



Determination of root species composition in grass-legume mixtures by Fourier Transform Infrared (FTIR) spectroscopy

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Abstract

Background and aims Determining belowground species composition is vital in understanding inter-specific competition or niche complementarity in grass-legume mixtures. This study explored the potential of Fourier Transform Infrared (FTIR) Spectroscopy to determine root composition in a three-species grass-legume mixture containing perennial ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.), and red clover (*Trifolium pratense* L.) under field conditions.

Methods Artificial root mixtures (Artmix) ($n=66$) and pure root (Pure) samples ($n=90$) of three species were prepared using roots collected from a field experiment in Denmark. Two FTIR models were calibrated using Artmix and Pure samples, and validated with leave-one-out and blind validation (BV) methods. The Artmix FTIR model was used to determine the belowground species composition of mixed root

biomass samples, and we related the belowground and aboveground species compositions.

Results FTIR spectra ($600 - 4000 \text{ cm}^{-1}$ wavenumbers) of three species were successfully differentiated in PCA and cluster analysis. Both Artmix and Pure FTIR models exhibited strong predictive performance in calibration (high R^2 [≥ 0.96], excellent RPD [> 5], and low RMSEP [< 5]). During BV, the Artmix model showed marginally better performance (RMSECV = 3–5; Bias = -1 to 2) than Pure model (RMSECV = 3–8; Bias = -6 to 2).

Conclusion This study showed that FTIR spectroscopy is a promising approach to determine species-specific roots in grass-legume mixtures. It has the potential to give improved insight into species complementarity and thus could be used to design mixtures aiming at improving nutrient use efficiency, yield stability, and soil C sequestration potential.

Keywords FTIR Spectroscopy models · Grass-legume mixtures · Root differentiation · Niche complementarity

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Introduction

Grasslands that contain grass and legume species are a key component in dairy crop rotation systems in Northwestern Europe (Cong et al. 2018; Jensen et al. 2024, 2025; Lüscher et al. 2014; Soussana et al. 2010). Owing to the niche complementarity,

facilitation and resource partitioning, these grass-legume mixtures often show higher aboveground biomass yields compared to the pure stands of the same species (Finn et al. 2013; Grange et al. 2021; Nyfeler et al. 2009; Sanderson et al. 2013). Above- and belowground interspecies relations are generally assumed to be equally important in ensuring the high performance of the mixtures (Dirks et al. 2021; Hadir et al. 2024; Streit et al. 2019). Although many studies focus on aboveground biomass formation in grass-legume mixtures, studies on belowground biomass are limited. So far, root species identification and quantification have been one of the major challenges in studying the belowground interspecies relations in grass-legume mixtures. A rapid and comparably inexpensive method for root species identification and quantification is lacking, as many available methods, such as visual morphology inspection, DNA-based techniques, and radioisotope labeling, often require comprehensive training, extensive resource utilization, and longer processing times (Rewald et al. 2012).

The utilization of Infrared spectroscopy (IR) for root taxa identification has been recognized as more advantageous and precise compared to other methods, due to its non-destructive nature, rapid analysis of large sample sets, non-use of chemicals, minimal sample preparation, and minimal sample materials requirement (Rewald et al. 2012; Straková et al. 2020). In plant science, several studies have demonstrated the potential of IR spectroscopy methods, such as Near-infrared reflectance (NIR) spectroscopy (Lei and Bauhus 2010; Locher et al. 2005; Roumet et al. 2006) and Fourier transform Infrared (FTIR) spectroscopy (Hadir et al. 2024; Legner et al. 2018; Naumann et al. 2010; Rewald and Meinen 2013; Straková et al. 2020; Streit et al. 2019) as a rapid and successful tool for plant root differentiation. However, with the progressive improvements in IR spectroscopy methods, FTIR has become more popular due to its high accuracy and rapid measurements (Rewald et al. 2012). Compared to NIR, FTIR can display more detailed spectra between 600–1500 cm^{-1} wave numbers, which is termed the “fingerprint region”, a highly characterized area for a particular substance, benefiting accurate identification and differentiation of substances, and has higher signal-to-noise ratios (Clarke et al. 2002; Rewald et al. 2012).

The FTIR spectroscopy method employs the mid-IR spectrum (400–4000 cm^{-1}) and reports an interferogram (the outcome of reflected waves), which is Fourier-transformed to a transmission spectrum or an absorption spectrum (Berthomieu and Hienerwadel 2009; Rewald et al. 2012). Studies have proven the successful application of FTIR spectroscopy in plant root analyses, such as discriminating crop and weed species (Meinen and Rauber 2015); analyzing root proportions of winter wheat (*Triticum aestivum* L.) and winter faba bean (*Vicia faba* L.) in a mixed cropping system (Streit et al. 2019); differentiating spring wheat and faba bean (Hadir et al. 2024); and quantifying root species composition in peatlands (Straková et al. 2020). Further, reports are also available indicating the successful differentiation of cultivars of the same species (in pea [*Pisum sativum* L.], maize [*Zea mays* L.], and oat [*Avena sativa* L.]) using FTIR spectroscopy (Legner et al. 2018; Naumann et al. 2010).

Although studies on plant root analysis using FTIR spectroscopy are available, research using FTIR for root quantification and identification in grass-legume mixtures is limited. For example, Dirks et al. (2021) tested the species-specific root composition using an FTIR model that contained only two species: white clover and perennial ryegrass. Yet to our knowledge, no studies have attempted to determine root species composition of diverse grass-mixtures, with higher levels of diversity or with multiple species of the same functional group. Furthermore, there is a lack of methodological comparisons of how FTIR models are best designed with regards to accuracy and workload. Grassland mixtures containing grass and clover are widespread, and many studies are ongoing to develop better mixtures regarding yield gain, yield stability, and other benefits, such as enhancing soil C storage (Jensen et al. 2025; Mortensen et al. 2025). The information about root species composition in grass-legume mixtures is important in developing grassland mixtures for enhancing belowground species complementarity. Therefore, the present study tested the application of FTIR technology to determine the species-specific root proportions in grassland mixtures using a three-species grassland mixture that contains perennial ryegrass, white clover, and red clover using two different approaches to establish the calibration models. Subsequently, we demonstrated how the FTIR model can be used to quantify belowground species composition in root biomass samples from a

previous study involving a grass-clover mixture of the same three species, where the aboveground species composition also was quantified.

