Innovations in the management of herbaceous grasslands of dehesas: remote sensing of pasture quality and new forage perennial legumes

Innovaciones para la gestión de pastos herbáceos de dehesa: teledetección de la calidad del pasto y nuevas leguminosas forrajeras perennes

Jesús Fernández Habas

Supervisors:
Pilar Fernández Rebollo
Pedro Sánchez Zamora

PhD Thesis
Cordoba, May 2023

Programa de doctorado: Ingeniería agraria, alimentaria, forestal y de desarrollo rural sostenible.

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TITULO: *Innovations in the management of herbaceous grasslands of dehesas: remote sensing of pasture quality and new forage perennial legumes*

AUTOR: *Jesús Fernández Habas*

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TÍTULO DE LA TESIS: Innovations in the management of herbaceous grasslands of dehesas: remote sensing of pasture quality and new forage perennial legumes

DOCTORANDO: Jesús Fernández Habas

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

Dra. **Pilar Fernández Rebollo**, Profesora Titular del Departamento de Ingeniería Forestal y Dr. **Pedro Sánchez Zamora**, Profesor Titular del Departamento Economía, Sociología y Política Agraria, directores de la presente Tesis Doctoral

INFORMAN:

Que la Tesis Doctoral titulada "Innovations in the management of herbaceous grasslands of dehesas: remote sensing of pasture quality and new forage perennial legumes" de la cual es autor Jesús Fernández Habas, Ingeniero de Montes, ha sido completada con éxito. El doctorando ha alcanzado y completado los objetivos planteados en este trabajo de investigación demostrando su capacidad como investigador. Como resultado se han publicado cinco de los seis capítulos que abordan los objetivos específicos como artículos científicos en revistas indexadas de alto impacto, todas ellas del Primer Cuartil, estando dos de ellas dentro del Primer Decil de su categoría.

Por tanto, cumple sobradamente los requisitos establecidos por el Reglamento 57/2020 de los Estudios de Doctorado de la Universidad de Córdoba (Art. 53) para la defensa de la tesis doctoral por la **modalidad de compendio de publicaciones**.

Publicaciones en revistas científicas indexadas:

- **Fernández-Habas, J**., Fernández-Rebollo, P., Gallardo-Cobos, R., Vanwalleghem, T., and Sánchez-Zamora, P. (2022). **A Farmer's Perspective on the Relevance of Grassland-Related Innovations in Mediterranean Dehesa Systems**. Forests, 13, 1182. Metrics 2021: IF: 3.282; JIF QUARTILE: Q1; JIF RANK: 14/69 (Forestry); Citations Google Scholar: 2.

- **Fernández-Habas, J**., Hidalgo-Fernández, M. T., Leal-Murillo, J. R., Méndez, P., Quero, J. L., Vanwalleghem, T., and Fernández-Rebollo, P. (2021). **Effects of two water regimes on morphological traits, nutritive value and physiology of three *Bituminaria bituminosa* varieties from the Canary Islands**. Journal of Agronomy and Crop Science, 208, 413-426. Metrics 2021: IF: 4.153; JIF QUARTILE: Q1; JIF RANK: 17/90 (Agronomy); Citations Google Scholar: 4.

- **Fernández-Habas, J**., Real, D., Vanwalleghem, T., and Fernández-Rebollo, P. (2023). **Lanza® Tedera Is Strongly Suppressed by Competition from Lolium multiflorum and Is Best Adapted to Light-Textured Soils**. Agronomy, 13, 965. Metrics 2021: IF: 3.949; JIF QUARTILE: Q1; JIF RANK: 18/90 (Agronomy); Citations Google Scholar: 1.

- **Fernández-Habas, J**., García-Moreno, A. M., Hidalgo-Fernández, M. T., Leal-Murillo, J. R., Abellanas-Oar, B., Gómez-Giráldez, P. J., ... and Fernández-Rebollo, P. (2021*)*. **Investigating the potential of Sentinel-2 configuration to predict the quality of Mediterranean permanent grasslands in open woodlands**. Science of the Total Environment, 791, 148101. Metrics 2021: IF: 10.754; JIF QUARTILE: Q1 (First decile); JIF RANK: 26/279 (Environmental sciences); Citations Google Scholar: 23.

- **Fernández-Habas, J**., Cañada, M. C., García-Moreno, A. M., Leal-Murillo, J. R., González-Dugo, M. P., Abellanas-Oar, B., ... and Fernández-Rebollo, P. (2022).

Estimating pasture quality of Mediterranean grasslands using hyperspectral narrow bands from field spectroscopy by Random Forest and PLS regressions. Computers and Electronics in Agriculture, 192, 106614. Metrics 2021: IF: 6.757; JIF QUARTILE: Q1 (First decile); JIF RANK: 4/59 (Agriculture, Multidisciplinary); Citations Google Scholar: 11.

Además, ha participado activamente en la difusión y transferencia de los resultados mediante seminarios, artículos de divulgación y participación en congresos nacionales e internacionales y realizado todas las actividades formativas obligatorias del programa de doctorado, así como actividades complementarias y voluntarias que han contribuido a su formación como investigador.

El doctorando ha realizado dos estancias de tres meses en el extranjero. Una estancia en el “Institute of Grassland Science” de la University of Göttingen (Alemania) bajo la supervisión del Dr. Johannes Isselstein y una segunda estancia en el “Centre for Applied Ecology "Prof. Baeta Neves" (CEABN)” de la University of Lisbon (Portugal) bajo la supervisión del Dr. Miguel Bugalho. Estas estancias han permitido al doctorando adquirir conocimientos necesarios para el desarrollo de la Tesis, así como mejorar su internacionalización.

Por tanto, esta Tesis cumple los requisitos necesarios establecidos en el Art. 54 a), b) y c) por el Reglamento 57/2020 de los Estudios de Doctorado de la Universidad de Córdoba para la **obtención de la mención internacional**.

Por todo ello, se autoriza la presentación de la tesis doctoral

Córdoba, 30 de mayo de 2023

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- **Fernández-Habas, J**., Fernández-Rebollo, P., Gallardo-Cobos, R., Vanwalleghem, T., and Sánchez-Zamora, P. (2022). **A Farmer's Perspective on the Relevance of Grassland-Related Innovations in Mediterranean Dehesa Systems**. Forests, 13, 1182. Metrics 2021: IF: 3.282; JIF QUARTILE: Q1; JIF RANK: 14/69 (Forestry); Citations Google Scholar: 2.

- **Fernández-Habas, J**., Hidalgo-Fernández, M.T., Leal-Murillo, J.R., Méndez, P., Quero, J.L., Vanwalleghem, T., and Fernández-Rebollo, P. (2021). **Effects of two water regimes on morphological traits, nutritive value and physiology of three *Bituminaria bituminosa* varieties from the Canary Islands**. Journal of Agronomy and Crop Science, 208, 413-426. Metrics 2021: IF: 4.153; JIF QUARTILE: Q1; JIF RANK: 17/90 (Agronomy); Citations Google Scholar: 4.

- **Fernández-Habas, J**., Real, D., Vanwalleghem, T., and Fernández-Rebollo, P. (2023). **Lanza® Tedera Is Strongly Suppressed by Competition from *Lolium multiflorum* and Is Best Adapted to Light-Textured Soils**. Agronomy, 13, 965. Metrics 2021: IF: 3.949; JIF QUARTILE: Q1; JIF RANK: 18/90 (Agronomy); Citations Google Scholar: 1.

- **Fernández-Habas, J**., García-Moreno, A. M., Hidalgo-Fernández, M. T., Leal-Murillo, J. R., Abellanas Oar, B., Gómez-Giráldez, P.J., González-Dugo. MP., and Fernández-Rebollo, P. (2021*)*. **Investigating the potential of Sentinel-2 configuration to predict the quality of Mediterranean permanent grasslands in open woodlands**. Science of the Total Environment, 791, 148101. Metrics 2021: IF: 10.754; JIF QUARTILE: Q1 (First decile); JIF RANK: 26/279 (Environmental sciences); Citations Google Scholar: 23.

- **Fernández-Habas, J**., Carriere-Cañada, M., García-Moreno, A. M., Leal-

Murillo, J.R., González-Dugo, M.P., Abellanas Oar, B., Gómez-Giráldez, P.J. and Fernández-Rebollo, P. (2022). **Estimating pasture quality of Mediterranean grasslands using hyperspectral narrow bands from field spectroscopy by Random Forest and PLS regressions**. Computers and Electronics in Agriculture, 192, 106614. Metrics 2021: IF: 6.757; JIF QUARTILE: Q1 (First decile); JIF RANK: 4/59 (Agriculture, Multidisciplinary); Citations Google Scholar: 11.

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- **Fernández-Habas, J.,** Hidalgo-Fernández, M. T., Leal-Murillo, J. R., García-Moreno, A. M., Reina-Belmonte, J. A., Quero, J. L., Fernández-Rebollo, P., Vanwalleghem, T., Méndez, P. **Respuesta y uso del agua de tres variedades de Bituminaria bituminosa bajo dos tratamientos de riego: resultados preliminares.** Oral communication. VIII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Cordoba (Spain) 08/04/2019-11/04/2019.

- **Fernández-Habas J**., Hidalgo-Fernández M.T., Leal-Murillo J.R., García-Moreno A.M., Reina-Belmonte J.A., Quero J.L., Fernández-Rebollo P1., Vanwalleghem T., and Méndez P. **Drought resistance and water use efficiency of three genotypes of Bituminaria bituminosa: preliminary results.** Poster communication. 28TH General Meeting of European Grassland Federation. Helsinki (Finland) 18/02/2020-19/02/2020.

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in permanent grasslands of open woodlands; preliminary results. Oral communication. 21st Symposium of the European Grassland Federation. Kassel (Germany) 17/05/2021-19/05/2023.

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- **Fernández-Habas, J**., Carriere Cañada, M., García Moreno, A.M., Leal-Murillo, J.R., González-Dugo, M.P., Abellanas Oar, B., Gómez-Giráldez, P.J., Fernández-Rebollo, P. **Satellite remote sensing of pasture quality in Dehesas: potential and future prospects**. Poster communication. IV Congreso ibérico DehesaMontado. Badajoz (Spain) 15/06/2022-16/06/2022.

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- **Fernández-Habas, J**., Fernández-Rebollo, P., Abellanas Oar, B., Leal-Murillo, J.R., Hidalgo-Fernández, M.T., García Arnés, J. **Uso de imágenes multiespectrales e hiperespectrales para estimar la calidad de los pastos herbáceos mediterráneos**. Poster comunication. 5ª Reunión Ibérica de Pastos y Forrajes, Huelva-Loulé (Spain-Portugal). 17/04/2023-20/04/2023.

- Fernández-Rebollo, P., **Fernández-Habas, J**., Leal-Murillo, J. R., Hidalgo-Fernández, M. T., Real, D. **Posibilidad de uso de *Bituminaria bituminosa* var. *lanza* como cultivo forrajero en sistemas ganaderos mediterráneos.** Oral comunication. 5ª Reunión Ibérica de Pastos y Forrajes, Huelva-Loulé (Spain-

Portugal). 17/04/2023-20/04/2023.

Dissemination articles

- Fernández-Rebollo, P., **Fernández-Habas, J**., Leal-Murillo, J.R., and García-Moreno, A. M. (2021). **Teledetección y modelos de predicción de la calidad bromatológica de los pastos para una gestión eficiente**. Tierras Ovino, 35, 70-74.

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List of acronyms

A_{area}: Net photosynthesis per area

ADF: Acid detergent fibre content

BAM: *Bituminaria bituminosa* var. *albomarginata*

BBT: *Bituminaria bituminosa* var. *bituminosa*

BBCH: Biologische Bundesanstalt Bundessortenamt und Chemische Industrie

BCC: *Bituminaria bituminosa* var. *crassiuscula*

BFE: Backward feature elimination

BOA: Bottom Of Atmosphere

CAP: Common Agricultural Policy

CHIME: Copernicus Hyperspectral Imaging Mission for the Environment

CI: Confidence intervals

CP: Crude protein content

DMH: Distance of Mahalanobis

DW: Deficit-watered

EDOM: Enzyme digestibility of organic matter

ES: Ecosystem services

ESA: European Space Agency

ET: Evapotranspiration

GHG: Greenhouse gases

g_{Sarea}: Stomatal conductance per area

HNV: High Nature Value

IPCC: Intergovernmental Panel on Climate Change

LAR: Leaf area ratio

LMF: Leaf mass fraction

LOO: Leave-one-out

LOOcv: Leave-one-out crossvalidation

LVs: Latent variables

NDF: Neutral detergent fibre content

NIRS: Near-infrared spectroscopy

MSE: Mean Squared Error

OBB: *Out-of-bag*

OR: Odds ratio

PLS: Partial Least Squares Regression

PRESS: Predicted Error Sum of Squares

RCP: Representative Concentration Pathway

RF: Random Forest

RMF: Root mass fraction

RMSE: Root mean square error

RPD: Ratio of the standard deviation/ Ratio of Performance to Deviation

RSWC: Relative soil water content

RWC: Relative leaf water content

SE: Standard error

SEP: Standard error of prediction

SLA: Specific leaf area

SMF: Stem mass fraction

Spec-field: Field in-situ pasture canopy reflectance recorded with an ASD FieldSpec Spectroradiometer

Spec-lab: Reflectance of dried and ground pasture samples recorded in laboratory with an ASD LabSpec 5,000 spectrometer and High-Intensity Muglight, model-A122100

SWIR: Short-wave infrared

UAVS: Unmanned aerial vehicles

Vis-NIRS: Visible-Near-Infrared

WUE: Water-use efficiency

WW: Well-watered

Resumen

Las dehesas son sistemas agrosilvopastorales caracterizados por una alta multifuncionalidad y capacidad de proveer múltiples servicios ecosistémicos. En este sistema, creado y mantenido como un bosque abierto para la cría de ganado en extensivo, los pastos herbáceos mediterráneos juegan un papel central como principal fuente de alimento. Este servicio de aprovisionamiento se ve amenazado por las altas variaciones espaciotemporales en la producción de biomasa y calidad nutritiva de los pastos, debidas en gran medida a la alta estacionalidad y variabilidad interanual de las precipitaciones en climas mediterráneos. Estas altas variaciones plantean un reto para los ganaderos, que tienen que lidiar con el efecto de sequías, baches alimenticios y recursos muy variables para alimentar al ganado de forma rentable. Las crisis socioeconómicas y los ya patentes efectos del cambio climático agravan esta situación al causar inestabilidad en los mercados, incremento en el coste de los alimentos para el ganado y un clima más errático. En este contexto, se requieren innovaciones que contribuyan a incrementar la resistencia y resiliencia a esas amenazas y a mejorar la eficiencia en manejo de los pastos. Esta tesis doctoral ha estudiado dos innovaciones con potencial para implementarse en el manejo de los pastos de dehesa; el uso de la leguminosa perenne *Bituminaria bituminosa* y la teledetección de la calidad nutritiva del pasto. A continuación, se resumen los principales resultados de seis capítulos en los que se ha investigado la relevancia, potencial y limitaciones de dichas innovaciones.

El **Capítulo II** estudió la opinión de los ganaderos de dehesa sobre la relevancia que pueden tener algunas innovaciones relacionadas con el manejo de los pastos. Los resultados mostraron que los ganaderos consideran muy relevantes las innovaciones dirigidas a incrementar la productividad de los pastos y su resistencia a sequías como por ejemplo la mejora de los pastos con mezclas de semillas y el uso de especies resistentes a la sequía. Sin embargo, innovaciones tecnológicas relacionadas con la agricultura de precisión como el uso de GPS para el ganado o el uso de teledetección parecen ser menos relevantes para los ganaderos de dehesa. Esto podría indicar baja aplicabilidad al contexto de las dehesas o la existencia barreras que dificulten su uso, pero también la necesidad de avanzar en su desarrollo e información sobre su potencial.

Ante el potencial de la leguminosa perenne *Bituminaria bituminosa* como una especie resistente a la sequía y la demanda de tales especies, el **Capítulo III** estudió en un

experimento en invernadero, características y respuesta a dos regímenes hídricos diferentes de las tres variedades de *B. bituminosa*: var. *albomarginata*, var. *crassiuscula* y var *bituminosa*. Las tres variedades mostraron una reducción de biomasa similar como respuesta a una reducción del 50% del riego. Sin embargo, var. *albomarginata* mostro menor producción de biomas (aérea y radical) en ambos tratamientos de riego que las otras dos variedades. El pobre desarrollo de var. *albomarginata* podría indicar susceptibilidad de esta variedad a las condiciones iniciales de alto contenido de agua en el suelo impuestas en este experimento. Esto podría limitar el establecimiento y desarrollo de var. *albomarginata* en casos de saturación periódica del suelo, especialmente al comienzo de la estación de crecimiento. Var. *crassiuscula* y var. *albomarginata* mostraron menor área específica de hoja que var. *bituminosa*, lo cual puede suponer una importante adaptación a condiciones de alta intensidad lumínica y altas temperaturas. Además, rasgos morfológicos como la proporción hoja/tallo de var. *crassiuscula* (0.8) y var. *albomarginata* (1.06) indicaron mejor aptitud forrajera de esas variedades que var. *bituminosa* (0.38). Las tres variedades mostraron valores adecuados de contenido en proteína y digestibilidad que indican potencial para mejorar la calidad nutritiva de los pastos de dehesa.

En base a los resultados del **Capítulo III**, el **Capítulo IV** investigó, en un ensayo en macetas, el efecto de suelos con distinta textura (franco arenoso vs. arcillosa) en condiciones de alto contenido de agua en el suelo en el desarrollo de la nueva variedad de *B. bituminosa* var. Lanza® que combina atributos de var. *crassiuscula* y var. *albomarginata.* Este estudio también testó el efecto de la competencia con *Lolium multiflorum* en ambos suelos en el desarrollo de Lanza®. Los resultados mostraron que Lanza® puede desarrollarse en ambos suelos sin un efecto significativo en la producción de biomasa. Sin embargo, se observó un desarrollo más lento y menor producción de raíces finas (<2 mm) (44%) en suelos arcillosos, lo cual podría indicar preferencia de esta especie por suelos de textura arenosa. La competencia con *L. multiflorum* redujo drásticamente (86%) la producción de Lanza®. La biomasa aérea de Lanza® también fue superior, en un 60%, a la producción total de Lanza® y *L. multiflorum* cultivados juntos. Estos resultados mostraron que *B. bituminosa* podría tener baja habilidad competitiva y su establecimiento podría fallar en condiciones de alta competencia con gramíneas anuales.

En el **Capítulo V** se testó el efecto de una reducción de la precipitación anual del 33% en producción, calidad nutritiva y fenología *B. bituminosa* cv Lanza® en un experimento en campo establecido en Córdoba desde febrero de 2021 a julio de 2022. Lanza® demostró ser resistente a una reducción anual de la precipitación del 33%. Sin embargo, la estacionalidad de las precipitaciones en ambos años influenció la perdida de hoja en verano y por tanto la capacidad de Lanza® de proveer forraje de calidad durante la época estival. Lanza® establecida como monocultivo al final del invierno fue tan productiva como alfalfa en condiciones de secano en el primer año (2,700 kg ha^{-1}). Las lluvias tardías en mayo, junio y julio permitieron a tedera mantener hoja verde durante todo el verano mientras que alfalfa perdió la hoja a finales de julio. Una distribución adecuada de las precipitaciones hasta abril del segundo año y las suaves temperaturas invernales permitieron a Lanza® alcanzar grandes producciones (9,000 kg ha^{-1}) sin un efecto de la reducción de precipitación. Sin embargo, la alta acumulación de biomasa y las condiciones de mayor sequía en mayo-julio provocaron una pérdida de hoja generalizada en Lanza®. En esas condiciones, alfalfa fue un 52% menos productiva bajo precipitación reducida (2,215 kg ha^{-1} en control vs 1,074 kg ha^{-1} bajo precipitación reducida). De acuerdo con los resultados del **Capítulo IV**, la competencia con especies espontáneas anuales tuvo un gran impacto en el rendimiento de Lanza® dando lugar a producciones muy bajas (<250 kg ha^{-1}). Lanza® mostró una calidad nutritiva adecuada como forrajera durante la época estival, especialmente en hoja (PB>13%), aunque durante el segundo año, las plantas de Lanza® sin cortar desde establecimiento desarrollaron tallos gruesos y lignificados que junto con la menor proporción hoja/tallo causó una gran pérdida de calidad nutritiva. Comparada con alfalfa, Lanza® mostró una fenología temprana para los estadios fenológicos desde emergencia de la inflorescencia hasta maduración del fruto, con una floración temprana y larga (desde principios de abril hasta mediados de mayo) e importantes solapes entre floración, desarrollo y maduración del fruto. Esta floración temprana podría ser una característica ventajosa en ambientes con sequias tempranas. La competencia con anuales retrasó la fenología de Lanza® y la reducción de precipitación indujo emergencia de la inflorescencia y floración más tempranas (~10 días), mientras que alfalfa únicamente mostró un retraso en el inicio del desarrollo y maduración del fruto.

En base a los resultados de los **Capítulos III-V**, *B. bituminosa*, y en particular cv Lanza®, muestra características adecuadas para usarla como especie forrajera durante la época estival en dehesas. Próximos estudios deben investigar medidas dirigidas a reducir la competencia durante el establecimiento de Lanza® en monocultivos y testar especies acompañantes adecuadas para incluir esta especie en mezclas multiespecíficas. Es necesario seguir investigando la respuesta de Lanza® en años de distinta meteorología en diferentes estaciones y localizaciones, así como su interacción con el manejo para definir estrategias de manejo adecuadas bajo condiciones climáticas variables.

Con respecto al uso de teledetección para evaluar la calidad de los pastos, el **Capítulo VI** investigó el potencial de la configuración del satélite Sentinel-2 para predecir la calidad nutritiva de pastos mediterráneos de dehesa. Se calibraron modelos "Partial Least Squares Regression" (PLS) para predecir concentración en proteína bruta (PB), fibra neutro detergente (FND), fibra ácido detergente (FAD) y digestibilidad enzimática de la materia orgánica (DEMO). Se emplearon tres conjuntos de datos diferentes: i) mediciones de reflectancia de pasto molido y seco en estufa realizadas en laboratorio con espectrorradiómetro y remuestreadas a la configuración de Sentinel-2 (Spec-lab), ii) medidas de reflectancia de pasto *"in situ"* con espectrorradiómetro y remuestreadas a la configuración de Sentinel-2 (Spec-field), y iii) reflectancia de imágenes Sentinel-2 corregida a niveles por debajo de la atmósfera (Sentinel-2 BOA). Se obtuvieron valores medios de R^2_{test}=0.68 y RPD_{test}=1.82 al utilizar Spec-lab, lo cual sugiere que esa podría ser la máxima precisión alcanzable utilizando la configuración espectral de Sentinel-2, ya que factores como suelo desnudo y la estructura de la cubierta vegetal no influyen en Spec-lab. R^2_{test} disminuyó 0.11 al utilizar Spec-field y 0.18 al emplear Sentinel-2 BOA. Los estadísticos para FND utilizando Spec-lab fueron algo menores, con valores medios de R^2_{test}=0.64 y RPD_{test}=1.73. Para FAD y DEMO, solo las predicciones hechas con Spec-lab produjeron valores aceptables. Los modelos ajustados con datos Sentinel-2 BOA mostraron una habilidad predictiva moderada para evaluar PB, con valores medios R^2_{test}=0.50 y RPD_{test}=1.54. En general, las bandas de las regiones del "red-edge" y SWIR fueron las más determinantes para predecir PB y FND. Estos resultados indicaron que el potencial de Sentinel-2 estaría limitado a estimaciones de PB de precisión moderada que podrían permitir hacer evaluaciones cualitativas de la calidad del pasto en dehesas.

El **Capítulo VI** estudió el potencial de la misión de alta prioridad de la Agencia Espacial Europea "Copernicus Hyperspectral Imaging Mission for the Environment (CHIME)" para estimar la calidad de pastos mediterráneos. Se emplearon los algoritmos de "Machine learning" PLS y "Random Forest" (RF), y datos reflectancia de pasto adquiridos "in situ" remuestreados a una resolución espectral de 10 nm para similar las características espectrales de CHIME. Los resultados mostraron una habilidad predictiva prometedora para evaluar FND y especialmente PB, con valores medios de R^2_{test}=0.82, RMSE=2.23% y RPD=2.47. El desarrollo de futuros satélites hiperespectrales como CHIME, podrían mejorar la estimación de calidad de pastos mediterráneos permitiendo evaluaciones cuantitativas de PB.

El desarrollo de conjuntos de datos extensos y diversos es esencial para reducir la incertidumbre de las estimaciones, mejorar su precisión y entender el verdadero potencial de la teledetección satelital de la calidad de los pastos mediterráneos. La combinación de modelos empíricos y físicos junto con algoritmos de “deep learning” y datos hiperspectrales pondría suponer un avance significativo. Sin embargo, la resolución espacial de los satélites no comerciales sigue siendo una limitación, especialmente en paisajes heterogéneos como las dehesas.

La integración de las innovaciones estudiadas en esta tesis doctoral podría mejorar la información sobre la variación espacial y temporal de la calidad del pasto y reducir la ocurrencia de baches alimenticios en dehesas, contribuyendo de esa forma a asegurar el futuro de este sistema multifuncional.

Abstract

Dehesas are “human-shaped” multifunctional ecosystems that provide important ecosystem services. In dehesas, the herbaceous Mediterranean grasslands play a central role as the main source of feed for livestock in extensive systems. However, this provisioning service is challenged by the strong spatio-temporal variations in biomass production and pasture quality due to a large extent to the high seasonality and inter-annual variability of rainfall in Mediterranean climates. This poses serious drawbacks to the management of grasslands of dehesas as farmers must deal with droughts, feed gaps and highly variable resources to feed livestock cost-effectively. Socio-economic crises and the already patent effects of climate change are crucial drivers leading to more unstable markets, the rise of the cost of feed for livestock and a drier and more erratic climate. In this context, there is a need for innovations that could contribute to increasing the resistance and resilience to such threats and provide information for a more efficient and informed management of herbaceous grasslands of dehesas. This PhD thesis has studied two innovations with potential to be used in the management of Mediterranean grasslands of dehesas; the use of the perennial legume *Bituminaria bituminosa*, and the remote sensing of pasture quality.

Chapters II studied the relevance that dehesa farmers attribute to grassland-related innovations in the management of grasslands of dehesas. The results showed that those innovations aimed at increasing the productivity of grasslands and their resistance to drought such as the use of seed mixtures and drought-resistant species were considered highly relevant by the farmers. However, high-tech innovations such as remote sensing and GPS collars were given lower relevance, which could denote low applicability to the context of dehesas or the existence of barriers hindering the adoption but also a need for further development and better information on their potential.

