over time. For each harvest, we also analysed the root exudation profiles, using untargeted liquid chromatography–time of flight–mass spectrometry (LC-qToF-MS) and the microbial community composition (bacteria and fungi) of the rhizosphere soil. For these measures, we hypothesised (3) that temporal shifts in PSF effects in early growth stages depend on how plants alter their rhizomicrobiome through changes in their root exudation profile.

2 | MATERIALS AND METHODS

2.1 | Experimental design

We set up a microcosm experiment including two phases: the conditioning and feedback phase. Soil used in the experiment was collected from Lange Dreef te Driebergen, The Netherlands and characterised as holtpodzol sandy loam with a particle size distribution: 2% <0.002 mm, 11% 0.002–0.063 mm, 84% >0.063 mm, with ~3% organic matter, 1150 mg/kg N, 61 mg P_2O_5/100 g, 2.4 mmol K/kg and pH 5.9. Seeds of *H. lanatus* L. (Poaceae), a fast-growing perennial grass, were purchased from Cruydt-Hoeck, Nijeberkoop, The Netherlands, and seeds of *J. vulgaris* Geartn. subs. *vulgaris* (syn. *Senecio jacobaea* L.; Asteraceae) a biennial forb species, were collected from a population of wild plants growing in a natural grassland near the village Wolfheze, The Netherlands (52°0′18″ N, 5°47′30″ E). Both plant species co-occur frequently in (semi)natural grasslands in the Netherlands. Seeds of both species were surface-sterilised (1 min in 2.5% sodium hypochlorite solution and rinsed with water afterwards) and germinated for 2 weeks on sterile glass beads in a temperature-controlled climate chamber set at 24°C light (16 h), 20°C dark (8 h), and 60% relative humidity.

In the conditioning phase, eighty 1-L pots (10 × 10 × 11 cm) were filled with 1 kg sieved (1 cm mesh) and homogenised soil. Forty seedlings of *H. lanatus* and 40 seedlings of *J. vulgaris* were transplanted to individual pots and randomly placed in a climate-controlled greenhouse set at 21°C light (16 h), 16°C dark (8 h), and 60% relative humidity. In Weeks 1–3 20 mL, in Weeks 4–6 50 mL, and in Weeks 7–10 70 mL of demineralised water was added to each pot every second day. After 10 weeks of soil conditioning, we collected the conditioned soil of each pot and homogenised the soils by sieving (mesh size 1 cm) per plant species (Figure 1).

In the feedback phase, we used *J. vulgaris* grown in tissue cultures to preclude variation in root exudate profiles due to genetic differences. In a climate room, *J. vulgaris* cuttings from a single genotype were asexually propagated in tissue culture using MS medium (Murashige and Skoog medium) with 100 mg/L benzylaminopurine (BAP) (16:8 h light:dark photoperiod, 20°C). After 4 weeks, the cuttings were grown in MS medium without BAP for 10 days to produce roots. The genotype that was propagated was formerly collected from Meijendel (Wassenaar), The Netherlands. Seeds of *H. lanatus* were again purchased from Cruydt-Hoeck, Nijeberkoop, The Netherlands. Seeds for germination were treated as described above. We filled two L-pots (diameter: 12.3 cm, height: 13 cm) with 1.62 kg of sterile soil from the same field (γ-irradiated >25 kGy; Synergy Health) and 0.18 kg of conditioned soil, resulting in a 9:1 ratio. We then planted one individual per pot of *J. vulgaris* on

J. vulgaris - conditioned soil ('*Jacobaea* home soil') and on *H. lanatus*—conditioned soil ('*Jacobaea* away soil'). Furthermore, we planted one individual of *H. lanatus* on *H. lanatus*—conditioned soil ('*Holcus* home soil') and on *J. vulgaris*—conditioned soil ('*Holcus* away soil'). Plants that died within the first 10 days were replaced by new seedlings or cuttings. Afterwards, plants were left to establish for 2 weeks before the first harvest. Pots were watered every second day (Weeks 3–7; 50 mL and in Weeks 8–10; 70 mL) with demineralised water. The pots were kept in the greenhouse under the same conditions as above. We grew three replicate pots for each plant/soil combination (4) and time point (8) resulting in 96 pots. We destructively harvested plants for eight consecutive weeks always on Tuesdays between 8 and 10 AM (2–4 h after sunrise; February–April 2018; Wageningen, Netherlands: 51°58′12.00″ N, 5°40′0.01″ E) (Figure 1). During each harvest we collected root exudates and rhizosphere samples for molecular identification of soil microbial community and weighed root and shoot biomass (details below).

2.2 | Root exudate collection

To capture root exudate compounds, alive roots were carefully separated from the soil by continuous and gentle rinsing with deionized water until roots were separated from mineral particles (protocol adapted after; Oburger et al., 2014). This method might cause potential root damage and thus leaking of cell contents, although the effects on exudate composition is not yet clear (Williams et al., 2021). The plants' roots were submerged for 10 min into 100 mL of deionized water in glass flask wrapped with aluminium foil to avoid any light effects. Again, roots were gently rinsed with deionized water. After the washing procedure, roots were then placed in the final sampling solution (100 mL deionized water, containing 0.01 g/L Micropur classic (Katadyn) and kept under greenhouse conditions for the entire sampling period (4 h). Thereafter, the sampling solution was filtered through 7 µm (Whatman folded filters, Ø 150 mm, 595½; Sigma-Aldrich) to remove remaining soil particles and further filtered through a sterile 0.2 µm syringe filter (Whatman Puradisc 30 syringe filters; Sigma-Aldrich) with a cellulose acetate membrane. The samples were stored at −20°C and lyophilised at −80°C. After root exudate collection, roots and shoots were separated, dried for 48 h at 70°C, and weighed.

2.3 | Solid phase extraction of root exudates and sample processing

The freeze-dried root exudates were dissolved in 2 mL of 5% methanol (LC-MS grade) in ultrapure water and sonicated for 10 min at ambient temperature in an ultrasonic bath, followed by a centrifugation step at 6000*g* for 10 min (after (Strehmel et al., 2014). The supernatant was transferred in a fresh 2 mL tube. For every sample, a SPE cartridge packed with C18 column material (Chromabond 200 mg/3 mL; Macherey-Nagel) was conditioned with 1 mL of pure methanol followed by 1 mL of 2% formic acid in water. The dissolved root exudates were transferred from the 2 mL tube to the conditioned column. The column was washed with 1 mL ultrapure water followed by one elution step with 2% formic acid in pure methanol. The eluates were evaporated to dryness using a Speed Vac at 40°C and resolved in 150 µL 70% methanol followed by sonification and centrifugation (10 min, 6000*g*). Finally, the supernatant was transferred in a LC glass vial.

We performed chromatographic separation of all samples by injecting 2 µL on a Thermo Scientific Dionex UltiMate 3000 (Thermo Scientific Dionex) UPLC unit, equipped with a C18 column (Acclaim RSLC 120 C18, 2.2 µm, 120 Å, 2.1 × 150 mm; Thermo Fisher Scientific). We applied the following binary elution gradient at a flow rate of 0.4 mL/min and a column temperature of 40°C: 0–2 min, 95% A (water and 0.05% formic acid), 5% B (acetonitrile and 0.05% formic acid); 2–12 min, 5%–46% B; 12–19 min, 46%–95% B; 19–22 min, 95% B; 22–25 min, 95%–5% B; 25–30 min, 5% B.

