

Article

Xanthan- and Gelatine-Based Composites Used as Nursery Groundcovers: Assessment of Soil Microbiology and Seedling Performance

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Abstract: In light of the significant impact of climate change, it is imperative to identify effective solutions to promote afforestation and reforestation operations, which are often constrained by a low survival rate. To mitigate the impact of weed competition and enhance water availability, biodegradable groundcovers comprising xanthan gum and gelatine were developed and evaluated over the course of the growing season in a nursery setting on narrow-leaved ash (*Fraxinus angustifolia*) and alder (*Alnus glutinosa*) in 3.5 L pots. The results demonstrated a beneficial impact of all groundcovers, particularly the gelatine-based ones, on both the aboveground and belowground growth rates. The efficacy of weed competition was controlled using gelatine-based groundcovers in the case of ash trees. Furthermore, the gelatine-based groundcover altered the soil physiochemical characteristics and affected the bacterial community while maintaining its role in increasing the soil nitrogen pool. In contrast, the xanthan gum-based groundcover was demonstrated to enhance microbial richness and diversity, with an augmented contribution to the soil nitrogen pool. However, further trials with diverse tree species and soil conditions are necessary to gain a more comprehensive understanding of these effects.

Keywords: xanthan; gelatine; groundcovers; soil; agriculture; forestry; mulch



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1. Introduction

Extreme events, such as floods and droughts, are becoming more common every year with a continuous reduction in recurrence intervals [1–5]. In 2023, southern Europe witnessed starkly contrasting meteorological conditions: the Iberian Peninsula faced widespread drought alert conditions according to the Combined Drought Indicator (CDI), while Italy endured heavy rains that caused extreme flooding in Emilia Romagna, resulting in estimated damages of EUR 9 billion [6,7]. The principal consequence of drought is a reduction in the photosynthetic activity of plants, as measured by the satellite-based fraction of the Absorbed Photosynthetically Active Radiation (fAPAR) anomaly indicator,

which was two times lower than normal in Spain in June 2023. Furthermore, the probability of wildfires increased to a level deemed a “high-to-extreme danger level” across the majority of the Mediterranean region during summer 2023 according to the European Forest Fire Information System (EFFIS) [6,8]. The second consequence is desertification, which consists of soil degradation due to the increased frequency, intensity, and duration of drought periods, accompanied by correlated negative effects such as soil sealing, higher surface temperatures, increased wildfire propagation, and sand and dust aerosols [9–11]. Approximately 25% of the total area of 1.68 million km² in southern, central, and eastern Europe is characterized by high to very high sensitivity to desertification, with an increase in the period between 2008 and 2017 of more than 177,000 km² [12]. Desertification may lead to a spiralling process, as unprotected soil is more susceptible to erosion and has reduced water infiltration, which increases its sensitivity to floods [13]. Desertification may also be amplified by land abandonment and lack of vegetation management as the higher risk of fires further contributes to erosion and soil degradation [14].

On the other hand, land management and afforestation/reforestation activities can reverse desertification, leading to proper soil restoration [12,14–16]. Moreover, afforestation and reforestation are also major tools to achieve both the climate change adaptation of land ecosystems and climate change mitigation [17,18]. In areas with a low risk of desertification, proper land and forest management is essential to reduce the wildfire risk with the catastrophic consequences of their propagation [19,20]. One of the challenges related to reforestation and afforestation activities is the low survival rate of plants in the first years, which is highly variable, with values ranging from 25 to 95% [12,21–23]. The main factors associated with the establishment success are vegetative material selection and the combined impact of weather (especially in terms of water availability [21,23]) and competition by aggressive weeds [24,25]. To facilitate plant acclimation to the planting site, it is essential to use high-quality seedlings, which led to the “target seedling concept”, i.e., to target specific physiological and morphological seedling characteristics that can be quantitatively linked with reforestation success [26]. Producing high-quality containerized seedlings requires adequate substrate and container choice and proper seedling management [27]. One of the key management decisions is weed management, which can be achieved through the use of herbicides, manual weeding, and the use of groundcovers [28]. Chemical weeding is a cost-effective solution whose applicability is increasingly restricted by EU regulations and environmental concerns and that may lead to damage in seedlings [29]. Manual mechanical weeding offers an alternative method that requires multiple operations throughout the growing season, entailing considerable costs [30]. Groundcovers, i.e., covering the soil around a seedling to prevent weed proliferation, is emerging as an attractive alternative [28,30]. The most common groundcover, widely applied in agriculture, consists of mulching films, which can be made of polyethylene, polypropylene woven mats, and, more recently, also biopolymers such as polylactic acid [31] and thermoplastic starch (e.g., Mater-Bi®) [32]. Alternatively to polymeric films, woodchips derived from pruning or forest operations can also be used. However, the main drawbacks of current commercial solutions include (i) the need to remove plastic films after a 5-year period, (ii) the high costs and uncertain durability of biodegradable polymers, and (iii) the challenges in applying woodchips in windy and steep areas [30].

Soil is crucial for maintaining the biosphere, but fluctuating precipitation patterns and soil drying can significantly impact soil functions. The composition and activities of beneficial microorganisms for plants are influenced by various soil conditions [33,34]. Several factors affect the dynamics of soil carbon, with soil moisture playing a pivotal role in plant development and microbial activity [35], affecting soil carbon dynamics such as litter decomposition, carbon output, heterotrophic respiration, and carbon input [36]. Plant roots and

soil microorganisms actively affect their surroundings by secreting extracellular polymeric substances (EPSs), which regulate soil moisture content [37]. Furthermore, plant-produced mucilage influences the rhizosphere soil's mechanical and hydrological properties, shaping soil dynamics [38]. Additionally, management techniques that induce physical or chemical modifications may alter the metabolic processes of soil microorganisms [39]. Groundcovers improve soil structure, increase organic matter content, and support beneficial microbial communities. This leads to better nutrient cycling and availability, ultimately enhancing agricultural productivity [40]. The aspect of moisture conservation is crucial for sustaining plant growth and mitigating drought stress [41]. Therefore, it is essential to thoroughly monitor potential changes in soil microbial communities and soil physiochemical parameters resulting from soil management strategies before implementing large-scale applications.

Considering that in the EU, the Green Deal sets the objective of planting 3 billion new trees by 2030, this challenge requires the availability of high-quality seedlings and cost-effective nursery operations. In this context, to further investigate the potential use of biopolymers for the production of groundcovers that combine soil protection, the inhibition of weed germination, and reduction in moisture loss and biodegradability, this work aims to assess the possible use of two biopolymers (xanthan gum and gelatine) combined with wood fibres as groundcovers (or topsoil covers) for potted seedlings in nursery applications. Despite the possible interest of using such biopolymers for groundcovering applications, particularly due to the ability of xanthan gum to improve soil water regulation properties [42,43], no other literature on this topic could be found. The effect of various formulations of fully bio-based xanthan gum and gelatine composites, used as pot-scale groundcovers, on early seedling performance was determined and the efficacy of these experimental materials was compared with a commercial biodegradable alternative and a locally made woodchip groundcover. Starting from these considerations, the present work aims to answer the following research questions:

1. Can the developed groundcovers improve early plant performance in terms of vegetative status and growth?
2. Are they effective in controlling weed proliferation?
3. Do they have any influence on the community composition and activity of the soil microbiota?
4. How do they perform compared to commercially available alternatives?