In light of the potential of the perennial legume *Bituminaria bituminosa* as a drought-resistant species and the demand for such species in dehesa systems, **Chapter III** studied in a pot experiment traits and response to two different water regimes of the three varieties of this species from the Canary Islands: var. *albomarginata*, var. *crassiuscula* and var *bituminosa*. The three varieties reported similar aerial biomass reduction (~50%) under 50% watering shortage. However, var. *albomarginata* showed lower biomass production (shoot and root) under both water regimes than the other two varieties. The poor root development of var. *albomarginata* could denote susceptibility and slower development under the initial high soil water content imposed in this experiment. This might limit its establishment and development in cases of periodic soil water saturation, especially at the beginning of the growing season. Var. *crassiuscula* and var. *albomarginata* had a lower specific leaf area than var. *bituminosa* which is an important adaptation to conditions of high light intensity and temperatures. In addition, the morphological traits such as the leaf to stem ratio of var. *crassiuscula* (0.80) and var. *albomarginata* (1.06) indicated better forage aptitude of these varieties than var. *bituminosa* (0.38). All varieties showed good values of crude protein and digestibility with potential to improve the pasture quality of Mediterranean grasslands.

Based on the results of **Chapter III**, **Chapter IV** investigated in a pot experiment the effects of soils with contrasting textures (loamy sand vs. clay) in conditions of high soil water content on the development of the newly developed variety of *B. bituminosa* var. Lanza® which combines attributes from var. *crassiuscula* and var. *albomarginata*. This study also tested the effect of competition from *Lolium multiflorum* in both soil types on the development of Lanza®. The results showed that Lanza® can develop in both soil types without significant effects on biomass production. However, slower development and lower thin root (<2 mm) production (44% reduction) in clay soil might denote a preference of this species for light-textured soils. Competition from *L. multiflorum* strongly reduced (86%) the dry mass production of Lanza®. Shoot DM production of Lanza® grown without competition was 60% higher than the total shoot DM production of the mixture of Lanza® and *L. multiflorum*. These results showed that *Bituminaria bituminosa* might have low competitive ability and could fail to establish in environments with strong competition from annual grasses.

In **Chapter V** the response of *Bituminaria bituminosa* cv Lanza® to 33% rainfall reduction and competition in terms of yield, forage quality, and phenology was tested in a field experiment carried out from February 2021 to July 2022 in Cordoba, south Spain. Lanza® proved to be resistant to a 33% annual rainfall reduction, although seasonality of rainfall in both years influenced leaf shedding, and thus its ability to provide green forage over the summer season. Monoculture of Lanza® established in late winter was as productive as alfalfa in rainfed conditions in the first year (2,700 kg ha^{-1}). Late spring and summer rainfall allowed Lanza® to maintain green leaf over the summer season, while alfalfa shed leaf by the end of July. During the second year, suitable distribution of rainfall until April and a mild winter temperature allowed a high production of tedera (9,000 kg ha^{-1})

with no significant effect of rainfall reduction on biomass. However, due to high biomass accumulation and drier conditions in May-July, Lanza® did not maintain green leaf over the summer. In these conditions, alfalfa DM yield was 52% lower under reduced rainfall (2,215 kg ha^{-1} in control vs 1,074 kg ha^{-1} reduced rainfall). In agreement with **Chapter IV**, competition from annual grasses and forbs had a strong effect on the yield of Lanza® in field conditions, leading to a very low production (DM yield<250 kg ha^{-1}). Lanza® showed suitable forage quality as an out-of-season forage, especially in the leaf fraction (CP>13%), although uncut Lanza® plants developed thick and lignified stems that together with higher leaf shedding caused a great decline in shoot forage quality in the second year. Rainfall reduction had little or no impact on forage quality. Compared to alfalfa, Lanza® showed more advanced phenology for the reproductive stages from inflorescence emergence to ripening, with early and long flowering (from early April to mid-May) and important overlaps between flowering, fruit development and ripening. This early flowering of Lanza® could be an advantageous characteristic in environments with early drought onset. Competition delayed the phenology of Lanza®, and rainfall reduction induced earlier inflorescence emergence and flowering (~10 days), while for alfalfa only a delayed beginning of development and maturity of fruit was observed.

Based on the results from **Chapters III** to **V**, *B. bituminosa*, and in particular cv Lanza®, shows suitable characteristics to be used as an out-of-season forage in dehesas. Future studies should investigate measures aimed at reducing early competition to successfully establish *B. bituminosa* in monoculture and test suitable partner species to include it in multispecies mixtures. Further research is needed to test the response of tedera to years of contrasting meteorology in different seasons and locations, and its interaction with management to define optimal management strategies under variable meteorological conditions.

Concerning the use of remote sensing to assess pasture quality, **Chapter VI** investigated the potential of Sentinel-2 configuration to predict forage quality of Mediterranean grasslands of dehesas. Partial Least Squares Regression (PLS) models were calibrated to predict crude protein content (CP), neutral detergent fibre content (NDF), acid detergent fibre content (ADF) and enzyme digestibility of organic matter (EDOM). Three different reflectance datasets were used: (i) laboratory measurements of reflectance of dry and ground pasture samples resampled to Sentinel-2 configuration (Spec-lab), (ii) field in-situ measurements of grasslands canopy reflectance resampled to Sentinel-2 configuration

(Spec-field), and (iii) Bottom Of Atmosphere (BOA) Sentinel-2 imagery. Mean R^2_{test}=0.68 and RPD_{test}=1.82 were obtained using Spec-lab, suggesting that this might be the maximum accuracy achievable using Sentinel-2 spectral configuration as Spec-lab reflectance is not influenced by canopy, or soil effects. Mean R^2_{test} decreased by 0.11 with Spec-field and by 0.18 when Sentinel-2 reflectance was used. Statistics for NDF with Spec-lab data were lower with mean R^2_{test}=0.64 and mean RPD_{test}=1.73. For ADF and EDOM, only predictions made with Spec-lab produced acceptable results. Models built with Sentinel-2 BOA reflectance showed a moderate predictive ability to assess CP, with mean R^2_{test}=0.50 and RPD_{test}=1.54. Overall, the bands from the red-edge region, and the SWIR regions showed the highest contribution to estimating CP and NDF. These results indicated that the potential of Sentinel-2 configuration might be limited to predictions of moderate accuracy of CP that could allow qualitative assessments of pasture quality in dehesas.

Chapter VII studied the potential of the forthcoming high-priority mission candidate of the European Space Agency, Copernicus Hyperspectral Imaging Mission for the Environment (CHIME) to assess pasture quality of Mediterranean grasslands. The machine learning algorithms PLS and Random Forest (RF) and field spectroscopy CHIME-like data at 10 nm of spectral resolution were used to estimate the pasture quality. The results showed promising ability to assess NDF and especially CP with mean values of R^2_{test}=0.82, RMSE=2.23% and RPD=2.47. Future hyperspectral satellites such as CHIME, could improve the assessment of pasture quality of Mediterranean grasslands allowing quantitative estimations of CP.

The development of large and diverse datasets is essential to reduce the uncertainty of the estimations, improve accuracy and understand the true potential of satellite remote sensing of pasture quality of Mediterranean grasslands. The combination of empirical and physical-based models coped with deep learning algorithms and hyperspectral data could mean a breakthrough in satellite remote sensing of pasture quality. However, the spatial resolution of the non-commercial satellites remains a limitation, especially in heterogeneous landscapes such as dehesas.

The integration of the innovations studied in this PhD Thesis could improve the information on the spatial and temporal variation of pasture quality and reduce the occurrence of feed gaps in dehesas, and thus contribute to ensuring the future of this multifunctional system.

CHAPTER I. GENERAL INTRODUCTION

BACKGROUND AND CONTEXTUALISATION

Mediterranean grasslands of dehesas: importance and characteristics

Grasslands, including open grasslands, woody grasslands, savannas and open woodlands ecosystems cover 40% of the Earth's terrestrial surface and Earth's 70% of the global agricultural land, which makes them one of the largest terrestrial ecosystems (Bardgett et al., 2021; Buisson et al., 2022; Suttie et al., 2006). Mediterranean grasslands in particular are represented in South Africa, California, Chile, southern Australia and in the Mediterranean basin itself (Cosentino et al., 2014). In these areas, they have played a major role in the development of human societies (Jouven et al., 2010). Mediterranean grasslands have sustained the production of livestock in very limited soil and climatic environments allowing the livelihood of humans. In the last decades, there has been a growing effort to recognise the role of Mediterranean grasslands to provide multiple ecosystem services. Some of them are the provision of animal feed, the rich biodiversity of these ecosystems, carbon storage and wildfire prevention (Porqueddu et al., 2016; Schils et al., 2022; Varela and Robles-Cruz, 2016).

This multifunctional value is strongly related to livestock grazing and human-mediated disturbances as key factors to perpetuate these ecosystems, their role in the landscape and to generate their high plant diversity (Bugalho et al., 2011; Cosentino et al., 2014; Myers et al., 2000; Porqueddu et al., 2016; Sternberg et al., 2003). For centuries, humans have made use of livestock and fire to shape Mediterranean landscapes which has led to a diverse mosaic of ecosystems. In the Iberian Peninsula, oak open woodlands are one of the most emblematic "human-shaped ecosystems" that have resulted from centuries of coexistence with human-mediated disturbances in Mediterranean environments (Bugalho et al., 2011). Oak open woodlands of the Iberian Peninsula, known as dehesas in Spain and Montado in Portugal are a savanna-like ecosystems with a sparse tree cover (5%-70% canopy cover) of Holm oak (*Quercus ilex*) and Cork oak (*Quercus suber*) and less frequently other species such as *Quercus pyrenaica*, *Olea europaea* and *Pinus pinaster* (Bugalho et al., 2011; Moreno and Pulido, 2008; Olea and Miguel-Ayanz, 2006). The understory is composed mainly of Mediterranean grasslands in combination with a heterogenous mix of patches of shrub, forest, fallows and cereal crops (in long rotation) in which the tree cover can vary considerably depending on the terrain and land use intensity. This system covers 3.1 million hectares in the southwest of the Iberian Peninsula (Moreno and Pulido, 2008) (Figure 1) being protected by the Habitat Directive, as habitat 6310 “Dehesas with evergreen Quercus spp” (European Commission, 1992, 2013). It is worldwide recognised as an example of a High Nature Value (HNV) farming system for its integration of land use and biodiversity conservation, which leads to a unique multifunctionality (Plieninger et al., 2021).

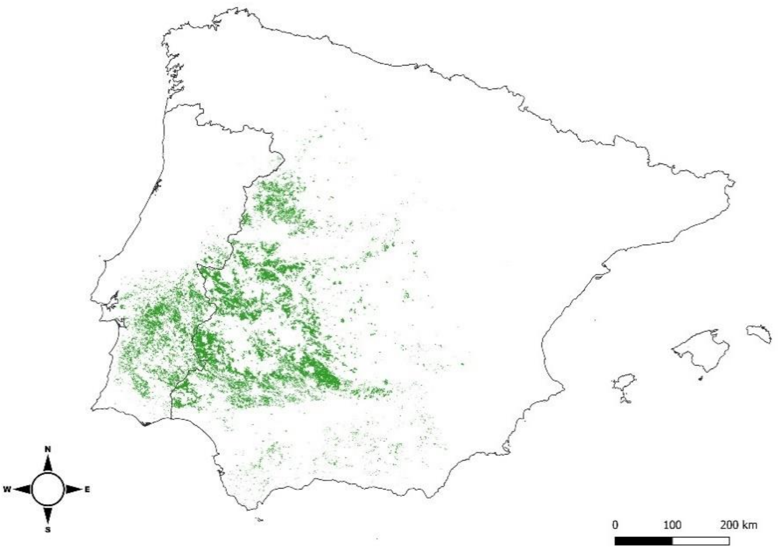

Figure 1. Area of dehesa in the Iberian Peninsula. Source: Copernicus Land Monitoring Service, 2018: https://land.copernicus.eu/pan-european/corine-land-cover/clc2018.

Dehesas are, therefore, agrosilvopastoral systems devoted to livestock raisin in extensive systems (Pinto-Correia et al., 2011). Oak trees are usually defined as “ecological engineers” in dehesas (Moreno and Pulido, 2008). They have crucial importance in the system to complement herbaceous grasslands in feed production by providing acorns and brows for an animal while providing other commercial products such as wood and cork (Pinto-Correia et al., 2011; Olea and Miguel-Ayanz, 2006). Oak trees are also a cornerstone in the provision of regulating ecosystem services from dehesas for example by enhancing carbon storage (Reyna-Bowen et al., 2020) and promoting plant and animal diversity (López-Sánchez et al., 2016). Crops are often important to provide feed for livestock, especially in key moments of the year as a complement to the seasonal production of grasslands (García-Moreno et al., 2016; Olea and Miguel-Ayanz, 2006).

In dehesas, Mediterranean grasslands play a central role as the main feed source for livestock (García-Moreno et al., 2016). They are composed mainly of annual species in communities of such as Helianthemetalia and Thero-Brometalia (Olea and Miguel-Ayanz, 2006) with high plant diversity, reaching values of 135 spp/0.1 ha and 45 species/m^2 (Marañon, 1985). The productivity and dynamics of these grasslands are determined mainly by the Mediterranean climatic conditions. Annual dry matter (DM) production of Mediterranean grasslands in dehesas can range from 1,000 to 3,500 kg ha^{-1} with an average production of 1440 kg ha^{-1} (García-Moreno et al., 2016; Olea and Miguel-Ayanz, 2006; Olea et al.,1989). As demonstrated by previous studies, the production of dehesas's grasslands shows strong intra- and inter- annual variability caused mainly by the seasonality and variability of rainfall between seasons and years but also affected by: i) the seasonal temperatures, with limiting high temperatures in summer and low temperatures in winter; ii) soil physicochemical characteristics, mainly sandy sallow soils of pH from acid to neutral, low organic matter, but highly variable along the dehesa area; iii) topography with high differences between slopes and valley bottoms and iv) management, showing a positive response to phosphorus fertilisation and improvement with legume-rich mixtures (García-Moreno et al., 2016; Hernández-Esteban et al., 2019; Moreno and Pulido, 2008; Olea and Miguel-Ayanz, 2006; Olea et al.,1989; Vázquez-De-Aldana et al., 2008).

The intra- annual seasonality results in an irregular distribution of biomass production with two peaks of production of 60-70% in spring and 15-25% in autumn, very low production of 5-15% in winter (highly dependent on the cold temperatures) and no production during the summer season (García-Moreno et al., 2016; Olea et al.,1989). As an example of the strong variations in production, Olea et al., (1986) reported differences of 250% (average of 5 years) in dehesa grasslands of the same region (Extremadura, Spain). Regarding the inter-annual variability, Vázquez-De-Aldana et al., (2008) recorded differences up to 100% in annual biomass production in a dehesa of western Spain.

This strong intra- and inter- annual variability also affects the quality of Mediterranean grasslands, which is frequently overlooked despite its importance in the main function of dehesas grassland's to provide animal feed (Vázquez-De-Aldana et al., 2008). Apart from the above-mentioned factors determining the variability of productivity, quality is also strongly affected by species, functional group composition, and phenology. The crude protein content (CP) increases with the proportion of legumes due to their higher content of CP compared to grasses and forbs. Their superior forage quality motivate the promotion of legumes in Mediterranean grasslands (Olea et al., 1986). The inter- annual variability of pasture quality in Mediterranean grasslands ranges from 20% of CP and 65% of digestibility of organic matter (DOM) in autumn to values of about 6% CP and 45% DOM in summer (García-Moreno et al., 2016; Olea et al.,1989). The decrease in quality is especially important after main the phenological stages of shoot development and flowering. Within the same season, differences in species composition, phenology among species and communities and topography can lead to strong spatial variability with values of CP and DOM ranging from 8.5% to 14% and from 55.2% to 63.3%, respectively in spring (Olea et al.,1989; Vázquez-De-Aldana et al., 2008). In virtually every year, the CP during the summer season drops below 8%, which is assumed to be a critical value in ruminants' diet as ingestion decreases drastically as a result of limited microbial activity due to nitrogen deficiency in the rumen (Mathis and Sawyer, 2007).

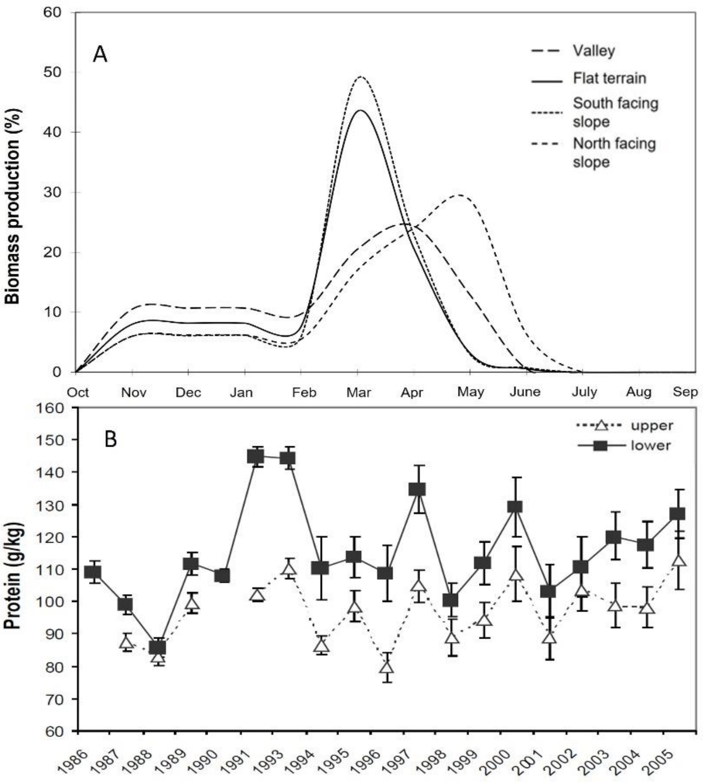

Figure 2. A: Growth rate curve of Mediterranean grasslands of dehesa as affected by topography. Source: Fernández-Rebollo et al., (1997). **B:** Concentration (g/kg) of protein in Mediterranean grasslands of dehesa in the upper and lower slope zones from 1986 to 2005. Source: Vázquez-De-Aldana et al., (2008).

Several implications for management arise from the described characteristics of the Mediterranean grasslands of dehesas concerning how to manage these grasslands for livestock raising in extensive systems:

i) The inter- and intra- variability of production and quality pose a drawback to planning the management of dehesas' farms. Farmers must cope with limited resources and strong variability in their availability to maintain the enterprise.

ii) The feed gap of the summer season imposes a major limitation on livestock production due to the low productivity and quality of grasslands, which can limit the carrying capacity of the dehesa farms.

Feed gaps can be defined as periods when the feed offer (in terms of either quality or quantity, or both) does not meet the demands of livestock (Moore et al., 2009). As explained by Moore et al., (2009), feed gaps are farm-specific phenomena as it depends on the interaction of: (i) the feed quantity and nutritive quality of all the resources available on the farm; (ii) the intake of the livestock; and (iii) the farmer’s objectives and management. Summer feed gaps are inherent to Mediterranean systems, although their duration and severity vary over years. As the production period of annual species in dehesas is short due to their strategy to overcome summer drought by drought escape, in years of low spring rainfall and high temperatures the period of production of dehesas ’grasslands shorten, leading to a longer and more severe summer feed gap. Following Granda Márquez de Prado et al., (2017) and Moore et al., 2009 we can differentiate three types of feed gaps: 1) feed gaps at the end of the summer and beginning of autumn when the pasture offer and quality is minimal (15 September-15 October), 2) period from senescence of grasslands at the end of spring/beginning of summer to end of summer (15 June – 15 September) and 3) irregular feed gap from the beginning of Autumn to February if there is a delay for the first autumn rainfall and the winter is cold (15 October – 15 February). The first type of feed gaps is certain every year in Mediterranean environments and can be defined as a regular feed gap. The two latter feed gap types are more irregular and can vary in frequency, severity and length depending on the spring and autumn rainfall.

Traditionally, dehesa farmers have coped with these feed gaps by increasing the in-farm resources i.e. haymaking, and forage crops, for regular and irregular feed gaps (Granda Márquez de Prado et al., 2017) and by making use of external resources in transhumance and transtermitant systems for regular feed gaps (Joffre et al., 1988; Ruiz and Ruiz, 1986). The structure of the landscape of dehesas is itself an ingenious strategy to overcome the feed gaps of Mediterranean climates by diversifying the feed sources to provide acorns and browse at key moments of the year (Joffre et al., 1988).

Dehesas in a changing world: the threat of global change and opportunities for innovations

The profound socioeconomic changes that the dehesa systems experienced over the second half of the past century led to an increase in dependency on concentrates, decoupling of livestock and agricultural systems and abandonment of transhumance systems (Garrett et al., 2020; Joffre et al., 1988; Moreno and Pulido, 2008; Ruiz and Ruiz, 1986). This was further aggravated by the increase in stocking rates due to a shift of the Common Agricultural Policy to coupled payments based on livestock heads (Moreno and Pulido, 2008; Pinto-Correia and Azeda, 2017). According to previous studies, approximately, between 25% and 40% of feed demands are covered by supplementary feedstuff in a dehesa with a 0.25-0.33 livestock units/ha of stocking rate (Escribano et al., 1996; Lifebio dehesa, 2018), being highly variable depending on forage resources, livestock type and management of the farm. These percentages are normally higher in dehesas with cattle compared to ones with sheep because of the higher nutritive demands of cattle and better adaptation of sheep to dehesa’s conditions.

The climate change projections for 2030 onwards predict an overall decrease of the mean annual rainfall (between -10% and -30% depending on the climate change scenario) (Figure 2), an increase in the inter- and intra- annual variability of rainfall, an increase of temperatures and summer days in the Mediterranean region (EEA, 2017; European Environment Agency, 2022; Giannakopoulos et al., 2009; Giorgi and Lionello, 2008). The last IPCC report alerted of extreme agricultural droughts being 150% to 200% more likely under the 2ºC increase scenario (IPCC, 2021). Therefore, the frequency and severity of feed gaps in dehesa systems are expected to increase due to the effects of climate change. This will worsen the uncertainty in farm management and increase the dependency on supplementary feeding for livestock.

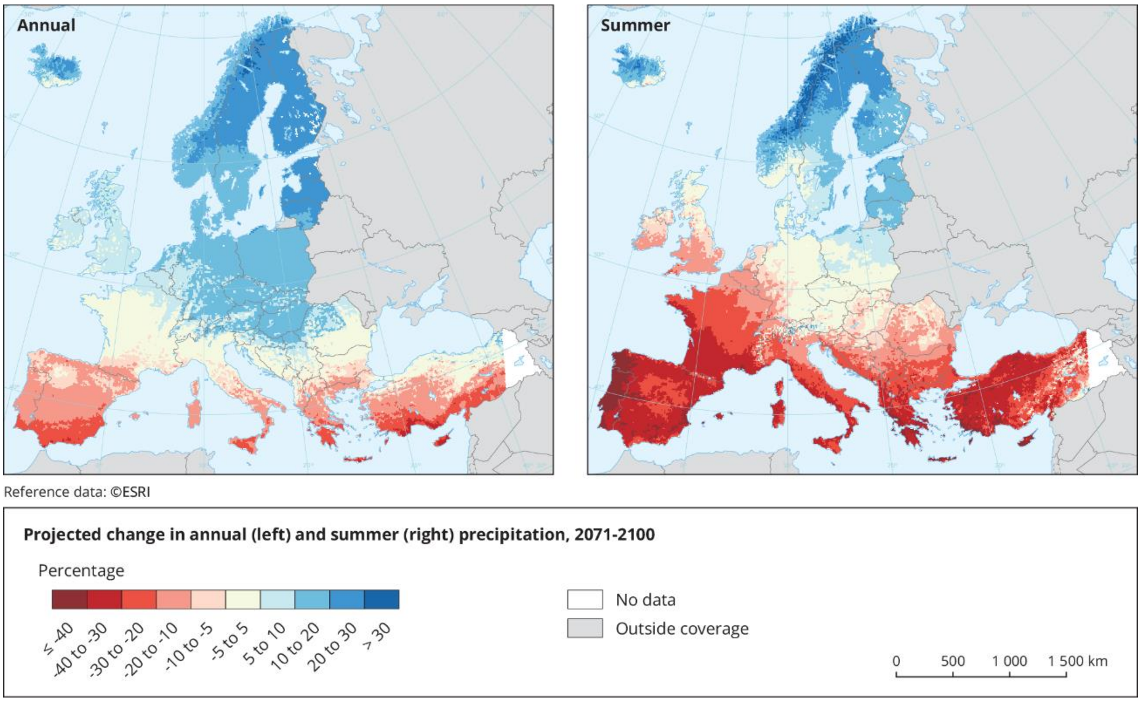

Figure 3. Projected changes in annual (left) and summer (right) rainfall (%) under the scenario RCP 8.5 in the period 2071-2100 compared to the reference period1971-2000 for the forcing scenario RCP 8.5. Based regional climate models simulations from the EURO-CORDEX initiative (EURO-CORDEX, 2023; European Environment Agency, 2022). Source: European Environment Agency, 2022: https://www.eea.europa.eu/data-and-maps/figures/projected-changes-in-annual-and-6.

In the current globalised world, even traditional HNV farming systems such as dehesas have become highly vulnerable to global social and economic changes (Jansen et al., 2009; Plieninger and Bieling, 2013). Maybe the most recent example is the Ukrainian war, which has caused a 40% rise in the prices of concentrates in Spain (MAPA, 2023). This together with the severe drought in the Iberian Peninsula since spring 2022 (Faranda et al., 2023) had a synergetic effect, raising the costs of dehesa farms to face the feed gap of the last summer and autumn. This situation might be a glimpse of future scenarios under the effects of climate change and the instability of global markets due to international conflicts (Sternberg, 2012; Werrell et al., 2015). These pressures add to the long-lasting impacts in dehesas systems of i) lack of competitiveness with intensive crop and livestock systems, ii) ill-suited Common Agricultural Policy regulations lacking relevance and adaptation to the dehesa context, iii) increasing labour costs iv) poor recognition of ecosystem services provision and v) rural outmigration and population ageing (Plieninger et al., 2021).

In this context, innovations are needed to tackle these challenges in order to ensure persistence and foster the adaptation of dehesas to future scenarios (Garrett et al., 2020). These innovations should contribute to increasing the efficiency in use of the highly variable and heterogeneous resources and improve the resilience and self-sufficiency towards the previously mentioned impacts. In addition, they must contribute, to preserving and enhancing the provision of ecosystem services (ES) in line with the increasing awareness of the importance of ES provision by farming systems for the human-wellbeing. In this Thesis, we adopt the definition of innovation of the Food and Agriculture Organisation of the United Nations (FAO), for agricultural innovation as *"the process whereby individuals or organizations bring new or existing products, processes or ways of organisation into use for the first time in a specific context in order to increase effectiveness, competitiveness, resilience to shocks or environmental sustainability and thereby contribute to food security and nutrition, economic development or sustainable natural resource management"* (FAO, 2018).