Metabolites were analysed on a LC-qToF-MS (Bruker impact HD; Bruker Daltonik) with an electrospray ionisation source operated in negative mode. Instrument settings were as follows: capillary voltage, 2500 V; nebuliser, 2.5 bar; dry gas temperature, 220°C; dry gas flow, 11 L/min; scan range, 50–1500 *m/z*; acquisition rate, 3 Hz. We used sodium formate clusters (10 mM solution of NaOH in 50/50% [vol/vol] isopropanol/water containing 0.2% formic acid) to perform mass calibration. For further annotation mass spectra (MS^2) of selected pooled samples were collected in positive and negative MSMS mode.

2.4 | LC-MS data processing and metabolite prediction

We followed the LC-MS data processing protocol described in (Ristok et al., 2019) with minor changes. We converted the LC-qToF-MS raw data to the mzXML format by using the CompassXport utility of the DataAnalysis vendor software. Subsequently, we trimmed each data file by excluding the same non-informative regions at the beginning and end of each run using the msconvert function of ProteoWizard v3.0.10095 (Chambers et al., 2012). We performed peak picking, feature alignment, and feature group collapse in R v3.3.3 (RStudio Team, 2020) using the Bioconductor packages 'xcms' (Benton et al., 2010; Smith et al., 2006; Tautenhahn et al., 2008) and 'CAMERA' (Kuhl et al., 2012). We used the following 'xcms' parameters: peak picking method 'centWave' (snthr = 10; ppm = 5; peakwidth = 4, 10); peak grouping method 'density' (minfrac = 0.5; bw = 6, 3; mzwid = 0.01); retention time (rt) correction method 'symmetric'. We used 'CAMERA' to annotate adducts, fragments, and isotope peaks with the following parameters: extended rule set (https://gitlab.com/R_packages/chemhelper/-/tree/master/inst/extdata); perfwhm = 0.6; calcIso = TRUE; calcCaS = TRUE, graphMethod = lpc. Finally, we collapsed each annotated feature group, hereafter referred to as 'metabolite' which is described by mass-to-charge ratio (*m/z*) and rt, using a maximum heuristic approach. In detail, this means that the intensity values of the feature that most

often displayed the highest intensity across all samples represent the feature group. We performed preprocessing with 'xcms' and 'CAMERA' for each species and sampling season. We merged all created feature lists by rt and *m/z* values. For each feature, we allowed for a rt window of 10 s and a mass deviation of 5 ppm.

2.5 | Soil sampling, DNA extraction, and sequencing

Samples for molecular analysis were collected from the rhizosphere soil before root exudate collection. Therefore, plant roots were gently shaken and the soil adhering to roots was carefully brushed from the roots, homogenised by mixing, collected in an Eppendorf tube and immediately frozen in liquid nitrogen and stored at −80°C before DNA-extraction. DNA was extracted from 0.75 g of soil using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories) following the manufacturer's protocol and the quantity of DNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Polymerase chain reactions (PCRs) were performed using approximately 100 ng of DNA primers ITS4ngs and ITS3mix targeting the ITS2 region of fungal genes (Tedersoo et al., 2015) and the primers 515F and 806R (Apprill et al., 2015; Caporaso et al., 2012; Parada et al., 2016) targeting the V4 region of the 16S RNA gene in bacteria were used. The PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA) and adapters and barcodes were added to enable multiplexing with Nextera XT DNA library preparation kit set A (Illumina). The final PCR product was purified again with AMPure beads and quantified using a Nanodrop spectrophotometer before equimolar pooling. Pooled libraries were sequenced using Miseq PE250 technology at McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada. Extraction negatives were used and further sequenced. A mock community, containing 10 fungal species, was included to investigate the accuracy of the bioinformatics analysis.

Bacterial sequences were analysed using the Hydra pipeline (Hollander, 2017) and fungi using the PIPITS pipeline (Gweon et al., 2015). In both pipelines, sequences were paired using VSEARCH and quality was filtered using standard parameters of the pipelines (i.e., min overlap 20 bp, primers need to be exact matches, quality score over 28 used). For fungi, the ITS2 region was extracted using ITSx (Nilsson et al., 2015). Short reads (<100 bp) were removed, and sequences were clustered based on a 97% similarity threshold using VSEARCH. Afterwards, chimeric sequences were removed by comparing with the UNITE uchime database. The representative fungal sequences were identified using the RDP classifier against the UNITE database (Nilsson et al., 2019). Bacterial sequences were identified using the SINA classification tool with SILVA database.

Only OTUs belonging to bacteria or fungi were kept in the analysis (protists, plants, archaea, mitochondria, and chloroplast were removed). For both bacterial and fungal OTU tables, samples with less than 1000 reads or more than 80 000 reads were removed and OTUs present in less than three samples with relative abundance of less than 0.05% were removed. These cut-off values were derived from inspection of mock communities consisting of 10 fungal species cumulative sum scaling was used to normalise the data.

2.6 | Statistical analysis

We used linear models to test the effects of plant growth (time), soil conditioning (soil) and its interaction on shoot and root biomass. Effect of PSF on plant biomass were calculated as: ln(plant dry mass (g) in 'home soil') at time *x* − ln(plant dry mass (g) in 'away soil') at time *x*, where ln is natural logarithm. Thus, negative values indicate that plants grow better in 'away soil'. The feedback effect was calculated separately for each replicate (i.e., ln('home soil' replicate 1) − ln('away soil' replicate 1), etc.). We further used comparisons of means for treatment-specific effects (Tukey's HSD test; $p < 0.05$). Tukey's tests were performed using the multcomp package (Hothorn et al., 2008).

To test the effects of duration of plant growth and soil condition on rhizosphere bacterial and fungal community compositions and on root exudate composition, we ran permutational multivariate analysis of variance (based on Bray–Curtis dissimilarities, 999 permutations) using the adonis2 function in the vegan package (Oksanen et al., 2020). For visualisation, we applied a nonmetric multidimensional (NMDS) analysis (using the metaMDS function in the vegan package; Oksanen et al., 2020) of the dissimilarities (based on Bray–Curtis dissimilarities) in root exudate and rhizosphere microbial community composition using the ggplot2 package (Wickham, 2021). Root exudate diversity was

TABLE 1 Linear model: table of *F* and *p* values on the effects of time and soil type on shoot and root biomass of *Jacobaea vulgaris* and *Holcus lanatus*.

Factor	Shoot biomass *J. vulgaris*			Shoot biomass *H. lanatus*			Root biomass *J. vulgaris*			Root biomass *H. lanatus*		
	df	*F* value	*p* Value	*df*	*F* value	*p* Value	*df*	*F* value	*p* Value	*df*	*F* value	*p* Value
Time	7	**111.74**	**0.001*** **	7	**79.85**	**0.001*** **	7	**28.97**	**0.001*** **	7	**9.14**	**0.001*** **
Soil	1	1.03	0.317	1	**9.57**	**0.004***	1	0.38	0.541	1	**4.70**	**0.038***
Time × soil	7	1.11	0.381	7	0.35	0.922	7	0.39	0.902	7	0.72	0.654
Residuals	32			32			32			32		

Note: 'Time' was used as categorical factor. Significant results ($p < 0.05$) are highlighted in bold.