Methodology

Experimental site

This study was conducted in a long-term field experiment at Foulumgaard Experimental Station, Aarhus University, Denmark (56°29'N, 09°34'E). The average annual rainfall and the temperature of the experimental site are 673 mm and 8.2°C, respectively (means of 1991–2020). The soil is loamy sand, and the soil type is Typic Hapludult (Soil Survey Staff 2014). The seed mixture consisted of 82% perennial ryegrass, 16% white clover, and 4% red clover and was sown at a seeding rate of 26 kg ha⁻¹ (Jensen et al. 2024). More details are provided in Jensen et al. (2025). For FTIR model building, root samples from perennial ryegrass, white clover, and red clover were collected in October 2024 from unfertilized grass-clover swards established by undersowing in spring barley harvested as a whole crop. After calibrating and validating the FTIR model, we used root samples and aboveground species composition data from a previous study (Jensen et al. 2024) within the same experiment. This allows us to test the application of the model based on 1- to 4-year-old mixed grass-clover swards with two N-application rates (0 and 300 kg total-N ha⁻¹ in cattle slurry). The aboveground species composition data are based on the total herbage yield (sum of three cuts) in 2020, while the root samples were based on the sampling conducted after the 3rd cut in 2020 (mid-September).

FTIR model building

Sample collection and preparation

The experimental field (spring barley with grass-clover mixture undersown) was divided into sections, and five to six living plants per species were randomly sampled, representing all growth stages. During this process, two sets of samples were collected to obtain separate sample sets for calibration and blind validation. The roots were sampled by gently digging up individual plants up to 20 cm in depth,

ensuring that the above- and belowground biomass belonged together, i.e., that the roots were connected to the main stem. Root samples were stored at 2°C until further processing. Root washing was conducted using a 425-µm sieve, with all loose root fragments, dead roots, and additional organic particles on the root samples being carefully removed using forceps during the washing. The washed roots were then oven-dried at 60°C for 48 h and milled using an oscillating ball mill.

Preparation of artificial root mixtures

For external validation and calibration purposes, artificial root mixtures were prepared using ball-milled roots of perennial ryegrass, white clover, and red clover. Artificial mixtures were designed using a ternary diagram (Fig. 1) and spreading all mixtures evenly to cover the entire range of possible root compositions across the three species. In total, 50 mg of 66 artificial root mixtures and monocultures were used for the calibration process, and the concentration of each species in artificial root mixtures ranged from 0 to 100% on a dry mass basis (Supplementary Table 1). Three physical replicates per prepared mixture were

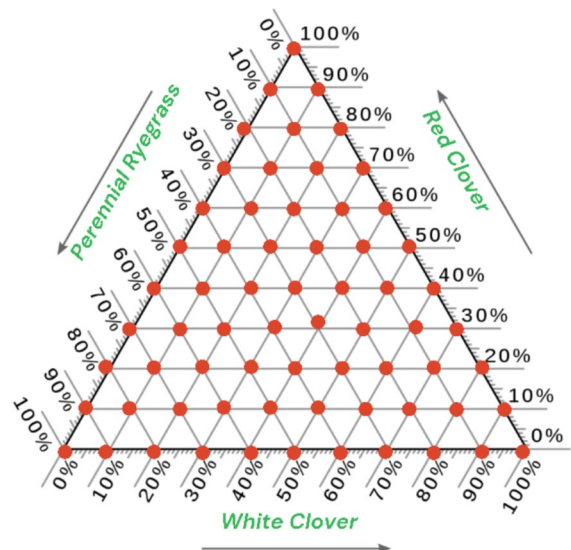


Fig. 1 Ternary diagram, illustrating the composition of artificial root mixtures and monocultures (in the corners) of perennial ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.), and red clover (*Trifolium pratense* L.), which were used for the calibration model, using three replicates of each mixture or monoculture

used during FTIR measurements, resulting in 198 samples. Another 20 artificial root mixtures based on new genotypes not included in the calibration set were prepared as an isolated data set for the model validation (Supplementary Table 2).

Pure root samples for model building

Following the suggestion of Straková et al. (2020) to use the pure root samples for model building, we used thirty physical replicates of each species to build a model using pure root samples, resulting in a total of 90 spectra for the model. The root materials used for the pure root model were also utilized to obtain the spectra for species identification, as described below.

FTIR Measurements

The spectra were recorded using a Bruker Invenio S FTIR spectrometer (Bruker Optics, Germany) equipped with an HTS-XT microplate reader and a liquid nitrogen-cooled MCT (LN-MCT) detector. The wells of a reusable 24-well microplate were carefully filled with root samples (in the form of dry powder), with approximately 50 mg of material placed in each well. During sample preparation, particular attention was paid to ensuring that the surface of each sample was as smooth and level as possible. Spectra were recorded using the OPUS software (Version 8.7.31, Bruker 2021) between 600 and 4000 cm^{-1} wavelengths with 4 cm^{-1} resolution and an average of 60 scans. We selected these parameters following available reports to ensure adequate peak separation and to enhance signal-to-noise ratio (SNR) (Meinen and Rauber 2015; Naumann et al. 2010; Straková et al. 2020). The three replicates of each artificial root mixture sample were measured, and the average spectrum of each mixture was considered for multivariate data analysis. Thirty physical replicates were measured in samples containing 100% of each species (pure root samples). The peaks of the spectra of pure root samples were recognized using the “Peak picking” option in OPUS software (Version 8.7.31, Bruker 2021).