In particular, this Thesis focuses on two of these potential innovations concerning grasslands of dehesas: i) the potential of a new perennial forage legume to increase resilience, and resistance to drought and ii) the use of satellite remote sensing of pasture quality to improve the efficiency of management through more informed and effective decision-making.

Perennial legumes

In a review of grasslands of Mediterranean-climate zones, Porqueddu et al., (2016) identified *"the potential of perennial species to increase forage production stability against a more erratic climate*" as a research priority of primary importance. They alerted of the need for species that could be less susceptible to climate change and that could counteract the effects of a possible shift on communities' composition to species of low palatability due to climate change (Belgacem and Louhaichi, 2013 seen in Porqueddu et al., 2016). Legumes have always played a pivotal role in Mediterranean grasslands thanks to their ability to fix atmospheric N_2 and superior forage quality. These functions will become even more important in the future to reduce the need for concentrates and inorganic fertilizers in low-input and low-cost farming systems (Porqueddu et al., 2016). They can also benefit the provision of multiple ecosystem services, especially those related to soil health and the provision of feed for livestock (Meena et al., 2018; Sulas, 2005). However, important implications arise from the contrasting strategies of annual versus perennial legumes to face summer drought. Annual legumes follow a strategy of dehydration escape that allows them to complete their life cycle and produce seeds before the onset of drought. Conversely, perennial legumes that exhibit drought resistance follow strategies of dehydration avoidance and tolerance mainly, which combine for summer dormancy under severe drought conditions (see Volaire, 2018). Consequently, in Mediterranean environments, annual species have a short productive period and reach senescence at the end of spring while perennials can delay leaf senescence and shedding. Perennials, once established, could also provide forage in early autumn before the emergence of annuals due to the faster regrowth (Annicchiarico et al., 2013; Ergon et al., 2018). This has important implications as drought-resistant perennial legumes can extend the growing season and provide "out-of-season" forage during feed gaps of summer and early autumn when annuals are senescent and have low forage quality. The use of perennials summer active or drought-resistant species has been recognised as a promising and suitable management strategy to face feed gaps in Mediterranean systems (Moore et al., 2009). Also, the use of perennial can have positive impacts on relevant ecosystems services such as soil organic carbon storage and soil erosion reduction, as pure stands of perennial legumes that do not have to be re-established annually could reduce the tillage frequency compared to annual crops (Annicchiarico et al., 2013; Ergon et al., 2018). However, there are very few species that can meet the requirements to provide "out-of-season" forage under the severe summer droughts of the Mediterranean climate (Annicchiarico et al., 2013). Despite the need and potential of this species the high species diversity of the Mediterranean Basin is still to be fully explored for this purpose, and the options for farmers remain restricted to a few commercial annual and perennial legumes (Lelièvre and Volaire, 2009; Porqueddu et al., 2016).

Alfalfa (*Medicago sativa* L) is one of the most used perennial legumes worldwide. It can persist over 3-4 years before being included in crop rotation, however, it is poorly adapted to grazing and requires irrigation to achieve suitable production in summer in Mediterranean climates. Other perennial legumes suited to Mediterranean climates are birdsfoot trefoil (*Lotus corniculatus*), sainfoin (*Onobrychis viciifolia* Scop.) and sulla (*Hedysarum coronarium* L). Especially, sainfoin and sulla are being reappraised because of their high palatability, beneficial condensed tannins concentrations for animal health, high N-fixing potential and rusticity (Hayot Carbonero et al, 2011; Malisch et al., 2017; Molle et al., 2003; Re et al., 2014). However, sulla is best suited to deep calcareous soils, shows low survival after the second year of establishment and has high specificity for rhizobium (Sitzia et al., 2018; Sulas et al., 1999). Sainfoin cannot withstand temperatures at or above 35 °C due to insufficient carbohydrate concentrations in taproots to sustain growth under thermal stress (Kallenbach et al., 1996).

In the seek for drought-resistant species to provide out-of-season forage for Mediterranean farming systems, the perennial legume *Bituminaria bituminosa*, known by the common name tedera, has drawn increasing attention in the last decade (Cosentino et al., 2014; Porqueddu et al., 2016; Raeside et al., 2012; Real et al., 2014). *Bituminaria bituminosa* is native to the Mediterranean Basin and the Canary Islands, having several varieties adapted to different conditions which offer opportunities for breeding programmes and the development of ideotypes (Melis et al., 2018; Méndez et al., 1991; Pazos-Navarro et al., 2011). Especially, the varieties from the Canary Islands described by Mendez et al., 1991: var *albomarginata* from Tenerife and Lanzarote, var. *crassiuscula* from Mount Teide and var. *bituminosa* spread over the whole archipelago and Iberian Peninsula show adaptations to cold temperatures (var. *crassiuscula*) and very low rainfall <200mm (var. *albomarginata*) that make them suitable candidates for ideotypes the need of Mediterranean farming systems. This species have been traditionally used in the Canary Islands to feed livestock (Méndez et al., 1991; Ventura et al., 2009) and is tolerant to heavy grazing (Gutman et al., 2000; Sternberg et al., 2000; Sternberg et al., 2006). The potential of this species has motivated research on drought resistance and other attributes for Mediterranean farming systems in Spain, Italy, Israel and more recently in Australia (Martínez-Fernández et al., 2012; Melis et al., 2018; Oldham et al., 2013; Pecetti et al., 2007; Real et al., 2014; Sternberg et al., 2006). Despite of the content of two furanocoumarins (psoralen and angelicin) (Pecetti et al., 2007) and condensed tannins present in leaves, no signs of ill health such as photosensitization or bloat have been reported in grazing animals (Oldham et al., 2015; Oldham et al., 2013; Sternberg et al., 2006; Sternberg et al., 2000) showing that is suitable for feeding livestock. *Bituminaria bituminosa* shows a wide range of physiological and morphological adaptations to drought. At physiological level, this species shows strong stomatal regulation to reduce water loss (isohydry) and osmotic adjustment that allows maintenance of leaf turgor and functional photosynthesis at low leaf water potential (-6.5 MPa) (Foster et al., 2015; Foster et al., 2012, 2013; Martínez-Fernández et al., 2012; Pang et al., 2011). These physiological strategies cope with morphological adaptations such as a deep tap root, leaf hairs and a pronounced paraheliotropism (Foster et al., 2015; Foster et al., 2012, 2013; Pang et al., 2011). Australian researchers in collaboration with Spanish and Italian researchers started a prolific development program to seek ideotypes and inform the potential of this species (Oldham et al., 2013; Real et al., 2014). Recently a new variety has been developed under the trademark of Lanza® as a result of this breeding programme (Real, 2016), which might be a commercial alternative for rainfed livestock systems of Mediterranean areas. In light of the potential of this new perennial legume to be used in the Mediterranean grasslands of dehesas, this Thesis investigated morphological and physiological traits, forage quality, response to future rainfall reduction and to soil type of *Bituminaria bituminosa*.

Remote sensing

Together with feed gaps and inter- annual variability, intra- annual spatial variability of grasslands of dehesas pose a major challenge in their management. Differences in slope, aspect, species composition and management lead to large variations in biomass production and pasture quality within the same farm (García-Moreno et al., 2016; Olea et al., 1989; Vázquez-De-Aldana et al., 2008). Farmers must cope with this variability in a low-input and low-cost farming system to allocate resources and plan management in an efficient manner (Granda Márquez de Prado et al., 2017). Unlike intensive farming systems, extensive systems do not have full control of the variables that can be managed to optimise the system, such as controlled diet and movement of animals for example. This contributes to the unbalance in management costs and final prices between intensive and extensive livestock systems. Although this unbalance must be matched by internalising provision of ecosystem services (namely payment for ecosystems services or result based eco-schemes by CAP) (Bugalho et al., 2011; Guimarães et al., 2023; Pinto-Correia et al., 2022; Rolo et al., 2020), extensive systems such as dehesas must seek for innovations to improve their efficiency in the management of resources.

The fast development of new sensors and platforms to provide and manage information in the last years in the so-called “agriculture 4.0” offers the opportunity to implement some of these technologies in extensive farming systems. One of the technologies that has experienced a big development in the last two decades is remote sensing. Remote sensing consists of acquiring information of the physical and chemical characteristics of an area or object without being in physical contact with it (Elachi and Van Zyl, 2021).

The physical basis of optical remote sensing consists of the interaction of the target of study with the electromagnetic spectrum. The reflectance in the different regions of the electromagnetic spectrum captured by an instrument can be related to the physical and chemical characteristics of the target. Different instruments can be used, from field spectrometers to sensors in unmanned aerial vehicles (UAVS) and satellites.

Remote sensing has been extensively applied to the monitoring and management of grasslands to assess manly biomass production, phenology species composition and management (see Ali et al., 2016 for a review). This could allow monitoring grasslands status without *in situ* methods that are normally labour intensive and expensive (Ali et al., 2016), especially for extensive farming systems. This might be the main reason hindering the continuous monitoring of pasture status in extensive farming systems such as dehesas compared to for example dairy systems with temperate grasslands. However, the development of satellite technology in the last decades has provided a unique opportunity for continuous monitoring of grasslands in extensive farming systems (Ali et al., 2016; Reinermann et al., 2020). Compared to the use of UAVS, satellites that offer open freely available data can reduce the cost of data acquisition as they capture information regularly at the expense of lower resolution and therefore reduced accuracy in the estimations. Although there are commercial satellites that provide high spatial resolution images such as Worldview-3 (ESA, 2023c) and Pleiades Neo (ESA, 2023a), the cost of the images is a limitation to its use in spatiotemporal monitoring of grasslands at farm level. The launch of Sentinel-2 in 2015 meant a breaking point in satellite remote sensing as has significantly improved the spatial resolution of previously used satellites such as Landsat. Sentinel-2 was developed by the European Space Agency (ESA), it provides freely available data worldwide with a revisiting time of 5 days and carries a multispectral sensor with 13 spectral bands: four bands at 10 m, six bands at 20 m and three bands at 60 m spatial resolution (ESA, 2023b). Previous studies in temperate grasslands have shown promising results in the assessment of pasture quality parameters using field spectroscopy and Sentinel-2 (Askari et al., 2019; Pullanagari et al., 2012; Pullanagari, et al., 2013; Pullanagari et al., 2013; Raab et al., 2020). The upcoming stream of new satellite sensors such as the forthcoming high-priority mission candidate of the European Space Agency, Copernicus Hyperspectral Imaging Mission for the Environment (CHIME) (Rast et al., 2019), together with the application of machine and deep learning algorithms bring the opportunity to improve the monitoring of grasslands at large scale and high accuracy (Morais et al., 2021; Pullanagari et al., 2021).

Previous PhD Thesis and research have addressed the remote sensing of dehesas. Gómez-Giráldez, (2021) studied the net primary production of grasslands, phenology, and other hydrological dynamics in dehesas. Carpintero García, (2021) focused on the hydrological balance in dehesas. Portuguese researchers have also used remote sensing to assess biomass and soil organic carbon of sown biodiverse pastures in Montado systems (Morais et al., 2023; Morais et al., 2021). However, remote sensing of pasture quality has been largely overlooked worldwide (Ali et al., 2016), especially in Mediterranean grasslands. Very few studies have addressed the potential of remote sensing to assess pasture quality in Mediterranean grasslands (Lugassi et al., 2019; Serrano et al., 2018). Pasture quality is essential in the main function of Mediterranean grasslands of dehesas of provision of animal feed. Parameters typically used to determine pasture quality are crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), enzyme digestibility of organic matter (EDOM) and metabolizable energy. Information on these parameters is indispensable for estimating carrying capacity, adjusting stocking rates and assessing the need for supplementary feeding, especially in Mediterranean grasslands due to their strong temporal and spatial variation in pasture quality (Vázquez-De-Aldana et al., 2008). Current methods rely on time-consuming field sampling and subsequent expensive laboratory analysis which are limited on spatial representation and the information cannot be used to make decisions in real-time. These limitations lead to no practical use of pasture quality analyses in dehesa farms.

Based on the opportunity that brings Sentinel-2 and future hyperspectral satellites such as CHIME, and the need for innovations to improve the monitoring and assessment of pasture quality of Mediterranean grasslands, this PhD Thesis studied the potential of remote sensing to assess pasture quality in Mediterranean grasslands of dehesas.

OBJECTIVES

The overall objective of this PhD thesis is to inform the potential of two relevant innovations in the management of herbaceous grasslands of dehesas to improve the efficiency of management and resistance to drought in a more erratic and drier future climate: i) remote sensing of pasture quality and ii) the new forage perennial legume *Bituminaria bituminosa*. To achieve this goal, the following specific objectives were addressed:

1. Investigate the relevance that dehesa farmers attribute to grassland-related innovations and identify the factors that might be linked to the adoption of these innovations.
2. Explore the differences in morphological and physiological traits, nutritive value, and response to water shortage of the three varieties of *Bituminaria bituminosa* of the Canary Islands
3. Study the response of the new improved variety of *Bituminaria bituminosa* cv Lanza® to different soil types, competition, and rainfall reduction under future scenarios of climate change in terms of biomass production, morphological traits, phenology, and forage quality.
4. Investigate the potential of Sentinel-2 configuration to assess pasture quality in Mediterranean grasslands of dehesas.
5. Explore the potential of the future mission candidate of the European Space Agency, Copernicus Hyperspectral Imaging Mission for the Environment to improve the assessment of pasture quality in Mediterranean grasslands of dehesas.

STRUCTURE OF THE THESIS

The thesis is structured in nine chapters, one chapter of general introduction and objectives followed by six chapters addressing the specific objectives of the thesis. Chapter eight covers the overall discussion and future research arising from the thesis. Chapter nine exposes the final conclusions of the thesis. The six chapters addressing the specific objectives are organised following the standard structure of a research article. Five of them reproduce published research articles in scientific journals.

- **Chapter II** addressed the specific objective one to offer an introductory perspective of the relevance that dehesa farmers attribute to potential grassland-related innovations including remote sensing and drought-resistant species:

Fernández-Habas, J., Fernández-Rebollo, P., Gallardo-Cobos, R., Vanwalleghem, T., and Sánchez-Zamora, P. (2022). A Farmer's Perspective on the Relevance of Grassland-Related Innovations in Mediterranean Dehesa Systems. Forests, 13, 1182. https://doi.org/10.3390/f13081182

- **Chapter III** covers the specific objective two to provide an overview of the differences between the different varieties of *Bituminaria bituminosa* of the Canary Island:

Fernández-Habas, J., Hidalgo-Fernández, M. T., Leal-Murillo, J. R., Méndez, P., Quero, J. L., Vanwalleghem, T., and Fernández-Rebollo, P. (2022). Effects of two water regimes on morphological traits, nutritive value and physiology of three Bituminaria bituminosa varieties from the Canary Islands. Journal of Agronomy and Crop Science, 208, 413-426. https://doi.org/10.1111/jac.12485

- **Chapter IV** explores the effect of competition from an annual grass (*Lolium multiflorum*) and contrasting soil types (loamy sand vs. clay) on the new variety of *Bituminaria bituminosa* cv Lanza® in a pot experiment within the specific objective three:

Fernández-Habas, J., Real, D., Vanwalleghem, T., and Fernández-Rebollo, P. (2023). Lanza® Tedera Is Strongly Suppressed by Competition from *Lolium multiflorum* and Is Best Adapted to Light-Textured Soils. Agronomy, 13, 965. https://doi.org/10.3390/agronomy13040965

- **Chapter V** studies the response of the new variety of *Bituminaria bituminosa* cv Lanza® to competition in field conditions and 30% rainfall reduction in terms of biomass, forage quality and phenology as part of the specific objective three:

Fernández-Habas, J., Real, D., Vanwalleghem, T., and Fernández-Rebollo, P. (2023). Yield, forage quality and phenology of the new forage perennial legume *Bituminaria bituminosa var. albomarginata* cv. Lanza® in response to rainfall reduction and competition. *In preparation for submission*

- **Chapter VI** addresses the specific objective four to investigate the potential of Sentinel-2 configuration to assess pasture quality on dehesas ’grasslands:

Fernández-Habas, J., Moreno, A. M. G., Hidalgo-Fernández, M. T., Leal-Murillo, J. R., Oar, B. A., Gómez-Giráldez, P. J., ... and Fernández-Rebollo, P. (2021). Investigating the potential of Sentinel-2 configuration to predict the quality of Mediterranean permanent grasslands in open woodlands. Science of the Total Environment, 791, 148101. https://doi.org/10.1016/j.scitotenv.2021.148101

- **Chapter VII** covers the specific objective five by studying the potential of the future mission candidate CHIME to improve the assessment of pasture quality in Mediterranean grasslands:

Fernández-Habas, J., Cañada, M. C., Moreno, A. M. G., Leal-Murillo, J. R., González-Dugo, M. P., Oar, B. A., ... and Fernández-Rebollo, P. (2022). Estimating pasture quality of Mediterranean grasslands using hyperspectral narrow bands from field spectroscopy by Random Forest and PLS regressions. Computers and Electronics in Agriculture, 192, 106614. https://doi.org/10.1016/j.compag.2021.106614

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CHAPTER II. A farmer's perspective on the relevance of grassland-related innovations in Mediterranean dehesa systems

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Abstract

Grasslands are of key importance for the provision of ecosystem services (ES). Suitable management is essential to guarantee their persistence and functionality. There is a growing interest in innovations such as new technologies aimed at facilitating and improving the management of grasslands while increasing their provision of ES. The uptake of innovations by farmers is a complex process, and relevant socio-economic or technological factors that are crucial to farmers are often overlooked. This information can be useful for increasing the adoption of these innovations through the design of public policies to facilitate it. This paper analyses the relevance of the main innovations that can be applied to the management of the grasslands of *Dehesa* farms for the farmers and the factors that might affect this relevance. Through questionaries, we gathered information on the relevance that farmers give to the selected innovations and analysed it by cumulative link models. The results show that innovations aimed at increasing the biomass production of grasslands and resilience such as the use of seed mixtures and the use of forage drought-resistant species are considered highly relevant by dehesa farmers. However, high-tech innovations such as GPS collars were poorly rated which could denote low applicability to the context of dehesas or the existence of barriers hindering the adoption but also a need for further development and better information on their potential. Characteristics of the farmer and farm such as age, education level, and stocking rate seem to be related to the relevance given to some of the innovations. These results provide insightful information for the implementation and research of relevant grassland-related innovations in the context of Mediterranean dehesa/montado systems, as well as for the design of policies supporting them.

Keywords: Policy design, innovation adoption; innovation process; agronomy-related innovations; technological innovations; cumulative link models; farmers' characteristics

INTRODUCTION

Grasslands are of key importance for the provision of ecosystem services (ES). Their preservation and sustainable management are essential to guarantee their multifunctionality (Schils et al., 2022). Several factors challenge the multifunctionality of grasslands. Among the most important ones are abandonment, land-use change, and climate change (Bardgett et al., 2021; Schils et al., 2022). In this context, there is a growing interest in innovations aimed at increasing the resilience of grasslands and, the efficiency, and sustainability of their management while maintaining or increasing the provision of ES (Bardgett et al., 2021; Berckmans, 2017; Jaurena et al., 2021). According to Food and Agriculture Organization of the United Nations (FAO), agricultural innovation *"is the process whereby individuals or organizations bring new or existing products, processes or ways of organization into use for the first time in a specific context in order to increase effectiveness, competitiveness, resilience to shocks or environmental sustainability and thereby contribute to food security and nutrition, economic development or sustainable natural resource management"* (FAO, 2018).

The development and implementation of innovations are especially relevant in High Nature Value (HNV) farming systems (Rolo et al., 2020), where the balance between land-use and biodiversity conservation depends on sustainable and active management (Bugalho et al., 2011; Lomba et al., 2020). One of the most representative and important HNV systems is the dehesas system of the Iberian Peninsula, also known as *Montado* in Portugal (Pinto-Correia, et al., 2011). *Dehesa* is a savanna-like ecosystem composed of scattered oak trees and pastures (Moreno and Pulido, 2008). This agrosilvopastoral system covers 3.1 million hectares in the Iberian Peninsula and is recognised as one of the most biodiverse and multifunctional systems in Europe (Bugalho et al., 2011; Moreno and Pulido, 2008; Plieninger et al., 2021). This ecosystem is protected by the Habitat Directive, as habitat 6310 *"Dehesas with evergreen Quercus spp"*(European Commission, 2013; Habitats Ditective, 1992). Dehesa farms are devoted to livestock breeding in extensive systems that rely on rain-fed grasslands to feed the animals (Moreno and Pulido, 2008). This farming systems also depend on acorn production from holm oaks (*Quercus ilex* sub. *ballota*) to feed the livestock, especially for Iberian pigs (Rodríguez-Estévez et al., 2009). Mediterranean grasslands of dehesa farms are subjected to high inter- and intra- annual variability of rainfall driven by the Mediterranean climate that contributes to their unique and valuable characteristics, but also makes the management of resources challenging (Marañón, 1991; Olea and San Miguel-Ayanz, 2006). Traditional knowledge and adaptation to the resources of farmers have coped with the limiting factors leading to a unique farming system (Plieninger et al., 2021). However, the new socio-economic context, together with threats such as climate change, and diseases put this farming system at risk, which increases the need for innovations to secure the provision of ES in the future. The inter- and intra- annual variability of the rainfall is being exacerbated by climate change (IPCC, 2022). Its effect on Mediterranean grasslands is reduced productivity, higher uncertainty, prolonged regular feed gaps, and more frequent irregular feed gaps (Moore et al., 2009). This is reflected in other ecosystem services such as diversity, erosion control, and carbon sequestration (Cerda et al.,1998; Ghahramani and Moore, 2013; Matías et al., 2021; Moore and Ghahramani, 2013). Since Mediterranean grasslands are the base of dehesas farming systems, it also affects the profitability of the farms (Iglesias et al., 2016), making them more dependent on concentrates and jeopardising the sustainability of the enterprises (Ghahramani and Moore, 2013; Moore and Ghahramani, 2013). In addition, the lack of profitability of extensive farming systems is frequently aggravated by inefficient management of the resources.

Research efforts have been directed at developing innovations that could increase the resilience of Mediterranean grasslands to face such threats while maintaining their ability to provide multiple ES. Plant breeding and genetic selection led to important agronomic innovations, such as the use of seed mixtures to increase the productivity of grasslands while promoting biodiversity (Hernández-Esteban et al., 2019a; Hernández-Esteban et al., 2019b), or the use of drought-resistant species that could reduce the effects of feed gaps in Mediterranean farming systems (Real et al., 2014). Important technological innovations opportunities have arisen from the development of new technologies in the last decades, such as remote sensing, GPS-collars, and virtual fencing have been proposed as powerful tools that can contribute to more efficient and sustainable management in Mediterranean grasslands (Fernández-Habas et al., 2022; Fernández-Habas et al., 2021; Gómez-Giráldez et al., 2019; Anderson et al., 2014; Chebli et al., 2022; Jouven et al., 2010). Recent research projects such as AGROFORWARD (Agroforward, 2022) and Inno4Grass (Inno4Grass, 2022) have significantly contributed to the identification and promotion of grassland-related innovations.

Despite the wide range of potential innovations that have been proposed for the management of grasslands, little is known about the preferences and needs of farmers regarding these innovations. The innovation adoption process can be grouped into three general phases; initiation, adoption, and implementation (Damanpour and Schneider, 2006). Having information on the relevance of the innovations for farmers and their attitude towards them is crucial for successful adoption (Jitea et al., 2021), and should be an inherent step in the co-innovation process in any sector. Within the phase of initiation, it can help to recognise the need of farmers (potential adopters) but also in the implementation phase, it can provide useful information on the users' acceptance (Pichlak, 2015). This information could provide insights into the compatibility and complexity (Pichlak, 2015; Rogers, 1995) of grassland-related innovations in the context of dehesa. This is essential as it could help to focus and optimise research efforts on the needs of farmers (Tey and Brindal, 2012) to fulfil the demand for innovations that could solve the challenges that dehesa farms are facing. Also, a better knowledge of how the characteristics of the farms and farms might affect their perception of them is essential to inform the innovation adoption process (Damanpour and Schneider, 2006; Mwangi and Kariuki, 2015; Pichlak, 2015) and to understand which profile of farmers are willing to apply certain innovations (Tey and Brindal, 2012). For public policies supporting innovations, such as the Common Agricultural Policy (CAP), having information on the perception of farmers of candidate innovations is of major interest, especially in the light of the new reformed CAP for 2023-2027, which will bring about important changes through eco-schemes (Arzeni et al., 2021; Brunori et al., 2013; Jitea et al., 2021).

This study is aimed at investigating the relevance that dehesa farmers attribute to several grassland-related innovations and identifying the factors that might be linked to the adoption of these innovations. The results can be useful for prioritising research and for the design of policies targeted at improving the profitability and sustainability of dehesas ecosystems through the innovation process.

MATERIAL AND METHODS

Selection of grassland-related innovations

The selection of relevant permanent grassland-related innovations in the context of dehesa farms was based on information collected within the project Sustainable Permanent Grassland Systems and Policies, Super-G (https://www.super-g.eu/). A list of relevant innovations in the context of grasslands in Dehesa farms was produced. This was done based on expert knowledge. These experts were selected based on their expert criteria as permanent advisors of the Super-G project. The proposed innovations were verified with published research concerning innovations related to the management of grasslands in the context of dehesa farms (Table 1), including both innovations that are already being implemented and those presenting potential to be implemented. Eventually, twelve permanent grassland-related innovations were selected to be evaluated by the farmers (Table 1).

Table 1. Permanent grassland-related innovations selected to be evaluated by the dehesa farmers.

