

The Potential for Analyzing the Correlation Between the Compressive Stress Resistance of Red Clover Seeds and the Environmental Conditions during Seed Maturation Using FT-IR Spectroscopy

Gergely Zoltán Macher^{*,a}, Marcell Lócsi^a, Anett Bedő^a, Eszter Kókai^a, Dalma Bódizs^a, Szilveszter Gergely^b

^aDept. of Applied Sustainability, Széchenyi István University, Hungary, Győr

^bDept. of Applied Biotechnology and Food Science, Budapest University of Technology and Economics, Hungary, Budapest
 macher.gergely.zoltan@sze.hu

The paper aims to examine the connection between environmental conditions during seed maturation and the ability of seeds to compressive stress, supported by statistically significant findings. The study involves using Fourier transform infrared (FT-IR) spectroscopic analysis on red clover seeds under varying pressure levels, specific tensions, and selected quality parameters. The methodology relies on a general FT-IR spectroscopic approach, with spectral comparisons made against results from pressure-induced rupture. Through this research, new methods for seed testing are intended to be informed by differential and correlation results across technical parameters and different measurement settings. The analyses indicated a noticeable difference of several 10 N in visible features between seed maturation stages and compressive tolerance. Given the novelty of the aspects being explored, one primary influencing factor is identified as the scarcity of available literature, which also serves as a limitation of this research. The obtained results have potential applications for analysts, agricultural specialists, consultants, and experts involved in seed management and distribution.

1. Introduction

The red clover, scientifically known as *Trifolium pratense* L., is a perennial legume in the *Fabaceae* family. It is the second most important forage legume globally, after *Medicago sativa* L. (Annicchiarico et al., 2015). This European-origin fodder plant is primarily used for pasture and hay in cool climates across Europe and North America (Putnam and Orloff, 2014). Forage crops like red clover play a crucial role in the economic and nutritional value of dairy, beef, and other livestock products, which are essential for global food supply and human nutrition (Fergus and Hollowell, 1960). Red clover is versatile, as its leaves, stems, and flowers contain isoflavonoid phytoestrogens like formononetin, biochanin A, daidzein, and genistein. Traditionally, teas or tinctures made from flowers have been used to address upper respiratory issues and provide mild sedative, antispasmodic, and anti-rheumatic effects (Sezik et al., 1997).

Attenuated total reflectance (ATR) Fourier-transform infrared spectroscopy is an effective technique for evaluating a material's resistance to compressive stress. It does this by subjecting the material to compressive stress and monitoring changes in its vibrational spectrum (Farro et al., 2023). ATR-FTIR spectroscopy can also analyze molecular alterations, such as conformational changes, providing valuable insights into both the mechanisms and performance of a material's compressive stress resistance. This spectroscopic technique has been widely used in plant research for many years, including studying microalgae to optimize cultivation conditions (Kiss et al., 2018), quantifying lignin content for fungal wood pretreatment, and discriminating plant (Holden et al., 2024) pathogenic fungi (Vieira et al., 2024). The ATR FT-IR spectra of hemp-based products exhibited similar macronutrient compositions. Additionally, FT-IR measurements were utilized to track potential changes in the biochemical components of peanut plant leaves when the seeds underwent pre-soaking in a solution containing copper oxide nanoparticles (Suresh et al., 2016). FT-IR analysis revealed the strongest

signals at approximately $2,923\text{ cm}^{-1}$, $1,636\text{ cm}^{-1}$, and $1,033\text{ cm}^{-1}$, representing lipid, protein, and carbohydrate levels in the leaf samples. Further examination of the FT-IR spectra could enable measurement of alterations in chemical components and secondary structure of proteins. The study indicated that using nanoparticles through a pre-soaking technique has the capacity to induce changes in biochemical components without markedly impacting plant development.

Red clover is a valuable plant species with potential benefits for human health and livestock farming. However, its cultivation in Hungary has declined as agricultural programs prioritize other clover varieties. This has resulted in lower market prices compared to 2022, despite recent growth in red clover seed production, which still relies on imports due to limited domestic cultivation (Çölgeçen et al., 2011). Red clover provides superior fodder quality compared to *Medicago sativa L.* and is often grown as an intercrop due to its pest resistance and ability to thrive in various soil types, improving meat and milk quality (Ulloa et al., 2003). Research is also exploring the industrial and pharmaceutical applications of this plant (Çölgeçen et al., 2020). Uneven seed maturation poses a challenge, making consistent field emergence important, with typical yields ranging from 400-600 kg per hectare in Europe (Boller, 2010). The coloration of red clover seeds is primarily determined by genetic factors, although environmental influences are still being debated. Various studies have examined the factors influencing the coloration of red clover seeds. While some theories propose that environmental factors contribute to this phenomenon, multiple studies have refuted this idea, leading to ongoing debate among agricultural and environmental scientists. Bortnem and Boe (2003) indicates that seed color is predominantly determined by two genetic loci. Specifically, if the loci are homozygous and recessive, the seed color will be yellow.