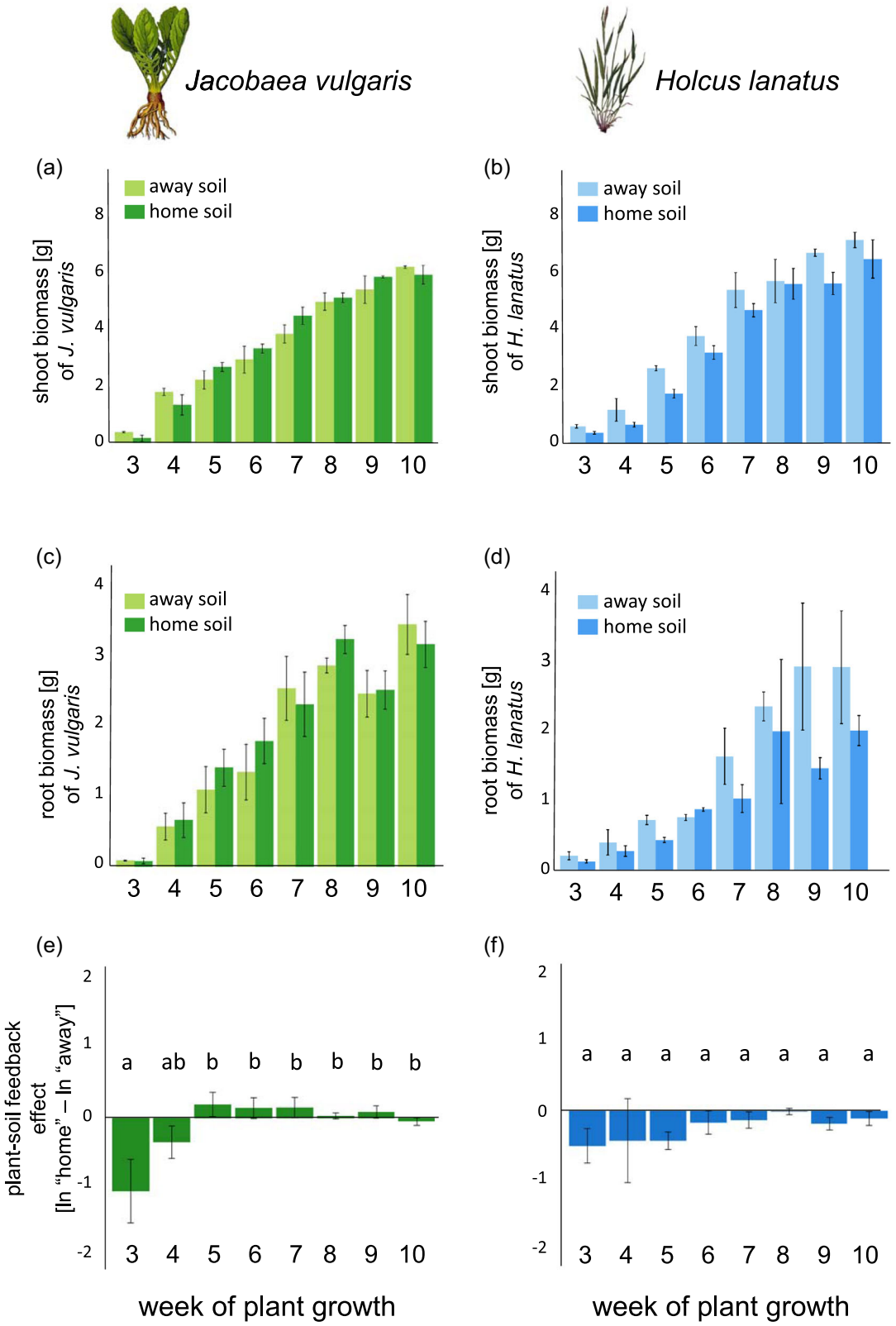


FIGURE 2 The effects of plant growth and soil conditioning ('home' and 'away') on shoot biomass of (a) *Jacobaea vulgaris* and (b) *Holcus lanatus*, root biomass of (c) *J. vulgaris* and (d) *H. lanatus*, and plant-soil feedback effect of (e) *J. vulgaris* and (f) *H. lanatus*. Values are mean ± SE. Bars with different letters vary significantly (Tukey's HSD test, $a < 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 PERMANOVA model: table of R^2, F values, and p values for the effect of time and soil type on root exudate, baterial, and fungal community compositon of *Jacobaea vulgaris*.

	Root exudate composition				Bacterial community composition				Fungal community composition			
Factor	df	R^2	F value	p Value	df	R^2	F value	p Value	df	R^2	F value	p Value
Time	7	0.5	6.60	**0.001*****	7	0.28	2.63	**0.001*****	7	0.23	1.80	**0.011***
Soil	1	0	1.50	0.154	1	0.09	5.86	**0.001*****	1	0.03	1.80	0.091
Time × soil	7	0.20	2.81	**0.001*****	7	0.14	1.26	**0.031***	7	0.19	1.50	**0.039***
Residuals	32				32				31			

Note: 'Time' was used as categorical factor. Significant results ($p < 0.05$) are highlighted in bold.
Abbreviation: PERMANOVA, permutational multivariate analysis of variance.

TABLE 3 PERMANOVA model: table of R^2, F values, and p values for the effect of time and soil type on root exudate, baterial and fungal community compositon of *Holcus lanatus*.

	Root exudate composition				Bacterial community composition				Fungal community composition			
Factor	df	R^2	F value	p Value	df	R^2	F value	p Value	df	R^2	F value	p Value
Time	7	0.47	5.19	**0.001*****	7	0.28	2.93	**0.001*****	7	0.23	2.19	**0.001*****
Soil	1	0.02	1.87	0.068	1	0.15	11.26	**0.001*****	1	0.08	5.26	**0.001*****
Time × soil	7	0.10	1.13	0.277	7	0.15	1.52	**0.005****	7	0.23	2.14	**0.001*****
Residuals	31				31				30			

Abbreviation: PERMANOVA, permutational multivariate analysis of variance.

based on the presence/absence of distinct metabolites. Fungal and bacterial diversity was estimated using the Simpson's diversity index. All statistics were performed within the R statistical environment (version 4.1.2; RStudio Team, 2021).

To disentangle the temporal effects of root exudates and rhizosphere microbiomes on the variation in PSF, we further performed path analysis. This was done for the two plant species separately. Based on our hypotheses, we used both direct and indirect paths from time to the variation in PSF using 'home soil' and 'away soil' root exudates and rhizosphere microbiomes (fungi and bacteria; conceptual figure, Figure S2). Given that root exudates and the rhizosphere microbiome can affect each other, we tested path relations in both directions (i.e., root exudates affecting rhizosphere microbiomes and rhizosphere microbiomes affecting root exudates) in our models. As all path models with the directional path from root exudates to rhizosphere microbiomes had lower AIC, our final reporting of path model results is based on this pathway. Nevertheless, we also report the path coefficients from rhizosphere microbiomes to root exudates. In our path models, we used diversity metrics for root exudates and rhizosphere microbiomes instead of their compositional variation (based on NMDS scores) to test our hypothesis to explain temporal shifts in the strength of PSF during the plant growth. Moreover, path models with dissimilarity indices were unsuitable based on Fisher's C statistics (Shipley, 2009) (details in Table S3). For the overall assessment of path model fits, Shipley's test of d-separation was used which computes Fisher's C statistics based on chi-square distribution (Shipley, 2009). We ran all our path models in the piecewiseSEM package (Lefcheck, 2016).