Building of calibration and validation models

The OPUS software was used to analyze the data, and the QUANT 2 package was used to build the calibration model (Version 8.7.31, Bruker 2021). The

spectra were preprocessed before performing any further analysis. Offset correction, 1st derivative (9 smoothing points), and vector normalization were the selected preprocessing methods to set the minimum absorbance value to zero, highlight the steep edges of peaks in a spectrum, and address the difference in the thickness of samples, respectively (Meinen and Rauber 2015; Straková et al. 2020; Streit et al. 2019). Only the spectral range between 600–1800 cm^{-1} was included in the multivariate data analysis and model building, as this fingerprint region is distinctly characterized for a given species (Rewald et al. 2012; Skrabal 2012). The principal component analysis (PCA) and cluster analysis (Ward’s method with Manhattan distance) were performed to define and group the three species using the spectra of pure root samples (Kemper et al. 2022; Robledo and Cuestas 2020).

Two calibration models were constructed using either artificial root mixtures (artificial mixture model) or pure root samples only (pure root model). The pure root model contained 30 spectra per species, resulting in a total of 90 spectra for the model, whereas the artificial mixture model contained 66 spectra. The calibration models were built using the Partial Least Square Regression (PLSR), where spectra were the independent variables (x) and the percentages of each species were the dependent variables (y). The models were separately validated using the leave-one-out method and a blind validation method (Meinen and Rauber 2015; Straková et al. 2020; Streit et al. 2019). In the leave-one-out method, each sample from the calibration set is sequentially excluded and used as the calibration datum, repeating this process until all the samples in the calibration set have been used as the calibration datum (Standard method OPUS 8.7.31). Since the artificial mixture model consisted of 66 mixtures, it was validated 66 times using the averaged spectrum (average of three replicates) of all the individual artificial root mixtures. Similarly, the pure root model was validated 90 times as it contained 90 spectra.

In blind validation, the performance of the two developed models was tested and evaluated using an isolated dataset based on 20 artificial root mixtures with known percentages of each species. This dataset was obtained from different root samples from the same field. The model optimization was performed automatically using the “optimize” option in OPUS

software (Standard method OPUS 8.7.31). Further, the number of PLS factors included in the calibration of the model (rank) was minimized to consider a ratio of 1:10 (approximately) between the number of samples and rank in the calibration data sets for the whole process of model development to avoid model overfitting (Lei and Bauhus 2010).

Application test: determining belowground species composition at the field level

Belowground sampling

The auger method (inner diameter of the core: 5.5 cm) with a core-sampling machine was used to sample the roots to 10 cm depth, and six soil cores per plot were randomly sampled in the in-row area of grass-clover swards. The grass-clover was established by undersowing in spring barley and the row distance was 12 cm. More details about the root sampling strategy within a plot are available in Jensen et al. (2024). The collected samples were stored at 2 °C until further analysis. Then, the roots were washed on a 425- μ m sieve using tap water until they were free from soil and extraneous organic materials. The cleaned roots were then oven-dried at 60 °C for 48 h, and a representative sample per plot was prepared by mixing six replicates. Oven-dried root samples were ball-milled and root species compositions were measured using the FTIR model. For the present study, we limited our test to the samples from the in-row area in 0–10 cm depth, as only enough oven-dried root material was left for this specific depth and location.

Aboveground sampling

The grass-clover swards were cut four times a year. For the present study, we only considered the sum of the first three cuts, as we sampled the roots after the 3rd cut. The perennial ryegrass, white clover, red clover, and weeds were manually separated from a 300 g sub-sample and oven-dried at 60 °C for 48 h to determine the aboveground species composition. More information about harvesting, aboveground yield, and species composition is available in Jensen et al. (2024). The harvested material had a small proportion of unsown species, which were ignored in this study.

Results

Spectra of root samples and root differentiation using PCA and cluster analysis

The spectral pattern of perennial ryegrass, white clover, and red clover roots showed similar peak distributions between 3700 and 3000 cm^{-1} and 3000 and 2800 cm^{-1} , yet with different peak heights, where perennial ryegrass had lower absorbance levels than white clover and red clover roots. Further, the spectra of perennial ryegrass, white clover, and red clover roots showed clear peak distributions of different heights in the fingerprint region (between 1800 and 600 cm^{-1} wavenumbers), showing their highest peaks at 1129, 1120, and 1118 cm^{-1} , respectively (Fig. 2b, c). Within the range of 1800 and 1450 cm^{-1} , white and red clover showed a similar peak distribution with approximate absorbance levels (about 1.40 and 1.42, respectively), and perennial ryegrass showed a lower absorbance level (~ 1.16).

The heterogeneity of the perennial ryegrass, white clover, and red clover roots was explored using PCA analysis and cluster analysis (Fig. 3). The first two PCA components captured 80% of the total variance in the spectral data of all three species, where PC 1 and PC 2, respectively, captured 61% and 19% of the variance. The PCA results showed three distinct groups, i.e., perennial ryegrass, white clover, and red clover (Fig. 3a). Following the pattern of PCA results, the dendrogram (Fig. 3b) also revealed three distinct clusters for the three species. The white clover and red clover clustered closely, whereas the perennial ryegrass cluster was more distant from the white clover and red clover. The intraspecies heterogeneity in all three species was considerably lower than the interspecies heterogeneity.

Building of quantification models: calibration and validation

The coefficient of determination (R^2), root mean square errors of prediction (RMSEP), and residual predictive deviation (RPD) were used to check the quality of the calibration and validation models. A good model generally has a high R^2 value close to 1, a low root mean square error of prediction (RMSEP), a low root mean square error of cross-validation (RMSECV), a high Residual Predictive Deviation

