Biochemical analysis of subterranean clover seed coat by FTIR-ATR

Carmen S P Teixeira[1], John G H[2], Notburga G[3] and Derrick D M[1]

[1] Dryland Pastures Research Group, Field Research Centre, Lincoln University, New Zealand
[2] Seed Research Centre, Department of Agricultural Sciences, Lincoln University, New Zealand
[3] University of Natural Resources and Life Sciences (BOKU), Institute of Biophysics, Austria

Abstract
Subterranean clover (*Trifolium subterraneum*) is an important legume in resilient crop systems in Australia and regenerative pastures in New Zealand. It provides high-quality biomass and nitrogen fixation during late winter and early spring. The hardseeds of subterranean clover have a seed coat which is impermeable and prevents germination even when moisture and temperature conditions are ideal. Ecologically it enables the regeneration and persistence of subterranean clover under a broad range of management and environmental conditions, supplying seeds for germination at the right time of the year and through the development of soil seed banks. Suberin, an extracellular tissue sealing polymer and lignin have been proposed to be the main seed coat biochemical components of hardseeds in subterranean clover. The classic procedures for assessing hardseededness are laborious, costly and involve sample destruction. The Fourier transform infrared microspectroscopy (FTIR) with Attenuated Total Reflectance (ATR) lens, which is a non-destructive, rapid and *in situ* technique was used to characterise the biochemical profile of the seed coat from six subterranean clover cultivars. The seed coat spectra showed various bands attributed to molecular vibrations of mainly aromatics (1500 -1200 cm^{-1}) and polysaccharides (1025 cm^{-1}). In addition, cutin, cellulose and tannins in the coat outer layer are important to confer hardseededness. The maximum percentage of hardseededness (HSmax) was assessed using top of paper germination test. HSmax ranged from16 to 96%. The FTIR-ATR technology can be broadly applied to study the biochemical profile of other important dormant legumes.

Keywords
Cuticle, lipids, spectroscopy, seed dormancy, technology.

Introduction
Subterranean clover (*Trifolium subterraneum,* sub clover) is an important legume in resilient crop systems in Australia and regenerative pastures in New Zealand (Nichols et al., 2023). It provides high-quality biomass and nitrogen fixation during late winter and early spring. The hardseeds of sub clover have a seed coat which is impermeable and prevents germination even when moisture and temperature conditions are ideal. Seedhardness is conferred by the chemical composition of the seed coat that influences permeability to soil moisture (Slattery et al., 1982; Vishwanath et al., 2013). These biochemical aspects are explored through two complementary methodologies, the Fourier transform infrared microspectroscopy (FTIR) and the Attenuated Total Reflectance (ATR) techniques. In combination, these methods were used to quantify the presence of different biochemical compounds in sub clover seeds based on previous studies with different biological tissues (Busch et al., 2010; Littlejohn et al., 2015; Schwanninger et al., 2004). These approaches offer the possibility of rapid and non-destructive *in situ* quantification of compounds such as lignins (Prats-Mateu and Gierlinger, 2017), suberin (Zeier and Schreiber, 1999) and cutins (Yan et al., 2009), which are known to influence the degree of permeability of cell walls. The FTIR-ATR technique was used to test genotype effects by scanning the seedccoat tissue from seeds harvested in field for six cultivars. The aim was to investigate and associate the variation in maximum hardseedeness by assessing the chemical compounds present in the seed coat.

Methods
Seeds
Sub clover seeds from six different cultivars ('Antas', 'Denmark', 'Leura', 'Monti', 'Narrikup' and 'Woogenellup') were harvested from the Field Research Centre experimental area (-43.6470, 172.4680 , 11 m a.s.l.) during 2015 and 2016. Burrs and seeds were excavated and then processed manually. A subsample of seeds per plot was selected from the June sowing date (S2). The maximum percentage of hardseededness (HSmax) was assessed using top of paper germination test and ranged from16 to 96% (Teixeira et al., 2021). The seeds were kept at 4 ºC and sealed bags prior the tests. The seeds were fixed in microscope slides on their longitudinal axis. To reduce potential optical or distorting effects during measurements, the scans were taken evenly across points on the seed cuticle (Teixeira et al., 2018). Removal of dust and potential contaminants was done by blowing pressurized air for 15 seconds on each sample.