3 | RESULTS

3.1 | Plant biomass and PSF effects

Shoot and root biomass increased significantly over the experimental period for both plant species (Table 1; Figure 2a–d). In Weeks 3 and 4, shoot biomass of *J. vulgaris* was higher (Week 3: +43%, Week 4: + 24%) in 'away soil' than in 'home soil' (Figure 2a), whereas from Week 5 onwards, *J. vulgaris* shoot and root biomass values were slightly higher in 'home soil' (Figure 2a,c). Thus, a strong negative 'home soil' feedback in the early growth phase (<−1) could be observed, but the strength changed over time ($F = 3.99$; $p = 0.010$). The negative feedback effect rapidly diminished, and became slightly, but not significantly, positive (Figure 2e). Shoot biomass of *H. lanatus* was higher in 'away soil' over the entire 10 weeks of the experiment (Figure 2b). Root biomass was mostly higher in 'away soil' except in week 6 (ranging between +2 and +45% increase) (Figure 2d), resulting in a negative 'home soil' feedback effect that did not significantly change over time ($F = 0.52$; $p = 0.807$; Figure 2f). However, for *H. lanatus* the strength of the negative feedback diminished over time.

3.2 | Root exudate composition and diversity

NMDS analysis revealed strong compositional changes of root exudate composition over time for both *J. vulgaris* and *H. lanatus* (Tables 2 and 3; Figure 3). Differences in soil conditioning of both plant species did not affect the root exudate composition (Tables 2 and 3; Figure 3). However, the interaction of time and soil conditioning was significant for *J. vulgaris* (Tables 2 and 3; Figure 3a). Further, the diversity of root exudates

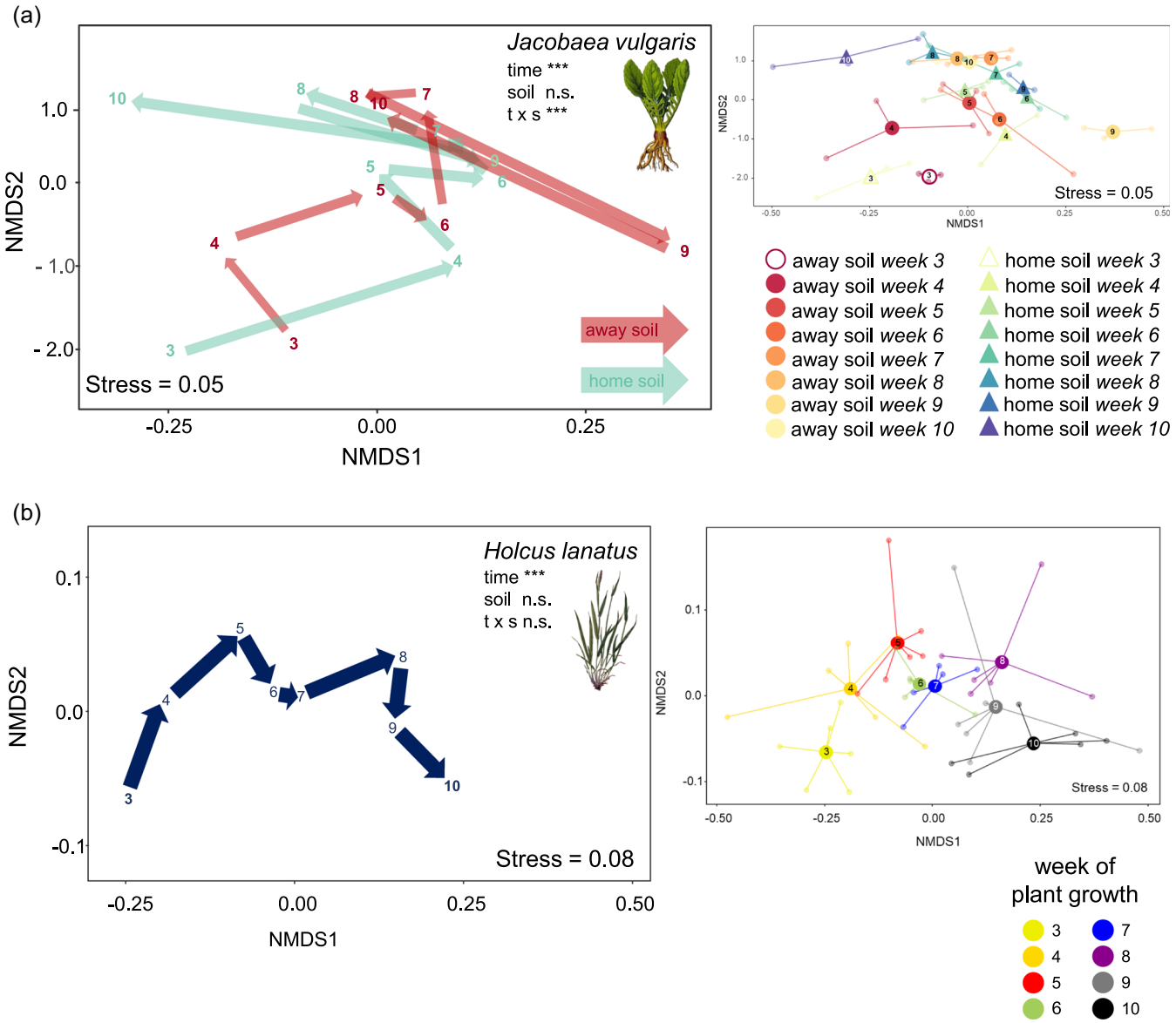


FIGURE 3 Nonmetric multidimensional scaling (NMDS) plots based on Bray–Curtis dissimilarity. The effects of (a) plant growth and soil conditioning on root exudate composition of *Jacobaea vulgaris* (red arrows = 'away soil' (*Holcus lanatus*—conditioned soil), turquoise arrows = 'home soil' (*J. vulgaris*—conditioned soil)), (b) plant growth on root exudate composition of *H. lanatus* within both soils. Big panels of (a) and (b) display temporal shifts of root exudate composition, whereas in small panel of (a) circles = 'away soil' (*H. lanatus*—conditioned soil), triangles = 'home soil' (*J. vulgaris*—conditioned soil), in small panel of (b) displays the same temporal shifts including individual samples (small dots); the large dots represent averaged centroids. Stress values are given for each NMDS. Asterisks represent significance levels (n.s. = not significant; $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$). Each root exudate composition had a total sample size of 48. [Color figure can be viewed at wileyonlinelibrary.com]

increased significantly over time for both plant species (Tables S1 and S2; Figure S1a,d) but soil conditioning did not affect root exudate diversity significantly (Table S1 and S2).