2. Materials and Methods

2.1. Materials

Commercial xanthan gum, with purity >91% and molecular weight (M_W) of $1 \cdot 10^6$ g/mol, was provided by Galeno srl (Prato, Italy); wood fibres STEICO® Flex-036, with an aspect ratio in the range 22.5–75 mm/mm, were provided by STEICO SE (Feldkirchen, Germany); vegetable glycerol (or glycerine), with a purity >98% and a M_W of 92.1 g/mol, produced by Farmalabor srl (Assago, Italy) was used as a plasticizing agent; citric acid monohydrate (CA), with a purity of 99.5% and a M_W of 210.14 g/mol, was supplied by Riedel-de Haën GmbH (Seelze, Germany) and used as crosslinking agent. Casein, with M_W of 23,700 g/mol, was supplied by Thermo Fisher Scientific Inc (Waltham, MA, USA) and used in the solution as protective coating. GELITA IMAGEL® LB, a type B gelatine powder with Bloom 113 (gel) and viscosity of 2.29 mPa·s (6.67%, 60 °C), was purchased from GELITA AG (Eberbach, Germany); tannic acid chestnut powder, with M_W of 1701.19 g/mol, was used as a crosslinking agent and was obtained from W. Ulrich GmbH (Eresing, Germany); sodium hydroxide (NaOH) was purchased from WHC GmbH (Schweitenkirchen, Germany) and used as microbeads for pH adjustment. Desalinated water with a conductivity of 15 μ S/cm at 20 °C was used for the material preparation.

2.2. Sample Preparation

Xanthan-based composites were prepared according to the detailed description reported in [42,44]. Glycerol and xanthan gum were mixed in a 1.2:1 weight ratio, then hot water ($T = 60\text{ }^{\circ}\text{C}$) was added and mixed for 3 min until reaching a homogeneous solution. Wood fibres (reduced in size using a laboratory grinder for 30 s) were added to the compound that was further mixed until homogeneity was reached. Citric acid was finally added as a crosslinking agent. Samples were then manually spread in the form of discs with diameters of 16 cm, dried at room temperature, and thermally treated at $165\text{ }^{\circ}\text{C}$ for 3.5 min in an oven to allow the crosslinking of xanthan gum. A protective layer based on an alkaline solution of casein was applied on the top side of each sample before the field tests. The casein coating was prepared according to the methodology described by Picchio et al. [45]. First, casein (10 wt%) was dissolved in 1M NaOH solution at $40\text{ }^{\circ}\text{C}$ under magnetic stirring; glycerol (50 wt% of casein) was then added, followed by incorporating tannic acid (10 wt% of casein) previously dissolved in 500 μL of 1M NaOH. The solution was then mixed for 1 h before use.

Gelatine-based composites were prepared starting from two different batches (A and B): batch A was composed of 12 wt% of the total amount of water, while batch B was composed of the 88 wt%. For the preparation of the tannic acid mixture (batch A), tannic acid was dissolved in water at room temperature and NaOH was added while stirring continuously until a pH of 9 was obtained. For the preparation of the gelatine mixture (batch B), gelatine was soaked in water at room temperature and, after 15–30 min of soaking time, heated to $55\text{ }^{\circ}\text{C}$ with constant stirring and stirred for a further 45–90 min until the gelatine was completely dissolved. Similarly to batch A, NaOH was added to the mixture to obtain a pH of 9.

Batch A was then gradually added to batch B under continuous stirring. Wood fibres were added to the mixture and stirred. This final mixture was poured into a mould and air-dried for two weeks at room temperature in front of an industrial fan before further processing. Finally, these sheets were cut into discs with an external diameter of 160 mm and an internal diameter of 30 mm, and cut in two halves, with a thickness of $(8 \pm 2)\text{ mm}$. The composition of xanthan-based and gelatine-based composites is listed in Table 1.

Table 1. Compositions of the innovative composites before drying (at the wet state).

Sample	Xanthan Gum [wt%]	Gelatine [wt%]	Glycerol [wt%]	Citric Acid [wt%]	Tannic Acid [wt%]	Fibres [wt%]	Water [wt%]
Xanthan-based composites	3.5	-	4.2	2	-	3.5	86.8
Gelatine-based composites	-	15	-	-	2	12	71

A commercial groundcover, Thermodisc[®] TD-16 produced by MYC-5 (Girona, Spain), composed of 90% natural fibres and 10% synthetic fibres, being fully biodegradable in 6–18 months (according to the producer technical data sheet), was used as a reference. Woodchips produced by chipping wood residues of the nursery, and applied as a 4 cm layer, were also applied and taken as a reference. The list of the tested samples is reported in Table 2.

Table 2. List of the applied groundcovers with the main technical properties.

Treatment	Material	Thickness [mm]	Grammage [g/m ²]
XW	Xanthan gum + wood fibres	3	900
GW	Gelatine + wood fibres	8	2400
NSF	Natural + synthetic fibres (Thermodisc®)	3	375
WC	Woodchips	40	8000
Control	No groundcover	-	-

2.3. Experimental Techniques

2.3.1. Evaluation of Groundcover Effects on Seedling Performance

The nursery study was conducted at the Central Nursery of the public company Forestal Catalana, SA, in Sant Feliu de Buixalleu (Girona, Spain; 41.729817, 2.571216), specialized in producing high-quality forest seedlings. The groundcovers were applied in 3.5 L pots with 16 cm in diameter at the top, on two species with high interest for forest restoration, narrow-leaved ash (*Fraxinus angustifolia*) and alder (*Alnus glutinosa*), both from Spanish Provenance Region 10—Coastal Catalonia. Seedlings were transferred from 300 (ash) or 200 (alder) cm³ containers to the 3.5 L pots just before installing the groundcover. The substrate was composed of forest soil (35%), composted pine bark (35%), blond peat (25%), and sand (5%), to which a slow-release fertilizer, Basacote® (NPK 16/8/12 + 2 MgO), was added at a rate of 3 kg/m³ substrate. The management during the vegetative period (2023) consisted of regular irrigation with sprinklers until water percolation. The irrigation dose was adapted to the daily temperatures and evapotranspiration. In summer, irrigation was applied every 1–2 nights.

For each species, a split-plot design with 27 trees per treatment was arranged in 3 blocks, giving a total of 135 experimental trees per species (see Figure 1). In each block, the treatments were randomly distributed in groups of 9 seedlings, arranged in a 3 × 3 quincunx design with seedlings 25 cm apart. Buffer trees in the perimeter of each block were also used to reduce possible edge effects (see Figure 2).

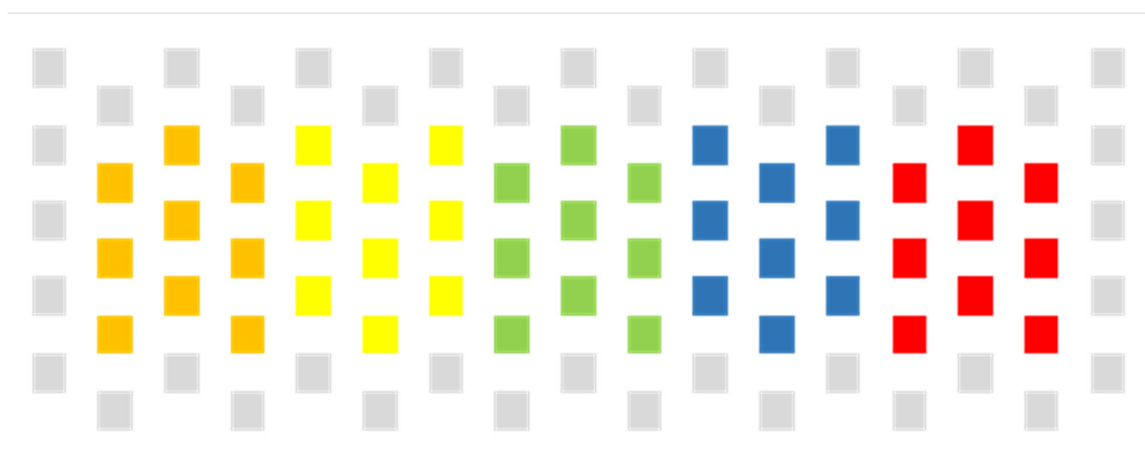


Figure 1. Arrangement of the experimental design of one block, for one species. The grey items indicate buffer trees while the rest of the colours correspond to experimental trees, randomly assigned to the five experimental treatments.