The Fourier transform infrared–attenuated total reflectance (FTIR-ATR) spectroscopy
FTIR-ATR spectroscopy was used to assess properties of the seed outer surface (cuticle). An average of 4 measurement points (~ 150 µm apart) were selected. In each point one spectrum was acquired using a Hyperion 2000 microspectrometer (Bruker Optics, Ettlingen, Germany) in a wavenumber range of 4500–600 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans per sample. The pressure arm of the instrument was set to level 1 to apply a constant pressure. The background spectrum was taken for each measurement at a position of the IR-window outside the seed coat tissue. A total of 300 spectra was obtained as 75 seeds were measured. Data acquisition was performed with OPUS software package (Bruker) and spectra data processing (baseline correction) and analysis were performed with the softwares Unscrambler X (CAMO, 2018). A principal component analysis (PCA) was used to reduce the dimensionality of the original data from thousands of variables to a few critical ones. (Gierlinger, 2017). The first two PCs (1 and 2) were considered the main sources of variability in the dataset and the scores of PC1 and PC2 were plotted against each other to establish potential relationships between spectra and cultivars (Abidi et al., 2014).

Results
The application of FTIR-ATR methods enabled the detection of different biochemical compounds in the cuticle of sub clover seeds. Figure 1 shows the mean spectra of the seed coat cuticle of the six sub clover cultivars across the various band intensities. The seed coat spectra were composed of different bands attributed to functional groups, mainly of aromatics (1500 -1200 cm^{-1}) and polysaccharides (1025 cm^{-1}). In addition, cutin, cellulose and tannins in the coat outer layer were important to confer hardseededness (Table 1).

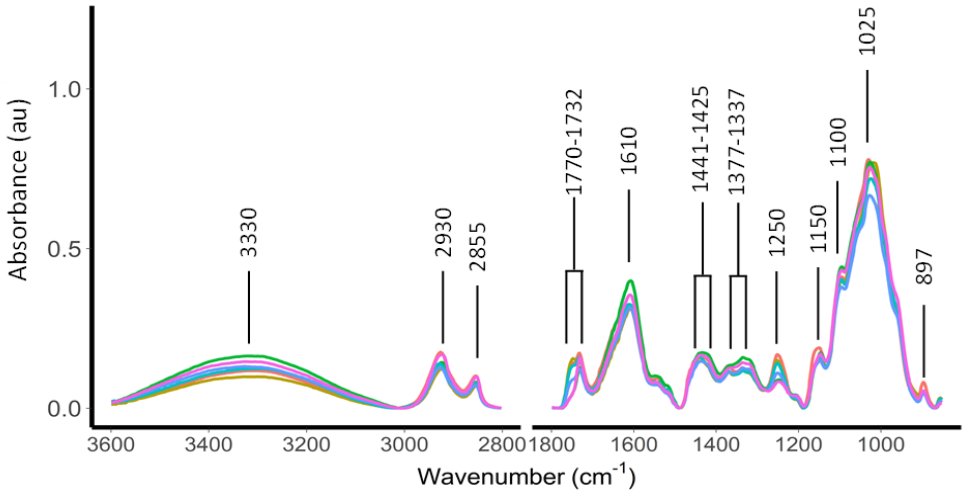

Figure 1. Mean ATR-FTIR spectra (baseline corrected normalised) of seed coat cuticle of sub clover cultivars 'Antas' (orange line), 'Denmark'(brown), 'Leura' (green line) , 'Monti' (light blue) , 'Narrikup' (dark blue), and 'Woogenellup' (pink) sown in July 2015 (S2) and produced in Field Research Centre, Lincoln, New Zealand. The IR regions between 2800-1800 and 850-600 cm^{-1} were omitted due to absence of significantly different bands.

For all cultivars the mean absorbance of wavenumber 1610 cm^{-1} was high (0.4), which indicates a high amount of tannin, and aromatic compounds present in the seed coat cuticle The mean absorbance of 897 cm^{-1} described as C-H ß-linkage of cellulose, was higher (P=0.004) for 'Antas' (0.1) than for the other five cultivars (mean of ~ 0.08). No clear absorbance band was found at 1515cm^{-1}, from which it was concluded that structural components such as lignin play a minor role in sub clover cuticle.

Table 1. IR assignments of the main vibrations in the FTIR-ATR spectra of sub clover seed coat cuticle.