Short denomination	Description	References
Sow seed mixtures	Sowing of seed mixtures to improve grasslands' productivity, quality, and ecosystem services such as pollination and nitrogen fixation. Mixtures of annuals mainly consisting of legumes.	(Hernández-Esteban et al., 2019; Moreno et al., 2015; Porqueddu et al., 2005; Rolo et al., 2020; Hernandez-Esteban and Moreno, 2017)
Drought resistant species	Search for drought-resistant grassland species adapted to the dehesa environment that could develop satisfactorily in a future scenario of reduced rainfall. It can reduce the impacts of regular feed gaps during the summer season by providing out of season forage	(Bell et al., 2016; Descheemaeker et al, 2014; Moore et al., 2009; Porqueddu et al., 2005; Real et al., 2014; Edwards et al., 2019; Rolo et al., 2020; Norton, et al., 2016; Volaire et al., 2014)
Knowledge grassland performance	Increasing the knowledge of farmers about productivity, quality, and phenology of grasslands species in dehesa farms through Apps, seminars, websites, or manuals The intrinsic complexity of Mediterranean grasslands due to high variability and diversity could difficult their efficient management. Increasing the knowledge in key aspects such as the dynamics of phenology and quality of the different types of grasslands/species could help farmers with a more efficient management and inform them in the search for suitable complementary forage crops.	(García-Moreno et al., 2016; González and Maya B , 2015; Hanrahan et al., 2017)
Monitoring soil	Monitoring and assessment of soil health through field indicators Soils of dehesa systems are essential to sustain ecological and economic functions such as pasture production for feeding livestock and regulating water dynamics. Since the management has a direct impact on soil health, field indicators can be used to assess the impact of management on soil health status and its effect on farm sustainability.	(Escribano et al., 2018; Jónsson et al., 2016; Pulido et al., 2017; Eekeren and Philipsen, 2020)

Grasslands fertilisation	Improving fertilisation of *Dehesa* grasslands and development of suitable fertilisation guidelines according to soil and fertiliser type. Application dates of nitrogen fertilisation are determinant to achieving the desired outcomes and avoiding negative effects such as legume depletion. Phosphate fertilisation is essential to maintain and promote the legume content of Mediterranean grasslands and thus improve their quality.	(Porqueddu et al., 2005)
Manure slurry outputs	Development of tools to make efficient use of manure and slurry generated on the farms. It could minimise the nitrogen loss and the need for external inputs. In dehesas systems the extensive production of ruminants and monogastric livestock (pigs mainly) is sometimes combined with more intensive phases such as fattening of lambs, finishing of beef cattle, or breeding of piglets. The manure and slurry produced in these phases could be integrated in the management of the grasslands of the farm.	(De Miguel et al, 2015; Lassaletta et al., 2021; Tarrasón et al, 2014)
GPS collars	GPS collars and associated Apps to obtain information on the localisation and behaviour of livestock. Farmers could use this information to save time in the surveillance and localisation of the animals as well as to derive information on the status of the animals.	(Bailey et al., 2021; Berckmans, 2017; Chebli et al., 2022; Jouven et al., 2010; Moreno et al., 2015; Rolo et al., 2020)
Virtual fencing	Technology based on collars attached to the animals that emit a tone and an electric pulse when they approach a pre-determined virtual fence. It could substitute the use of physical fences, improve grassland utilisation, and allow easier management of short-duration rotational grazing.	(Campbell et al., 2021; Jouven et al., 2010; Marini et al, 2018; Moreno et al., 2015; Rolo et al., 2020)
Remote sensing	Use of drones and satellites to obtain information on biomass production, quality, and composition of grasslands that could be used for the management of the farm. This technology can provide information in nearly real-time on key attributes of the grasslands that can help the farmer in the decision-making.	(Ali et al., 2016; Fernández-Habas, et al., 2021; Gómez-Giráldez et al., 2019; Reinermann et al., 2020)
Software grass growth	Software and models to forecast the grass growth and biomass production in the short-term based on information on the current stage and weather forecast. It could provide useful information to plan practices such as early sowing at the beginning of autumn and make estimations on forage needs in the short-term to feed livestock. It could also allow a more informed grazing management to for example increase stocking rate if higher grass growth is forecasted.	(Huson et al., 2020; AgriSearch, 2022; Moore et al.,1997; Donnelly et al., 1998)

Software GHG emissions	Software and models to assess the GHG emissions of the farm based on the management and provision of recommendations on how to reduce them. There is a growing interest in assessing the GHG emissions of farming activities. This is expected to have an impact on the design of public policies but also on the consumers' preferences. The possibility of quantitatively estimating the GHG emissions at farm level could help extensive farmers to differentiate their products from those with higher GHG emissions.	(Aguilera et al., 2021; Topp et al., 2017; Reyes-Palomo et al., 2022)
Dissemination research	Dissemination and divulgation of research on grasslands through websites, seminars, manuals, advising organisations, and courses. Establishing communication channels between research and dehesa farmers can increase the effectiveness, competitiveness, resilience, and environmental sustainability of dehesa farms, and thereby could mean a potential innovation in this context.	(Porqueddu et al., 2020; Inno4Grass, 2022; Mwangi and Kariuki, 2015)

Data collection

Information on the relevance of grassland-related innovations for dehesa farmers was gathered by questionaries completed by these farmers from the Andalusia region (Spain). With the aim of targeting farmers performing active management of their farms and showing the intention of implementing innovations, the questionaries were delivered to be filled out by farmers participating in five seminars related to the management of dehesa farms and the answers were collected at the end of the seminars. The topics of the seminars were: i) Pruning of holm oaks in dehesa farms (20/02/2019), ii) Management of sheep in extensive farming systems (21/02/2019), iii) Marketing and commercialisation of dehesa products (13/11/2019), iv) Techniques for oak regeneration in dehesas, (21/11/2019) and v) Organic beef cattle production in dehesas (19/02/2020). These seminars cover relevant issues in the context of dehesa systems from which arise the need for innovations and attract farmers concerned about the improvement of the management of their farms. Additionally, the questionary was also passed to the farmers of the Super-G farm network in Spain (https://www.super-g.eu/farm-networks/#table-spain) that are collaborating in benchmarking and testing innovations in grasslands.

To avoid ambiguity, and to promote a high participation rate, the questionary was designed to prioritise its concision and simplicity. It consisted of a first section aimed at collecting information about the farm and farmer characteristics: Farm size, livestock type, total livestock units by livestock type, age of the farmer, gender, and education level.

The second section focused on ranking the relevance that the proposed innovations could potentially play on the management of the farm to respond to their needs. They were asked to answer the central question *What could be the relevance of the following innovations for the management of grasslands in your farms?* by giving a score to each innovation on the Likert scale from 1 (irrelevant) to 5 (very relevant). At the begging of each seminar, the questionaries were handed and explained to the participants. In the case of farmers from the Super-G farm network, the questionary was explained in person or by telephone interview before sending/handing the questionary. A total of 55 farmers completed the questionary (average response rate of 45%). After removing incomplete questionaries and those from farming systems different to dehesa farms, the responses of a final set of 42 questionaries were analysed.

Statistical analysis

Cumulative link models, also known as ordinal regression models (Agresti, 2003; R. Christensen, 2015; Christensen, 2019; Christensen, 2015, 2019; Christensen, 2019; McCullagh, 1980), were used to analyse the differences in the relevance given to the innovations and its relationship with farmer and farms characteristics. Cumulative link models allow for the analysis of response variables following an ordered finite set of categories. In this case, the response variable (relevance of innovations) follows an ordinal scale from 1 (irrelevant) to 5 (very relevant). Cumulative link models with logit link (McCullagh, 1980) were fitted using the “ordinal” package of R (Christensen, 2019). This package also allows fitting cumulative link mixed models, which are cumulative link models with normally distributed random effects (Christensen, 2019).

Firstly, the differences in relevance among the different innovations were tested by cumulative link mixed models following (Christensen, 2019) and (Mangiafico, 2022). To do so, the score given by all the farmers to the twelve innovations (504 scores) was set as the response variable, the innovation as the predictor variable, and the farmer as the random effect. The reason for setting farmer as random responds to the fact that each farmer might rank the innovations higher than another one. Significant differences among groups of innovations were tested by a post-hoc test with “emmeans” R package (Lenth et al., 2019; Mangiafico, 2022).

To further understand the influence of farm and farmer characteristics on the relevance given to each innovation, a cumulative link model was fitted for each innovation. The score given to the innovation was specified as the response variable. The predictor variables were farm size, livestock type, stocking rate, age of the farmer, and their education level. The stocking rate was calculated as the total livestock units divided by the farm hectares. Total livestock units were calculated based on the reference equivalence tables of the regional government of Andalusia (Consejería de Agricultura, Ganadería, 2006). The stocking rate was calculated with and without pigs. The calculated stocking rate without pigs was used to fit the models since the breeding of Iberian pigs in dehesas is partly dependent on an intensive phase and less reliant on the grassland production grasslands than sheep and cattle (Rodríguez-Estévez et al., 2009). Before fitting the models, the quantitative variables stocking rate and farm size were transformed into three categorical classes, based on their quantiles position: less than 25%, between 25% and 75%, and higher than 75% quantiles position (Maroto-Molina et al., 2018; Milán et al., 2006; Solano et al., 2000). Education level was divided into three categories: 1) From primary to secondary general education, 2) Professional qualification (secondary vocational education), and 3) From university to Ph.D (tertiary education). Farmer age was divided into three classes: 1) <35 years, 2) 35-55 years, and 3) >55 years. Kendall Tau was used to test multicollinearity among predictor variables. No strong correlation (<0.7) was found (Figure S 1) indicating no potential multicollinearity risk, therefore all the variables were used to fit the regressions. To graphically display the relationship between the predictor variables, a categorical principal component analysis was fitted with these variables using the “princals” function from “Gifi” R package (Mair et al., 2019).

At first, all the predictor variables were included in a baseline or saturated model. Then, a stepwise model selection based on the Akaike information criterion (AIC) was conducted using the “step” function of R to determine the model of the best fit and the most significant variables (Christensen, 2015a; 2015b; Schmitz et al., 2018). The AIC of the saturated and fitted model as well as the Nagelkerke´s pseudo R^2 and p-value of the best-fit model were calculated and reported. This procedure was followed to fit a model for each innovation (Table S1).

Following McKinley et al., (2020), coloured tables were used to represent the results of the cumulative link models (Table S1). The results of each model were represented in rows (one by innovation) and the predictor variables in columns. If the predictor variable was dropped from the saturated model, the corresponding cell was coloured in grey. These predictor variables that were kept after the stepwise model selection were coloured in green if the categories of this variable increased the odds of giving a high score to the innovation or in red if they decreased the odds of giving a high score (Table S1). The estimates, Odds ratio, and p-value of the levels of each predictor variable included in the best-fit models were also reported. Odds ratio (OR) is the measure of association between an exposure and an outcome (Szumilas, 2010). The OR associated with an increase in the exposure of one unit is calculated as the exponential function of the coefficients/estimate (e^{β}) of the ordinal logistic regression (Szumilas, 2010). In this case, the OR is used to compare the effect of the shift from a reference category on the odds of a higher innovation rating (OR>1) or lower innovation rating (OR<1). Based on the magnitude of the OR, the effect of the different variables in a model can be compared.

All models were best-fit and validated as recommended by Christensen (2015a; 2015b; 2019b). Assumptions of proportional odds and scale effects were met in all the best-fit models. Hessian number of the best-fit cumulative link models was always <10,000, indicating no sign of non-identifiable models (Christensen, 2015b). All statistical analyses were performed using the software R v. 3.6.1 (R Development Core Team, 2019).

RESULTS

Information about farmers and farms

Table 2 shows the structural variables and the number of observations collected by variable class. Except for “*Farmer education level*” the observations had an even distribution over the categorical classes created (Table 2). The average farm size is 343 ha, and showed high variability, from 15 ha to 1,400 ha. 48% of the farms belonged to the class of farm size (FS) medium (78-432 ha). Concerning the stocking rate, it presented an average value of 0.50 LU/ha, and 0.65 LU/ha when pigs were considered. Farm size correlated negatively (R=-0.65) with stocking rate (with and without pigs). The three main types of livestock in dehesa farms; sheep, cattle, and pigs were part of the enterprise in 71%, 43%, and 43% of the farms respectively. 55% of the farms had just one species as part of the enterprise. 33% of the farms had two species of livestock, being in almost all cases a combination between pig and a ruminant (sheep or cattle). In 12% of the farms, the three livestock species were present. The age class of <35 was the least represented, with 10 farmers (24%), while classes 35-55 and >55 had 17 and 15 farmers respectively. Of the 42 farmers, only 6 were female.

Table 2. Structural variables collected from the survey.

Variable	Classes	N
Farm size (FS) (ha)[a]		
small	<78	11
medium	78-432	20
large	>432	11
Stocking rate (LU/ha)		
Low	<0.30	13
Medium	0.30-0.72	18
High	>0.72	11
Stocking rate without pig (LU/ha)		
Low	<0.29	11
Medium	0.29-0.59	20
High	>0.59	11
Sheep	No	30
	Yes	12
Cattle	No	24
	Yes	18
Pig	No	24
	Yes	18
Farmer age (FA)	<35	10
	35-55	17
	>55	15
Farmer education level (FE)		
Prim	From primary to secondary general education	8
Prof	Professional qualification (secondary vocational education)	6
Uni	From university to PhD (tertiary education)	28

N: number of observations

[a]Indicates the total land owned/managed

Figure 1 shows the results of the categorical principal component analysis. The first five principal components explained 94% of the total variance. The first and second components were the most contributing components and were therefore selected to generate the loadings plot (Figure 1), which accounted for 27.1% and 24.8% of the variance respectively, 51.9% in total. The first component indicates a discrimination between farms specialized in sheep from those with a combination of cattle and pig. The second component explained differences in stocking rate, farm size, education level, and age. As commented before, stocking rate and farm size are negatively correlated, and there is an association between education level and farm size (Figure 1). University studies (FE Uni), with 67%, dominated the education level of the dehesa farmers that participated in this study. FE Uni was also the predominant education level across farmer age classes, 50% in <35, 71% in 35-55, and 73 % in >55. The same happened concerning the farm size, farmers owning large farms had mostly university studies (91%), being also the predominant education level of farmers owing medium and small farms with 60% and 55% having university studies respectively (data not shown).

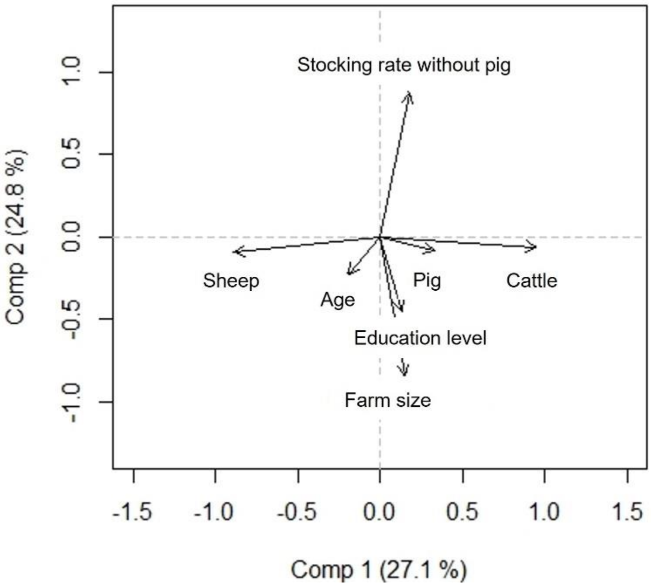

Figure 1. Loadings plot from categorical principal component analysis of structural variables.

Relevance of the innovations for dehesa farmers

There was a contrasting distribution of the scores given to the twelve innovations assessed (Figure 2). For the agronomy-related innovations *Sow seed mixtures, Drought resistant species, and Knowledge of grassland performance,* more than 57% of the farmers considered them highly relevant for the management of grasslands on their farms, showing an overall positive rating of these innovations. Conversely, technological innovations, *GPS collars, Virtual fencing, Remote sensing, Software grass growth,* and *software GHG emissions,* showed a more dispersed distribution of the scores, with just 31% or less of the farmers rating them as highly relevant. The rest of the innovations, *Grassland fertilisation, Monitoring soil, Manure slurry outputs, and Dissemination research* showed an overall trend to a positive rating with 40-48% of the farmers considering them as highly relevant. *Drought resistant species* was the innovation best rated while Software GHG emissions was the worst-rated innovation according to their relevance for the management of grasslands.

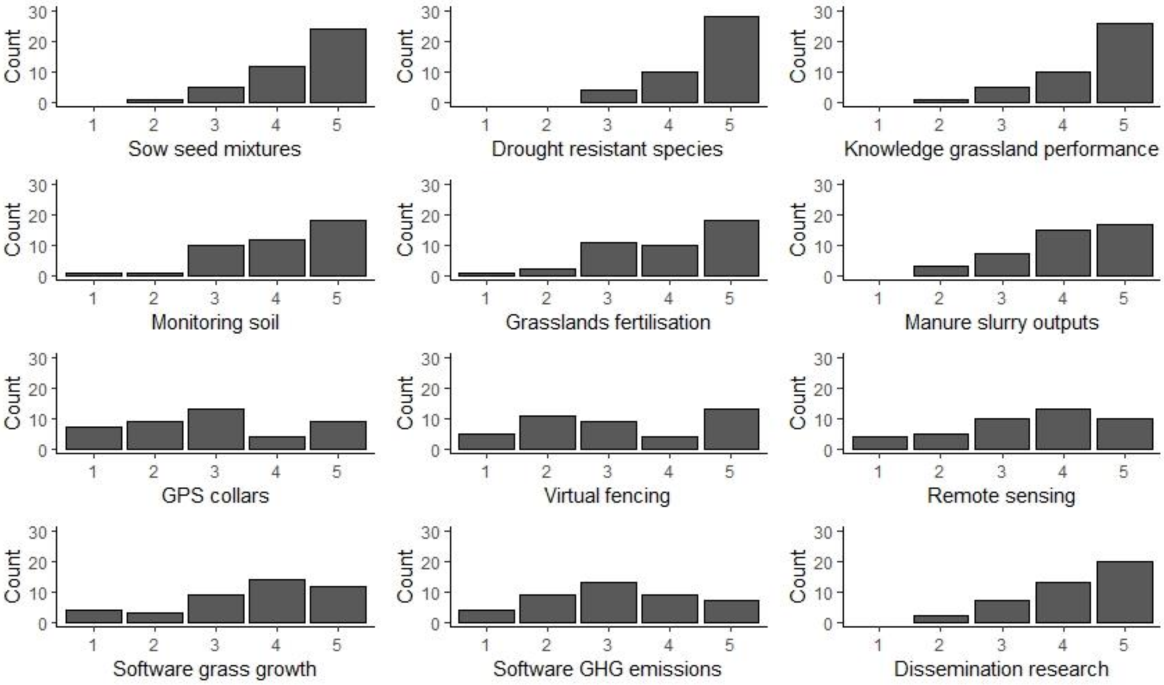

Figure 2. Histograms of relevance score given to grassland-related innovations in the Likert scale from 1 (irrelevant) to 5 (very relevant) (N=42).

According to the cumulative link mixed model, there was a statistically significant difference ($p<0.001$) in the relevance given to the innovations after controlling for the random effect of farmer. Figure 3 shows the homogeneous groups in which the innovations are grouped by post-hoc test. As outlined before, there were two main groups of innovations that showed statistical differences ($p<0.05$) in their relevance. A group of agronomy-related innovations consisting of *Sow seed mixtures, Drought resistant species,* and *Knowledge of grassland performance*, with a median score of 5, had a significantly higher relevance than *GPS collars, Virtual fencing, Remote sensing, Software grass growth,* and *Software GHG emissions*. Especially, *GPS collars, Virtual fencing,* and *Software GHG emissions*, with a median of 3, were significantly worse rated than the rest of the innovations (Figure 3). *Grassland fertilisation, Monitoring soil, Manure slurry outputs,* and

Dissemination research, with a median of 4, did not show significant differences with *Sow seed mixtures*, *Drought resistant species*, and *Knowledge of grassland performance*.

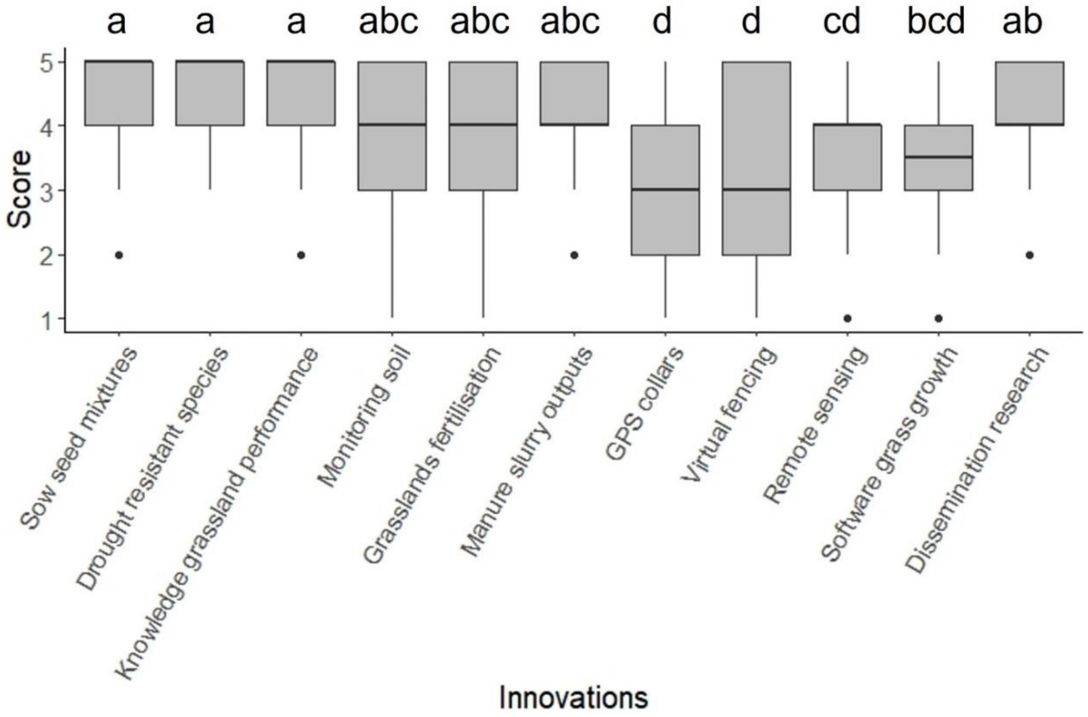

Figure 3. Boxplots of relevance given to grassland-related innovations. Different letters above the boxplots indicate significant differences in the relevance among innovations ($p<0.05$). Black centre line, median; box, interquartile range; box limits, lower and upper quartiles; whiskers, 1.5× interquartile range.

Influence of farmer and farm characteristics on the relevance given to the innovations

The random effect of farmer in the cumulative link mixed model resulted as significant ($p<0.001$), which justifies studying the effects of farm and farmer characteristics that might influence the relevance given to the different innovations. This was investigated by cumulative link models, whose results are shown in Table S1 and Table 3. For the innovations *Sow seed mixtures, Drought resistant species,* and *GPS collars,* all the predictor variables were dropped by stepwise model selection (Table S1), indicating that none of these variables influenced their relevance. The relevance score of the remaining innovations was influenced by one or more characteristics of the farm/farmer (Table S1). Results of the cumulative link models for these innovations are presented in Table 3.

Table 3. Results of the best-fit cumulative link models of the influence of farmer and farm characteristics on the relevance given to the innovations. For the description of variables see Table 1 (response) and Table 2 (predictor). Categories within predictor variables are presented in italics. Missing category within predictor variables are the reference category for the model. Estimates must be interpreted as the effect of the shift from the reference category on the score of the given innovation. Significance levels: <0.001***, <0.01**, <0.05*.

Innovation (Response)	**Variable (Predictor)**	**Estimate**	**Standard error**	**OR**	**z value**	**p-value**
Knowledge of grassland performance	Farmer age *35-55*	3.1	1.1	21.9	2.8	<0.01**
	Farmer age *>55*	2.7	1.1	14.6	2.5	<0.05*
	St. rate *medium*	2.4	1.0	10.9	2.4	<0.05*
	St. rate *high*	2.2	1.1	8.8	2.0	<0.05*
	Sheep *yes*	1.8	1.0	6.2	1.8	0.07
Monitoring soil	Farmer age *35-55*	2.1	0.8	7.8	2.5	<0.05*
	Farmer age *>55*	1.0	0.7	2.6	1.3	0.20
Grasslands fertilisation	St. rate *medium*	1.5	0.8	4.4	1.9	0.06
	St. rate *high*	0.6	0.8	1.8	0.7	0.48
	Cattle *yes*	-0.9	0.7	0.4	-1.4	0.15
	Pig *yes*	1.8	0.7	6.1	2.6	<0.01**
Manure slurry outputs	St. rate *medium*	-1.6	0.8	0.2	-1.9	0.05
	St. rate *high*	0.1	0.9	1.1	0.1	0.88
	Pig *yes*	1.4	0.6	3.9	2.2	<0.05*
Virtual fencing	Farm size *medium*	0.3	0.7	1.4	0.5	0.62
	Farm size large	1.5	0.8	4.6	1.9	0.06
Remote sensing	FE *prof*	-0.1	0.9	0.9	-0.3	0.74
	FE *uni*	1.5	0.8	4.5	2.0	<0.05*
	Pig *yes*	1.0	0.6	2.6	1.6	0.11
Software grass growth	Pig *yes*	1.1	0.6	3.1	1.9	0.05
Software GHG emissions	FE *prof*	3.1	1.2	21.4	2.5	<0.05*
	FE *uni*	2.3	0.9	9.7	2.5	<0.05*
	St. rate *medium*	-0.9	0.8	0.4	-1.2	0.22
	St. rate *high*	1.8	0.9	6.3	2.1	<0.05*
	Pig *yes*	3.4	0.8	31.0	4.2	<0.001***
Dissemination research	Farmer age *35-55*	2.1	1.0	7.9	2.0	<0.05*
	Farmer age *>55*	2.7	1.0	14.9	2.7	<0.01**
	St. rate *medium*	0.1	0.8	1.1	0.1	0.92
	St. rate *high*	2.7	1.1	14.3	2.4	<0.05*
	Sheep *yes*	2.3	0.9	9.5	2.4	<0.05*
	Pig *yes*	1.5	0.8	4.6	1.9	0.05

OR: Odds ratio; FE prof: Farmer education level corresponding to secondary vocational education; FE uni: Farmer education level corresponding to tertiary education; St. rate: Stoking rate. Innovations not shown correspond to those in which all the variables were dropped by stepwise model selection resulting in a null model (Table S1). Reference categories: *<25* for Farmer age; *low* for St. rate; *no* for Sheep, Cattle and Pig; *small* for Farm size and *Prim* for FE (Table 2).

Contrary to expectations, higher age classes were associated with higher scores of the innovations. For *Knowledge of grassland performance* and *Dissemination research* the age class 35-55 and >55 increased the odds of higher scores compared to age class <35 (Table 3). *Monitoring soil* showed also an increased the odds of a higher score for class 35-55. However, as expected, the education level of farmers was positively associated with high scores of the innovations in which this factor was significant (*Remote sensing* and *Software GHG emissions*) (Table 3). Concerning the characteristics of the farm, the stocking rate significantly affected the innovations *Knowledge of grassland performance, Software GHG emissions,* and *Dissemination research*. Higher stocking rates increased the odds of higher scores in these innovations. Finally, the livestock type also affected the relevance of some innovations, especially the presence of pigs favoured the positive rating for the innovations *Grasslands fertilisation*, *Manure slurry outputs,* and *Software GHG emissions* (Table 3).