Figure 2. A picture of the experimental design in the nursery. It is possible to recognize the following samples' blocks (from bottom to top): XW, GW, NSF, control, and WC. Meanwhile, the perimeter consists of non-experimental buffer trees.

The experiment started on 7 March 2023 and ended on 19 October 2023. At the beginning and end of the study, the basal diameter of the trees was measured in tenths of millimetres with a digital calliper (the measuring point was marked with a permanent marker to ensure consistent evaluation) and the total height in centimetres was taken with a measuring tape. The aerial tree volume was evaluated considering a conical tree shape. The aerial tree volume growth rate, the diameter growth rate and the height growth rate were evaluated on annual timeframe as the difference between the final and the initial values. At the end of the experiment, biomass allocation was assessed in 10 ash seedlings per treatment, using the following procedure: trees were carefully uprooted in the laboratory, and the substrate was gently rinsed with tap water without damaging the roots. All broken roots of each seedling were recovered. Thus, seedlings were cut into three components: the (i) stem; (ii) coarse roots (>2 mm thick); (iii) fine roots (<2 mm thick). Root thickness was measured using a digital calliper. Each component was placed in aluminum trays and dried at 65–75 °C for 72 h. Finally, dry components were weighed with an accuracy of 0.1 g (laboratory scale) to calculate the dry matter of each component and the aggregated root biomass, total seedling biomass, and root:shoot ratio (ratio of belowground biomass to aboveground biomass).

During the initial and final measurements, and in addition in late June, the vegetative status of the trees was monitored, assigning each tree to one of the following categories: A (alive and healthy); B (alive but showing vegetative problems such as basal sprouting, leaf chlorosis, and loss of apical shoot); C (dead). At the end of June and at the end of the experiment, weed proliferation in each pot was assessed through three categories: (i) none (no weeds); (ii) low (1–20% of pot mouth covered with weeds); (iii) high (>20% of pot mouth covered with weeds).

The treatment effect on annual aerial growth (diameter, height, volume) and on each biomass allocation component was assessed by one-way ANOVA at a 5% level of significance on a sample size of 27 trees in the first case and of 10 trees in the second. When the assumptions of normality or homoscedasticity were not met, the data set was transformed by either the square root, logarithm, or inverse function, while tables and figures show untransformed data. Pairwise differences between treatments were assessed

using the post hoc Tukey test. Statistical analyses were performed using R software release 4.3.1 (the R Foundation for Statistical Computing, 2023).

2.3.2. Determination of Soil Properties

At the experiment endpoint, soil samples were collected, leaving the top organic layer intact. Soil samples from three points in each pot and from three replicate pots were mixed and homogenized to obtain a representative sample (Replicate 1). Thus, 12 groundcover samples (3 sampling replicates for 4 materials) and 3 replicates from untreated control samples were collected. The soil samples were transported to the laboratory in dry ice to maintain their freshness and prevent any potential degradation during the transport. Upon arrival at the laboratory, the samples were promptly stored at $-20\text{ }^{\circ}\text{C}$ to halt any biological activity and preserve the soil's chemical composition.

Soil physical properties

Soil moisture content (%) was determined using 3 g fresh soil sample subjected to oven drying at $105\text{ }^{\circ}\text{C}$ for 12 h, ensuring accurate measurement (by using a scale with 0.01 g measurement accuracy) while minimizing organic matter oxidation [46]. Additionally, a portion of the soil was air-dried at room temperature (approximately $22\text{ }^{\circ}\text{C}$) for one week (until constant mass was attained) to provide a realistic scenario.

Soil chemical properties

Five grammes of previously air-dried or oven-dried soil was combined with 50 mL of deionized water and agitated at 200 rpm for one hour. Following agitation, the mixture underwent gravity sedimentation for 30 min before pH and oxidation reduction potential (ORP) measurements [47].

Additionally, the oven-dried soil samples were finely ground, and a precise quantity was subjected to analyses for carbon (C) and nitrogen (N) content using dry combustion in an Organic Elemental Analyzer (Flash 2000 CHNS/O Analyzers, Thermo Scientific, Waltham, MA, USA). The total C and N content was determined as the mean value from three experimental and three technical replicates, expressed in grammes per kilogramme of soil dry weight. Heterotrophic soil respiration was assessed by rehydrating 5 g of dried soil in a 125 mL glass vial with 1.25 mL of deionized water (at a ratio of 4:1, soil to water, w/v). The vials were sealed with a grey butyl septum and metal collar, then incubated at $25\text{ }^{\circ}\text{C}$ for 72 h [48]. Headspace CO_2 concentration was quantified using gas chromatography (HP7890A, Agilent Technologies, Santa Clara, CA, USA), and soil respiration rates were calculated and reported as micrograms of CO_2 per gram of soil per hour [49].

2.3.3. Microbiological Analysis

Community-level physiological profiling

A Biolog EcoPlateTM (Hayward, CA, USA) assay was used to generate community-level physiological profiling (CLPP) for the microbiota in all the soil samples. Soil samples were prepared according to Koner et al. [50]. In brief, 5 g of the soil was thoroughly mixed in 45 mL sterile saline solution and after gravitational settling, the top layer was diluted to obtain the required dilution of 10^{-3} , which was used as an inoculum for the assay. An inoculum of 100 μL of the dilution was inoculated into each well of the EcoPlate and incubated at $25 \pm 2\text{ }^{\circ}\text{C}$ for 7 days and every day, data were measured at 595 nm using a plate reader (Infinite F200, TECAN, Männedorf, Switzerland). From the data, endpoints such as the average well colour development (AWCD) and substrate average well colour development (SAWCD) were assessed. The measured optical density (OD) was normalized by subtracting the OD values of the control (water) and the initial OD value (after adding the samples to the wells [51]).

Dominant and differentially abundant phylotype determination in soils

DNA was extracted from triplicate soil samples obtained from the experimental site. The DNeasy® PowerSoil® Pro kit (QIAGEN N.V. Venlo, The Netherlands) was used for the extraction and purification of DNA from approximately 0.3 g of soil samples, following the manufacturer's instructions. Subsequently, the integrity and purity of the DNA were assessed using 1.5% agarose gel electrophoresis. The quantification of DNA was performed using a fluorescence-based Qubit™ dsDNA High Sensitivity assay (Invitrogen, Carlsbad, CA, USA).