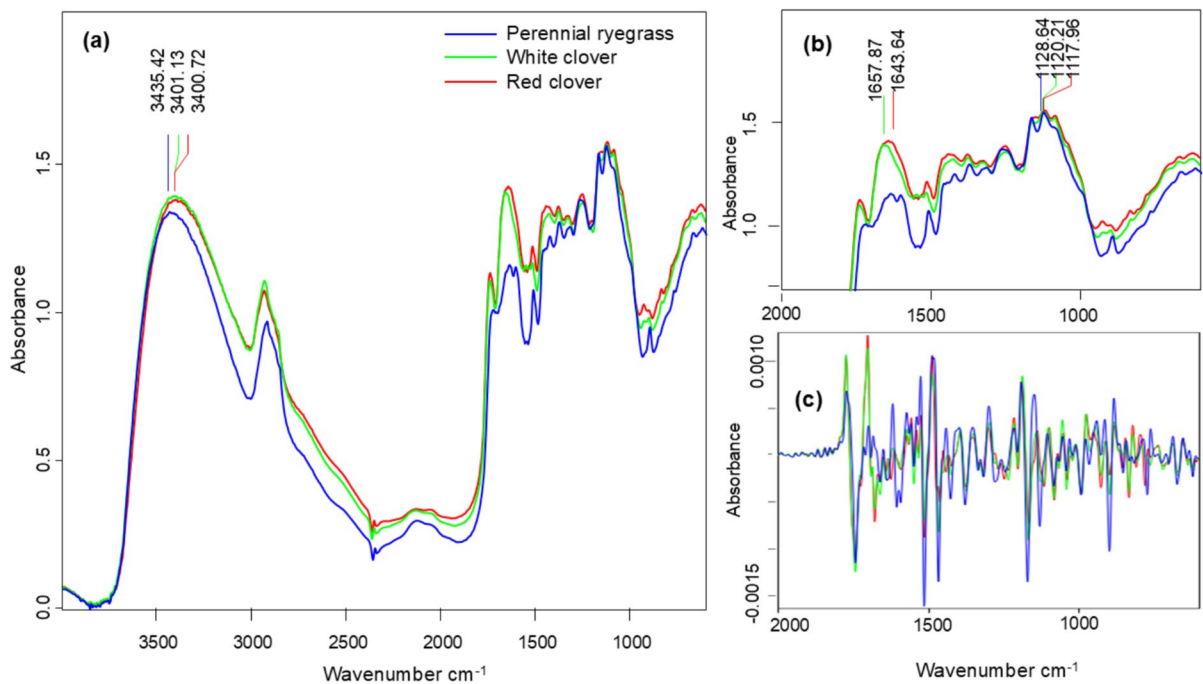


Fig. 2 Mean FTIR spectra of perennial ryegrass, white clover, and red clover root samples after offset correction and vector normalization (a), an enlargement of the "fingerprint region"

(1800–600 cm⁻¹) (b), and fingerprint region after transformation by the first derivative (c)

(RPD), and a bias close to 0. In general, R^2 values between measured proportions of perennial ryegrass, white clover, and red clover and predicted proportions based on calibration models in both artificial mixtures (Fig. 4a) and pure root samples (Fig. 4b) were approximately 0.97 and 0.99 in all three species. The RPD values for the calibration of all three species ranged from 5 to 6, whereas the RMSEP values ranged from 4 to 5 for the artificial mixture model. However, the RPD values for all three species in the pure root model (Fig. 4b) were higher (ranging from 21 to 49), while the RMSEP values were lower (ranging from 1 to 2) than those of the artificial mixture model.

Table 1 shows the statistical parameters of the leave-one-out and blind validation methods for the artificial mixture model and pure root model in perennial ryegrass, white clover, and red clover. The same statistical parameters used in the calibration model evaluation process were applied again in the validation model quality checking; however, here, the root mean square errors of validation (RMSECV) were used instead of RMSEP. In addition, the "bias" of the

model was also calculated. The R^2 values of perennial ryegrass, white clover, and red clover in the leave-one-out validation were the same as those in the calibration in both artificial mixture and pure root models. The RPD of all three species in the leave-one-out validation of the artificial mixture model showed a similar range to that of the calibration model, whereas the pure root model showed lower values (19–35) compared to the calibration model (21–49). In the artificial mixture model, the RMSECV values for all three species in the leave-one-out validation ranged from 4 to 5, while those of the pure root model were relatively lower, ranging from 1 to 2.5. The biases in perennial ryegrass, white clover, and red clover in both the artificial mixture model and the pure root model ranged from 0.01 to 0.07.

Similar to the leave-one-out method, the blind validation of the artificial mixture model also showed high R^2 values for perennial ryegrass, white clover, and red clover of 0.97, 0.99, and 0.96, respectively. However, the R^2 values for perennial ryegrass, white clover, and red clover in the pure root model were lower compared to the blind validation of the