DISCUSSION

This study intended to put in perspective the research and development of grassland-related innovations in the context of dehesa farms by directly asking dehesa farmers about the relevance that a selection of innovations could play on the management of grasslands in this farming system.

The values of the structural variables (Table 1) show that the questionaries covered the variability of the dehesa farms in terms of, size, stocking rate, and enterprises, in agreement with previous studies that have characterised the typology of dehesa farms (Gaspar et al, 2007; Maroto-Molina et al., 2018; Milán et al., 2006; Reyes-Palomo et al., 2022; Gaspar et al., 2008)

Relevance of the studied innovations and relationship with farmer and farm characteristics

Results showed that there were two main groups of innovations with contrasting scores of relevance. A first group of agronomy-related innovations with high relevance, in which can be found innovations directly related to the performance of the pasture such as *Sow seed mixtures, Drought resistant species* and their fertilisation and quality such as *Grassland fertilisation, Manure slurry outputs,* and *Knowledge of grassland performance.* In the second group, with the lowest relevance, were included high-tech innovations related to the monitoring of livestock and grazing management such as *GPS collars*, *Virtual fencing*, and impact assessment tools, for example, *Software GHG emissions*. dehesa farms rely heavily on pasture performance to feed livestock, which justifies the relevance given to innovations directly related to the improvement of the performance of grasslands and increasing their resilience to threats like climate change. It is worth remarking on the relevance given to the innovation *Drought resistant species*. It reflects the perception of farmers of the strong dependence on rainfall and its seasonality to feed the livestock in rain-fed farming systems (Hughes et al., 2022; Lamega et al., 2021), highlighting the impacts of feed gaps on the profitability of the farms and how this is being aggravated by climate change (Ghahramani and Moore, 2013; Iglesias et al., 2016; Moore and Ghahramani, 2013). This innovation together with *Sow seed mixture* were considered of high relevance for the management of grasslands, irrespectively of the characteristics of the farm/farmer (Table S1). Given the correspondence between the demand for innovations like *Sow seed mixture* and *Drought resistant species* and their demonstrated potential to increase the resilience and performance of grasslands in extensive grazing farming systems (Hernández-Esteban, et al., 2019a; Hernández-Esteban, et al., 2019b; Thomas et al., 2021), policies such as CAP should promote and facilitate its implementation. In the face of the new reformed CAP for 2023-2027, these innovations have been specifically pinpointed in a published list of potential agricultural practices that eco-schemes could support (European Comission, 2021). The recently published IPCC Sixth Assessment Report provides conclusive evidence of the special vulnerability of Mediterranean systems to the already patent climate change effects (IPCC, 2022). Among other solutions, this report has pointed out the use of "*drought-resilient ecologically appropriate plants*" as one of the land-based solutions to combat desertification (IPCC, 2022). In this context, the novel drought-resistant perennial fodder legume, *Bituminaria bituminosa* (Fernández-Habas, et al., 2021; Real et al., 2014), has been proposed as a suitable alternative for Mediterranean farming systems. An improved cultivar of this species (Real, 2022), is being tested within Super-G project to be included in dehesas systems to increase their resilience to climate change effects (Fernández-Habas et al., 2021).

Innovations that can be labelled as high-tech (*GPS collars, Virtual fencing, Remote sensing, Software grass growth,* and *Software GHG emissions*) seem to be of low relevance for dehesa farmers. The poor score given to them points at two main reasons i) low applicability and/or interest of these innovations in the context of dehesas and ii) the initial stage of development in the S-shaped innovation curve (Pichlak, 2015; Rogers, 1995). In the case of GPS collars, with the current functionalities offered by this technology, it may result in low interest for dehesa farmers. Dehesa farms are delimited and subdivided by physical fences where the typical grazing method is rotational grazing with long grazing and resting periods (low frequency of rotation). Therefore, farmers have control of the movement of the animals and do not need to spend too much time and resources on the localization of the herd. Conversely, localization of livestock in non-fenced high-mountain grasslands can be a resource- and time-consuming task, in which GPS collars could make a difference in management optimisation (Bailey et al., 2021). For GPS collars to be implemented and widespread technology in dehesas, further functionalities should be developed. Some of them could be calving/lambing detection based on animal behaviour analysis through machine learning algorithms or information on the energy demands based on activity monitoring (Bailey et al., 2021; Borchers et al., 2017; García et al., 2020; Miller et al., 2020). Similarly, virtual fencing could not be suitable or necessary for the grazing method of dehesas and could adapt better to strip or ration grazing methods typical of dairy farming systems of temperate grasslands (Umstatter et al., 2015; Verdon et al., 2021). Innovations like *Remote sensing* and *Software grass growth* can be an example of innovation at an initial stage of development S-shaped innovation curve. These technologies have been implemented in the management of grasslands in other latitudes, for example, GrassCheck (AgriSearch, 2022; Huson et al., 2020) in Northern Ireland and Pastures from Space (Department of Primary Industries and Regional Development, 2022) in Australia. However, in Mediterranean grasslands, this technology is still to be developed. Although recent studies have shown potential for its use in dehesa farms (Fernández-Habas et al., 2021; Gómez-Giráldez et al., 2019; Lugassi et al., 2019; Serrano et al., 2021) there is a need for further optimisation of the models and, especially, platforms and applications that could make this technology accessible and usable by farmers. This need aligns with the lack of dissemination and information transference of the research being developed in this and similar high-tech innovations as could indicate the fact that *Dissemination research* was highly rated (Figure 2 and Figure 3). Therefore, the poor relevance score given to these innovations may result from the combination of a deficient development of the innovations (and associated platforms and Apps facilitating its use) and the lack of information on their potential. To facilitate the implementation and user acceptance, the proposed innovations have to be perceived as profitable (Tey and Brindal, 2012) reliable, and predictable by farmers, otherwise their willingness to adopt them could mean a barrier difficult to overcome (Hogg and Davis, 2009). This is especially important in the case of technological applications, for which it seems to lack transference of research results to practical use in the context of dehesa systems. The low productivity and high dependency on the rainfall of Mediterranean grasslands could contribute to the low interest in high-tech innovations.

It is worth highlighting also the relevance of *Dissemination research* which points to the need for a direct and closer relationship between advisers and researchers (Inno4Grass, 2022). For example, Porqueddu et al., (2020), showed that farmers from Sardinia (Italy) considered the direct relationships with advisers and researchers as the most reliable sources to adopt innovations. They also found that seminars, field days, or visiting innovative farms are inspirational events that encourage them to adopt innovations (Porqueddu et al., 2020).

The association between higher age classes and better rating of some innovations contrasts with previous studies reporting a negative correlation between age and willingness to innovate (Larson et al., 2008; Walton et al., 2008). However, other studies have found a positive relationship between farmer age and adoption (Isgin et al., 2008; Mwangi and Kariuki, 2015). In this case, the positive relationships might be associated with the higher education level of farmers in the superior age classes (see 3.1. Information about farmers and farms). According to the farm and farmer characteristics, it seems that larger farms, are owned by older farmers with university studies (Junta de Andalucía, 2017; Plieninger et al., 2004), and this profile could show a higher interest and demand for certain innovations. This aligns with previous research reporting a positive association between older farmers with higher education levels and the adoption of agricultural technologies (Tey and Brindal, 2012). This is confirmed by the relationship found between the education level and the higher relevance score given to some high-tech innovations such as *Software GHG emissions* and *Remote sensing* (Table 3) (Tey and Brindal, 2012). However, these results must be carefully interpreted and confirmed by future research since they might be also affected by the unbalanced observations in the three classes of farmer education level, with 66.6% having tertiary education (Table 2). Plieninger et al. (2004), in a study on dehesa managers attitudes toward management, regeneration, and conservation of dehesas also reported that most of the responders (57.5%) had attended college.

The fact that farmers managing higher stocking rates tended to provide higher scores of relevance for *Knowledge of grassland performance, Software GHG emissions,* and *Dissemination research* could be related to the specialisation of these farmers and therefore a higher need for innovations to optimise and facilitate the management. Finally, the presence of pigs as part of the enterprise was related to a higher relevance of innovations concerning the management of manure, CHG emissions, and fertilisation. This might show the need for better management and integration of the residues and the impact of this type of livestock on dehesa farms.

Study limitations and future research

This study focused on grassland-related innovations in dehesa systems. However, oak trees play a central role in this agroforestry system, being described as ecosystem engineers (Moreno and Pulido, 2008; Plieninger et al., 2003; Reyna-Bowen et al., 2020; Hidalgo-Galvez et al., 2022). Therefore, to fully cover the innovations of interest for dehesa farmers, those affecting the management of oak trees should be considered. One of the main threats to dehesa systems is the lack of tree regeneration due to intensification and livestock/wild ungulates browsing (López-Sánchez et al., 2016; López-Sánchez et al., 2021) and oak mortality due to the root-rot disease caused by *Phytophthora cinnamomi* (Fernández-Habas et al. 2019). Previous studies have highlighted the demand for tree-related innovations in agroforestry systems across Europe (Rolo et al., 2020; Caceres et al., 2017). Future research should complement the results presented in this study with information on the preference and relevance of tree-related innovations to contribute to the integrative view of innovations and policies that should be applied in agroforestry systems (Plieninger et al., 2021). Apart from management-related innovations, there is also a need for socioeconomic innovations in the marketing and commercialisation of products from dehesa farms (Escribano et al., 2020; Moreno et al., 2015; Rolo et al., 2020). The value-added of these products and ecosystem services associated with their production, is frequently, not fully reflected in the final prices, which is essential to improve the profitability and economic sustainability of these farms (Campos et al, 2018; Gaspar et al., 2016; Rolo et al., 2020). Higher revenues and profitability of dehesa farms could provide a suitable environment for the adoption of innovations. According to the stakeholders' perspective of HNV agroforestry systems, the low profitability of these systems is putting at stake their sustainability and persistence (Jitea et al., 2021; Rolo et al., 2020), and therefore hindering the adoption of innovations. Governance strategies and public policies should respond to this challenge, and the way through seem to be the compensation for the public ecosystem services provided by these systems (Campos et al, 2018; O'Rourke et al, 2016; Strohbach et al, 2015). Since the objective of this study was not to produce a complete list of innovations, we are aware that several relevant innovations such as those related to water harvesting and minimum tillage are missing from this analysis and must be addressed in future research.

Results from this study have shown that characteristics of farm/farmers affect the relevance of the innovations for farmers. This information can be useful to target farms and farmers that will have a higher willingness to implement certain innovations, thus facilitating the innovation adoption process (Pichlak, 2015; Tey and Brindal, 2012). Future research should also investigate the opportunity cost and risk associated with the adoption of the proposed innovations since these are certainly factors driving the willingness to implement innovations. This paper also highlights the importance of co-creating innovations (Fielke et al., 2018; van Ewijk and Ros-Tonen, 2021) for an efficient co-innovation process that avoids the risk of investing resources in the development of innovations that are not useful or demanded by farmers.

Some farm and farmer characteristics not included in this study might also be affecting the preference and relevance of innovations for dehesa farmers. For example, belonging to social groups or producer organizations has been found to promote information sharing and technology adoption (Mwangi and Kariuki, 2015). Farms association and cooperative membership are key features that might influence both relevance and adoption of innovations in dehesa farms (Manda et al., 2020), and should, therefore, be taken into account in future research. Also, farm characteristics like tree density could mean a technical limitation for the implementation of innovations such as remote sensing for grasslands due to high canopy cover impeding grass cover monitoring. Finally, it is worth remarking, that the results and conclusions derived from this study apply to grassland-related innovations in the specific case of *Dehesa*s. These results should not be extrapolated to different farming systems, in which the relevance of the studied innovations could differ considerably.

The aforementioned limitations indicate that more detailed studies with a higher sample are necessary to confirm the findings of this study and go deeper into the factors affecting the relevance given to the innovations. However, the presented results emphasise the importance of consulting farmers in order to develop useful, applicable, and meaningful innovations for dehesa farming systems and to inform the policies supporting them.

CONCLUSIONS

Concerning the objectives of this study the conclusions are:

- Innovations aimed at increasing the performance and resilience of grasslands such as the use of new seed mixtures to improve the performance and diversity of grasslands and the adoption of new forage drought-resistant species are considered highly relevant by dehesa farmers. Considering the potential of these measures to improve: i) the profitability of the farms, ii) their resilience to face current and future threats like increasing droughts and iii) their ability to provide ES; these types of innovations should be targeted by policies.
- High-tech innovations were, overall, poorly rated by dehesa farmers. This might denote low applicability to the context of dehesas or the need for further development of the innovations and better information on their potential.
- Dissemination of research results is demanded by dehesa farmers and could be essential to promote the innovation process.
- Famer and farm characteristics such as education level, and stocking rate seem to be related to the relevance given to some of the innovations and could play an important role in the willingness to adopt them.

This study provides insightful results that can inform the implementation and research of relevant innovations in the context of Mediterranean grasslands and *Dehes*a-like systems, as well as contribute to the relevance of policies aimed at developing and implementing these innovations. Future research should confirm and complement these findings to contribute to integrated approaches for the innovation process and policy design in dehesas and similar agroforestry systems.

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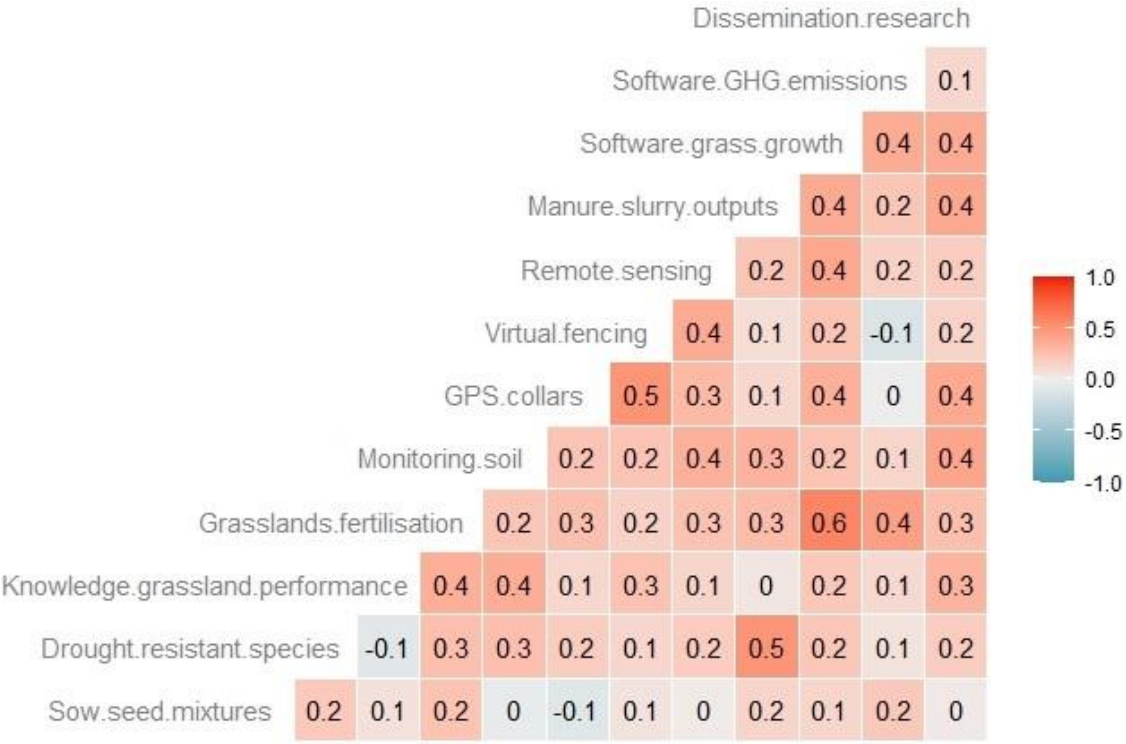
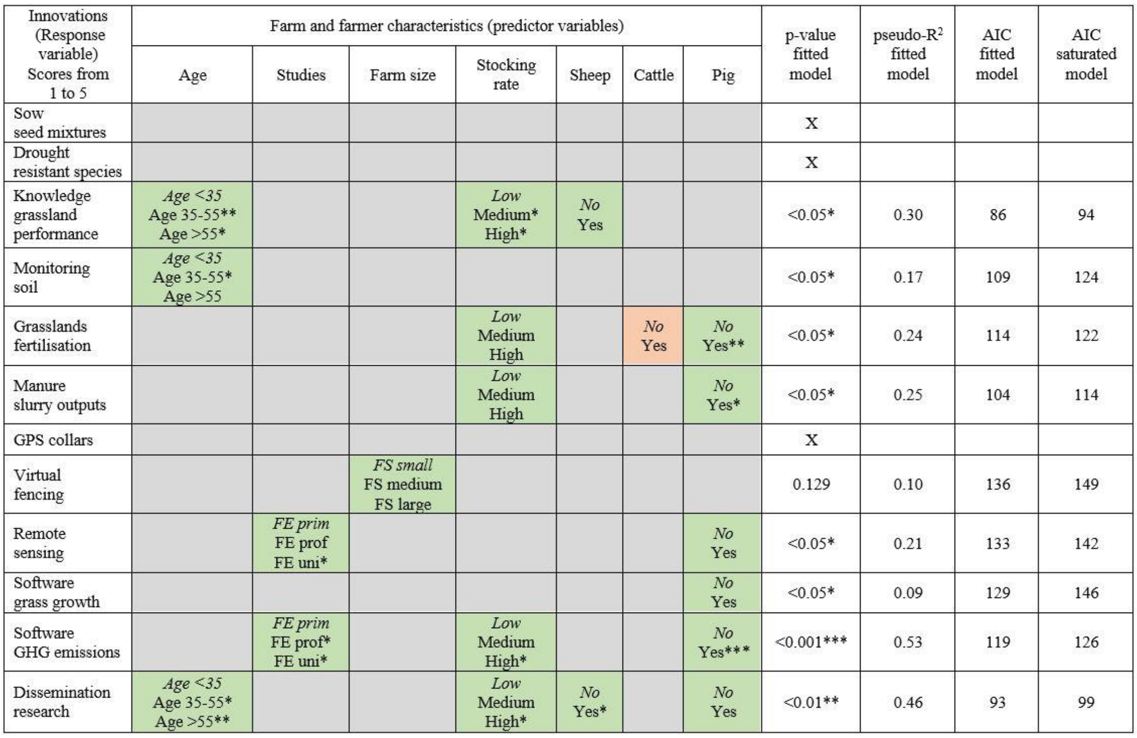
Supplementary Material



	Drought.resistant.species	Knowledge.grassland.performance	Grasslands.fertilisation	Monitoring.soil	GPS.collars	Virtual.fencing	Remote.sensing	Manure.slurry.outputs	Software.grass.growth	Software.GHG.emissions	Dissemination.research
Dissemination.research											
Software.GHG.emissions											0.1
Software.grass.growth										0.4	0.4
Manure.slurry.outputs									0.4	0.2	0.4
Remote.sensing								0.2	0.4	0.2	0.2
Virtual.fencing							0.4	0.1	0.2	-0.1	0.2
GPS.collars						0.5	0.3	0.1	0.4	0	0.4
Monitoring.soil					0.2	0.2	0.4	0.3	0.2	0.1	0.4
Grasslands.fertilisation				0.2	0.3	0.2	0.3	0.3	0.6	0.4	0.3
Knowledge.grassland.performance			0.4	0.4	0.1	0.3	0.1	0	0.2	0.1	0.3
Drought.resistant.species		-0.1	0.3	0.3	0.2	0.1	0.2	0.5	0.2	0.1	0.2
Sow.seed.mixtures	0.2	0.1	0.2	0	-0.1	0.1	0	0.2	0.1	0.2	0

Figure S 1. Kendal Tau correlation coefficients among innovations

Table S 1. Influence of farmers and farm attributes on the relevance of permanent grasslands-related innovations. Each row corresponds to the best-fit cumulative link model for the innovation (response variable) being the columns the predictor variables included in the saturated models. Grey cells indicate that the corresponding attribute did not influence the relevance given to the innovation and was dropped in the stepwise model selection. Green and red colours indicate the variables that were kept in the stepwise model selection. Green indicates that the levels influencing the relevance of the innovation increased the odds of giving a high score to the innovation and red decreased the odds of giving a high score. Reference category in italics. X indicates that the null model yielded better AIC than any other combination of variables based on the likelihood-ratio test. AIC of the saturated and fitted model, and Nagelkerke´s pseudo R^2, p-value of the fitted model are reported. Significance levels: <0.001***, <0.01**, <0.05*. For description of variables see Table 1 and Table 2.



Innovations (Response variable) Scores from 1 to 5	Farm and farmer characteristics (predictor variables)							p-value fitted model	pseudo-R^2 fitted model	AIC fitted model	AIC saturated model
	Age	Studies	Farm size	Stocking rate	Sheep	Cattle	Pig				
Sow seed mixtures								X			
Drought resistant species								X			
Knowledge grassland performance	*Age <35* Age 35-55** Age >55*			*Low* Medium* High*	*No* Yes			<0.05*	0.30	86	94
Monitoring soil	*Age <35* Age 35-55* Age >55							<0.05*	0.17	109	124
Grasslands fertilisation				*Low* Medium High		*No* Yes	*No* Yes**	<0.05*	0.24	114	122
Manure slurry outputs				*Low* Medium High			*No* Yes*	<0.05*	0.25	104	114
GPS collars								X			
Virtual fencing			*FS small* FS medium FS large					0.129	0.10	136	149
Remote sensing		*FE prim* FE prof FE uni*					*No* Yes	<0.05*	0.21	133	142
Software grass growth							*No* Yes	<0.05*	0.09	129	146
Software GHG emissions		*FE prim* FE prof* FE uni*		*Low* Medium High*			*No* Yes***	<0.001***	0.53	119	126
Dissemination research	*Age <35* Age 35-55* Age >55**			*Low* Medium High*	*No* Yes*		*No* Yes	<0.01**	0.46	93	99

CHAPTER III. Effects of two water regimes on morphological traits, nutritive value and physiology of three *Bituminaria bituminosa* varieties from the Canary Islands

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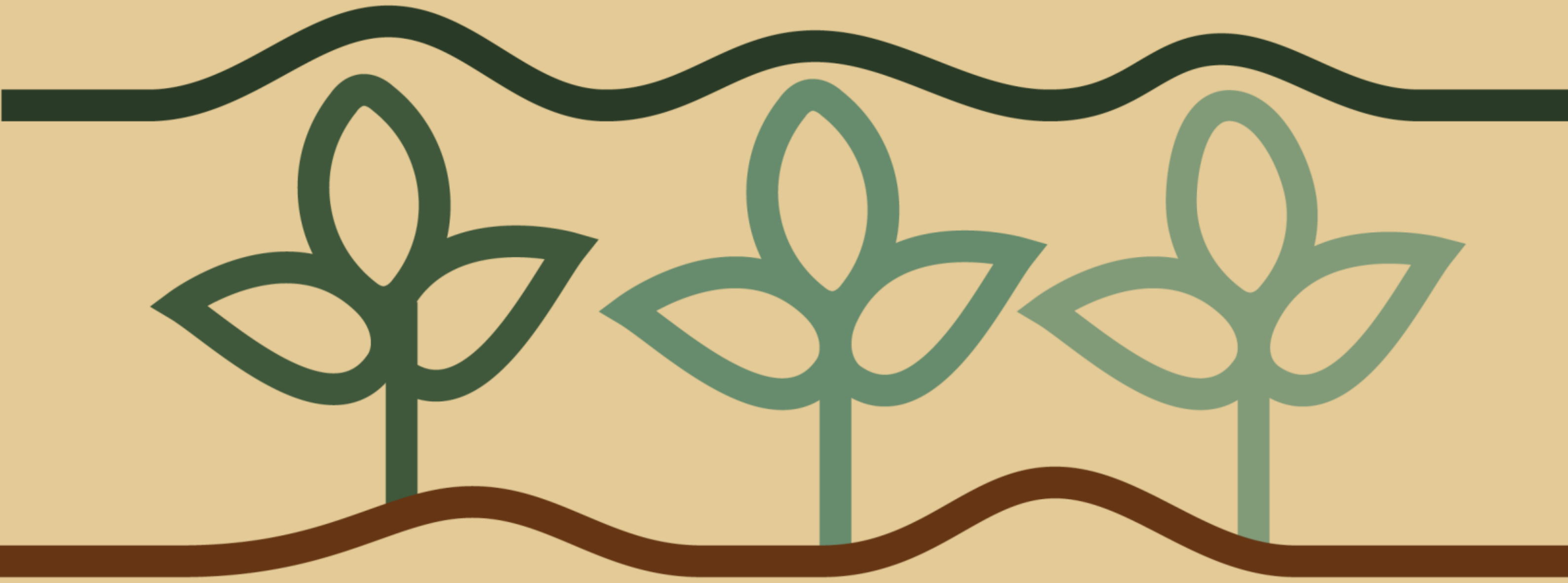

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Abstract

Morphological traits, nutritive values and physiological responses to two different water regimes of three *Bituminaria bituminosa* varieties: var. *albomarginata*, var. *crassiuscula* and var. *bituminosa* were evaluated in a greenhouse experiment. Two water regimes were imposed for 63 days; well-watered (WW) plants and deficit-watered (DW) plants, both starting from a high soil water content (dripping point). The three varieties showed similar aerial biomass reduction under reduced watering, 50% for var. *albomarginata,* 51% for var. *bituminosa* and 43% for var. *crassiuscula.* Var. *albomarginata* showed lower shoot biomass under both water regimes than var. *bituminosa* (56.2 % in WW plants and 55.2% in DW plants) and var. *crassiuscula* (52% in WW plants and 57.8% in DW plants). This lower shoot biomass could be attributed to the high initial soil water content imposed in this experiment, affecting early development. This hypothesis is supported by the lower root biomass production of var. *albomarginata* and its distribution. The DW treatment of this experiment was not sufficiently restrictive to cause morphological modifications, whilst of the forage quality variables analysed, only ash was affected. Var. *crassiuscula* and var. *albomarginata* had a lower specific leaf area (239 cm^2 g^{-1} and 235 cm^2 g^{-1}, respectively) than var. *bituminosa* (352 cm^2 g^{-1}), which might represent an important adaptation to high light intensity and temperature conditions. The values of stem mass fraction (SMF) and leaf mass fraction (LMF) for var. *crassiuscula* (SMF=0.36 and LMF=0.28) and var. *albomarginata* (SMF=0.35 and LMF=0.36) indicated better forage aptitude of these varieties than var. *bituminosa* (SMF= 0.50 and LMF=0.19). All varieties showed good values of crude protein and digestibility, although important differences were found between leaf and stem. According to the studied morphological, nutritional and physiological traits, var. *albomarginata* showed the best aptitude for being introduced as permanent grasslands in some Mediterranean farming systems. However, the possible susceptibility of var. *albomarginata* to high water content in the soil could limit its introduction. These results help to inform the potential use of these three Canarian *B. bituminosa* varieties to improve Mediterranean rainfed grasslands of extensive farming systems.