Approximately 20 ng μL^{-1} (~200 ng) DNA samples were sent to the STABvida genomic sequencing centre (Caparica, Portugal) for sequencing on the Illumina MiSeq platform, utilizing MiSeq Reagent Kit v3 for 300 bp paired-end sequencing of amplicons, with each sample generating between 50,000 and 100,000 reads. The library construction involved the utilization of primer sets 341F/785R [52] and ITS1f/ITS2 [53], targeting the bacterial V3-V4 variable region of the 16S rRNA gene and the fungal ITS1 region among the 18S-5.8S rRNA genes, respectively. The DNA fragments underwent sequencing, and subsequent quality control measures were applied to the raw data generated. Utilizing QIIME2 release 2023.9, the process included sequence quality assessment, feature table establishment, and taxonomy classifications [54]. Denoising of the reads was accomplished through the implementation of the DADA2 plugin [55]. Subsequently, the reads were then trimmed and low-quality regions truncated, the reads were then dereplicated, and chimeras were filtered. The reads were structured into features, serving as units of observation, specifically operational taxonomic units (OTUs), and subsequently categorized by taxon using a trained classifier. The scikit-learn classifier was employed for training, utilizing the SILVA (138.1) database with a clustering threshold of 99% similarity, and the UNITE (release 9) database with a dynamic clustering threshold. Classification criteria dictated that only OTUs containing a minimum of 10 sequence reads were considered significant. The raw reads were deposited in the NCBI SRA database for accessibility (PRJNA1140590).

Alteration in soil nifH gene copy number

The qPCR analysis utilized universal primer sets targeting the 16S rRNA and *nifH* genes, using primer pairs 341F and 518R, and polF and polR, respectively [56]. Extracted DNA samples were diluted 1:10 in nuclease-free water to reduce possible inhibition. Each 20 μL reaction mixture comprised 10 μL of SYBR green master mix (BioRad, Hercules, CA, USA), 1.25 μL of each primer (5 pmol), 4.0 μL template DNA, and 3.5 μL PCR-grade water. The CFX96 Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA) facilitated the amplification under specified thermal conditions: an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C (55 °C for *nifH*) for 60 s, followed by extension at 72 °C for 60 s and a subsequent melting curve analysis. The fluorescence signal during the extension step was captured, and threshold cycle (C_t) values were obtained. Relative fold changes in *nifH* abundance using $2^{-\Delta\Delta C_t}$ were performed as described by Geissler et al. [57].

3. Results and Discussion

3.1. Evaluation of Groundcover Effects on Seedling Performance

The vegetative status (Figure 3) of both plant species at the end of the growing season was predominantly good for both species, with no dead seedlings. In the case of ash, the only treatment leading to more than 11% of trees with vegetative problems was GW, amounting to 23%. In the case of alder, vegetative problems affected only 4% of seedlings in GW and WC, and 7% of NSF.

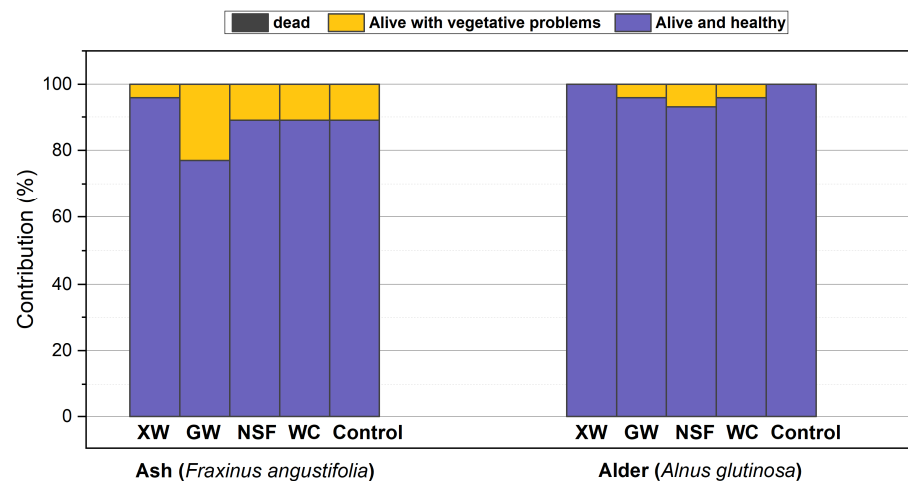


Figure 3. Results of vegetative status evaluations in the final measurement. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

The results for weed proliferation (Figure 4) show a different trend for ash and alder, with greater weed proliferation for ash, likely due to its lower shading capacity than the former. The best weed control results were obtained for ash trees with treatment GW in June and NSF in October. For alder trees, the treatments were almost equivalent in June, while the best results in October were observed with NSF and GW. The weed distribution within the pots (Figure S1) indicated that GW was very effective, as only a few weeds grew through the groundcover, mainly around the top perimeter, in the centre, and along the cut radius of the groundcover. Instead, the reduced efficacy of XW, probably due to its lower thickness and by a shrinkage in the first months, was evident as weeds grew throughout most of the pots, without any preferential region.

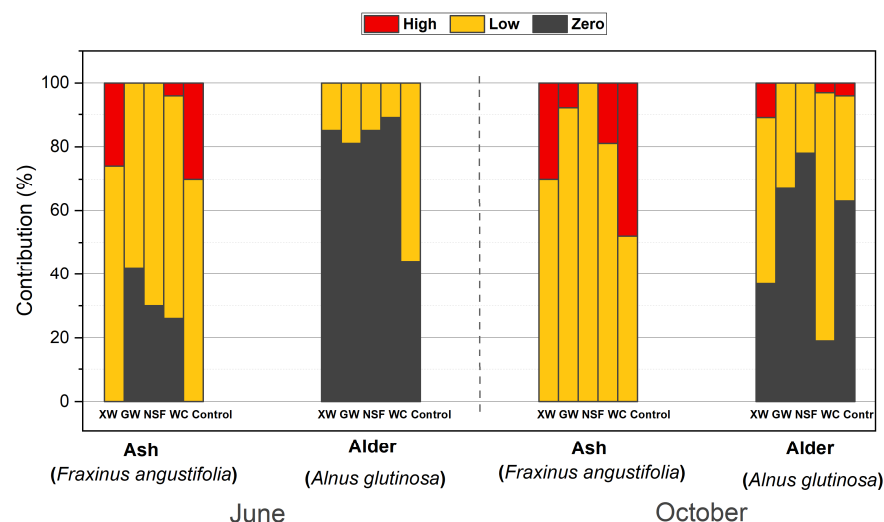


Figure 4. Results of weed proliferation evaluation performed in June (left) and October (right). XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

With regard to seedling growth rate (Table 3), treatment GW resulted in better ash diameter and aerial growth rate than all other treatments, doubling the volume growth rate. For alder, GW and WC led to higher aerial volume growth rate than the control, while XW and NSF did not show significant differences compared to any treatment. The aerial volume

growth rate of alder is more than twice that of ash and this different behaviour might also be influenced in various ways by the four treatments applied to control weed proliferation.