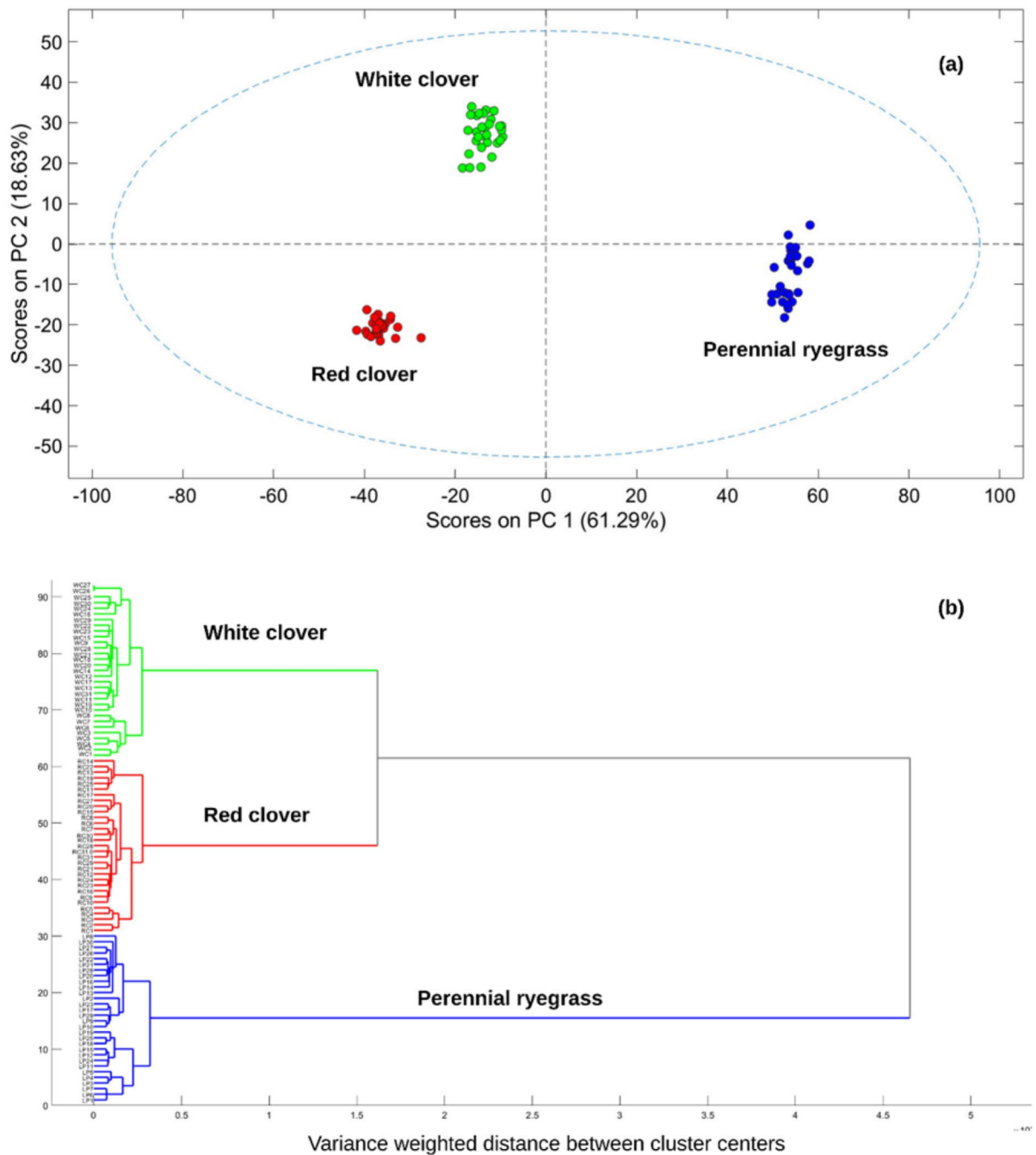


Fig. 3 PCA analysis (a) and cluster analysis (b) of preprocessed spectra (offset correction, vector normalization, 1st derivative) of perennial ryegrass, white clover, and red clover root

samples. In cluster analysis, Ward's algorithm with Manhattan distance was used between 1800–600 cm^{-1} . Each species had 30 physical replicates

artificial mixture model, showing the lowest of 0.89 in red clover. In the artificial mixture model, the RPD values for perennial ryegrass and white clover

(6.12 and 8.53, respectively) were relatively higher than those of the leave-one-out method, whereas the RPD of red clover in the blind validation model and

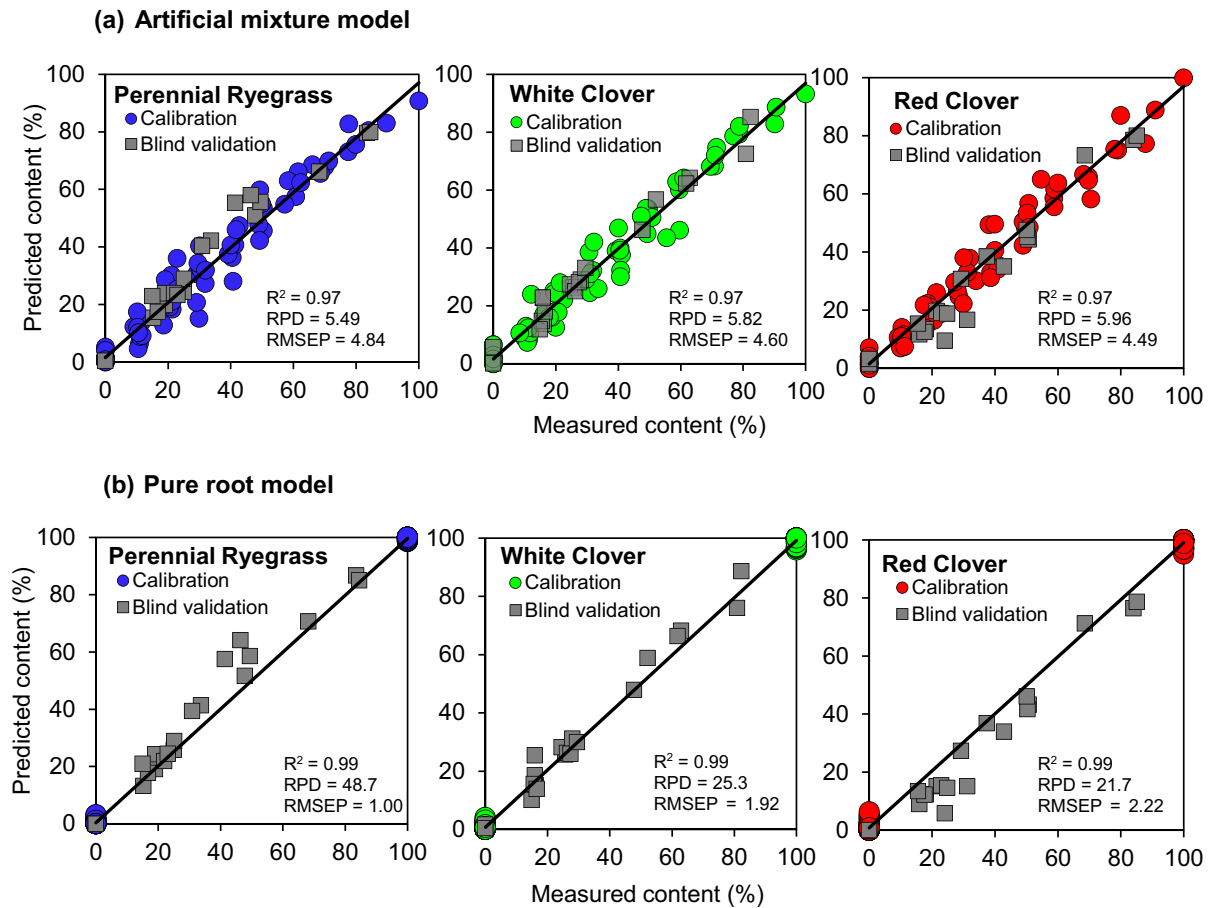


Fig. 4 Calibration parameters and predicted content (%) versus measured content (%) of perennial ryegrass, white clover, and red clover root samples in artificial mixture model (a) and pure root model (b). Spectra were preprocessed with offset correction, vector normalization, and 1st derivative. The

coefficient of determination (R^2), root mean square errors of prediction (RMSEP), and residual predictive deviation (RPD) were used to check the quality of the calibration model. Data points from blind validation are also included to illustrate their distribution within both models