Keywords: Roots, water-use, morphology, biomass production, net photosynthesis, crude protein.

INTRODUCTION

Bituminaria bituminosa (L.) C.H. Stirton is a perennial legume widely distributed in the Mediterranean basin and Macaronesia and traditionally used as forage crop for livestock in the Canary Islands, where it is known as "*tedera"* (Méndez, Fernández, and Santos, 1990; Ventura, Castañon, and Mendez, 2009). Varieties from this archipelago are adapted to its large climatic diversity, with annual rainfall varying from 150 mm up to 800 mm. In this study the following varieties were investigated: *B. bituminosa* var. *albomarginata* (BAM), *B. bituminosa* var. *crassiuscula* (BCC) and *B. bituminosa* var. *bituminosa* (BBT). These varieties were selected based on their different morphology and distribution in the Canary Islands (Méndez et al., 1990) which may be a source of adaptation to drought stress. Although *B. bituminosa* is known for its high content of metabolites such as furanocoumarins and isoflavonoids (Pecetti, Tava, Pagnotta and Russi, 2007; Pistelli et al., 2003), previous studies have demonstrated that it is consumed by livestock in nature (Sternberg, Gishri and Mabjeesh, 2006) and safe to feed sheep maintaining liveweight in a diet of BAM and BCC (Oldham et al., 2015). Ventura et al. (2009) studied the intake of the three varieties of *"tedera"* by goats and found preference for fresh *"tedera"* versus alfalfa hay, except in summer, when alfalfa hay was preferred due to a higher concentration of secondary compounds of *"tedera"* during this season. The same authors showed that the intake of *"tedera"* in summer could be increased by hay making.

In recent years there has been a growing interest in these varieties of *B. bituminosa* because of their drought resistance and good forage aptitude, especially BAM, which has been established as the most drought- resistant variety (Martínez-Fernández, Walker, Romero, Martínez-Ballesta, and Correal, 2012; Raeside et al., 2012). Its aptitude as a fodder plant in Mediterranean-like climates has been tested in Australia and Israel, showing promising results (Oldham et al., 2013; Real, Oldham, Burgel, Dobbe and Hardy, 2017; Sternberg et al., 2006). The large genetic diversity of *B. bituminosa* is a promising source for breeding programs (Foster, Ryan, Real, Ramankutty, and Lambers, 2013; Pazos-Navarro et al., 2011). Significant advances have been made by Australian and Spanish researchers in selecting basic material for breeding programmes to produce improved lines of good forage aptitude adapted to different environmental conditions of arid lands (Pazos-Navarro et al., 2011; Raeside et al., 2012; Real and Verbyla, 2010). Indeed, a new variety, named *Lanza*, has recently been registered as a result of these breeding programmes.

The Mediterranean basin is expected to be especially vulnerable to climate change (IPCC, 2018). Lower precipitation associated with a higher uncertainty of inter-annual distribution together with increases in temperature are forecast for the 21st century (Giannakopoulos et al., 2009; Giorgi and Lionello, 2008). Gang, et al. (2015), found a decreasing trend of net primary productivity of grasslands in Europe from 1981 to 2010 and an overall decline in water use efficiency in woody savannas and non-woody grasslands in response to climate change from 2000 to 2013 (Gang et al., 2016). The expected climatic variability challenges the pasture productivity and hence the capacity to sustain livestock production. In this context, perennial legumes with drought resistance, dehydration tolerance, and consequently steady forage production, such as *B. bituminosa*, are of key importance for sustaining extensive farming systems in the Mediterranean region such as dehesas (Bennett, Ryan, Colmer, and Real, 2011;; Hernández-Esteban, López-Díaz, Cáceres and Moreno, 2019; Melis, Franca, Re, and Porqueddu 2018; Porqueddu et al., 2016).

Although summer drought is a limiting factor for forage production in the Mediterranean region (Cosentino, Gresta, and Testa, 2014), soil water saturation and wet conditions frequently occur at early stages of plant establishment and development during the autumn and winter months, especially in lower topographies (Ceballos and Schnabel, 1998; Lozano-Parra, Schnabel, and Ceballos-Barbancho, 2015; Maneta, Pasternack, Wallender, Jetten and Schnabel, 2007; Maneta, Schnabel, Wallender, Panday, and Jetten, 2008). Both limiting factors are expected to increase under future climate conditions, as projections show that droughts could start earlier in the year and last longer (Beniston et al., 2007; Giannakopoulos et al., 2009), whereas wet conditions may increase in late autumn and winter due to increases in precipitation extremes (Giorgi and Lionello, 2008).

While the adaptation of *B. bituminosa* to drylands has been proven successful (Suriyagoda, Real, Renton, Lambers, and Ryan, 2013), susceptibility to wet conditions and unsuccessful development during wet winters has also been reported (Raeside et al., 2012; Real and Verbyla, 2010). This reflects the need to investigate the response of *B. bituminosa* varieties under different soil moisture conditions to ensure their successful introduction into pastures of Mediterranean farming systems in the face of climate change.

The morphological traits and nutritive value of *B. bituminosa* have been widely studied in Spain, Italy and Australia (Correal, Hoyos, Real, Snowball, and Costa, 2008; Melis et al., 2018; Méndez et al., 1990; Méndez, Santos, Correal, and Ríos, 2006; Muñoz and Correal, 2000Porqueddu, Dettori, Falqui, and Re, 2011; Raeside et al., 2012). However, to our knowledge, some traits such as leaf mass fraction or leaf area ratio and leaf and stem-nutritive value have not been investigated for Canarian varieties. These traits may have important implications since they influence forage aptitude (Abd El Moneim, Khair and Rihawi, 1990; Méndez et al., 2006).

This study aims to assess the response in terms of (i) physiology, (ii) biomass and morphological traits of shoot/root and (iii) nutritive value of leaves and stems of the three recognised Canarian varieties of *B. bituminosa* under two different water regimes in greenhouse controlled conditions: high soil water content at the beginning of the growth cycle, followed by high or low irrigation regimes (well-watered or deficit-watered).

This study could further inform the potential of these three varieties to adapt to future Mediterranean climatic conditions and elucidate the role that upcoming commercial varieties could play in improving pastures and thereby sustaining livestock production.

MATERIALS AND METHODS

Plant material

Seeds from the three varieties of *B. bituminosa* were collected from wild populations of the Canary Islands (Spain) (Table 1). Tedera is a selfpollinated diploid (2n=20) species (Pazos-Navarro et al., 2011). This together with the geographical isolation of the populations guaranteed no outcrossing pollination among varieties. BAM is native to the island of Lanzarote, where it grows in semi-arid coastal habitats with not more than 200 mm annual rainfall, having a five to six month-long hot and dry season and high relative humidity due to maritime influence. BCC grows in the National Park of Cañadas del Teide (2200m a.s.l.) with 500 mm of annual rainfall (including snow), showing winter dormancy and the growing season during spring and mild summer (Martínez-Fernández et al., 2012).

Table 1. Population and descriptive location data for the three varieties used. *B. bituminosa* var. *albomarginata* (BAM); *B. bituminosa* var. *bituminosa* (BBT); *B. bituminosa* var. *crassiuscula* (BCC).

Variety	Population	Location	Average Rainfall (mm)	Altitude (m)	Mean temperature (°C) hottest/coldest months	Average annual evapotranspiration (mm)
BAM	Malpaso	Lanzarote	150-250	250-280	24/16	1293
BCC	Chavao-Cañadas	Tenerife	500	1900-2200	22/8	1585
BBT	Tamarco	Tenerife	400	400	22/15	1208

Previous research has pinpointed a biannual behaviour of this variety outside its native habitat at Mount Teide (Melis et al., 2018; Méndez, unpublished data). BBT is widespread on all the Canary Islands and the Mediterranean basin with 200-800 mm of annual rainfall during warm and dry summers (Table 1).

The seeds were scarified by nicking the outer seed coat using a surgical scalpel (Beard, Nichols, Loo, and Michael, 2014) and germinated in 90-mm Petri dishes with wet filter paper (Foster et al., 2015; Pecettzi, Tava, Pagnotta, and Russi, 2007;). Once germinated, the seeds were grown in trays (17 days). Subsequently, each seedling was transplanted into individual plastic six-litre cylindrical pots 37 cm high, with a 16.80 cm and 12.5 cm diameter at the top and bottom of the pot respectively and holes at the bottom to allow free drainage of water. Each pot was filled with a mix of commercial peat (Gramosemi GF-Anz./Verm from Gramoflor) and sand (9:1 v/v) with 2 g of 19-19-19 NPK fertiliser per pot. The soil had a pH of 7.31 (1/2.5), 2.96 g $100g^{-1}$ organic matter content, 0.647 (mmhos cm^{-1}) of electrical conductivity and cation exchange capacity of 20.69 meq 100 g^{-1}. Water was provided daily at a rate of 50 ml per pot, and the temperature set at 22°C/7°C (day/night) for 13 days in the greenhouse to ensure successful establishment and acclimatisation before the treatments were imposed.

Experimental design

The experiment was conducted at the greenhouse of the University of Cordoba, Cordoba (Spain). The experiment comprised a complete randomised design to analyse two treatments (water regimes), and three *B. bituminosa* varieties with six pots per variety and treatment, having a total of 36 pots. The water regimes imposed were: high soil water content at the beginning followed by a high irrigation regime, well-watered plants (WW); and high soil water content at the beginning followed by a reduced irrigation regime, deficit-watered plants (DW). On the first day of the experiment, all pots were watered with 2,279 ml of water to the point of dripping (~1.17 g g^{-1}) to achieve even starting conditions of high soil water content. Then, WW plants were manually watered every day with 50 ml (2.3 l m^{-2}) for the first 47 days. For the rest of the experiment (16 days), when the plants had a higher demand for water due to an increase in daily mean temperature, watering was increased to 75 ml (3.4 l m^{-2}). In the second treatment, watering was reduced by ~50%, with the DW plants being watered every second day with the same amount of water and following the same irrigation strategy as the WW plants. These watering regimes did not produce drainage in either WW or DW plants. At the end of the experiment, each WW plant received ~2,279 ml of water at initial watering, plus ~3,550 ml (263 l m^{-2}) for 63 days while DW plants received ~2,279 ml plus ~1,775 ml (183 l m^{-2}). This initial watering represents around half of the average autum rainfall of Cordoba, whilst the water supplied for 63 days is about 33% higher (WW) or 33% lower (DW) than the average spring rainfall registered in Cordoba. The greenhouse temperature was kept in the range of 30 °C /15 °C (day/night) during the experiment. The experiment started on April 1st, 2019 when all plants had approximately seven leaves (after 13 days of acclimatisation in the greenhouse as mentioned in the previous section) and the plants were harvested on June 3rd, 2019 at the beginning of flowering. The duration of the experiment was 63 days.

Water use and physiological measurements

Four pots per variety and water regime were weighed three times per week from day 30 to the end of the experiment (day 63). Pot evapotranspiration, ET (ml) was estimated as $ET = PW_i - PW_j + I_{ij}$, where PW_i is the pot weight at day i (g), PW_j is the pot weight at day j (g) and I_{ij} the irrigation water provided between days i and j (ml). During the same period, relative soil water content was estimated as $RSWC = (SW_i - DSW)/(FSW - DSW)$, where SW_i is the soil weight at day i, DSW is dry soil weight and FSW is the soil weight at point of dripping. DSW was obtained by weighing and averaging the oven-dried soil (105°C/24h) of four pots. For FSW, the same four pots were previously watered to point of dripping and weighed discounting the weight of an empty pot. Since at day 30 the canopy of the plant already covered the entire pot surface, transpiration can account for most of the measured ET (Or, Lehmann, Shahraeeni, and Shokri, 2013; Ritchie, 1972).

At day 46, four fully expanded leaves of each plant were removed (12:00-14:00 h) to measure the relative leaf water content (RWC). RWC was calculated as RWC = (FW – DW) / (SFW – DW) × 100, where FW is fresh leaf weight, DW is dry leaf weight and SFW is saturated fresh leaf weight. After recording FW, the leaves were immersed in 90-mm Petri dishes filled with deionised water for 24 h at 20 – 25 ºC and then weighed again to record SFW. DW was measured after oven-drying the leaves at 60ºC for 72h. Before drying, the leaves were scanned (EPSON 1640 XL) and the area was measured using the image analyser software ImageJ (https://imagej.nih.gov/ij/) for the later calculation of the specific leaf area (SLA, cm^2 g^{-1}). Net photosynthesis per area (A_{area}) and stomatal conductance per area (g_{Sarea}) were also measured at day 46 (09:00-12:00 h) of mid-height, fully expanded and undamaged leaves using a portable infrared CO_2 gas analyser (LiCor Li6400XT, Li-Cor, Inc., Lincoln, NE, USA) fitted with a 2-cm^2 leaf cuvette. PAR was set at 1000 µmol photon m^{-2} s^{-1}, flow rate at 500 µmol s^{-1}, [CO_2] to 400 ppm, with block temperature set at 25ºC. Water-use efficiency (WUE) was derived from the ratio A_{area}/g_{Sarea}. An example of the state of WW and DW plants at the moment of measurement of the physiological variables can be seen in Figure S1.

Biomass and trait measurement

At the end of the experiment, plants were cut at the soil surface. Aerial plant components were separated into stem and leaves, which were also split into green/senescent leaves. Pots were cut open and the soil was separated into three layers (0-10.5, 10.5-21, 21-31.5 cm). The soil was carefully removed from the roots using a 2-mm sieve. The roots of each layer were then split into thin roots (<0.5mm) and thick roots (>0.5mm) and immediately frozen. This threshold was chosen to explicitly emphasise more absorptive roots (Wang, Liu, Fang and Shangguan, 2020). A representative subsample of thin roots from the first layer and all thick roots from the three soil layers of four WW plants per variety were split and independently scanned (EPSON 1640 XL). Root length was calculated using WinRHIZO software (http://regent.qc.ca/) and then root length to weight ratio (m g^{-1}) was also calculated. Leaves, stems and roots were oven-dried for 72 h at 60ºC and dry mass (DM) recorded.

Root to shoot ratio was calculated as the total root DM/total shoot DM. The Leaf mass fraction (LMF), Stem mass fraction (SMF) and Root mass fraction (RMF) were calculated as the proportion of the total DM corresponding to each one of these plant components. SLA, (cm^2 g^{-1}) was calculated as the ratio between the leaf area and its dry mass. Leaf area ratio (LAR, cm^2 g^{-1}), the ratio of leaf area and total plant DM, was estimated as the product of SLA and LMF (Poorter and Remkes, 1990; Villar et al., 2004). Leaf to stem ratio was measured as total leaf DM/total steam DM. Senescent to green leaf ratio was calculated as senescent leaf DM/green leaf DM. The proportion of thin roots in each layer was estimated as the thin roots DM/total roots DM.

Nutritive value

Samples of green, fully expanded leaves and stems were independently analysed for four plants of each variety and treatment at the end of the experiment. These samples were ground and passed through a 1-mm sieve. Crude protein (CP), ashes, neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM) were determined by near-infrared spectroscopy (NIRS) using a portable LabSpec 5.000 spectrometer (350–2.500 nm; ASD Inc., Boulder, Colorado, USA) using IndicoPro 6.0 spectrum acquisition software (ASD Inc., Boulder, CO, USA). Spectral data of samples was recorded in the whole range of 350-2,500 nm by 1-nm step. Four replicates of each sample were scanned (each being an average of 50 internal scans). White reference scans (with a Spectralon panel) were taken between every sample scan. The final sample spectrum was obtained by averaging the four scans. The statistics of the NIRS equations used to predict nutritional values of samples in this study are presented in Supplementary material (Table S1). NIRS equations were calibrated based on 130 spectra of Mediterranean pastures and forage crops analysed by wet chemical methods at the Laboratory of Animal Nutrition of SERIDA (Villaviciosa, Spain). NIRS predictions were performed using WinISI software (Infrasoft International, Port Matilda, PA, USA).

Statistical analysis

Differences among varieties and treatments for each variable were tested by two-way ANOVAs (variety and water regimes as fixed factors). For those variables where soil layer (3 depth levels) and plant organ (stem or leaf) were included, differences were explored by three-way ANOVAs (variety, water regimes and depth/organ as fixed factors). Accumulated evapotranspiration and RSWC were tested by two-way ANOVA for each measurement date and the interactions of the factors were also considered. When differences were significant ($p<0.05$), post-hoc Tukey's test at the 0.05 probability level was carried out to test differences among means. The data was transformed, when needed, using either logarithmic or square-root transformation to meet normality and homoscedasticity assumptions. To summarise and analyse the covariation between variables, a principal component analysis (PCA) was performed with morphological and physiologic traits. Evapotranspiration and nutritive value parameters were not included in the analysis because of the different number of observations. Statistical analyses were performed using the software R v. 3.6.1 (R Development Core Team, 2019).

RESULTS

Biomass and morphological traits

The deficit-watered treatment significantly affected ($p < 0.05$, Table S2) biomass production and senescent to green leaf ratio of all varieties (Table 2). The morphological traits shown in Table 3 were modified not by the water regime but by the variety. BAM was the variety with the lowest biomass production for both water regimes, while no difference was found between BBT and BCC under each treatment (Table 2). Shoot biomass under the DW treatment as a percentage of the WW treatment was similar for the three varieties: 50% for BAM, 49% for BBT and 57% for BCC. The three varieties showed significantly different aboveground biomass allocation (Table S3).

Table 2. Dry mass production and senescent leaves to green leaves ratio at the end of the experiment (63 days) for B. bituminosa varieties under two water regimes.

Variety	Treatment	Shoot dry mass (g)	Root dry mass (g)	Total dry mass (g)	Senescent leaves: Green leaves (g g^{-1})
BAM	**WW**	6.0 ± 1.0 c	2.7 ± 0.4 c	8.7 ± 1.4 c	0.38 ± 0.10 b
	DW	3.0 ± 0.7 d	1.2 ± 0.4 d	4.2 ± 1.1 d	0.52 ± 0.03 a
BBT	**WW**	13.7 ± 1.4 a	5.9 ± 0.9 a	19.6 ± 1.9 a	0.33 ± 0.06 b
	DW	6.7 ± 1.4 b	3.1 ± 0.5 b	9.8 ± 1.8 b	0.35 ± 0.03 a
BCC	**WW**	12.5 ± 1.7 a	8.0 ± 1.3 a	20.5 ± 2.2 a	0.18 ± 0.04 b
	DW	7.1 ± 0.4 b	3.5 ± 0.3 b	10.6 ± 0.5 b	0.32 ± 0.04 a

Mean values ($n = 6$) not sharing a common letter differ significantly *($P < 0.05$)* according to Tukey’s test. Standard error is showed. BAM: *B. bituminosa* var. *albomarginata*; BBT: *B. bituminosa* var. *bituminosa*; BCC: *B. bituminosa* var. *crassiuscula*.

BAM had the highest leaf to stem ratio (1.06 g g^{-1}) followed by BCC (0.80 g g^{-1}) and BBT (0.38 g g^{-1}). Similarly, BAM had the highest LFM whereas BBT had significantly higher SMF than BCC and BAM (Table 3). The SLA and LAR were not modified by the water regime although it was different for each variety (Table S3). The SLA was significantly lower for BAM (235 cm^2 g^{-1}) and BCC (239 cm^2 g^{-1}) than for BBT (352 cm^2 g^{-1}). Concerning LAR, BBT showed significantly lower LAR than BAM (Table 3).

Table 3. Morphological traits at the end of the experiment (63 days) for the B. bituminosa varieties. Mean of the two water regime treatments is presented. Leaf to stem ratio, root to shoot ratio, Leaf mass fraction (LMF), stem mass fraction (SMF), root mass fraction (RMF), specific leaf area (SLA) and leaf area ratio (LAR)

Variety	Leaf:Stem ratio (g g^{-1})	Root: Shoot ratio (g g^{-1})	LMF (g g^{-1})	SMF (g g^{-1})	RMF (g g^{-1})	SLA (cm^2 g^{-1})	LAR (cm^2 g^{-1})
BAM	1.06±0.10 a	0.46±0.05 n.s.	0.36±0.02 a	0.35±0.02 b	0.29±0.02 b	235±10.0 b	86.9±7.3 a
BBT	0.38±0.04 c	0.47±0.04 n.s.	0.19±0.02 c	0.50±0.02 a	0.31±0.02 ab	352±17.0 a	64.1±4.8 b
BCC	0.80±0.03 b	0.59±0.06 n.s.	0.28±0.01 b	0.36±0.01 b	0.36±0.02 a	239±9.3 b	67.6±3.7 ab

Mean values (n = 6) not sharing a common letter differ significantly *($P < 0.05$)* according to Tukey's test. Standard error is showed. BAM: *B. bituminosa* var. *albomarginata*; BBT: *B. bituminosa* var. *bituminosa*; BCC: *B. bituminosa* var. *crassiuscula*. n.s.: no significant.

Although root to shoot ratio did not change for either variety or treatment (Table 3), significant differences were found for root biomass and its allocation throughout the profile (Table S4). Root biomass was reduced by the DW treatment and it was always the lowest for BAM (Table 2). There was a two-way interaction among varieties and depth (p=0.017, Table S4), revealing a different pattern of root biomass distribution throughout the profile (Figure 1: A). While BBT and BCC showed a clear stratified distribution of root biomass decreasing with depth, BAM showed similar root biomass throughout the soil profile. Root biomass in the lower layer was of similar magnitude for all three varieties and this pattern was not modified by the water regime. The proportion of thin roots (<0.5 mm) increased throughout the profile for all varieties, being 30%, 79% and 95% for upper, middle and lower levels, respectively (Figure 1: B). BCC invested more biomass in thin roots than BAM and BBT under WW treatment (82%, 65% and 62% respectively). However, under DW treatment, BCC and BAM showed a higher proportion of thin roots than BBT (72%, 71% and 56% respectively). BCC had a significantly shorter root length to weight ratio of thin roots (33.5 m g^{-1}) than BAM (60.1 m g^{-1}) and BBT (59.1 m g^{-1}). No differences were found for thicker roots (>0.5mm) with 1.1 m g^{-1} root length to weight ratio on average.

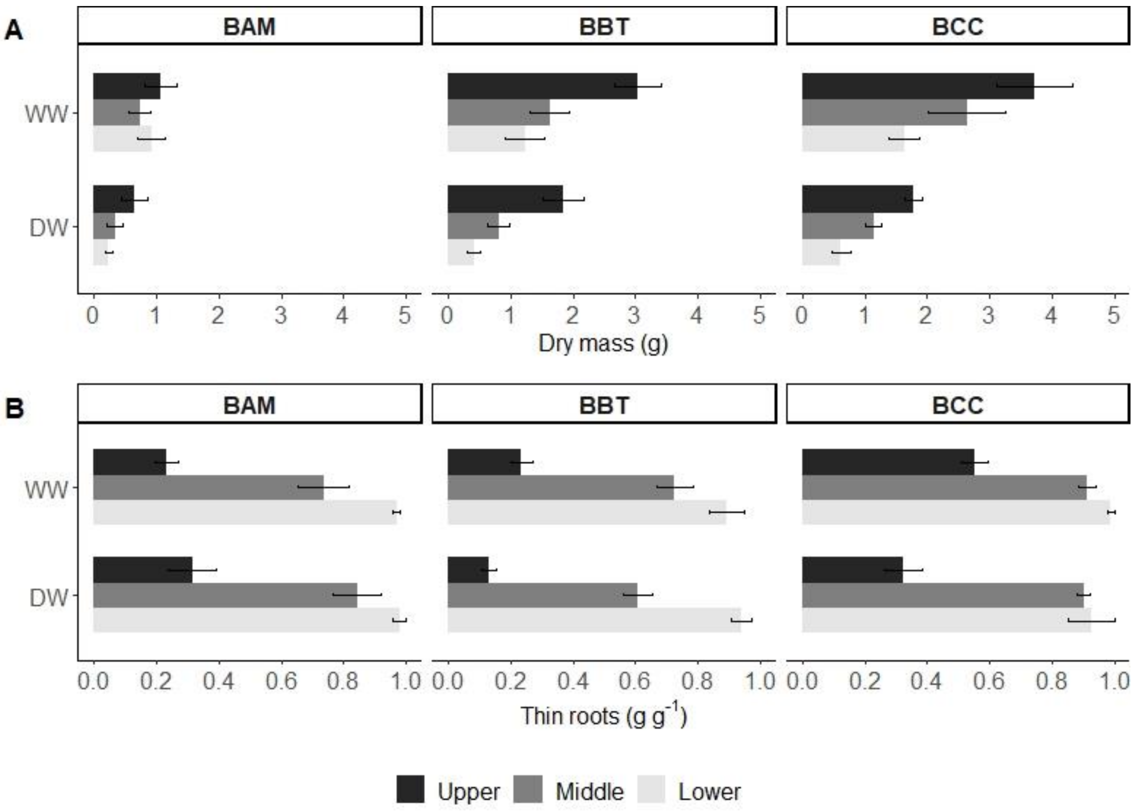

Figure 1. A: Root dry mass distribution along the three soil layers at the end of the experiment (63 days) for *B. bituminosa* varieties under two water regimes and **B**: Proportion of thin roots. Thin roots proportion down the three soil layers for *Bituminaria bituminosa* varieties. BAM=*B. bituminosa* var. *albomarginata*; BBT=*B. bituminosa* var. *bituminosa*; BCC= *B. bituminosa* var. *crassiuscula*; WW= well-watered; DW=deficit-watered. Mean values and standard errors (N=6).

Plant water use

The accumulated evapotranspiration over the measurement period showed a significant difference (p<0.001) between treatments. The total evapotranspirated water was 1,790 ± 25 ml under DW and 2,833 ± 102 ml under WW. Moreover, important differences were found when the accumulated evapotranspiration was evaluated at each measurement date. At the beginning, both treatments had similar evapotranspiration; however, from day 36 to the last measurement WW plants had significantly higher evapotranspiration than DW plants. Regarding differences by variety, BAM showed significantly lower evapotranspiration than BCC from the first measurement to day 40. Thereafter, there was an interaction that indicated lower accumulated evapotranspiration of BAM under WW treatment (Figure 2: A).