Table 3. Seedling annual growth rate. The asterisks indicate a significant difference between treatments. The letters indicate treatment grouping according to Tukey’s test. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

Tree	Treatment	Diameter Growth Rate [mm/year]	Height Growth Rate [mm/year]	Aerial Volume Growth Rate [cm ³ /year]
Ash	XW	8.2 ± 0.3 ^b	107.7 ± 4.6	45.8 ± 3.2 ^b
	GW	12.2 ± 0.5 ^a	103.0 ± 5.7	84.5 ± 7.8 ^a
	NSF	8.0 ± 0.3 ^b	99.0 ± 4.8	41.2 ± 2.5 ^b
	WC	7.7 ± 0.3 ^b	94.6 ± 5.2	38.0 ± 3.5 ^b
	Control	7.6 ± 0.2 ^b	104.6 ± 4.1	39.5 ± 2.4 ^b
	<i>p</i> -Value	<0.001 *	0.346	<0.001 *
Alder	XW	10.3 ± 0.3	134.8 ± 5.3	108.6 ± 6.4 ^{ab}
	GW	11.1 ± 0.5	143.0 ± 4.6	128.8 ± 9.0 ^a
	NSF	10.6 ± 0.6	134.5 ± 4.1	106.1 ± 8.7 ^{ab}
	WC	11.7 ± 0.5	136.2 ± 6.2	129.7 ± 8.1 ^a
	Control	10.3 ± 0.5	133.1 ± 4.5	99.8 ± 7.7 ^b
	<i>p</i> -Value	0.238	0.667	0.023 *

It was found no relation between weed proliferation and the total annual growth (Table 4): for both ash and alder, regardless of the weed proliferation level, the majority of plants (>85%) did not show any significant difference in the measured parameters. However, the total aerial volume was increased as a result of weed control in the case of ash (GW led to higher value than control) and alder (GW and WC led to higher value than control).

Table 4. Relation between weed proliferation levels (measured in June) and average growth of ash and alder (measured at the end of the growing season).

Tree	Growth Parameter	Weed Proliferation		
		Zero	Low	High
Ash	Diameter [mm]	13.4 ± 0.6	11.8 ± 0.2	11.7 ± 0.3
	Height [cm]	120.6 ± 4.6	117.0 ± 2.7	124.0 ± 6.8
	Aerial volume [cm ³]	60.8 ± 7.6	46.1 ± 2.4	48.7 ± 5.4
Alder	Diameter [mm]	15.9 ± 0.2	14.7 ± 0.4	-
	Height [cm]	173.8 ± 2.7	155.9 ± 6.8	-
	Aerial volume [cm ³]	122.7 ± 4.2	96.7 ± 6.6	-

The results of the biomass allocation test performed on ash trees (Figure 5 and Table S1) show that GW leads to the best outcomes in most indicators: higher stem biomass than any other treatment, higher total biomass than all treatments except for NSF, and higher root biomass than the control. Fine root biomass did not show significant differences between treatments. The root:shoot ratio of GW was significantly lower than that of NSF and WC.

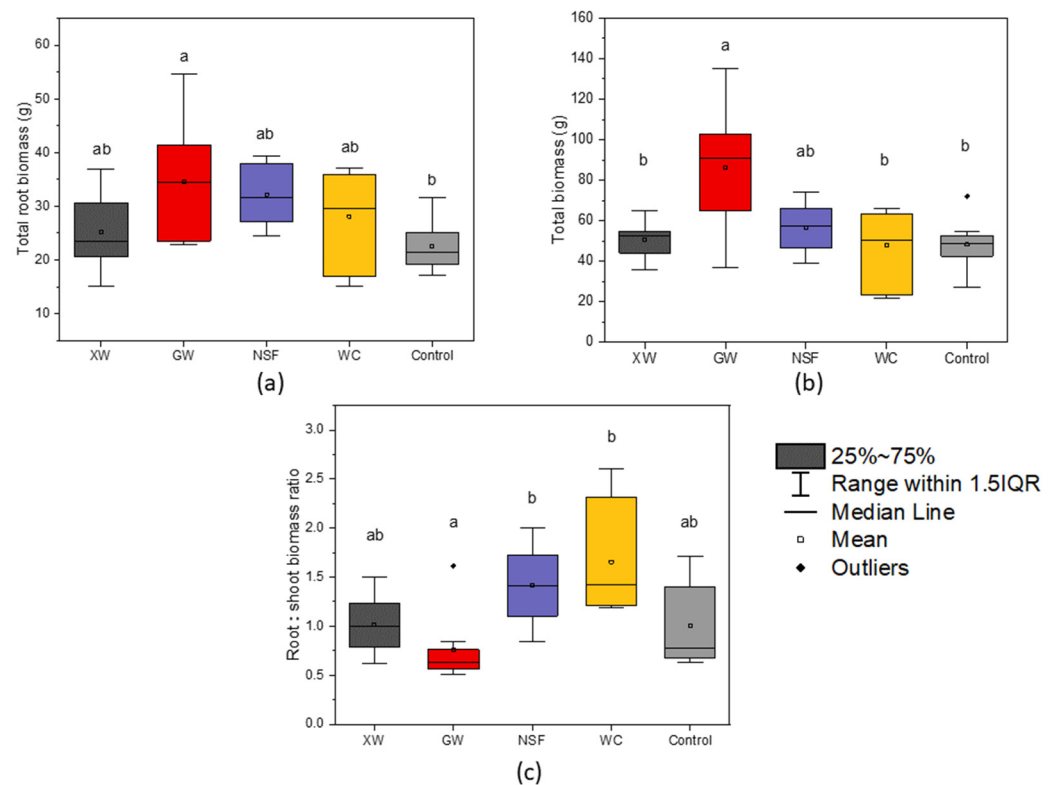


Figure 5. Results of ash biomass allocation: (a) total root biomass, (b) total biomass, (c) root:shoot biomass ratio. The letters indicate treatment grouping according to Tukey's test. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

3.2. Determination of Soil Properties

Soil Physico-Chemical Characteristics

According to the physical analysis of the soil samples presented in Table 5, no significant changes in moisture content were observed across the soil samples for both drying methods. The consistent moisture levels across all samples could be attributed to the placement of the groundcovers on the soil surface, which did not cause any irreversible changes to the soil. However, the pH of the GW-treated soil (pH = 6.1) significantly decreased ($p = 0.00063$) compared to the control (pH = 7.2) when the soil was dried at room temperature. Conversely, when dried in an oven, the pH of the soil in the control (6.7) significantly drops to pH 5.8 in GW- ($p = 0.00031$) and 6.2 in the WC-treated sets ($p = 0.0149$).

Table 5. Results of soil moisture and pH evaluation. The letters indicate treatment grouping according to Tukey's test. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

Samples	Moisture Content [%]		pH [-]	
	RT	Oven	RT	Oven
XW	34.4 ± 0.4 ^a	36.5 ± 21.4 ^a	7.2 ± 0.1 ^a	6.8 ± 0.02 ^a
GW	15.2 ± 4.7 ^a	31.9 ± 7.6 ^a	6.1 ± 0.3 ^b	5.8 ± 0.3 ^b
NSF	32.2 ± 12.5 ^a	43.4 ± 5.2 ^a	7.1 ± 0.2 ^a	6.7 ± 0.05 ^a
WC	27.1 ± 10.7 ^a	44.2 ± 10.6 ^a	6.7 ± 0.2 ^a	6.2 ± 0.05 ^b
Control	25.5 ± 8.5 ^a	43.0 ± 7.9 ^a	7.2 ± 0.2 ^a	6.7 ± 0.06 ^a

From the chemical analysis reported in Table 6, it can be observed that the ORP values significantly changed, with respect to the control, in soil sampled from GW and WC, while the addition of any of the groundcovers did not alter the soil nitrogen content significantly. However, the addition of WC (392.4 g/kg; $p < 0.0298$) significantly increased the total carbon content of the soils compared to the control (~316.1 g/kg). Furthermore, the heterotrophic soil respiration rate in the control soil (~28.5 $\mu\text{g CO}_2/\text{g}\cdot\text{h}$) significantly increased ($p < 0.0001$) with the addition of the groundcovers, including XW (~63.3 $\mu\text{g CO}_2/\text{g}\cdot\text{h}$), GW (~72.0 $\mu\text{g CO}_2/\text{g}\cdot\text{h}$), NSF (~39.7 $\mu\text{g CO}_2/\text{g}\cdot\text{h}$), and WC (~43.0 $\mu\text{g CO}_2/\text{g}\cdot\text{h}$). Heterotrophic soil respiration serves as an indicator of microbial community activity, reflecting crucial soil processes such as nutrient oxidation, mineralization, and solubilization [58–62]. These findings suggest that while nitrogen dynamics remain unchanged, some groundcovers significantly increased the soil carbon content and microbial activity in the soil.