3.3 | Soil microbial composition and diversity

Rhizosphere bacterial community composition changed significantly with plant growth for both plant species and the two differently conditioned soils (Table 2; Figure 4). Further, rhizosphere bacterial communities in 'home soil' and 'away soil' of both *J. vulgaris* and *H. lanatus* converged over time (Figure 4). This was particularly evident for composition of bacteria in *H. lanatus* where bacterial composition in the 'home soil' (*Holcus* growing in *Holcus* soil) remained relatively constant, while the composition in the 'away soil' (*Holcus* growing in *Jacobaea* soil) started differently but moved into direction of the home soil over time (Figure 4). The diversity (Simpson's diversity index) of bacterial communities of *J. vulgaris* was not affected by plant growth or soil conditioning. However, the bacterial diversity within the rhizosphere of *H. lanatus* was

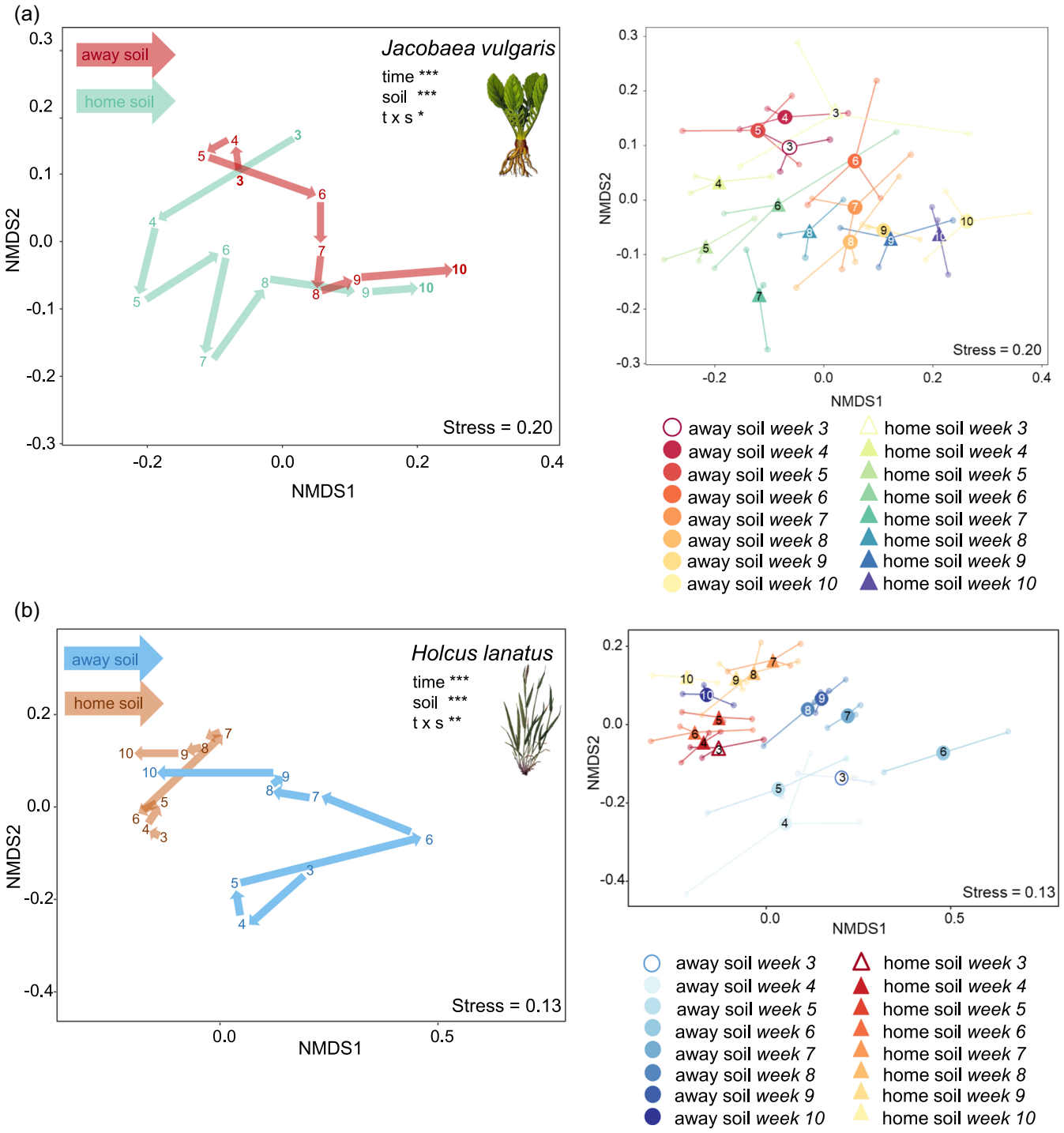


FIGURE 4 Nonmetric multidimensional scaling (NMDS) plots based on Bray–Curtis dissimilarity. The effects of (a) plant growth and soil conditioning on rhizosphere bacterial community composition of *Jacobaea vulgaris* (red arrows = 'away soil' (*Holcus lanatus*—conditioned soil), turquoise arrows = 'home soil' (*J. vulgaris*—conditioned soil)), (b) plant growth and soil conditioning on rhizosphere bacterial community composition of *H. lanatus* (blue arrows = 'away soil' (*J. vulgaris*—conditioned soil), brown arrows = 'home soil' (*H. lanatus*—conditioned soil). Big panels of (a) and (b) display temporal shifts of rhizosphere bacterial community composition, whereas small panels display the same temporal shifts including individual samples (small dots), and large dots represent averaged centroids. In small panel of (a) circles = 'away soil' (*H. lanatus*—conditioned soil), triangles = 'home soil' (*J. vulgaris*—conditioned soil), and in small panel of (b) circles = 'away soil' (*J. vulgaris*—conditioned soil), triangles = 'home soil' (*H. lanatus*—conditioned soil). Stress values are given for each NMDS. Asterisks represent significance levels (n.s. = not significant; $*p < 0.05$; $**p \leq 0.01$; $***p \leq 0.001$). Each bacterial community composition had a total sample size of 48. [Color figure can be viewed at wileyonlinelibrary.com]