Conversely, if the loci are heterozygous and dominant, the color will be light purple. When the loci are homozygous and dominant, the seed will appear purple. Additionally, the development of brown and red hues in red clover seeds is associated with seed aging. There is substantial research exploring the relationship between red clover seed color and seed quality (Veljjevic et al., 2017). Climate change is a major factor contributing to the variability in annual red clover yields. Management techniques, as well as abiotic and biotic elements, can impact seed production (Sadenova et al., 2022). Insufficient rainfall may restrict red clover production for grazing, while excessive rainfall during flowering and seed formation stages could diminish yields by adversely affecting pollination and creating unfavorable conditions for harvesting (Petkovic et al., 2017). Drought during flowering is a significant source of environmental stress, leading to a high number of seeds that are unable to germinate (Anderson et al., 2016). Prior research has demonstrated the importance of irrigation in enhancing seed yields, as evidenced by increased red clover seed production from irrigation treatments. These factors collectively influence the quality attributes of the seed, including its color, weight, and resistance. The optimal red clover seed measures between 1.5 and 2.1 mm in length and typically weighs between 1.8 and 2.2 mg while exhibiting yellowish-red coloration with a "fingerless glove" shape (Casler and Undersander, 2019). This research innovatively applies ATR-FTIR spectroscopy to study the compressive stress resistance of red clover seeds and how it relates to environmental conditions during seed maturation. While FT-IR spectroscopy has mainly been used to analyze the biochemical components of plant tissues, this study focuses on the physical resilience of the seeds. The goal is to explore how environmental stressors impact the structural and molecular properties of red clover seeds, providing deeper insights into seed quality and resistance.

2. Materials and methods

2.1 Sample Preparation

The red clover seeds under study are shown in Figure 1. Based on the color of the seeds, six color variants were separated. From each of these color shades, 10 seeds were selected and weighed. The weight of the seeds ranged from an average of 1.80 mg to 2.17 mg. The average weight of the entire sample group was $1.91 \pm 0.28\text{ mg}$.



Figure 1. Examined red clover (*Trifolium pratense*) seeds according to color shade

2.2 FT-IR Measurements

FT-IR spectra were obtained at ambient temperature using a PerkinElmer Spectrum 3 FT-IR spectrometer equipped with a PerkinElmer ATR Accessory containing a top plate with a single reflection diamond crystal and a pressure arm for applying force on the sample, pushing it onto the diamond surface. Spectra were recorded at 10 N increments from 0 N to 100 N to observe the behavior of the seeds. The sample holder was cleaned with 96 % ethanol between measurements. Reaching 100 N, the spectrum of substances released from the seeds was measured without any pressure. The PerkinElmer Spectrum IR spectroscopy software was utilized to compare the spectra against the reference library. Spectra that matched at least 80 % with those in the reference library were considered to be identified reference materials. First, the seed's resistance to compressive force was observed. As the compressive force increased, the seeds cracked at a defined pressure value.

2.3 Statistical analysis

To ensure the reliability and validity of the experimental results, we used various statistical methods to evaluate the data. We calculated descriptive statistics, such as the mean, standard deviation, minimum, and maximum values, to analyze the data. These statistics provided an initial understanding of the data's distribution and variability. By employing these techniques, we gained deeper insights into the data, allowing for a comprehensive assessment of its characteristics and underlying patterns. Additionally, we utilized regression analysis to investigate the relationships between a dependent variable and one or more independent variables. This method allowed us to model and analyze the influence of multiple factors on the dependent variable, offering a comprehensive understanding of how changes in the independent variables are associated with variations in the outcome. Regression analysis is valuable for identifying trends, making predictions, and elucidating the strength and nature of the relationships between the variables.

3. Results

The investigation of the resistance of red clover seeds to pressure observed that the differentiation of seeds according to color groups did not significantly affect the pressure resistance. The onset of resistance against pressure for the examined seeds was observed to be on average 31.7 ± 5.89 N. For the V-1 group, it was 38.0 ± 4.47 N; for the V-2 group, it was 30.0 ± 7.07 N. The difference falls within 10 N, making its significance minimal. For the V-3 group, this value was 34.0 ± 5.48 N, and for V-4, it was 32.0 ± 4.47 N. The smallest value was observed in the V-5 group: 22.0 ± 8.37 N, while the value for V-6 tended towards a similar value as V-3 (34.0 ± 5.48 N).