Table 6. Soil chemical characteristics. The letters indicate treatment grouping according to Tukey’s test. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

Samples	ORP [mV]		N [g/kg of Soil]	C [g/kg of Soil]	Heterotrophic Soil Respiration Rate [$\mu\text{g CO}_2/\text{g}\cdot\text{h}$]
	RT	Oven			
XW	-51.3 ± 8.5^c	-27.0 ± 1.0^c	12.0 ± 1.5^a	331.4 ± 29.7^{ab}	63.3 ± 1.1^b
GW	19.0 ± 9.9^a	34.5 ± 16.3^a	12.9 ± 1.7^a	306.4 ± 32.5^b	72.0 ± 2.0^a
NSF	-45.0 ± 9.0^{bc}	-25.3 ± 3.1^c	10.8 ± 0.4^a	305.6 ± 12.5^b	39.7 ± 1.4^c
WC	-23.7 ± 12.6^b	5.7 ± 2.3^b	10.0 ± 1.6^a	392.4 ± 21.2^a	43.0 ± 2.4^c
Control	-53.0 ± 9.5^c	-23.3 ± 3.8^c	11.2 ± 1.0^a	316.1 ± 37.1^b	28.5 ± 1.2^d

The nonmetric Multidimensional Scaling (nMDS) and clustering analysis (Figure S2) performed on the soil’s physico-chemical properties reveal two distinct groups. All samples, except for GW, form one cluster, indicating that the addition of groundcovers containing gelatine and tannic acid significantly alters the soil’s physico-chemical properties in a manner distinct from other groundcovers and the control set without groundcovers.

3.3. Microbiological Analysis

3.3.1. Community-Level Physiological Profiling

The analysis of average well colour development (AWCD) from the Biolog data (Figure 6a) shows a consistent increase in AWCD values across the sample set during the incubation period, suggesting a gradual increase in microbial metabolic activity in this period. At any incubation point, XW showed the highest AWCD, suggesting an overall enhancement of microbial metabolic activity over time, possibly due to improved soil conditions or increased microbial population density. Conversely, the WC and control showed the lowest AWCD, implying lower metabolic activity of soil microbes.

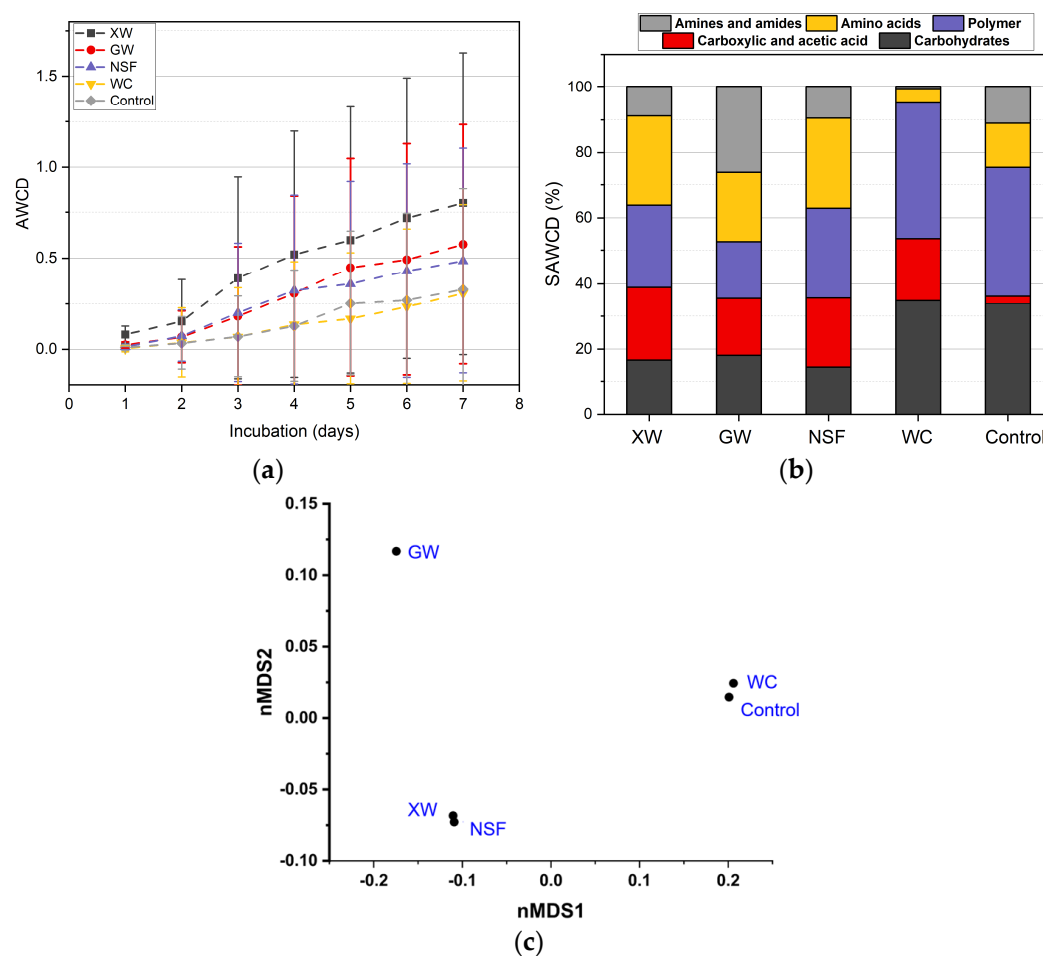


Figure 6. Results of community-level physiological profiling: (a) average well colour development, (b) substrate average well colour density, and (c) nonmetric Multidimensional Scaling (nMDS) of SAWCD values. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

Compared to the control group (~33.9%), the analysis of substrate average well colour density (SAWCD) at day 7 (Figure 6b) revealed a decline in carbohydrate utilization by the microbial community in all groundcover-treated soil samples except for WC (~35%). A similar trend is observed in the case of polymer utilization (control: ~39%, WC: ~41.8%). Instead, carboxylic and acetic acid utilization is notably higher in all groundcover-treated samples compared to the control group (control: 2.4%, others: >17.5%). Amino acid utilization also exhibited an increase in all samples except for WC (~4.1%) relative to the control (~13.8%). However, in the utilization of amines and amides by microbial communities, all treated samples demonstrate lower utilization rates (<9.5%) compared to the control (~11%), except for GW (~26.2%). These differences in substrate use patterns indicate varied effects of groundcover treatments on the composition and activities of the soil microbial community. The enhanced use of carboxylic and acetic acids suggests an increase in metabolic diversity and microbial adaptability resulting from the groundcover-treated soil samples. The lower utilization of carbohydrates and polymers, except in WC, could be attributed to the shift in the microbial metabolic potential. This pattern of carbohydrate utilization might reflect the changes in microbial metabolic diversity as a consequence of groundcover treatment.