IR region (cm-1)	Wavenumber (cm-1)	Assigned functional group	Description

3600-3000	3330	O–H and N–H group stretching vibration	polysaccharides, protein
3000-2800	2930 2855	CH2 asymmetric stretch CH2 symmetric stretch:	mainly lipid (unsaturated) with contribution from proteins, carbohydrates, nucleic acids; wax associated with cutin and cutan
1800-1500	1770 1745 1732 1610	C=O stretch C=O stretch esters C=O esters and lactones C=C stretch	Vinyl, phenil ester triglyceride and phospholipid cutin cutan
1500-1200	1441 1425 1377 1337 1250	C-H in plane deformation with aromatic skeletal vibrations (lignin) C-H bending CH3 bending vibration C-H2 rocking vibration C-N stretching vibration; N-H bending vibration	 alcohol lipids, proteins cellulose Aromatics, phenols
1200-700	1150 1100 1025 897	C-O stretch C-O and C-C Anti-symmetric in plane stretching band C-O stretch C-H ß-linkage of cellulose	alcohol secondary alcohols (hemi cellulose – cellulose) Pectin glucose, polysaccharides cellulose

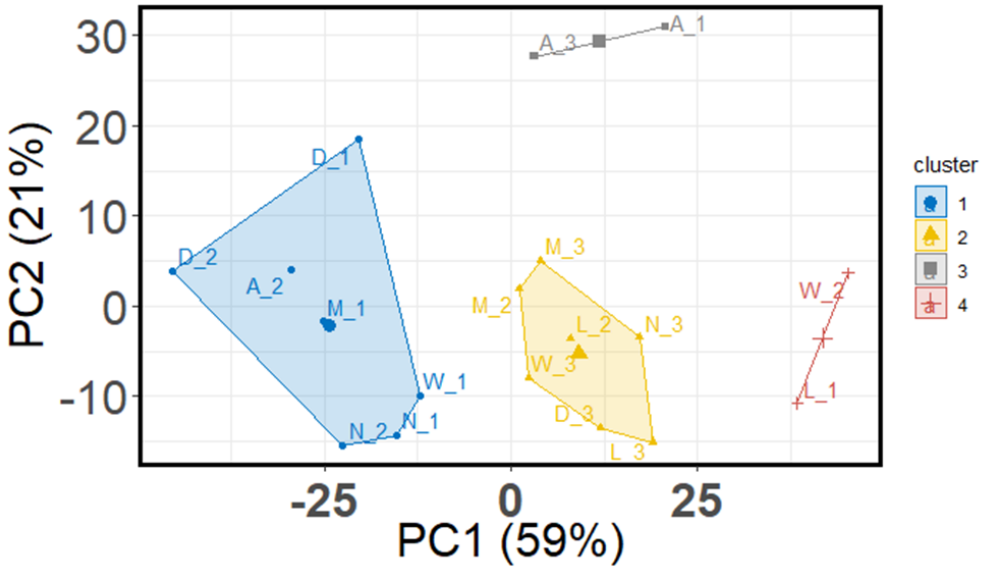

Figure 2. Principal component analysis (PCA) of FTIR spectra of the seed coat cuticle of sub clover cultivars 'Antas' (A), 'Denmark' (D), 'Leura'(L), 'Monti' (M), 'Narrikup' (N) and 'Woogenellup' (W). Numbers following letters represent the replicate. Cluster 1 (blue ○), cluster 2 (yellow, ▲), cluster 3 (grey, ■), cluster 4 (red ┼) represent the hierarchical clusters performed with the spectral data in the wavenumber range from 3600-2800 cm^{-1} and 1800–850 cm^{-1}.

PC's accounted for 80% of the total cultivar by replicate variability. The most distinct samples were 'Antas' (*brachycalycinum*, replicates 3 and 1) which was separated from the clusters 2 and 3, formed by 'Monti' (*yanninicum*), 'Denmark' and 'Narrikup'. The fourth cluster contained only one replicate of each of the subterraneums 'Leura and 'Woogenellup' (Figure 2). There was no clear grouping according to cultivars. The PC1 explained 59% of the cultivar differences. PC1 loadings display the main contribution of bands at 1610 cm^{-1}, 1337 cm^{-1}, 1250 cm^{-1}, 1150 cm^{-1} and 1025 cm^{-1}. The PC2 loadings had strong (>0.1) contributions from 1250 cm^{-1} and 1025 cm^{-1}. The other important contributions were from the lipids (2930 cm $^{-1}$, 2855 cm^{-1}) and tannins, cutin and suberin located between wavenumbers ~1730-1770 cm^{-1}. The mean absorbance of 897 cm^{-1} (assigned as cellulose, Table 1) was higher (P=0.004) for 'Antas' (0.10) than for the

other five cultivars (mean of ~ 0.08). The highest peak in the entire spectra was measured at 1025 cm^{-1} and was not different (P=0.15) among the cultivars.