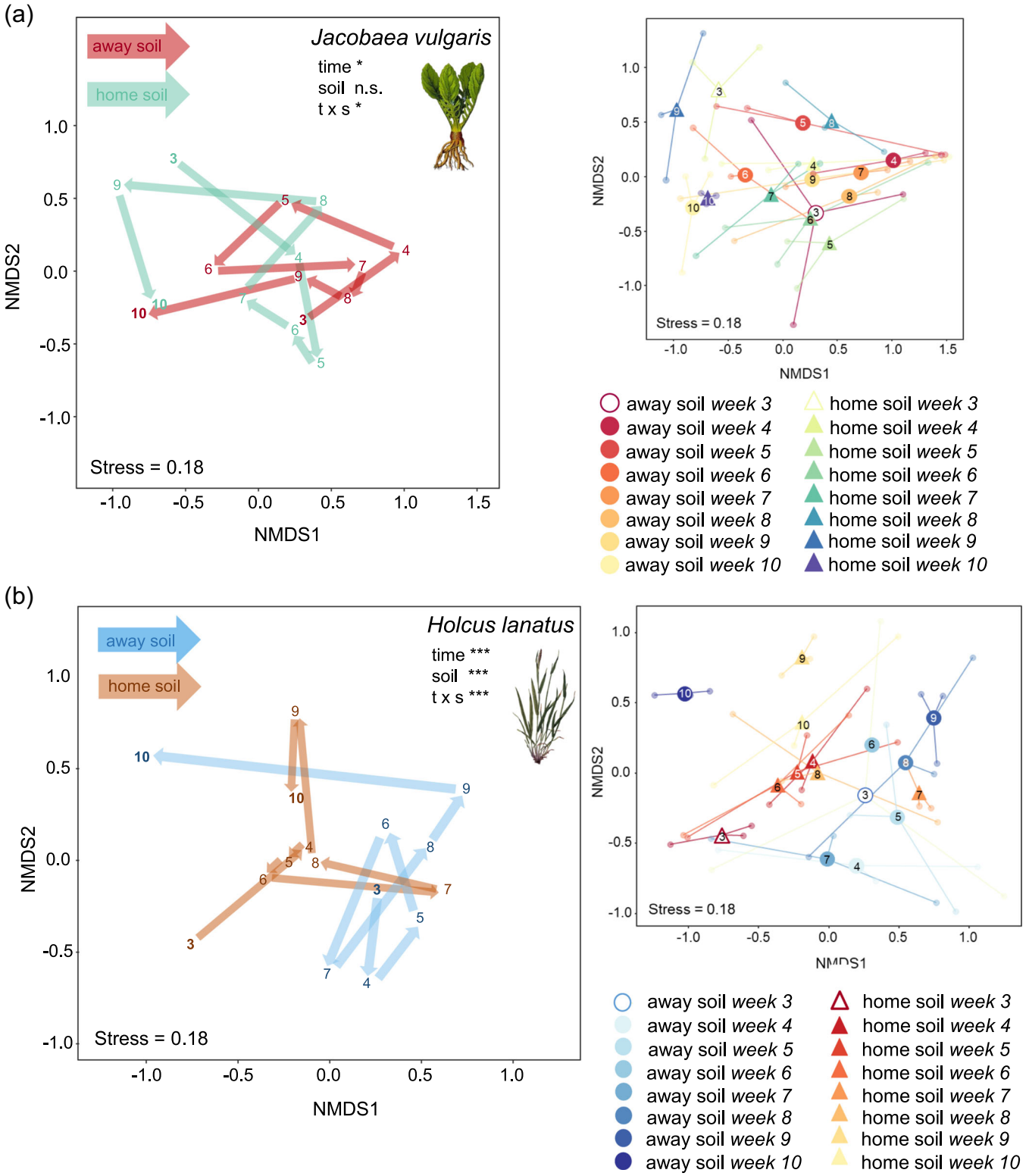
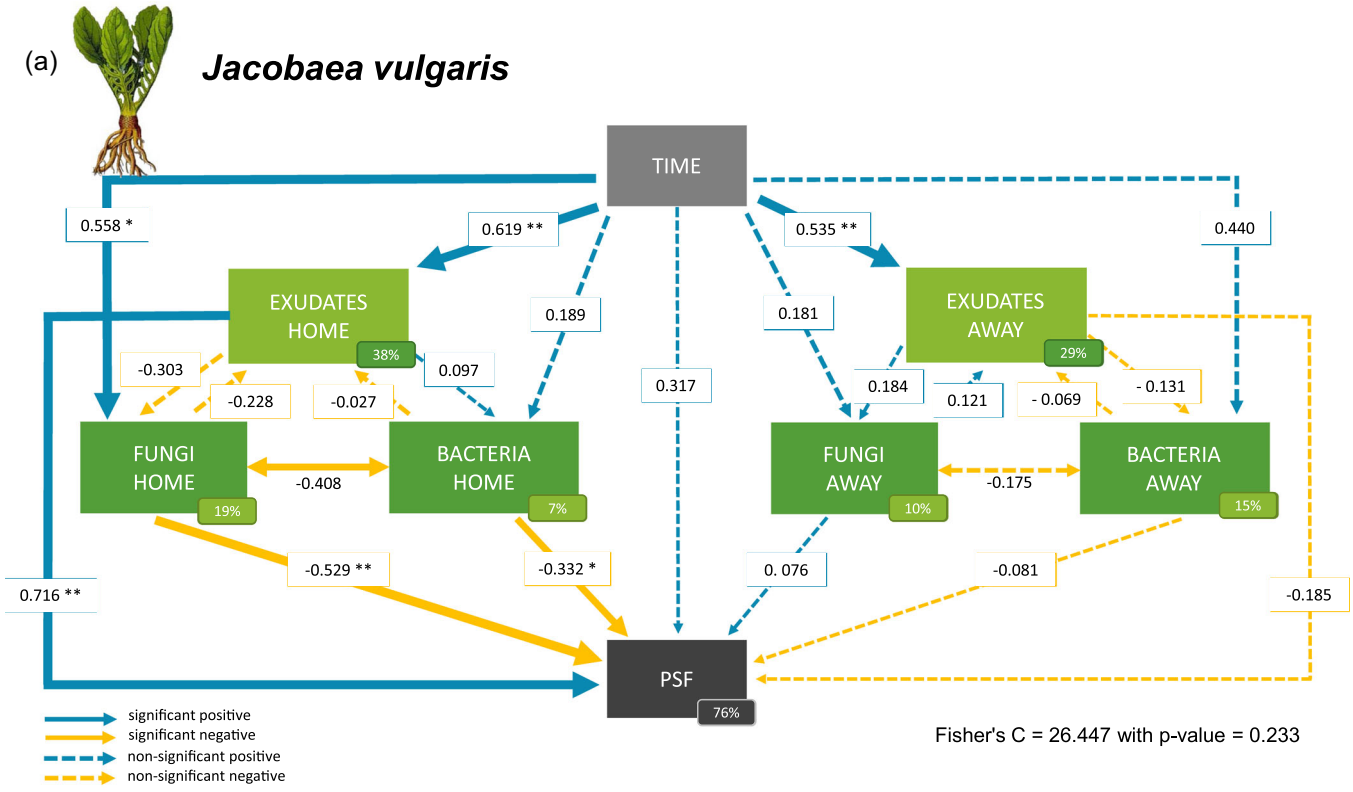


FIGURE 5 Nonmetric multidimensional scaling (NMDS) plots based on Bray–Curtis dissimilarity. The effects of (a) plant growth and soil conditioning on soil fungal community composition of *Jacobaea vulgaris* (red arrows = 'away soil' (*Holcus lanatus*—conditioned soil), turquoise arrows = 'home soil' (*J. vulgaris*—conditioned soil)), (b) plant growth and soil conditioning on soil fungal community composition of *H. lanatus* (blue arrows = 'away soil' (*J. vulgaris*—conditioned soil), brown arrows = 'home soil' (*H. lanatus*—conditioned soil)). Big panels of (a) and (b) display temporal shifts of rhizosphere fungal community composition, whereas small panels display the same temporal shifts including individual samples (small dots), and large dots represent averaged centroids. In small panel of (a) circles = 'away soil' (*H. lanatus*—conditioned soil), triangles = 'home soil' (*J. vulgaris*—conditioned soil), and in small panel of (b) circles = 'away soil' (*J. vulgaris*—conditioned soil), triangles = 'home soil' (*H. lanatus*—conditioned soil). Stress values are given for each NMDS. Asterisks represent significance levels (n.s. = not significant; *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Each fungal community composition had a total sample size of 48. [Color figure can be viewed at wileyonlinelibrary.com]