Table 1 Statistical parameters of the artificial mixture model and pure root model for perennial ryegrass, white clover, and red clover in leave-one-out validation and blind validation. The coefficient of determination (R^2), root mean square errors of

validation (RMSECV), residual predictive deviation (RPD), and bias were used to evaluate the validation performance of models

Model	Leave-one-out validation				Blind validation			
	R^2	RMSECV	RPD	Bias	R^2	RMSECV	RPD	Bias
Artificial mixtures ($n = 66$)								
Perennial Ryegrass	0.96	5.16	5.2	0.07	0.98	5.44	6.12	1.67
White clover	0.97	4.87	5.5	0.07	0.99	3.91	8.53	0.50
Red clover	0.97	4.76	5.6	0.05	0.96	5.80	5.75	-1.51
Pure roots ($n = 90$)								
Perennial Ryegrass	0.99	1.36	34.8	-0.04	0.92	6.72	3.58	4.25
White clover	0.99	2.49	19.0	0.05	0.97	3.95	6.14	1.45
Red clover	0.99	2.43	19.4	-0.01	0.89	7.89	3.08	-6.08

the leave-one-out model were within a similar range. In contrast, the RPD values for perennial ryegrass, white clover, and red clover in the pure root model decreased considerably in the blind validation model (3.58, 6.14, and 3.08, respectively) compared to the leave-one-out method. Further, RPD values of blind validation in the pure root model were also lower compared to those in the artificial mixture model for all three species. The blind validation showed higher “bias” values for all three species, in both artificial mixture and pure root models, whereas the pure root model showed the highest “bias” values for all three species. In the pure root model, among the three species, the red clover showed the highest “bias” value of -6.08 . In the artificial mixture model, the RMSECV values ranged between 3 and 6 for all three species, showing the highest RMSECV in red clover and the lowest in white clover. The RMSECV in the pure root model showed a similar pattern to the artificial mixture model but had relatively higher RMSECV values for all three species.

Figure 5 shows the deviation between predicted and measured values for three species under blind

validation for both models, including mean absolute deviation (MAD) values. No systematic deviation patterns were observed across species or models. However, the artificial mixture model generally exhibited slightly lower average deviations. The difference in MAD values between the two models was minor for perennial ryegrass and white clover, but more pronounced for red clover, where the pure root model had a lower MAD, indicating an underestimation of red clover percentage.

In our calibration and validation models, “predicted content (%)” sometimes showed values less than 0 (up to -5) when the “true content (%)” was 0, and values higher than 100 when the actual percentage was 100 (Supplementary Data). During the calibration process, the “predicted content (%)” values, which were less than 0, were set to 0, and values higher than 100% were set to be hundred. However, this modification did not significantly alter the result of the model statistics and agreed with the results of Straková et al. (2020) and Hadir et al. (2024), who also reported the same observation and performed the same adjustment.

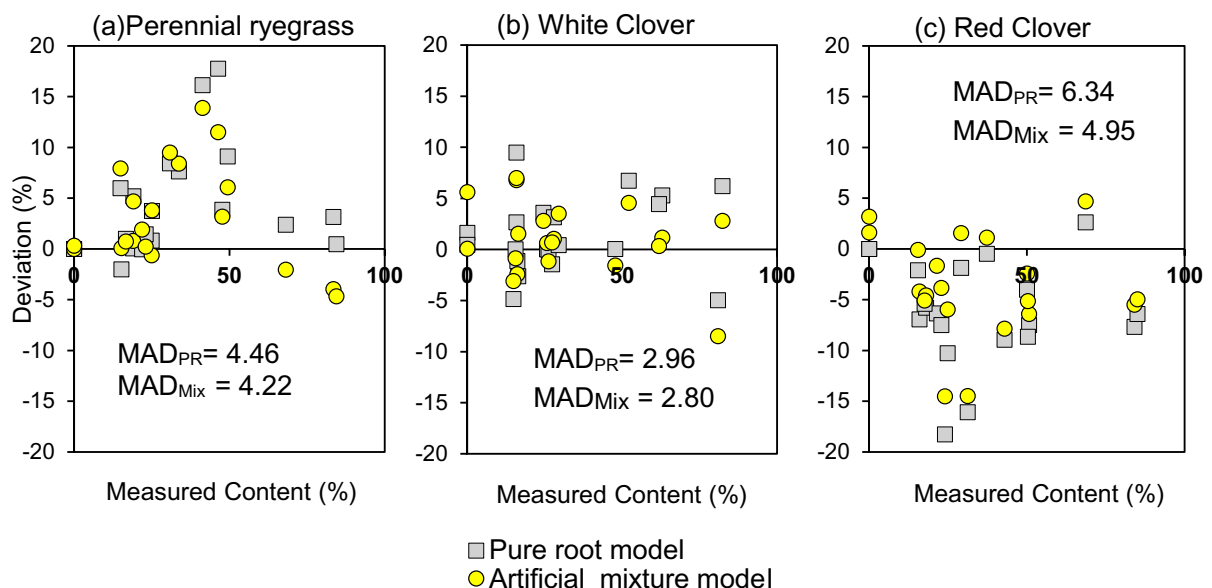
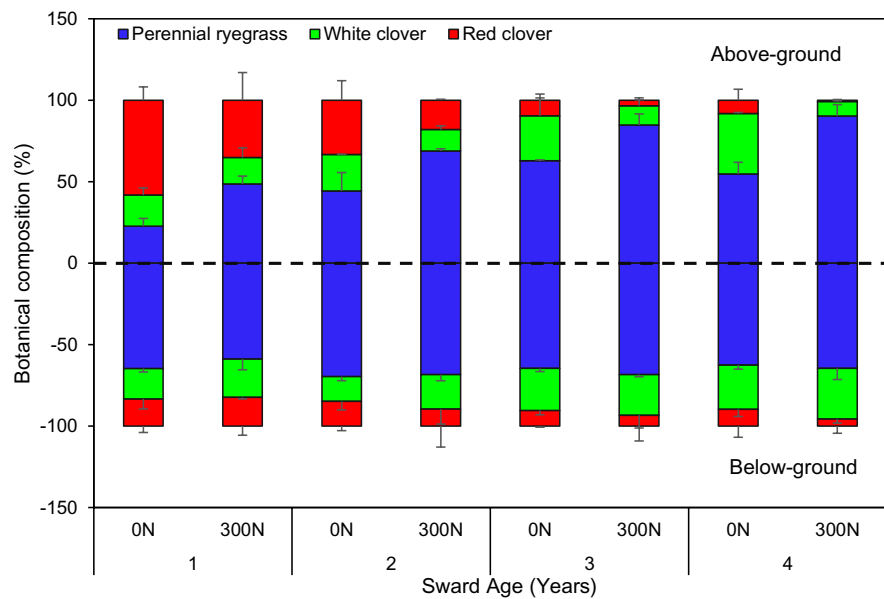