The V-5 group exhibited exceptional resistance with notably low values compared to other groups. The cracking and splitting of seeds were observed during the transition between 50 and 60 N. It was 62.0 ± 8.37 N for V-1; 64.0 ± 18.2 N, for V-2; and 68.0 ± 11.0 N for V-3. The value for the V-4 group was lower: 60.0 ± 10.0 N, while V-5 represented a local minimum: 56.0 ± 19.5 N. The value of the V-6 group was 64.0 ± 11.4 N, which resembled the V-2 group again. The FT-IR spectrum peaks ranged between $3,300$ and $2,800$ cm^{-1} , as well as below $1,700$ cm^{-1} , typically extending up to the zone between $1,000$ and 900 cm^{-1} . The peak at $3,291$ cm^{-1} indicates a hydrogen-bonded hydroxyl group ($-\text{OH}$). Two smaller peaks are also discernible at $2,920$ cm^{-1} and $2,852$ cm^{-1} , indicating asymmetric/symmetric stretching of methylene $\text{C}-\text{H}$ bonds. The seeds cracked under 60 N compressive force, and no significant differences were found in Figure 2 the fingerprint region of red clover seed spectra can be seen from V-1 to V-6 at 50 N compressive force. Isoflavones are typically found in red beet seeds (Coon et al., 2007), so the spectrum evaluation was based on this, and functional group correspondences were only assigned where they were clearly observed. The characteristic peaks of the spectrum (ranges provided using six spectra) are as follows: $1,731$ - $1,730$ cm^{-1} , $1,606$ - $1,603$ cm^{-1} ($\text{C}=\text{C}-\text{C}$ aromatic ring vibration), $1,420$ - $1,416$ cm^{-1} , $1,319$ - $1,320$ cm^{-1} , $1,023$ - $1,011$ cm^{-1} , 894 - 893 cm^{-1} (Nandiyanto et al., 2019). No literature reference to the spectrum of red beet seeds was found, so the results were not compared with a spectrum library. Since the seeds cracked under a compressive force of 60 N, the material inside the seed, which leaked out due to the crack and became measurable, was examined.

The spectrum of the material leaked from the interior of the seed is shown in Figure 3, marked with a black line. The results were compared with a spectrum library, and the spectrum shown in green provided the best match with the spectrum of *Flores Primulae cum Calybus*, it differs at peaks $1,603$ cm^{-1} and $1,238$ cm^{-1} . FT-IR analysis cannot determine each material inside the red clover seed but 1) high carbohydrate absorption site could be obtained within the range of $1,250$ - 900 cm^{-1} based on stretching vibrations of the $\text{C}-\text{O}-\text{C}$ group, which can be associated with subunits of carbohydrate, and 2) peak around at $1,740$ cm^{-1} is reported to correlate with lipids owing to $\text{C}=\text{O}$ ester group vibration of lipids and fatty acids (Coates, 2006).

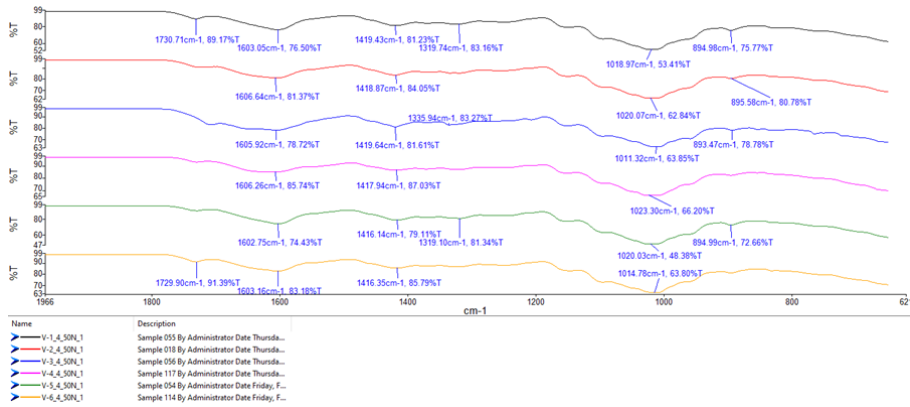


Figure 2: FT-IR spectrum of red clover seed (V1 → V6) at 50 N

The ATR FT-IR spectra of the material leaked and remained from the six different colored red clover seed groups, which were consistent with each other. Thus, the seed color was not identified as an influencing factor in this case.