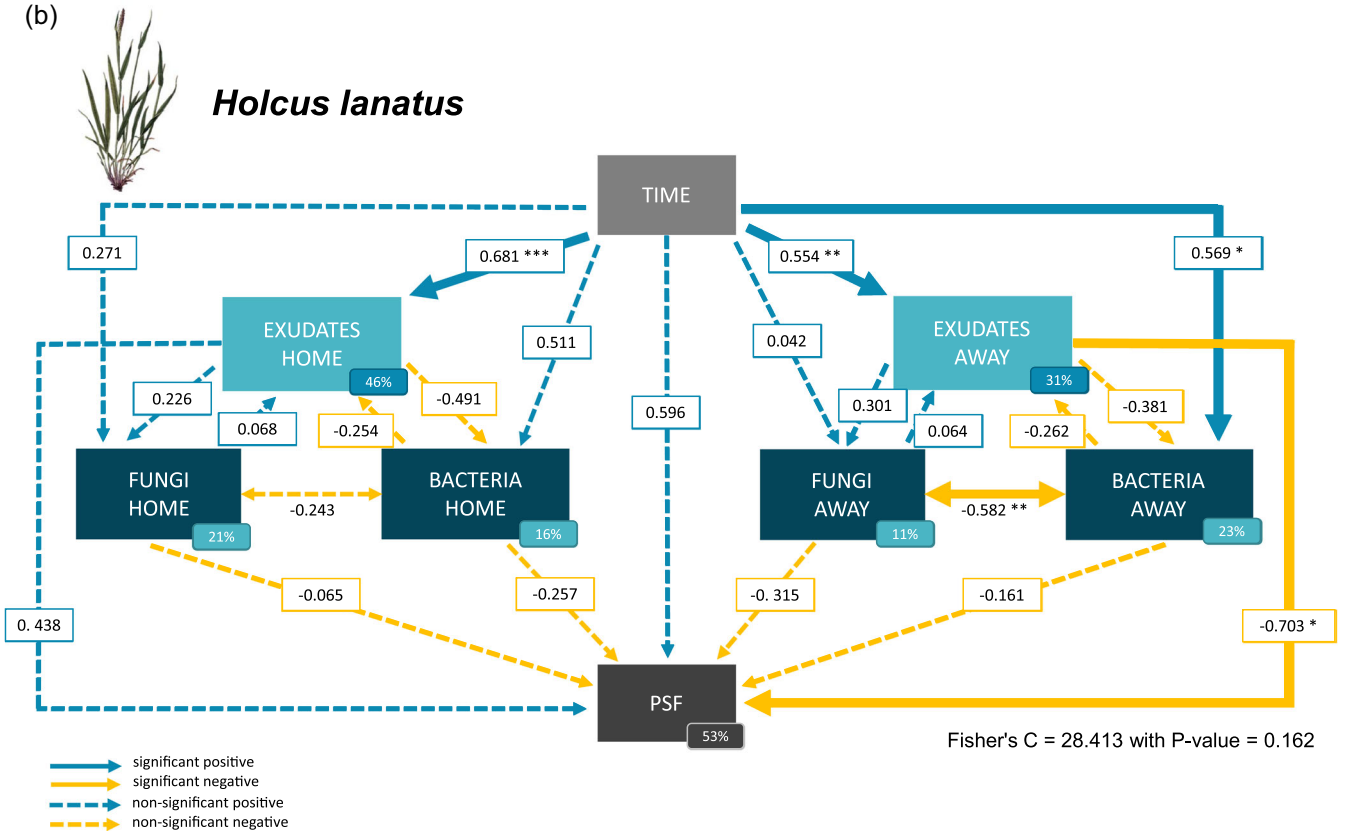


FIGURE 6 (See caption on next page)

significantly higher at Week 9 when grown in its 'home soil', and significantly lower in Week 6 only when grown in its 'away soil' (Tables S1 and S2; Figure S1b,e).

The fungal community in the rhizosphere of *J. vulgaris* was strongly affected by plant growth and to a lesser extent by soil conditioning (Table 2; Figure 5a). However, plant growth and soil conditioning led to strong compositional changes in fungal communities in the rhizosphere of *H. lanatus* (Table 3; Figure 5b). The diversity (Simpson's diversity index) of fungal communities in the rhizosphere of *J. vulgaris* was not significantly affected by plant growth or soil conditioning, however, it was significantly lower in 'away soil' for *H. lanatus* compared to 'home soil' (Tables S1 and S2; Figure S1c,f).

3.4 | Linkages between PSF, root exudate, and rhizosphere microbial diversity

Our path models suggest that temporal variation in PSF in both plant species over the growth period depends on temporal shifts in root exudate diversity (Figure 6), although the pathways differed between *J. vulgaris* (through home soil) and *H. lanatus* (through 'away soil'). More specifically, we found stronger positive effects of root exudate diversity on temporal increase in the slightly positive PSF of older *J. vulgaris* plants in 'home soil' (Figure 6a). By contrast, root exudate diversity in 'away soil' sustained negative PSF on *H. lanatus* during its growth (Figure 6b), however, the effect size of negative PSF tended to decrease over time (Figure 2f). Moreover, path models revealed that rhizosphere fungal and bacterial diversity were not influenced by root exudate diversity in either 'home soil' or 'away soil' for both plant species (Figure 6). However, we found that a moderate increase in fungal diversity over time in 'home soils' for *J. vulgaris* constrained its temporal shift towards neutral (slightly positive) PSF (Figure 6a). Direct effects of rhizosphere microbiomes on PSF in *H. lanatus* were further nonsignificant irrespective of 'home soil' or 'away soil' pathway (Figure 6b), whereas bacterial diversity in 'home soils' of *J. vulgaris* directly influenced (negatively) its PSF independent of its growth period (Figure 6a).

4 | DISCUSSION

In this study, we examined the temporal variation in PSF effects, root exudates and microbial communities within the rhizosphere of two common grassland species. Confirming our hypothesis, we found strong negative conspecific feedback effects for both plant species in their early plant growth stages. Notably, the negative conspecific PSF effect of *J. vulgaris* shifted in Week 5 to a neutral (slightly) positive PSF effect (Figure 2e). The strength of the conspecific PSF effects for *H. lanatus* also declined over time but the PSF of this species remained negative. These findings are consistent with previous studies and provide strong evidence that the strength of PSFs strongly depend on the plant growth stage of a plant (Bezemer et al., 2018; Hannula et al., 2021; Kardol et al., 2013). Emphasising the importance of considering such temporal shifts in PSF strength when designing and performing PSF experiments.