Fig. 5 The deviation values (difference between predicted and measured content) of perennial ryegrass (a), white clover (b), and red clover (c) root samples for the pure root model and the artificial mixture model based on blind validation. The Mean

Absolute Deviation (MAD) values for both the pure root (PR) and artificial mixture (Mix) models are indicated inside each graph

Fig. 6 Above- and below-ground species composition of a three-species grassland mixture for different sward ages and fertilization rates (0 and 300 kg total-N ha⁻¹ slurry application). The belowground data exhibits the root species composition in the 0–10 cm soil layer. The aboveground composition was measured by manual separation and is based on the sum of three cuts, while belowground composition was determined by the FTIR artificial mixture model. The figure contains the mean values of replicates, and error bars indicate the standard deviation



Application test: Determining belowground species composition at the field level with the artificial mixture model

Figure 6 indicates the above- and belowground species composition in a three-species grass-legume mixture. Aboveground, without N application, the red clover proportion was highest in 1st year-old swards (58%), and with increasing sward age and N application, the red clover proportion decreased gradually, showing the lowest proportion in the 4th year-old sward receiving 300 N kg ha⁻¹ (0.8%). In contrast, the perennial ryegrass proportion increased with N-application and sward age, showing its highest proportion of 90% in the 4th year-old sward under the 300 N kg ha⁻¹. The white clover proportion showed similar behavior to red clover for N application, with a relatively lower proportion under 300 N kg ha⁻¹. However, the white clover proportion increased with sward age under 0 N, showing the highest proportion in the 4th year-old sward (37%).

The proportion of red clover in the belowground root biomass generally decreased with increasing N-application rate and sward age, following a pattern similar to that observed in the aboveground species composition. Although the aboveground red clover proportion was high in the 1 year-old swards under 0 N kg ha⁻¹ (56%), perennial ryegrass was always the species present in largest proportions belowground at

both N-application rates and all sward ages (ranging from 59 to 70%). The white clover proportion in the belowground root biomass ranged from 19 to 31%, showing a general persistence under all sward ages and both N-application rates.

Discussion

The present study revealed that perennial ryegrass, white clover, and red clover roots can be differentiated and quantified using FTIR spectroscopy. Chemometric analysis of FTIR spectra is based on the variation in the chemical composition of roots of different species (Berthomieu and Hienerwadel 2009; Hadir et al. 2024; Kemper et al. 2022; Naumann et al. 2010; Straková et al. 2020; White et al. 2011). The peaks in spectra are linked to specific compounds or functional groups. The distinct peaks of all three species in the wavenumber range of 3700 and 3000 cm⁻¹ are attributed to the O–H and N–H functional groups, while the peaks between 2800 and 3000 cm⁻¹ are attributed to the fats and lipids (Artz et al. 2008; Legner et al. 2018; Naumann et al. 2010; Wilson et al. 2000). Further, the wavenumber range from 600 to 1800 cm⁻¹ contains hemicellulose and cellulose (900–1200 cm⁻¹), protein/lipid-associated peaks (1200–1500 cm⁻¹), and protein peaks (1500–1800 cm⁻¹) (Artz et al. 2008; Kacuráková et al. 2000; Naumann et al.

2010; Wilson et al. 2000). All three species had their highest peak at $\sim 1120\text{ cm}^{-1}$, which falls within the cellulose and hemicellulose wavenumber range, which is in line with the results of Legner et al. (2018), Meinen and Rauber (2015), Naumann et al. (2010), and Streit et al. (2019). The white and red clover are legume species and generally contain more protein than perennial ryegrass. This explains the higher absorbance in protein-specific peaks at 1485 and 1800 cm^{-1} compared to perennial ryegrass. Naumann et al. (2010) also reported a similar observation with pea and oat species, where pea had higher absorbance peaks within this wavenumber range than oat. In the present study, only the wavenumber range of 600 and 1800 cm^{-1} was used for PCA and cluster analysis, which showed clear differences between the three species. These results revealed that the polysaccharide, protein, and protein/lipid compounds in the three species used in this study play a crucial role in interspecies differences. Therefore, the peaks associated with these compounds presented within the 600 and 1800 cm^{-1} could effectively differentiate the three species used in this study, agreeing with the results of Meinen and Rauber (2015) and Rewald and Meinen (2013).