Furthermore, the nMDS analysis of the SAWCD values shows a distinct pattern, where GW showed a unique utilization pattern as compared to others (Figure 6c). The composition of GW, gelatine, and tannic acid may promote the proliferation of proteolytic and phenolic compound-degrading microorganisms. This could potentially change the community metabolism distinctly from the other treatment groups. Conversely, the similar utilization pattern observed in XW and NSF could be attributed to the similar properties of these groundcovers.

The calculated dominance index indicates an increased dominance in the metabolic potential of the microbial community in the control soil, which decreases significantly in the presence of all groundcovers, except for WC (Figure S3a). This decline in dominance is further corroborated by lower Simpson diversity (Figure S3b) and Shannon diversity (Figure S3c) indices within the microbial community of the control soil compared to other samples. Notably, there are no significant changes observed in the evenness index across all samples (Figure S3d), indicating no significant changes in the distribution of metabolic activities. The higher dominance index observed in the control and WC-treated soil samples implies an uneven distribution of microbial metabolic activities. This is likely driven by the preferential utilization of specific carbon sources on the EcoPlates, limiting the functional diversity of the microbial community [63]. The XW, GW, and NSF treatment sets display higher diversity in metabolic potential, reflected by the broader range of carbon source utilization on EcoPlates and functionally diverse microbial distribution. Moreover, the constant evenness among all the samples suggests that the metabolic potential of microbial communities is influenced by the groundcover treatments without an imbalance in functional activities.

3.3.2. Dominant and Differentially Abundant Phylotype Determination in Soils

The analysis of the top 10 most abundant bacterial classes ($\geq 1\%$ relative abundance on the average) in the soil samples shows that *Planctomycetes*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Phycisphaerae*, *Vicinamibacteria*, *Verrucomicrobiae*, *Anaerolineae*, *Bacteroidia*, *Parcubacteria*, and *Acidobacteriae* are dominant across all the samples. As shown in Figure 7a, *Planctomycetes* abundance is decreased in all the samples as compared to the control. Bacteria belonging to the class *Planctomycetes* play a crucial role in the nitrification process, converting ammonium into nitrite and nitrate, facilitating their uptake by plants [64]. Alpha and Gamma proteobacteria exhibit various plant growth-promoting properties, such as mineral solubilization and the synthesis of plant growth hormones [65]. Bacterial classes such as *Phycisphaerae*, *Vicinamibacteria*, and *Verrucomicrobiae* are associated with diverse nutrient cycling processes, while *Anaerolineae*, *Bacteroidia*, *Parcubacteria*, and *Acidobacteriae* contribute towards nutrient cycling and organic matter decomposition. Moreover, a decline in the abundance of *Planctomycetes* may disrupt carbon degradation and cycling mechanisms, potentially impacting soil organic matter decomposition and carbon sequestration. Changes in the abundance of specific bacterial classes, such as *Alphaproteobacteria* and *Gammaproteobacteria*, can significantly impact soil carbon cycling and decomposition processes [66,67]. Furthermore, the nMDS followed by the clustering analysis of the normalized abundance of the 16S amplicon (Figure 7c) highlights that GW-treated soil showed a distinct cluster from the other soil samples, suggesting a different bacterial community composition that may have been caused by the antibacterial qualities of tannic acid present in GW [68].

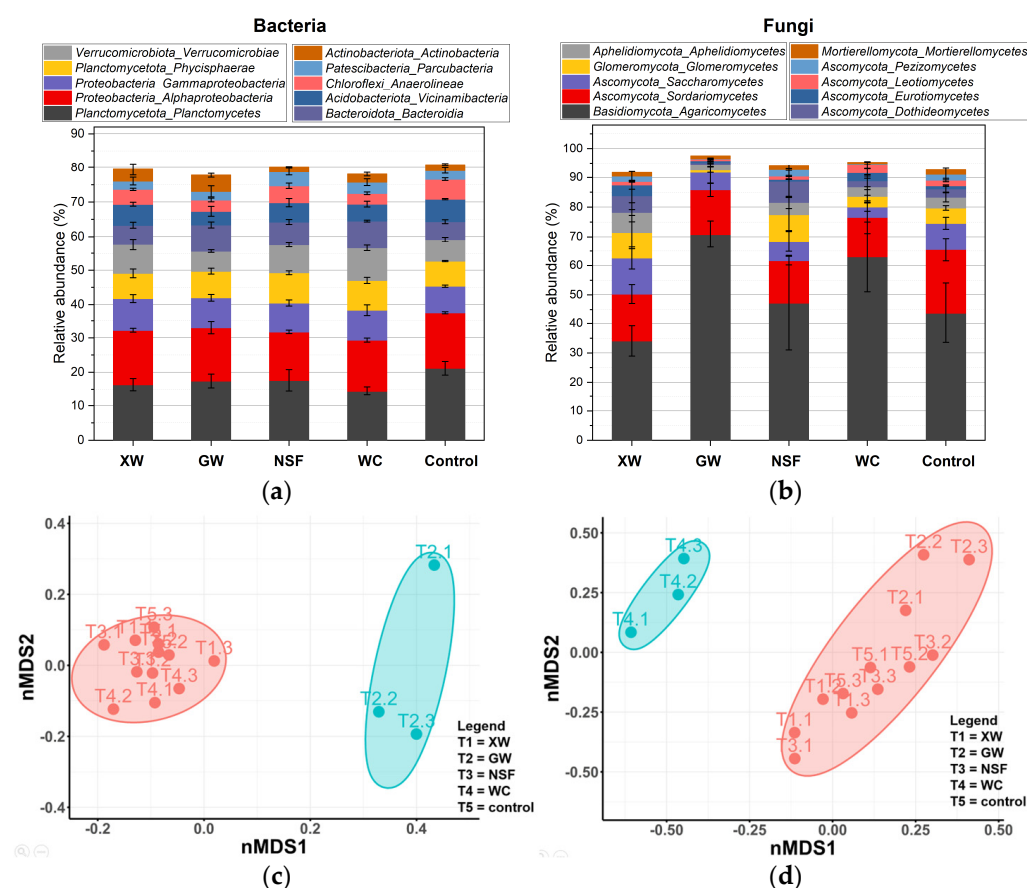


Figure 7. Results of phylotypes in soils: (a) bacteria, (b) fungi, and (c) nonmetric Multidimensional Scaling (nMDS) of bacteria and (d) of fungal communities as a result of groundcover treatment. The ellipse represents the 95% confidence interval. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermodisc®); WC: Woodchips; Control: No groundcover.

Similarly, the exploration of ITS sequences shows *Agaricomycetes* as the most abundant fungal class. Other abundant fungal classes include *Soradiomycetes*, *Saccharomycetes*, *Glomeromycetes*, *Aphleidiomycetes*, *Mortierellomycetes*, *Leotiomycetes*, *Eurotiomycetes*, and *Dothideomycetes*. The abundant *Agaricomycetes* fungi (maximized for GW) play a crucial role in carbon recycling through decomposing lignocellulose from plant residue [69]. *Sordariomycetes* are particularly important decomposers, breaking down complex polysaccharides and contributing to the production of soil organic matter [70,71]. *Saccharomycetes* are involved in fermentation processes and can be facilitated by leaf-decomposing fungi in specific carbon environments. *Glomeromycetes* are known to form symbiotic associations with plant roots, promoting plant growth and facilitating nutrient exchange [72]. However, no significant change in their abundance was observed between the samples.