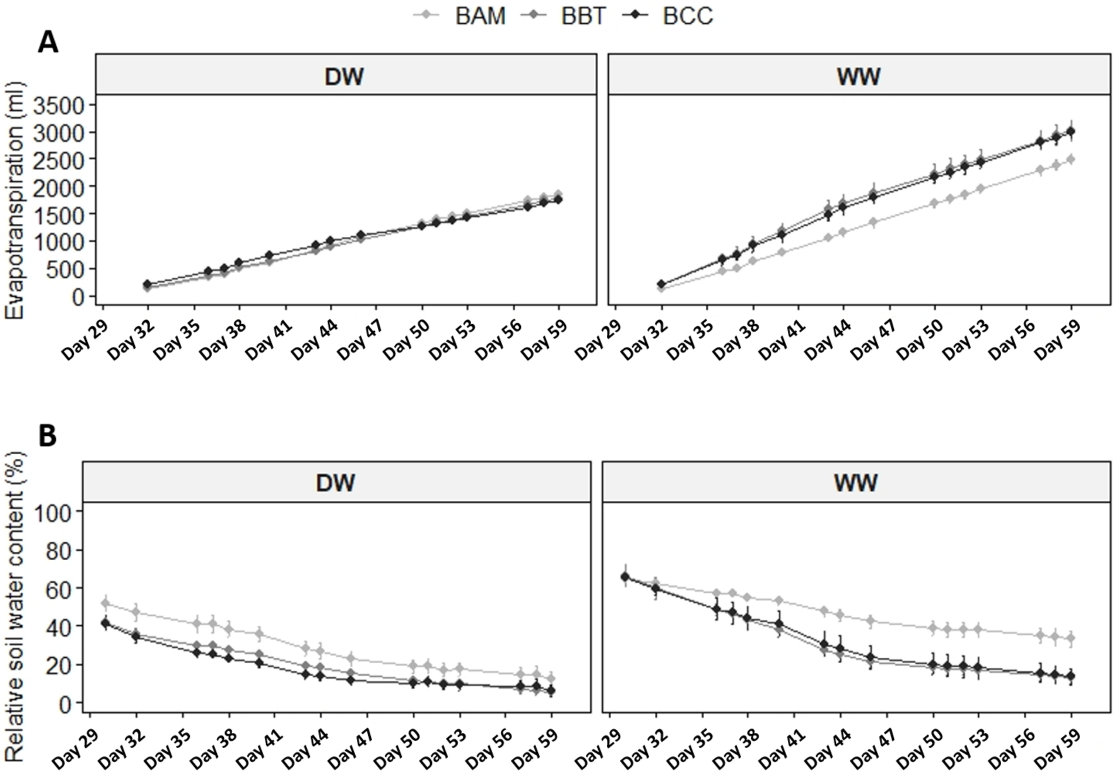

Figure 2. Evapotranspiration (ml) (**A**) and relative soil water content (RSWC) (%) (**B**) of *B. bituminosa* varieties under two water regimes WW= well-watered and DW=deficit-watered. BAM=*B. bituminosa* var. *albomarginata*; BBT=B. *bituminosa* var. *bituminosa*; BCC= *B. bituminosa* var. *crassiuscula*. Mean values and standard errors (N=4).

At the beginning, relative soil water content (RSWC) was 66% on average and 45% under WW and DW respectively (Figure 2: B), showing no differences among varieties. During the measurement period, RSWC was always significantly lower under DW. From days 36 to the last measurement BAM showed significantly higher RSWC than BCC and BBT. In DW treatment, RSWC decreased to a minimum value of around 12% at day 53 when it seemed to stabilise; this value was firstly reached by BCC (Figure 2: B).

Relative water content, photosynthesis and stomatal conductance

The deficit-watered regime did not affect the RWC (Table S5), as values are similar under both water regimes. However, BAM showed higher RWC than the other two varieties (Table 4). For A_{area}, the interaction of variety and treatment (Table S5) reflected the fact that BCC was the only variety affected by DW treatment. BAM was the variety with the highest A_{area}, although it showed similar values to BCC under WW treatment (Table 4).

g_{Sarea} was also higher for BAM and was reduced by DW treatment across the three varieties. WUE was not influenced by either variety or treatment.

Table 4. Relative water content (RWC) of the leaf, net photosynthesis per area (A_{area}), stomatal conductance per area (g_{Sarea}) and water use efficiency (WUE) for B. bituminosa varieties under two water regimes measured at day 46.

Variety	Treatment	RWC (%)	A_{area} (µmol CO_2 m^{-2} s^{-1})	g_{Sarea} (mmol H_2O m^{-2} s^{-1})	WUE (µmol CO_2 $(molH_2O)^{-1}$))
BAM	**WW**	85.5 ± 1.5 a	19.9 ± 1.6 a	192.5 ± 8.5 a	105.6 ± 11.3 n.s.
	DW	92.1 ± 1.0 a	15.5 ± 2.1 a	164.9 ± 20.4 b	104.9 ± 21.4 n.s.
BBT	**WW**	83.5 ± 2.5 b	6.0 ± 2.0 bc	54.7 ± 11.8 c	117.2 ± 18.0 n.s.
	DW	77.3 ± 4.7 b	4.7 ± 1.1 c	35.2 ± 4.3 d	124.7 ± 21.6 n.s.
BCC	**WW**	81.9 ± 3.4 b	13.3 ± 2.7 ab	75.5 ± 29.9 c	138.9 ± 28.4 n.s.
	DW	74.8 ± 4.2 b	2.5 ± 0.6 c	23.2 ± 5.3 d	86.3 ± 13.6 n.s.

Mean values (n = 6) not sharing a common letter differ significantly *($P < 0.05$)* according to Tukey's test. Standard error is showed. BAM: *B. bituminosa* var. *albomarginata*; BBT: *B. bituminosa* var. *bituminosa*; BCC: *B. bituminosa* var. *crassiuscula.* n.s.: no significant.

The first two axes of the PCA accounted for 33.0% and 24.1% of total variation, respectively. The three varieties showed clear clustering (Figure 3. A) while the treatments were more scattered on both axes (Figure 3. B). Overall, morphological traits were more important to explain the variability of both principal components than physiological ones. LMF, leaf to stem ratio and LAR had high negative loadings and high positive loading for root biomass for the first principal component. For the second principal component, root biomass, root to shoot ratio and thin roots proportion presented high positive loadings while SMF, SLA and senescent to green leaf ratio had high negative loadings. All biomass-related variables together with water use efficiency (WUE), RMF and root to shoot ratio showed covariation. LAR, LMF and leaf/stem ratio presented covariation with A_{area}, g_{Sarea} and RWC. Finally, SLA and SMF also showed covariation.

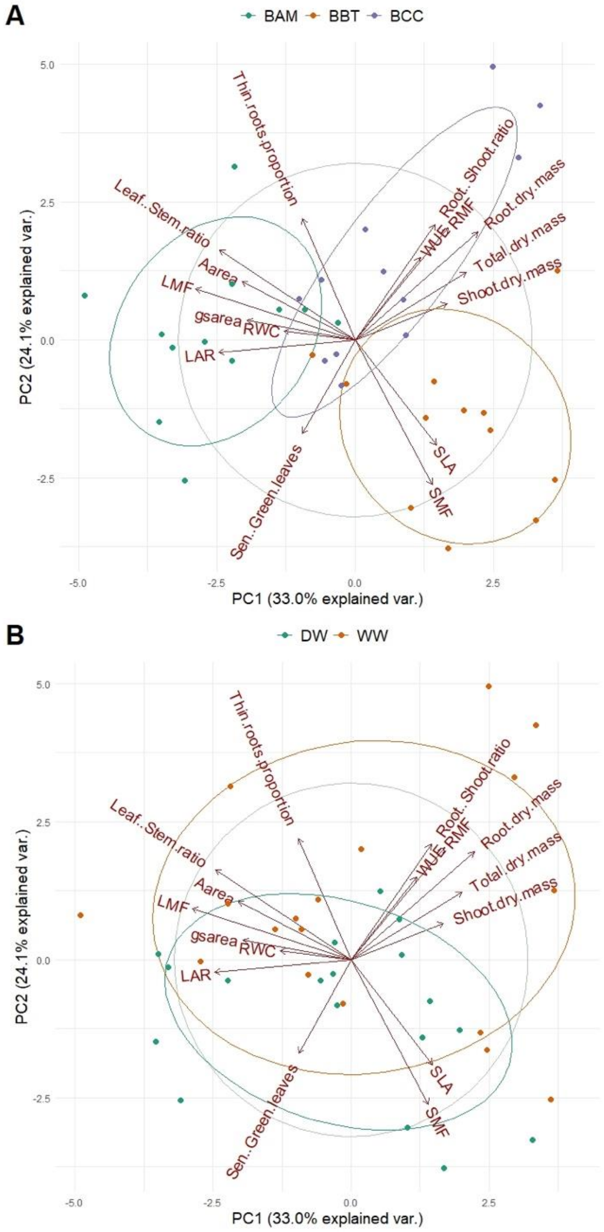

Figure 3. Principal component analysis (PCA) showing the two main axes of variability in morphological and physiological variables. Normal ellipse grouping observations by *B. bituminosa* variety: BAM=*B. bituminosa* var. *albomarginata*; BBT=*B. bituminosa* var. *bituminosa*; BCC= *B. bituminosa* var. *crassiuscula* (**A**) and water regime: WW= well-watered and DW=deficit-watered (**B**). Abbreviations: net photosynthesis per area (A_{area}), stomatal conductance per area (g_{sarea}), water use efficiency (WUE), Leaf mass fraction (LMF), stem mass fraction (SMF), root mass fraction (RMF), specific leaf area (SLA) and leaf area ratio (LAR), Senescent to green leaves ratio (Sen.green,leaves).

Nutritive value

Ash content was around 10-12% (leaf and stem weighed average across varieties), showing two interactions (Table S6): variety by organ (p=0.008) and organ by treatment (p=0.019). BBT ash content was significantly lower in the stem than in the leaf while BAM and BCC showed no difference in ash content between organs (Figure 4: A). The organ by treatment interaction reflected the effect of DW on the leaf ash content reduction whereas it had no effect on the stem (Figure 4: B).

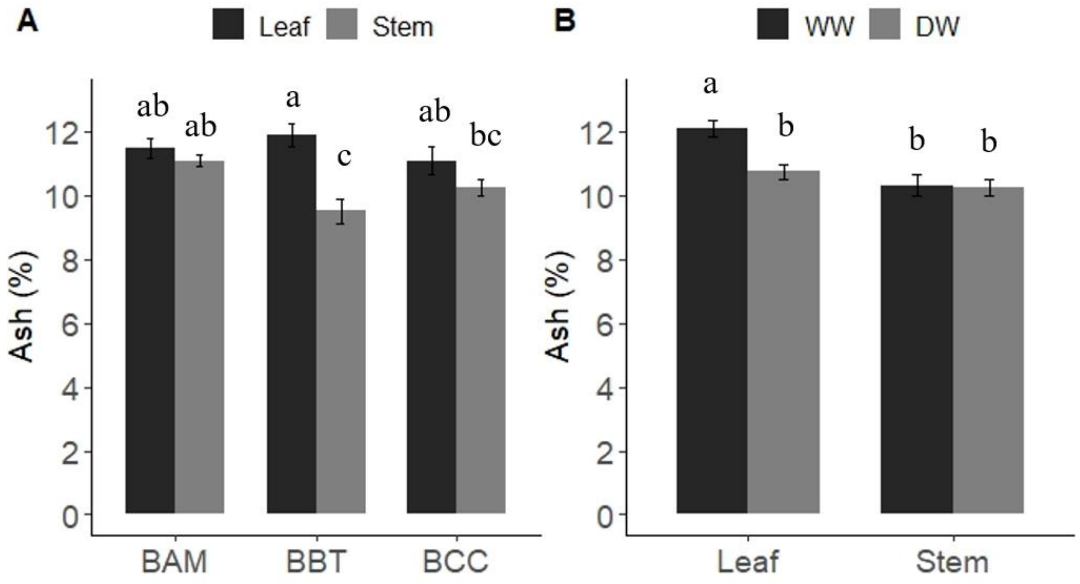

Figure 4. Ash content (%) in leaf and stem of *B. bituminosa* varieties (means of two water regimes) (**A**), and in leaf and stem under two water regimes WW= well-watered and DW=deficit-watered (means of three *B. bituminosa* varieties) (**B**) at the end of the experiment (63 days). BAM=*B. bituminosa* var. *albomarginata*; BBT=*B. bituminosa* var. *bituminosa*; BCC= *B. bituminosa* var. *crassiuscula*. Mean values and standard errors (N=4). Means not sharing a common letter differ significantly (P < 0.05) according to Tukey's test.

The other parameters analysed, EDOM, ADF, NDF and CP, were not affected by the water reduction imposed in this experiment (Table S6). The leaf EDOM was similar for all varieties, with an average value of 86.2%. Stem EDOM was significantly lower than in leaf, also being lower for BBT than for BAM and BCC, which had similar values (Figure 5: A). CP leaf values were around 16% across all varieties. However, a two-way interaction (Table S6) reflected BAM having higher stem CP than BCC whereas BBT had the same CP value as the other varieties (Figure 5: B). There was an interaction between variety and organ for NDF (Table S6). Although the NDF value for stem was similar for all varieties, 42.9% on average, the leaf NDF content was significantly higher for BAM than for BBT, while BCC showed no difference in leaf NDF compared to the other two varieties (Figure 5:C). As expected, ADF was significantly higher for stem than for leaf, although values were similar across the three varieties, 19.9% for leaf and 31.4% for stem on average (Figure 5:D).

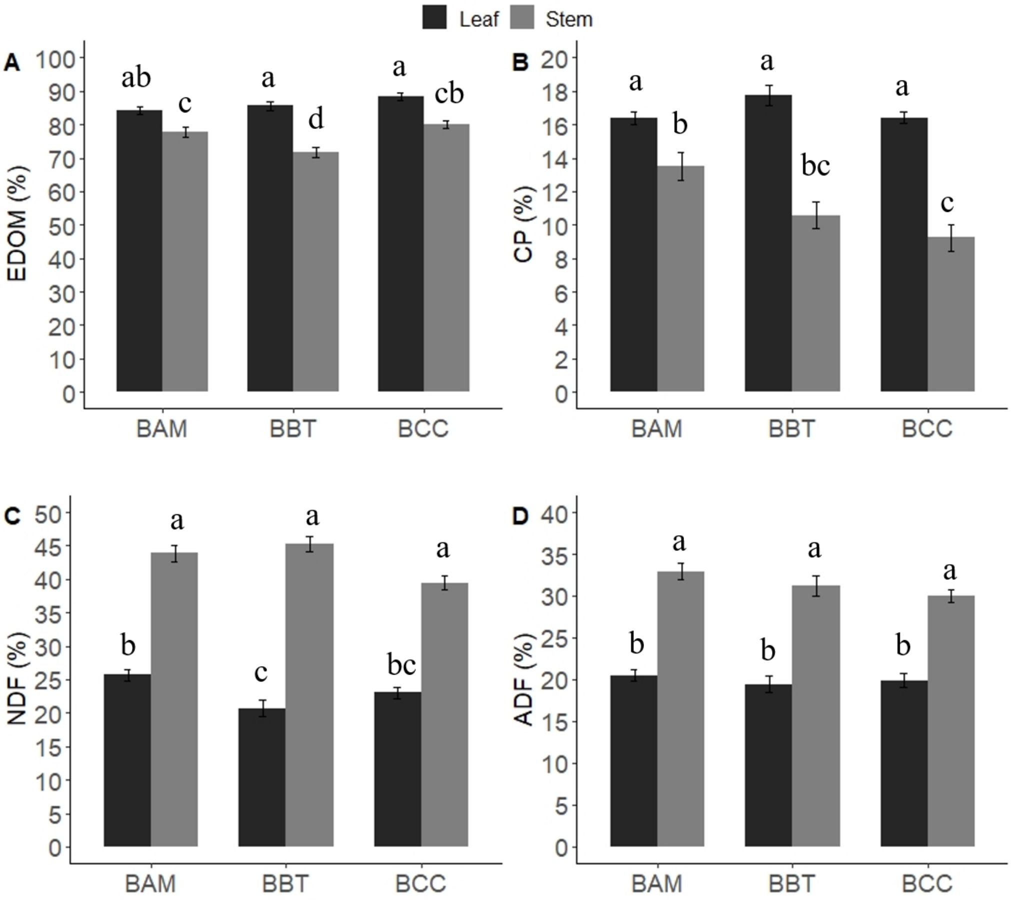

Figure 5. Enzyme digestibility of organic matter (EDOM) (**A**), crude protein (CP) (**B**), neutral detergent fibre (NDF) (**C**) and acid detergent fibre (ADF) (**D**) of leaf and stem of *B. bituminosa* varieties at the end of the experiment (63 days). BAM=*B. bituminosa* var. *albomarginata*; BBT=*B. bituminosa* var. *bituminosa*; BCC= *B. bituminosa* var. *crassiuscula*. Mean values and standard errors (N=4). Means not sharing a common letter differ significantly (P < 0.05) according to Tukey’s test.

DISCUSSION

The lower shoot biomass production of BAM under both treatments differs from previous studies where BAM was highly productive (Melis et al., 2018; Real et al., 2014). These contrasting results compared to previous studies (Foster et al., 2015; Foster, Ryan, Real, Ramankutty, and Lambers, 2012; Martínez-Fernández et al., 2012) could be explained by differences in experimental conditions, in particular, the high initial soil water content and differences in the water regimes. The relatively high initial soil water content could have affected the early development of BAM leading to a lower biomass production. BAM grows naturally in a very low-rainfall environment (< 200 mm) while BCC and BBT grow in environments with higher rainfall (Table 1) where periodic wet conditions may be expected during the growing season. BCC, in particular, which grows in the National Park of Cañadas del Teide (2200m a.s.l.) and receives part of the average precipitation (500 mm) as snow (Méndez et al., 1990; Raeside et al., 2012; Real et al., 2014), may have longer wet conditions due to snow melt. Real et al. (2014) also suggested that variability in wet conditions tolerance might be expected based on the high natural habitat variability of *B. bituminosa.*

This hypothesis is supported by the lower root biomass production of BAM and its distribution throughout the profile compared to BCC and BBT (Figure 1: A). In the same line, BAM was the only variety for which the WW treatment reduced the proportion of thin roots, as these are expected to be more susceptible to root rot. This poor root development limited the soil water use, as shown by the high RSWC maintained over the last growing period even for DW plants (Figure 2: B). Similarly, the evapotranspiration of BAM could be affected by the lower shoot biomass and by a root system that was unable to use the soil water content as much as BCC and BBT which developed larger root systems. *B. bituminosa* susceptibility to stem and root rot has been pointed out in previous studies (Martínez-Fernández et al., 2012; Real et al., 2014). Real et al. (2014) found *B. bituminosa* accessions were generally sensitive to wet conditions although four accessions showed tolerance. Raeside et al. (2012) also found BAM failed to persist and produce biomass during wet winters. These results have important agronomic implications since BAM susceptibility to wet conditions may limit its potential as a novel forage legume (Raeside et al., 2012) in areas with contrasting rainfall during the plant growing season. In the Mediterranean basin, clay/silty soils and high-rainfall periods could often contribute to periodic wet conditions that might limit BAM root system development and therefore its resistance to subsequent drought periods. This could also mean a competitive disadvantage of BAM in grassland mixtures at initial stages, since more tolerant species to initial high soil water content could dominate and outcompete BAM. For example, as shown in Figure 2: A, BAM under WW conditions showed lower accumulated evapotranspiration, while no differences between varieties were found under DW. Breeding programmes have used BCC to improve BAM cold-resistance (Real et al., 2014; Walker, Romero and Correal 2010); these crossed lines with BCC could also improve BAM susceptibility to wet conditions (Raeside et al., 2012). The efforts of the breeding programs to deliver wet conditions- and drought-resistant ideotypes (Real et al., 2014) will be crucial to overcoming this potential limitation of BAM in permanent grasslands of the Mediterranean basin.

The DW treatment of this experiment was not restrictive enough to cause SLA or other morphological modifications seen in previous studies (Foster et al., 2012; Foster et al., 2015; Martínez-Fernández et al., 2012). Only biomass-related variables and senescent to green leaf ratio contributed to differentiating between both treatments (Figure 3. B). However, BAM and BCC had a significantly lower SLA than BBT, which could indicate a thicker and/or denser leaf for these varieties. The higher SLA of BBT could also be related to the lower NDF content in leaf (which indicates the cell-wall material) of this variety (Figure 5:C). Khaled, Duru, Decruyenaere, Jouany and Cruz, (2006) also found a negative correlation between SLA and fibre content in grassland species. SLA values for the three varieties were higher than those reported by Martínez-Fernández et al. (2012) for pot-grown plants and lower than SLA values of plants grown in the field in the same study. As these authors state, these differences might be due to different temperature and light intensity exposure. Foster et al. (2013) and Martínez-Fernández et al. (2012) suggested that lower SLA in BAM might play an important role in its protection against light intensity and water loss. Since BCC and BAM showed lower SLA (i.e., thicker and/or denser leaves), these two varieties could be better adapted than BBT to high light intensity and temperature. A_{area} and g_{Sarea} values of BAM measured at day 46 (that was not the point of maximum drought stress) were associated with higher leaf RWC (Foster et al., 2015) which confirms the drought adaptation of this variety (Foster et al., 2013). The differences in A_{area} of BCC by treatment could also be influenced by its RSWC at measurement time (day 46) under DW treatment, which was the lowest of the three varieties (12% vs 15% and 23% for BBT and BAM, respectively) (Figure 2: B). The significant lower A_{area} recorded for BBT under WW treatment might indicate higher responsiveness to high temperature leading to earlier A_{area} reduction. The A_{area} and g_{Sarea} response of the different *B. bituminosa* varieties to increasing temperature and also light intensity could be further investigated in future research as it might play an important role in their adaptation to Mediterranean permanent grasslands.

In agreement with the results of this study, previous research has found little or no effect of moderate drought stress on the quality parameters of forage legumes (Komainda, et al., 2019; Kuchenmeister, Kuchenmeister, Kayser, Wrage and Isselstein, 2013). As Kuchenmeister et al. (2013) stated, selection of legume species and cultivation in mixture or monoculture may have more influence on quality parameters than drought stress. These studies reported values of CP for alfalfa between 19% and 27% and informed of minor effect of drought stress on CP content (Kuchenmeister et al. 2013; Staniak and Harasim, 2018). In this study, just green leaves were sampled for nutritive analysis. Although green leaves might have no differences in quality, it is worth noting that drought stressed plants with senescent leaves may have lower global quality (lower digestibility and CP content and higher NDF and ADF) than those with no senescent leaves.

Overall, BAM and BCC showed better forage aptitude than BBT due to the higher leaf proportion (Table 3). The leaf proportion is an important morphological value to assess the nutritive value of forage legumes (Abd El Moneim et al., 1990). Although BBT had similar leaf nutritive value to the other two varieties (Figure 4), the lower leaf to stem ratio of this variety (Table 3) makes it less suitable as forage since it reduces its palatability compared to BAM and BCC. As the PCA showed, the LMF, leaf to stem ratio (both higher in BAM) and the SMF clearly contributed to differentiating the three varieties, especially between BAM and BBT. The weighted average value of CP between stem and leaf of BAM (15%), BBT (12.5 %) and BCC (12.4%) were lower than concentrations found for Canarian varieties with values ranging from 15 to 17.7% for samples taken in summer (Ventura et al., 2009; Ventura, Méndez, Flores, Rodriguez and Castañon, 2000), although similar to CP values from 9.4 to 16.1% reported for Italian accessions (Pecetti et al., 2007). As Ventura et al. (2009) informed, these varieties have higher forage quality than alfalfa hay, which showed values of 11.8% CP, 57.4% NDF and 43.7% digestible organic matter. The weighted average concentrations of NDF (BAM 34.8%, BBT 38.4% and BCC 32.1%) and ADF (BAM 26.7%, BBT 28.1% and BCC 25.5%) were lower than previously evaluated concentrations of Canarian varieties and other for Italian accessions that reported values of NDF from 41.1 to 53.7% (Pecetti et al., 2007; Ventura et al., 2000; Ventura et al., 2009). These differences in NDF may be explained by the age of the plants sampled, three-year-old plants in the case of the Italian accessions (Pecetti et al., 2007). Evaluations of BAM nutritive value have shown CP to be highly variable depending on the accession analysed, with values ranging from 12.8% to 24.2% (Oldham et al., 2013; Raeside et al., 2012). The same studies presented lower values of NDF (25.4 - 32.8 %) and ADF (19.4 - 22.3 %) (Oldham et al., 2013; Raeside et al., 2012) although these differences may be accounted for by the earlier phenological stage. Mismatches in reproductive development of the three studied varieties could be observed due to the different meteorology in their native environment (Méndez et al., 1990). Although phenology was not the subject of study here, the three varieties showed signs of being at the beginning of the reproductive stage without considerable differences between them. Melis et al. (2018) found some differences in the date of the first flower appearance between *B. bituminosa* Spanish accessions, Sardinian accessions and *Bituminaria morisiana* all grown in Sardinia (Italy). However, no significant differences were found for accessions of the three varieties from the Canary Islands. The intrinsic morphological traits such as leaf and stem mass fraction seem to have stronger effects on the forage aptitude than possible mismatches of the phenology between the three varieties. CP (15.0%), NDF (34.8%) and ADF (26.6%) stem and leaf average concentration of BAM were very similar to the concentrations also evaluated by near infrared analysis for the same variety by Adriansz, Hardy, Milton, Oldham, and Real, (2017), CP (15.0%), NDF (37.6%) and ADF (26.6%). However, it would be worth investigating the drought stress effect on phenology of the studied varieties since it would directly affect their nutritive value.

Leaf ash content increased with WW treatment. This finding is consistent with other research that reported an increase in most leaf nutrient under well irrigated condition (Olivera-Viciedo et al., 2020), especially those nutrients that are passively uptaken (Wu, Liu, Wang, Zhang and Xu, 2012). Furthermore, ash content has been positively correlated with transpiration ratio (ratio of water transpired to carbon fixed) (Masle, Farquhar and Wong, 1992; Merah, Deleens, Souyris and Monneveux, 2001), and an increase in the later can occur under well irrigated condition. However, Masle et al. (1992) stated, that changes in the transpiration ratio induced by environmental factors (as atmospheric humidity or carbon dioxide concentration) do not cause a noticeable change in the mineral content. Leaf ash content can affect the response of the plant to water stress as some nutrients are involved in water flow regulation. For example, moderate potassium starvation inhibits the mechanism of stomatal closure and can cause tissue dehydration in water-stressed plants (Benlloch-González, Arquero, Fournier, Barranco and Benlloch, 2008). In fact, we found a positive correlation between leaf ash content and RWC (result not shown). The ash content may seem higher compared to previous studies Italian accessions (6.0-7.7%) (Pecetti et al., 2007); however, similar values in Spanish accessions (9.18-12.2%) are reported by Oldham et al., (2013) and SIA (2019). These Canarian varieties show higher CP and lower NDF and ADF content than Mediterranean rainfed grasslands, at a similar phenological stage (Henkin et al., 2011; Vázquez De Aldana, García-Ciudad, and García-Criado, 2008; Zarovali, Yiakoulaki, and Papanastasis, 2007). This higher nutritive value together with its drought and grazing tolerances make this species a promising fodder plant for Mediterranean grasslands (Oldham et al., 2013; Real et al., 2017; Sternberg et al., 2006).