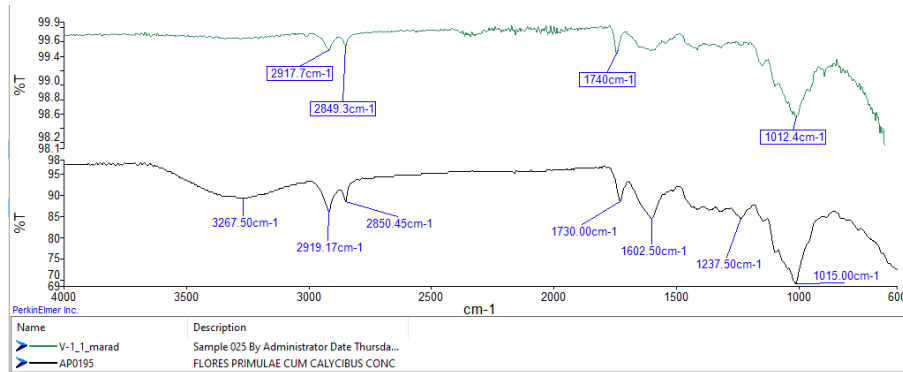


Figure 3: Liquid found inside the red clover seed (black), and the best matching spectrum from the spectrum library of Flores Primulae cum Calycibus (green)

4. Discussion

Based on the examination results, it can be concluded that the relationship between color affected by environmental influences and the network formed due to pressure resistance is non-linear, with no significant differences observed. An exception is the V-5 seed group, exhibiting an average deviation of 4.83 N from the norm. While the tolerance of the V-1 group is exceptional at the onset of resistance, the V-3 group is first in terms of seed cracking and splitting. The role of seed color followed a wave-like characteristic, wherein the V-2, V-4, and V-5 sample groups performed weaker in resistance, while the V-1, V-4, and V-5 groups were weaker in terms of splitting. Illustrating the relationship between seed color and pressure tolerance in red clover seeds, the linear regression equation is $y = -1.3143x + 36.267$, with a determination coefficient of 0.2052. The Pearson correlation coefficient is -0.453, indicating a moderate inverse relationship between the two factors. For seed cracking and splitting, the linear regression equation is $y = -0.6286x + 64.533$, with a determination coefficient of 0.083. The correlation coefficient in this case is -0.2880, indicating a weak inverse relationship.

The results of this study highlight the complex nature of tolerance, with differences between seed groups and variability in changing environmental conditions. Therefore, further research should emphasize deepening our understanding of tolerance and efforts to improve it (De Leander et al., 2013). In line with Atis et al. (2011), the findings shed light on the intricate relationship between seed color, pressure resistance, and cracking tendencies in red clover seeds. One notable finding is the non-linear nature of this relationship, suggesting that environmental influences impact seed color and pressure resistance in a complex and interconnected manner. While no significant differences were observed overall, the V-5 seed group stands out with a substantial average deviation from the norm, indicating potential variability within seed populations. This suggests a nuanced relationship between seed color and mechanical properties, warranting further investigation (Liu et al., 2020). According to Pirzado et al. (2021), the regression analysis further elucidates the relationship between seed color

and pressure tolerance, revealing a significant negative correlation. The determination coefficients indicate that while the relationship is moderate for pressure tolerance, it is weaker for seed cracking and splitting, suggesting additional factors influencing these phenomena. Zhao et al. (2024) highlight that a deeper understanding and enhancement of seed tolerance can contribute to the efficiency and sustainability of the agricultural sector, helping farmers improve yield averages and cope more effectively with environmental stresses.

5. Conclusions

The primary goal of our research was to investigate the general correlations between the environmental conditions of seed production and the seeds' tolerance to tension stress. The study assumed a connection between the color of red clover seeds and their resistance to external tension. Using ATR FT-IR analysis, we confirmed that the spectral response of seeds under compressive stress can be examined, allowing for a comparison between seed color and external influence. The Pearson correlation calculation showed an inverse, moderate relationship (-0.453) between seed color and pressure tolerance. For seed cracking and splitting, the correlation was inverse and weak (-0.288). The relationship between seed color and these factors is non-linear, with stronger connections in certain seed groups. The V-5 seed group differed from the average in terms of its relationship between cracking, pressure tolerance, and resistance onset.

Our results partially confirm our hypotheses. While genetic traits affect seed properties like color, the data also shows that darker seeds have lower compressive stress tolerance. In the future, we plan to expand this research by investigating correlations between seed composition and properties. FT-IR spectroscopy can detect lipid oxidation and protein changes in seeds, providing insights into stress resistance. This will improve our understanding of how treatments and environmental factors influence seed vitality and resistance. We also aim to examine tension stress resistance in seeds subjected to various conditions. The findings of this research can aid environmental and agricultural professionals involved in seed quality assessment and recommendations.

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