On the other hand, clustering of the normalized abundance of the fungal genus (Figure 7d) shows that WC-treated soil has a distinct fungal composition from the control soil. The presence of wood pieces in WC, due to their lignocellulosic composition, might have favoured the development of lignocellulolytic fungi [73], resulting in a distinct clustering pattern for these samples compared to others. The abundance of *Trichoderma* spp., *Fusarium* spp., *Aspergillus* spp., *Ganoderma* spp., *Pleurotus* spp., *Chaetomium* spp., *Sistotremastrum* spp., and *Mortierella* spp. further confirms this (Figure S4).

Moreover, the analysis of the bacterial classes across different treatments (Figure S5a) shows that most bacterial classes across the soil samples are shared, with some unique taxa to WC and control samples. Similarly, the fungal class follows the same pattern

(Figure S5b), with unique taxa to WC samples. This might explain the abundance of ligninolytic fungal taxa.

The differential abundance of bacterial classes (Figure S6) analyzed using the DESeq2 package in R shows significantly different ($p < 0.05$) abundances of some taxa between the GW-treated samples and other groundcover-treated samples. This difference may explain the different cluster of these GW-treated samples in the nMDS plot (Figure 7c). Moreover, the clustering of the remaining samples into a single group might denote almost similar bacterial abundance. This could highlight the effect of tannic acid on soil microbes. Similarly, WC groundcover-treated soil samples were shown to have a significantly different fungal taxon (Figure S7) as compared to other groundcover-treated samples. This confirms the clusters in the nMDS plot (Figure 7d). Furthermore, the high lignocellulose content in the WC selectively promoted some lignocellulose-degrading fungi (Figure S4), resulting in the different abundance and different clusters.

Additionally, the calculated alpha diversity of bacterial (Table S2) composition within the samples revealed no significant change in the diversity indices between them. However, the GW-treated soil samples were shown to have lower richness, Shannon, and Fisher indices compared to other groundcovers and even than the control soil. In contrast, the comparison of the fungal alpha diversity within the samples (Table S3) showed that GW caused a significant decline in fungal richness, Shannon, and Fisher indices. This could validate the previously hypothesized influence of tannic acid and confirms its effect within the GW groundcovers on microbial populations.

3.3.3. Alteration in Soil *nifH* Gene Copy Number

The qPCR analysis using the $2^{-\Delta\Delta C_t}$ method (Figure 8) shows that the GW-treated sets have no relative fold change in *nifH* gene copies from the control soil. Despite an observable increase in *nifH* gene copy numbers in all the groundcover-treated sets (except GW), the change seems to remain significantly unaltered ($p > 0.1$). Generally, higher copy numbers of the *nifH* gene, which is necessary for biological nitrogen assimilation, may indicate that the microbial population will be able to fix more nitrogen, indicating healthy soil [74]. This implies that the groundcovers do not have significant impacts on *nifH* gene copies in soil, suggesting no impact of them on soil nitrogen fixation.

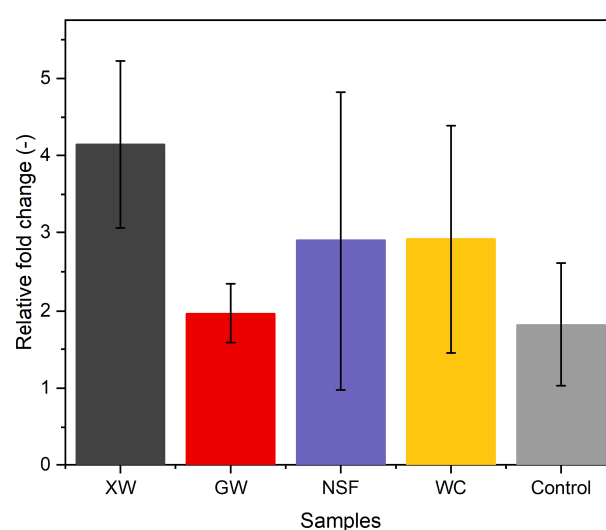


Figure 8. Assessment of relative fold change in *nifH* gene copies in the soil samples using the $2^{-\Delta\Delta C_t}$ method. XW: Xanthan gum + wood fibres; GW: Gelatine + wood fibres; NSF: Natural + synthetic fibres (Thermoflex®); WC: Woodchips; Control: No groundcover.

4. Conclusions

Innovative groundcovers based on xanthan gum (XW) and gelatine (GW) were tested for forest nursery applications and compared with two commercial solutions with regard to plant vegetative status, weed control, seedling performance, and soil properties. The treatments were applied to two different seedling types: ash and alder. For both seedlings, GW was the most effective treatment in terms of controlling weeds, and increasing ash aerial growth (surpassing all other treatments in terms of volumetric growth) and alder aerial growth (being the only one, together with woodchips, surpassing control treatment). XW was not efficient in impeding weed proliferation or in leading to a higher growth rate than the control, but it led to the best vegetative status. In the case of ash tree biomass allocation, the GW led to higher root biomass than the control, and to higher total biomass than XW, WC, and the control. It can be concluded that GW is a promising technique for increasing the nursery pot seedling quality. According to the endpoint evaluation of the soil physico-chemical properties, the groundcover based on gelatine (GW) modifies the overall characteristics of the soil (ORP, soil respiration, and pH) differently than the other groundcovers, including the control. This was further confirmed by the carbon source utilization pattern and distinct bacterial community structure in GW groundcover-treated soil. Further assessment of the groundcovers with different seedlings and soil types, as well as cost–benefit and LCA studies, should be conducted in the future to confirm the most favourable usage conditions for the innovative groundcovers.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su17031265/s1>, Figure S1: Results of weed distribution within the pot (observations performed in June); Figure S2: Nonmetric Multidimensional Scaling (nMDS) of soil's physico-chemical properties; Figure S3: Results of calculated (a) dominance, (b) Simpson index, (c) Shannon index, and (d) evenness; Figure S4: Relative abundance of *Trichoderma* spp., *Fusarium* spp., *Aspergillus* spp., *Ganoderma* spp., *Pleurotus* spp., *Chaetomium* spp., *Sistotremastrum* spp., and *Mortierella* spp. in soil samples; Figure S5: Shared and unique (a) bacterial and (b) fungal classes within the soils from different groundcover treatments; Figure S6: Pairwise comparison of bacterial classes in soil samples where red colour indicates significant ($p < 0.05$) difference: (a) T1 vs. T2, (b) T2 vs. T3, (c) T2 vs. T4, (d) T2 vs. T5; Figure S7: Pairwise comparison of fungal classes in soil samples where red colour indicates significant ($p < 0.05$) difference taxa: (a) T1 vs. T4, (b) T2 vs. T4, (c) T3 vs. T4, (d) T4 vs. T5. Table S1: Results of ash biomass allocation; Table S2: Alpha diversity calculated from the 16S sequences of the bacterial communities within the soil samples; Table S3: Alpha diversity calculated from the ITS sequences of the fungal communities within the soil samples.

Author Contributions: Conceptualization, F.V. and A.S.; methodology, J.C., J.B., A.S., A.A.C. and L.B.; validation, A.S. and F.V.; formal analysis, J.C., A.A.C., F.P. and G.C.; investigation, J.C., A.A.C., F.P., G.C. and L.R.; data curation, J.C., A.A.C. and F.V.; writing—original draft preparation, F.V., A.S., A.A.C. and J.C.; writing—review and editing, A.S., A.A.C., L.B., J.B., S.H., J.C. and A.D.; supervision, A.D., L.B., S.H. and J.C.; funding acquisition, A.D., L.B., S.H. and J.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are available on reasonable request to the corresponding authors.

Conflicts of Interest: Author Laura Ros was employed by the company Forestal Catalana SA. The remaining 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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