The calibration and validation models in this study were built using artificial root mixtures following previous studies (Dirks et al. 2021; Hadir et al. 2024; Lei and Bauhus 2010; Straková et al. 2020; Streit et al. 2019; Tong et al. 2016; White et al. 2011), and the pure root model was built following Straková et al. (2020). In our study, all three species in both artificial mixture and pure root models showed good model performance in calibration showing high R^2 (> 0.96), high RPD (> 4), and low RMSEP (< 5), agreeing with the reported model parameters of Lei and Bauhus (2010), Meinen and Rauber (2015), and Straková et al. (2020). The leave-one-out validation approach utilizes all the data points in the calibration model, leading to low bias, and the blind validation approach provides an unbiased estimate of the performance of the model on unseen data, helps to avoid overfitting, and offers a clear assessment of the generalization ability of the models. Therefore, we performed both validation approaches to test the models. Similar to the calibration, both models showed a good performance in the leave-one-out validation with high R^2 , high RPD, low RMSECV, and low “bias”. However,

in the blind validation model, the artificial mixture model showed a better performance than the pure root model, with lower bias values and higher RPD values for all three species. When comparing the calibration and validations of the artificial mixture model and pure root model, the pure root model showed the highest RPD in both calibration and leave-one-out validation. However, in the blind validation, the RPD values of the pure root model significantly decreased, resulting in lower values than those of the artificial mixture model for all three species. Since the blind validation helps to detect overfitting of a model, the higher RPD values in the pure root model in calibration and leave-one-out validation might be a result of overfitting, as it only used samples with 0 and 100% of a given species. According to Meinen and Rauber (2015), creating root sample mixtures representing possible combinations of species proportions from 0–100% is vital for constructing a quantification model with good RPD values. Therefore, the good RPD values in the artificial mixture model in blind validation for all three species might be a result of using the ternary diagram approach for making mixtures, which covers a reasonable amount of possible mixture combinations of perennial ryegrass, white, and red clover species. Therefore, we performed both validation approaches to test the models. The bias values in blind validation were higher than in the leave-one-out validation method in both models but were highest in the pure root model. In general, the absolute mean deviation (MAD) values of the artificial mixture model for the blind validation were slightly lower than those of the pure root models, indicating more precise and stable predictions.

Straková et al. (2020) proposed the use of models built from pure root samples, and our findings support the feasibility of this approach for grass-clover mixtures. However, the overall performance of the artificial mixture model in identifying and quantifying the roots of perennial ryegrass, white clover, and red clover suggests that incorporating more representative sample mixtures during calibration may help reduce the higher bias observed in the pure root model. Depending on the required level of accuracy, models can be developed using either pure roots or artificial mixtures. For three-species mixtures, fewer than 66 mixtures may be sufficient. In more complex grassland mixtures involving more than three species,

the pure root model may offer a practical advantage by reducing both complexity and workload compared to the artificial mixture approach.

In a demonstration of an application in this study, the aboveground proportions of perennial ryegrass, white clover, and red clover, reported by Jensen et al. (2024), showed that legumes dominate under unfertilized conditions but decline with increasing sward age and fertilizer application rates, allowing perennial ryegrass to become dominant. These findings align with previous studies (Brophy et al. 2017; Burchill et al. 2014; Suter et al. 2015). In contrast to the behavior of the aboveground species composition, the perennial ryegrass dominated belowground irrespective of the sward age and N application rate. Further, in general, the proportion of white clover roots was also relatively higher than that of red clover. This could be, because perennial ryegrass and white clover have shallow fibrous root systems, while red clover has a deep tap root system. Therefore, the dominance of perennial ryegrass and, subsequently, white clover roots in the present study might be a result of root morphology, as we only sampled roots up to 10 cm depth. However, belowground red clover proportions followed the general trend of aboveground, showing the lowest proportion of red clover roots in the 4th-year-old sward with 300 kg total-N ha⁻¹.

Our results confirmed the potential of using FTIR spectroscopy under field conditions. As White et al. (2011) reported that soil type, climate, and fertilization did not affect the interspecies spectra, these results can likely be extrapolated, despite the fact that in the present study, we did not compare the impacts of external factors, such as climate, soil type, and fertilization, while building the FTIR model for root differentiation and quantification. The successful differentiation and quantification of perennial ryegrass, white clover, and red clover roots in the present study using the FTIR model, regardless of fertilization and with calibration and validation sets including plants of different sward ages, also implies that the species characteristics have a higher impact on spectra.

Most of the studies used monocultures for obtaining root samples for building FTIR models (Dirks et al. 2021; Hadir et al. 2024; Streit et al. 2019). In the present study, individual plants with their attached roots were directly sampled from the grass-clover mixtures and used for model building. As the model building was a success in this study,

growing monocultures for obtaining root samples is not required for FTIR model building.

In most studies concerning the productivity of grass-legume mixtures, only the aboveground relations are considered, but this FTIR model for root differentiation and quantification can facilitate root-related studies in multispecies grasslands. For instance, information on root composition can empower studies related to belowground species complementarity, leading to the development of more productive grassland mixtures. Further, this model can be used to quantify the root composition in different soil depths, which will provide insights into resource acquisition, growth strategies, and the C sequestration potential of plant species in multispecies grasslands.

Conclusion

The present study indicated the potential of FTIR spectroscopy for identifying and quantifying species-specific roots in grass-legume mixtures under field conditions. Our study demonstrated that FTIR spectroscopy of root samples could sufficiently differentiate both distantly and closely related plant species. Calibration models built using both artificial mixtures and pure root samples performed reasonably well in quantifying the species-specific compositions under field conditions. Although using artificial mixtures slightly improved the overall generalized ability of the model compared to the pure root approach, the improvement was rather small considering the additional workload. However, the approach used for model building depends on the expected accuracy and the number of species to be differentiated. The FTIR models can be used to link above- and belowground species composition, opening potential for studies to tailor the design of grass-clover mixtures for the future.

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commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available online in a Zenodo repository: <https://doi.org/10.5281/zenodo.17017812>.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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