Beside temporal changes in PSF effects, the root exudation profile of both plant species strongly depended on the plant growth stage. Especially, *J. vulgaris* showed very distinct composition of root exudates in the first few weeks of growth, whereas they became temporally more similar when the plants grew larger (from Week 5 onwards). The root exudation profile of *H. lanatus* changed more gradually. Previous studies with common grass species, reported that plant biomass is positively correlated with carbon rhizodeposition and thus that root exudation increases when plants grow larger (Baptist et al., 2015). The exudation profile of *A. thaliana* also has been shown to strongly vary among different plant stages (Chaparro et al., 2013). The root exudate diversity of both plant species in our study increased considerably with the developmental stage of the plant individual, thereby emphasising that the process of root exudation is highly dynamic (Sasse et al., 2018). Interestingly, both the composition and diversity of root exudates did not differ between 'home soil' and 'away soil'. This indicates that the soil legacy of the previous plant (i.e., the changed soil microbiome) appears to have far less effect on the root exudation profile of the subsequent plant individual than the plant growth stage of the individual.

Plant root exudates have been shown to shape soil microbial communities in the rhizosphere (Oburger & Jones, 2018; Sasse et al., 2018; Steinauer et al., 2016). Soil microbes respond to these plant-derived metabolites which in turn determines their success to establish within the rhizosphere. Due to the continuous changes in root exudation profiles over plant growth, we expected that rhizosphere microbial communities would exhibit strong temporal dynamics. In our study, both rhizosphere bacterial and fungal community composition varied strongly on weekly basis. This is in line with previous studies, reporting temporal changes of soil and rhizosphere microbial communities at the scale of days (Zhang et al., 2011) to months (Hannula et al., 2019; Lauber et al., 2013). Our results can thus confirm that a seedling, a juvenile or an older plant shape their rhiosphere microbial community differently at least

FIGURE 6 Structural equation models of plant growth, plant-soil feedback (PSF), root exudate diversity (richness), Simpson diversity index of rhizosphere bacterial and fungal communities in 'home soil' and 'away soil' of (a) *Jacobaea vulgaris* and (b) *Holcus lanatus*. Numbers on arrows are standardised path coefficients. Single-headed arrows indicate directed relationships, and double-headed arrows indicate correlations. Blue lines and positive coefficient indicate positive relationships, whereas negative relationships are indicated in yellow and negative coefficients. Solid lines with path coefficients with asterisks indicate significant ($p < 0.05$) relationships, dotted lines indicate nonsignificant relationships. [Color figure can be viewed at wileyonlinelibrary.com]

for the two grassland species used in our study. Furthermore, the rhizosphere bacterial community composition of both plant species appeared to be different between 'home soil' and 'away soil'. For *J. vulgaris*, it seemed that the rhizosphere bacterial community was more dissimilar between 'home soil' and 'away soil' in the early stages of plant growth (Weeks 3–7) whereas they were more similar in a later plant developmental stage (Week 8–10). The rhizosphere bacterial community of *H. lanatus* was highly distinct between 'home soil' and 'away soil' at the start of the experiment, and converged only at Week 10. Moreover, for *H. lanatus*, the temporal pattern shows that in home soil, the rhizosphere bacterial community remains relatively constant over time (i.e., the rhizosphere bacterial community associated to *H. lanatus*), while in the away soil, the host plant *H. lanatus*, steers the rhizosphere bacterial community associated to *J. vulgaris* (the previous plant) towards the *H. lanatus* community. Why bacterial communities in both soils in which *J. vulgaris* was growing changed much more over time, remains further examination. We speculate that this is driven by changes in chemical composition in root exudates, but we cannot conclude this from our study. However, what our study shows is that host plants can affect the structure and development of rhizosphere bacterial communities over a period of several weeks. Similarly, the composition of the rhizosphere fungal community of both plant species differed among plant developmental stages, whereas 'home soil' and 'away soil' of *H. lanatus*, but not *J. vulgaris*, led to distinct rhizosphere fungal communities over the course of the entire experiment. These results are in line with previous studies, reporting that temporal changes in plant growth are leading mainly to changes in rhizosphere bacterial communities of *J. vulgaris* whereas rhizosphere fungal communities in grasses like *H. lanatus* showed to be affected stronger (Hannula et al., 2021).

Temporal changes in root exudate diversity seemed to strongly affect the variation in PSF in both plants. More specifically, the temporal variation in PSF in both plant species was mainly driven by the exudate diversity in *J. vulgaris*-conditioned soil. For *J. vulgaris*, increasing root exudate diversity in 'home soil' coincided positively with changes in PSF effects, whereas for *H. lanatus* this was true for its 'away soil'. Previous studies have indeed shown that *J. vulgaris* can shape a distinct rhizosphere environment potentially through specific exudation dynamics (Kowalchuk et al., 2006). Our results suggest that it could relate to the diversity of root exudates and their effects on the rhizosphere microbiome as revealed by our path models. Future experiments are required to establish causal relationships between root exudate diversity and the role of specific compounds in the exudates on rhizosphere microbiomes. This is particularly important for plant species such as *J. vulgaris*, where we know now that both root exudate diversity and PSF changes over its growth period.

In conclusion, this study demonstrates that the direction and magnitude of PSFs depends on plant growth stages. Especially, *J. vulgaris* showed strong directional changes in its PSFs—from negative to neutral (slightly positive)—in the early life stage, thereby highlighting the importance to consider temporal variability in PSF studies. We examined the importance of two potential key factors driving temporal shifts in PSF and found the root exudation profiles of both plant species to greatly depend on the plant growth stage. Furthermore, both rhizosphere bacterial and fungal community composition varied strongly on a weekly basis and were different between 'home soil' and 'away soil' (conspecific and heterospecific), which may also have contributed to temporal variation in PSF. Moreover, through linking these results in path-models, we could link shifts in PSF effects to temporal dynamics of root exudate diversity whereas changes of rhizosphere bacterial and fungal diversity effects on root exudate diversity and PSFs need to be investigated in more detail in future experiments. Furthermore, both shifts in root exudates and rhizosphere microbial communities could cause nonlinear shifts in PSF effects which would be recommended to test for in future studies. The importance of PSFs is increasingly recognised among ecologists and more detailed studies on root exudate metabolites (e.g., identity, concentrations, and individual functions) and their specific effects on temporal PSF effects together with how they associate with soil and rhizosphere microbiome are urgently needed to understand and predict the magnitude and direction of PSFs.

ACKNOWLEDGEMENTS

The authors would like to thank Martine Huberty for her support in growing *Jacobaea vulgaris* in tissue cultures. The authors thank Dr Benjamin Delory and an anonymous reviewer for several constructive suggestions on a previous version of the manuscript. This study was funded by a Vici grant from The Netherlands Organisation for Scientific Research (NWO VICI grant 865.14.006) to T. Martijn Bezemer. Madhav P. Thakur acknowledges the funding from the German Research Foundation (TH 2307/1-1). Alexander Weinhold, Henriette Uthe, and Nicole M. van Dam gratefully acknowledge support of iDiv to EcoMetEoR and MIE was funded by the German Research Foundation (DFG–FZT 118, 202548816).

DATA AVAILABILITY STATEMENT

Sequences created in this study were deposited in NCBI with accession number PRJNA925517. The data that supports the findings of this study are available in the supplementary material of this article. All data is available as supplementary information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Steinauer, K., Thakur, M.P., Emilia Hannula, S., Weinhold, A., Uthe, H., Dam, N.M., et al. (2023) Root exudates and rhizosphere microbiomes jointly determine temporal shifts in plant-soil feedbacks. *Plant, Cell & Environment*, 46, 1885–1899. https://doi.org/10.1111/pce.14570