Conclusion

The FTIR-ATR technique enabled the detection of biochemical compounds in the seed coat composition. The key biochemical compounds identified to confer seedhardness in sub clover were lipids, cutin, and polysaccharides. Unlike other species, suberin has not been definitively confirmed as the sole contributor to seed hardness in the seed coat cuticle. Furthermore, there is no single ideal cultivar when it comes to hardseededness. Instead, for each specific environment and management combination, there exist one or more genotypes that could meet the requirements of a sustainable production system. For example, for the temperate climate of New Zealand with mild (15-20 ^{o}C) soil temperatures, a low hardseededness cultivar with a seed coat rich in lipids and low in cellulose would be desirable. This would enable most seeds to germinate in the following autumn. Conversely in Australia, where soil temperatures are higher, the opposite chemical characteristics would be more desirable for seeds need to remain in the seedbank and germinate at cooler periods. The biochemical changes in the seed cuticle over time which occurs in the field ultimately define seedling recruitment and adaptation to an environment. These findings point out to the latent potential for cultivar selection and improvement from the wide genetic variability found in sub clover cultivars.

References

Abidi, N., Cabrales, L., Haigler, C.H., 2014. Changes in the cell wall and cellulose content of developing cotton fibers investigated by FTIR spectroscopy. Carbohydr. Polym. 100, 9–16.

Busch, S., Schmitt, K., Erhardt, C., Speck, T., 2010. Analysis of self-repair mechanisms of phaseolus vulgaris var. saxa using near-infrared surface-enhanced raman spectroscopy. J. Raman Spectrosc. 41, 490–497.

CAMO, 2018. The Unscrambler X - Multivariate Statistical and Analytical Software.

Gierlinger, N., 2017. New insights into plant cell walls by vibrational microspectroscopy. Appl. Spectrosc. Rev. 4928, 1–35.

Littlejohn, G.R., Mansfield, J.C., Parker, D., Lind, R., Perfect, S., Seymour, M., Smirnoff, N., Love, J., Moger, J., 2015. In Vivo Chemical and Structural Analysis of Plant Cuticular Waxes Using Stimulated Raman Scattering Microscopy. Plant Physiol. 168, 18–28.

Nichols, P.G.H., Smith, G.R., Moot, D.J., Ates, S., Porqueddu, C., Rios, E.F., Louhaichi, M., del Pozo, A., Kotze, N., Humphries, A.W. , Ghamkhar, K., Ryan, M., 2023. Annual clovers around the world: current status and future prospects. In: XXV International Grassland Congress,. Covington, Kentucky, USA., pp. 765–768.

Prats-Mateu, B., Gierlinger, N., 2017. Tip in–light on: Advantages, challenges, and applications of combining AFM and Raman microscopy on biological samples. Microsc. Res. Tech. 80, 30–40.

Schwanninger, M., Rodrigues, J.C., Pereira, H., Hinterstoisser, B., 2004. Effects of short-time vibratory ball milling on the shape of FT-IR spectra of wood and cellulose. Vib. Spectrosc. 36, 23–40.

Slattery, H.D., Atwell, B.J., Kuof, J., 1982. Relationship between Colour , Phenolic Content and Impermeability in the Seed Coat of various Trifolium subterraneum L genotypes. Ann. Bot. 50, 373–378.

Teixeira, C., Hampton, J., Moot, D., 2021. Phenological development of subterranean clover cultivars under contrasting environments. Ann. Appl. Biol.

Teixeira, C., Hampton, J.G., Gierlinger, N., Moot, D.J., 2018. Biochemical changes in subterranean clover seed coat after thermal treatment. Proc. 15th Eur. Soc. Agron. Conf.

Vishwanath, S.J., Kosma, D.K., Pulsifer, I.P., Scandola, S., Pascal, S., Joubes, J., Dittrich-Domergue, F., Lessire, R., Rowland, O., Domergue, F., 2013. Suberin-associated fatty alcohols in Arabidopsis: distributions in roots and contributions to seed coat barrier properties. Plant Physiol. 163, 1118–1132.

Yan, H., Hua, Z., Qian, G., Wang, M., Du, G., Chen, J., 2009. Analysis of the chemical composition of cotton seed coat by Fourier-transform infrared (FT-IR) microspectroscopy. Cellulose 16, 1099–1107.

Zeier, J., Schreiber, L., 1999. Fourier transform infrared-spectroscopic characterisation of isolated endodermal cell walls from plant roots: Chemical nature in relation to anatomical development. Planta 209, 537–542.