CONCLUSION

Water stress reduced biomass production equally for the three varieties. However, the initial soil water content affected the early development and biomass of BAM, which was reflected by the poor root system development. This denotes susceptibility of BAM to high soil water content which may limit its introduction to some Mediterranean farming systems, where periodic soil water saturation can be expected, especially at the beginning of the growing season. According to the morphological traits studied, BAM showed the best forage aptitude. The lower SLA of BAM and BCC is an important adaptation against high light intensity and temperature in arid environments. The three *B. bituminosa* native to the Canary Islands showed good nutritive value for their use to improve the quality of Mediterranean rainfed pastures.

The wide diversity of existing varieties and the progress of the breeding programmes developed in Spain and Australia based on Canarian *B. bituminosa* varieties bring forward the opportunity for the incorporation of these lines for the improvement of Mediterranean pastures. However, additional research through continuous monitoring of its use and long-term field experiments will be needed to confirm the proper adaptation of the improved lines and selected ideotypes to the wide range of Mediterranean environmental conditions.

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Supplementary material

Figure S 1. Plants of the B. bituminosa varieties at day 46 of experiment: B. bituminosa var. albomarginata (BAM); B. bituminosa var. bituminosa (BBT); B. bituminosa var. crassiuscula (BCC) under two water regimes well-watered (WW), deficit-watered (DW).

Table S 1. Statistics of NIR equations used for determination of nutritive value parameters.

Nutritive value parameter	Nº samples	Mean (%)[a]	Range (%)	SD [b]	SEC [c]	R^{2} [d]	SECV [e]	r^{2} [f]
Ash	130	7.63	4.45-13.10	1.79	0.386	0.95	0.651	0.87
CP	127	12.70	4.47-24.07	4.59	0.324	0.99	0.647	0.98
NDF	129	50.87	22.03-71.34	11.35	1.328	0,99	1.749	0.98
EDOM	127	58.91	38.45-92.56	12.20	1.257	0.99	2.171	0.97
ADF	128	30.91	13.63-50.70	7.11	0.767	0.99	1.170	0.97

[a] Mean value of the calibration set

[b] Standard deviation of the calibration set

[c] Standard error of the calibration set

[d] Coefficient of determination of the calibration set

[e] Standard error of the cross-validation

[f] Coefficient of determination of the cross-validation

Table S 2. Summary results of the two-way ANOVA evaluating the effects of variety, treatment and their interaction on dry mass production and senescent leaves to green leaves ratio.

Variable	Source of variation	d.f.	SS	MS	F	P
Shoot dry mass	**Variety (V)**	2	246.490	123.240	14.585	<0.001
	Treatment (T)	1	237.670	237.670	28.128	<0.001
	V x T	2	23.790	11.900	1.408	n.s.
	Error	30	253.490	8.450		
Root dry mass	**Variety (V)**	2	6.419	3.210	20.198	<0.001
	Treatment (T)	1	4.760	4.760	29.954	<0.001
	V x T	2	0.157	0.078	0.493	n.s.
	Error	30	4.767	0.159		
Total dry mass	**Variety (V)**	2	609.700	304.800	20.313	<0.001
	Treatment (T)	1	586.500	586.500	39.083	<0.001
	V x T	2	56.900	28.400	1.895	n.s.
	Error	30	450.200	15.000		
Senescent leaves: Green leaves	**Variety (V)**	2	2.152	1.076	3.112	n.s.
	Treatment (T)	1	2.148	2.148	6.212	0.0189
	V x T	2	0.622	0.311	0.899	n.s.
	Error	30	9.682	0.346		

d.f. = degrees of freedom, SS= sum of squares, MS= Mean of squares. Level of significance P = 0.05.

Table S 3. Summary results of the two-way ANOVA evaluating the effects of variety, treatment and their interaction on Leaf to stem ratio, root to shoot ratio, Leaf mass fraction (LMF), stem mass fraction (SMF), root mass fraction (RMF), specific leaf area (SLA) and leaf area ratio (LAR).

Variable	**Source of variation**	**d.f.**	**SS**	**MS**	**F**	**P**
Leaf: Steam ratio	**Variety (V)**	2	1.054	0.527	37.516	<0.001
	Treatment (T)	1	0.021	0.021	1.462	n.s.
	V x T	2	0.027	0.013	0.946	n.s.
	Error	30	0.422	0.014		
Root: Shoot ratio	**Variety (V)**	2	0.123	0.061	1.972	n.s.
	Treatment (T)	1	0.023	0.023	0.732	n.s.
	V x T	2	0.076	0.038	1.222	n.s.
	Error	30	0.933	0.031		
LMF	**Variety (V)**	2	0.174	0.087	26.187	<0.001
	Treatment (T)	1	0.000	0.000	0.036	n.s.
	V x T	2	0.006	0.003	0.829	n.s.
	Error	30	0.100	0.003		
SMF	**Variety (V)**	2	0.175	0.088	25.835	<0.001
	Treatment (T)	1	0.008	0.008	2.284	n.s.
	V x T	2	0.006	0.003	0.928	n.s.
	Error	30	0.102	0.003		
RMF	**Variety (V)**	2	0.033	0.017	3.427	0.0461
	Treatment (T)	1	0.010	0.010	1.97	n.s.
	V x T	2	0.018	0.009	1.829	n.s.
	Error	30	0.141	0.005		
SLA	**Variety (V)**	2	1.240	0.620	25.634	<0.001
	Treatment (T)	1	0.016	0.016	0.667	n.s.
	V x T	2	0.040	0.020	0.832	n.s.
	Error	30	0.726	0.024		
LAR	**Variety (V)**	2	0.580	0.290	4.052	0.0277
	Treatment (T)	1	0.023	0.023	0.322	n.s.
	V x T	2	0.005	0.002	0.035	n.s.
	Error	30	2.149	0.072		

d.f. = degrees of freedom, SS= sum of squares, MS= Mean of squares. Level of significance P = 0.05.

Table S 4. Summary results of the three-way ANOVA evaluating the effects of variety, treatment, depth and their interaction on roots dry mass and proportion of thin roots.

Variable	Source of variation	d.f.	SS	MS	F	P
Roots dry mass	**Variety (V)**	2	6.014	3.007	39.749	<0.001
	Treatment (T)	1	4.937	4.937	65.256	<0.001
	Depth (D)	2	4.478	2.239	29.595	<0.001
	V x T	2	0.143	0.071	0.943	n.s.
	V x D	4	0.970	0.242	3.205	0.017
	T x D	2	0.042	0.021	0.28	n.s.
	V x T x D	4	0.067	0.017	0.222	n.s.
	Error	90	6.808	0.076		
Thin roots: Total roots DM	**Variety (V)**	2	1.091	0.545	16.953	<0.001
	Treatment (T)	1	0.005	0.005	0.150	n.s.
	Depth (D)	2	14.047	7.023	218.289	<0.001
	V x T	2	0.271	0.135	4.204	0.018
	V x D	4	0.154	0.038	1.194	n.s.
	T x D	2	0.097	0.049	1.508	n.s.
	V x T x D	4	0.128	0.032	0.992	n.s.
	Error	90	2.896	0.032		

d.f. = degrees of freedom, SS= sum of squares, MS= Mean of squares. Level of significance P = 0.05.

Table S 5. Summary results of the two-way ANOVA evaluating the effects of variety, treatment and their interaction on relative water content (RWC) of the leaf, net photosynthesis per area (A_{area}), stomatal conductance per area (g_{Sarea}) and water use efficiency (WUE).

Variable	Source of variation	d.f.	SS	MS	F	P
RWC	**Variety (V)**	2	1.902	0.951	5.233	0.0117
	Treatment (T)	1	0.184	0.184	1.014	n.s.
	V x T	2	0.991	0.496	2.726	n.s.
	Error	30	5.090	0.182		
A_{area}	**Variety (V)**	2	25.837	12.919	23.491	<0.001
	Treatment (T)	1	6.945	6.945	12.629	0.001
	V x T	2	5.278	2.639	4.799	0.016
	Error	30	15.399	0.550		
g_{Sarea}	**Variety (V)**	2	0.376	0.188	49.843	<0.001
	Treatment (T)	1	0.032	0.032	8.575	0.007
	V x T	2	0.009	0.005	1.219	n.s.
	Error	30	0.102	0.004		
WUE	**Variety (V)**	2	3.060	1.528	0.298	n.s.
	Treatment (T)	1	4.230	4.229	0.824	n.s.
	V x T	2	10.170	5.087	0.991	n.s.
	Error	30	138.540	5.131		

d.f. = degrees of freedom, SS= sum of squares, MS= Mean of squares. Level of significance P = 0.05.

Table S 6. Summary results of the three-way ANOVA evaluating the effects of variety, treatment, organ and their interaction on enzyme digestibility of organic matter (EDOM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF)

Variable	Source of variation	d.f.	SS	MS	F	P
Ash	**Varierty (V)**	2	3.94	1.97	2.772	n.s.
	Treatment (T)	1	6.082	6.082	8.557	0.006
	Organ (O)	1	15.592	15.592	21.939	<0.001
	V x T	2	0.151	0.076	0.106	n.s.
	V x O	2	7.863	3.932	5.532	<0.001
	T x O	1	4.329	4.329	6.09	0.019
	V x T x O	2	0.226	0.113	0.159	n.s.
	Error	34	24.165	0.711		
EDOM	**Varierty (V)**	2	284.7	142.4	11.628	<0.001
	Treatment (T)	1	14.5	14.5	1.186	n.s.
	Organ (O)	1	1054.3	1054.3	86.115	<0.001
	V x T	2	22	11	0.898	n.s.
	V x O	2	105.5	52.7	4.308	0.021
	T x O	1	48	48	3.917	n.s.
	V x T x O	2	24.9	12.4	1.016	n.s.
	Error	34	416.2	12.2		
ADF	**Varierty (V)**	2	38	19	2.844	n.s.
	Treatment (T)	1	12	12	1.788	n.s.
	Organ (O)	1	1512.2	1512.2	226.23	<0.001
	V x T	2	3.4	1.7	0.257	n.s.
	V x O	2	13.5	6.7	1.01	n.s.
	T x O	1	6	6	0.903	n.s.
	V x T x O	2	7.4	3.7	0.557	n.s.
	Error	34	227.3	6.7		
NDF	**Varierty (V)**	2	1	0.5	7.174	0.003
	Treatment (T)	1	0.01	0.01	0.12	n.s.
	Organ (O)	1	34.65	34.65	495.78	<0.001
	V x T	2	0	0	0.027	n.s.
	V x O	2	1.17	0.59	8.396	<0.001
	T x O	1	0.05	0.05	0.703	n.s.
	V x T x O	2	0.29	0.15	2.107	n.s.
	Error	34	2.38	0.07		
CP	**Varierty (V)**	2	16888	8444	3.492	0.042
	Treatment (T)	1	2688	2688	1.112	n.s.
	Organ (O)	1	279027	279027	115.38	<0.001
	V x T	2	1184	592	0.245	n.s.
	V x O	2	29233	14616	6.044	0.006
	T x O	1	2353	2353	0.973	n.s.
	V x T x O	2	1986	993	0.411	n.s.
	Error	34	82226	2418		

d.f. = degrees of freedom, SS= sum of squares, MS= Mean of squares. Level of significance P = 0.05.

CHAPTER IV. Lanza® Tedera is strongly suppressed by competition from *Lolium multiflorum* and is best adapted to light-textured soils

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Abstract

Bituminaria bituminosa var. *albomarginata*, known as Tedera, is a promising forage for Mediterranean climates. An improved variety named Lanza® has been developed. Previous research suggests that soil water saturation in heavy-textured soils might affect its initial development. Competition from grasses could also compromise its successful establishment and persistence. We investigated the effects of soils with contrasting textures (loamy sand vs. clay) with a high soil water content and the competition from *Lolium multiflorum* on the development of Lanza® in a pot experiment. Dry mass (DM) production of Lanza® was strongly reduced (86%) when grown with L. multiflorum. Lanza® shoot DM was 60% higher than the total shoot DM production of the mixture of Lanza® and *L. multiflorum*. Soil type did not significantly affect the shoot and total root DM. However, a 44% reduction of the DM of thin roots and slower development was observed in clay soils, which may indicate a preference for light-textured soils. This study provides further information on the factors limiting the establishment and persistence of Lanza®. Future research should confirm these results at field scale and investigate measures aimed at reducing early competition in monocultures and functional complementarity with partner species in mixtures to successfully establish Lanza®.

Keywords: competitive ability, establishment, mixtures, forage legumes, *Bituminaria bituminosa*.

INTRODUCTION

Mediterranean grasslands and their rich provision of ecosystem services are strongly associated with livestock grazing in extensive and semi-extensive systems, in which small ruminants and beef cattle graze rain-fed grasslands and croplands (Cosentino et al., 2014). These systems are challenged by scarce summer rainfall, droughts, and high inter-/intra-annual rainfall variability, resulting in feed gaps and irregular feed availability (Bell et al., 2016; Moore et al., 2009). This leads to poor animal performance, inefficient farm management, and often, insufficient revenues to maintain the enterprise (Bell et al., 2008; Moore et al., 2009). Climate change is expected to exacerbate this situation due to an overall reduction of mean annual rainfall, its increased inter-/intra- annual variability, and more frequent and severe droughts (Giannakopoulos et al., 2009; Giorgi and Lionello, 2008; Ma et al., 2017). Among the options to cope with these issues and reduce their effects on farming systems of the Mediterranean regions, it has been proposed the use drought-resistant legumes, especially hard-seeded annual legumes and perennial legumes (Hernández-Esteban et al., 2019; Moore et al., 2009; Real et al., 2014; Thomas et al., 2021), and their integration in ley-farming systems (Edwards et al., 2019).

The use of perennial legumes in Mediterranean extensive livestock systems is recognised as one of the most promising measures to improve the environmental and economic sustainability of farms (Moore et al., 2009). Research efforts have focused on the search for species that could meet the requirements of Mediterranean livestock systems (Moore et al., 2021; Rognli et al., 2021). One of the most promising species is *Bituminaria bituminosa* C.H. Stirton, especially *var. albomarginata* from the Canary Islands, where is known by the common name Tedera (Mendez et al., 1991). Previous research has demonstrated that this species is productive under very low rainfall (<200 mm), is highly drought-resistant (Foster et al., 2012; Foster et al., 2015; Martínez-Fernández et al., 2012), has good forage quality (Fernández-Habas et al., 2021; Ventura et al., 2009; Ventura et al., 1991), and it is suitable for feeding livestock (Finlayson et al., 2012; Méndez et al., 1990; Oldham et al., 2015; Oldham et al., 2013). Recently, an improved variety has been developed as a result of a collaborative breeding programme led by Australian and Spanish researchers (Real et al., 2014). This new variety is registered under the trade mark of Lanza® and protected by the Plant Breeder´s Right Act 1994 in Australia (Real, 2016). It was bred by the Department of Primary Industries and Regional Development (DPIRD) as part of an initiative by the Future Farm Industries Cooperative Research Centre. Seed from Lanza® became marketable in Australia in 2019. The breeding program combined attributes from elite plants of var. *crassiuscula* and var. *albomarginata* to seek ideotypes with drought and cold tolerance (Real et al., 2014).

Recently, Real (2022a) demonstrated that this species could be established using machinery commonly used for cereal establishment and provided information about fertilization requirement (Real et al. 2022b) and herbicide tolerance (Real et al 2022c).

There is still research to be done to clarify the potential adaptation of this species to the wide range of environmental conditions of the Mediterranean Basin (Finlayson et al., 2012). Two main factors potentially affecting the adaptation of *B. bituminosa* are the soil texture and the ability to deal with competition during the establishment phase. Previous research suggested that soil water saturation could be unfavourable for *B. bituminosa var.*

albomargina (Fernández-Habas et al., 2021) and that it is unlikely to persist in environments with cold wet winters (Raeside et al., 2012). Real et al. (2014) found *B. bituminosa* to be overall sensitive to waterlogging, although it showed high variability in waterlogging tolerance. One of the essential characteristics for new perennial legumes to ensure successful adaptation in Mediterranean environments is having a broad range of soil and climatic adaptations (Real et al., 2011). Also, the slow early vigour of this plant coupled with competition might compromise successful establishment in temporary or permanent Mediterranean grasslands, where annual grasses and forbs might outcompete *B. bituminosa*. Low competitiveness has been reported as a major limiting factor to establish perennial legumes (Bell et al., 2005; Häring et al., 2008; Hogg and Davis, 2009). Field observations of *B. bituminosa* plantations in Australia have reported failure due to competition by weeds (Raeside et al., 2012). The cost of establishment and unreliable production in variable environments are the main barriers hindering the adoption of pasture legumes (Hogg and Davis, 2009; Lewis, et al., 2012), and therefore its study is essential for the successful introduction of new forage legumes such as Lanza® (Thomas et al., 2021).

In light of the potential of *B. bituminosa* as a new forage legume to fulfil the needs of Mediterranean livestock systems, we studied the effects of soil texture and competition. This study aimed at investigating the development of cv. Lanza® in i) interspecific competition with *Lolium multiflorum* and ii) in two types of soils of contrasting texture (loamy sand and clay) in a pot experiment in controlled conditions.

We hypothesise that:

Hypothesis 1: The interspecific competition of Lanza® with *Lolium multiflorum* will reduce Lanza® development.

Hypothesis 2: Heavy-textured clay soils in conditions of high soil water content could limit the development of the root system of Lanza® due to its water retention capacity, and hence reduce the biomass production of this species.

MATERIAL AND METHODS

Plant material

Lanza® seeds were provided by the Department of Primary Industries and Regional Development (DPIRD), Australia. To achieve fast and effective germination, the seeds were mechanically scarified by nicking the outer seed coat with a surgical scalpel (Carruggio et al., 2020). After scarification, the seeds were germinated in flat trays with a wet filter paper (Foster et al., 2015; Pecetti et al., 2007). Once germinated, the seeds were planted in seedlings trays and grown for approximately 30 days. When the seedlings had three leaves, they were transplanted into six litre, free draining individual plastic cylindrical pots of 37 cm high, with a 16.8 cm and 12.5 cm diameter at the top and bottom of the pot, respectively. *L. multiflorum* var. Tamtbo seeds were provided by FERTIPRADO (http://www.fertiprado.pt/es/). *L. multiflorum* seeds were directly planted in seedlings trays until they produced three leaves when they were transplanted into the same pots described before.

Experimental design

The experiment design was a completely randomised factorial design with two factors, two treatments per factor and six replicates. The factors were: level of competition and soil texture. For competition, the treatments were single Lanza® plants grown alone in pots (Control) and single plants of Lanza® grown in mixture with two plants of *L. multiflorum* (Competition). The soil textures were loamy sand (S) and clay (C) (Table 1). S soil was collected at the experimental farm located at Hinojosa del Duque, Cordoba, Spain (38° 29′ 46″ N and 05° 06′ 55″ W). This area is characterised by shallow Eutric Cambisols devoted mainly to livestock grazing of permanent grasslands in rangelands of dehesa ecosystems (Reyna-Bowen et al., 2020). This soil was fertilised with 50 kg P_2O_5 ha^{-1} two years before collecting the soil. C soil was collected at the experimental farm of University of Cordoba in the Campus of Rabanales, Cordoba, Spain (37° 55′ 01″ N and 04° 42′ 57″ W). These soils are deep Vertisol from the Guadalquivir valley typically devoted to cereal crops (Campillo García et al.,1993). The agronomic characteristics of both soils are described in Table 1. Both soils were mixed at 1/5 v v^{-1} with soil collected from natural populations of *B. bituminosa* to promote *Rhizobium* inoculation (soil data correspond to the soil already mixed). The water retention curves of both soils were determined in the laboratory to obtain the saturated volumetric water content (θ_s) (Table 1). Three undisturbed samples were directly extracted from the three different pots per soil type using 100 cm^3 volume core rings. The sandbox apparatus (Sand / Kaolin box, Eijkelkamp, ZG Giesbeek, The Netherlands) was used to obtain five pressure head points between saturation and −63.1 cm (Eijkelkamp, 2019). The sand/kaolin box was used to obtain two pressure head points at -100 and -199.5 cm (Eijkelkamp, 2016). Pressure head points between $-1x10^3$ cm and $-1.5x10^6$ cm were determined using the WP4-T Dew Point Potentiometer (Decagon Devices, Inc., NE Hopkins Court Pullman WA, USA) (Decagon, 2007). The resulting points were fitted to the Van Genuchten (1980) equation (1) with the RETC software (https://www.pc-progress.com/en/Default.aspx?retc) (Van Genuchten et al., 1991).

$$\theta = \theta_r + \frac{(\theta_s - \theta_r)}{[1+|\alpha\psi|^n]^m} \quad (1)$$

Where θ is the volumetric water content ($cm^3\ cm^{-3}$), θ_s is the saturated volumetric water content ($cm^3\ cm^{-3}$), θ_r is the residual volumetric water content ($cm^3\ cm^{-3}$), ψ is the matric potential (-cm), α, *n* and *m* are fitting parameters that determine the shape of the curve, being $m = 1\text{-}1n^{-1}$.

The resulting water retention curves of both soils and the corresponding fitting parameters of Van Genuchten's model are shown in Figure S1.

Table 1. Soil analysis of the two soil types used in the experiment.

Parameters	Units	Loamy sand (S)	Clay (C)
Sand	%	73	29
Silt	%	13	25
Clay	%	14	46
Bulk density	$g\ cm^{-3}$	1.48	1.02
Soil pH in water (1 2.5)	U. pH	7.1	8.12
Cation exchange capacity	$Meq\ 100g^{-1}$	11	31
Electrical conductivity 25 ºC (1:5)	$mS\ cm^{-1}$	0.16	0.22
Oxidable Organic matter content	%	1.97	3.21
N (Kjeldahl)	%	0.11	0.19
Available P (Olsen)	$mg\ kg^{-1}$	44	7
Exchangeable K (NH4Cl)	$mg\ kg^{-1}$	138	319
Exchangeable Ca (NH4Cl)	$mg\ kg^{-1}$	1.12	4.91
Exchangeable Mg (NH4Cl)	$mg\ kg^{-1}$	114	371
Exchangeable Na (NH4Cl)	$mg\ kg^{-1}$	102	118
Saturated volumetric water content	$cm^3\ cm^{-3}$	0.33	0.62

During the experiment, the soil water content of the pots was maintained between θ_s and 70% of θ_s (on average) by weighing the pots and watering periodically (with a watering cadence that fluctuated from three days at the beginning of the experiment to every day at the end). The experiment was conducted from 8 March to 21 July 2021 (135 days) in a shade house at the experimental farm of the University of Cordoba in the Campus of Rabanales, Cordoba, Spain (37° 55′ 01″ N and 04° 42′ 57″ W). The net of the shade house had a nominal shading factor of 60%. The mean of the daily mean temperatures, mean of the daily maximum, and mean of the daily minimum temperature during the experiment were 20.6, 28.3, and 12.9 ºC respectively (Figure S 2).

Phenology

From day 65 (12 of May) to the end of the experiment on day 135 (21 July) the phenology stage of Lanza® in both soil types and with and without competition was recorded. The phenological stages were recorded following the general scale of the Biologische Bundesanstalt Bundessortenamt und Chemische Industrie (BBCH) (Feller et al., 1994). The phenology of *L. multiflorum* at key phenological stages (from inflorescence emergence to senescence) was also recorded. The phytovolume of Lanza® plants was estimated by calculating the cylinder volume using the average diameter of two perpendicular measurements and plant height. Phytovolume was calculated at day 65 (12 of May) and day 135 (21 July).

Dry mass, morphological traits, and leaf nutrients content

At the end of the experiment, plants were cut to ground level. The shoot biomass of Lanza® was separated into stems and leaves. Roots were separated from the soil using a 2-mm sieve and divided into thin (<2 mm) and thick roots (2> mm). Thin root separation was not possible in Competition treatment since the roots of Lanza® and *L. multiflorum* were strongly intertwined (Figure S 3). Therefore, in Competition treatment, only thick roots of Lanza® were separated since *L. multiflorum* did not produce roots of >2 mm. Biomass was oven-dried at 60ºC for 72h and then weighed. Leaf to stem ratio was measured as leaf dry mass/steam dry mass in Lanza® plants grown in both treatments. The mean leaf area for Lanza® plants was calculated by scanning the leaves (EPSON 1,640 XL, Epson America, Inc., Long Beach, CA) before drying and then measuring the leaf area using the image analyser software ImageJ (https://imagej.nih.gov/ij/). When the leaf biomass of a plant was higher than 1.5 g, a random representative sample of leaves was scanned. The sample was always at least 15% of the total leaves' dry mass. From the ratio between the leaf area and its dry mass, the specific leaf area (SLA) (cm^2 g^{-1}) was also calculated for Lanza®. Finally, for Lanza® plants grown without competition (Control) in both soil types, the proportion of thin roots was estimated as thin roots dry mass/total roots dry mass.

Samples of leaves of Lanza® plants of all treatments were ground and passed through a 1-mm sieve and then analysed for the following nutrients: *N*, *P*, *K*, *Ca*, *Mg* and *S*. The ratios of *C/N* and *N/P* were also calculated. Concentrations of *N*, *C*, and *S* were analysed at the Central Service for Research Support (SCAI) of the University of Cordoba using a LECO macro combustion instrument, model CNS928 (LECO Corporation, Lakeview Ave, MI, USA). The concentrations of *P*, *K*, *Mg* and *Ca* were analysed at the Soil Science Unit of the University of Cordoba. *K* was analysed with a flame photometer Jenway™ PFP7 (Cole-Parmer Ltd, Staffordshire, UK). *Ca* and *Mg* were measured with an Atomic Absorption Spectrometer Perkin Elmer AAnalyst 200 PerkinElmer, Inc, Waltham, MA, USA). *P* was analysed with a absorbance microplate reader BioTek PowerWave HT Microplate (BioTek® Instruments, Inc., Winooski, Vermont, USA).

Physiological measurements

Physiological measurements were taken the day before harvesting the plants, on day 134 (20 July). Net photosynthesis per area (A_{area}) and stomatal conductance per area (g_{Sarea}) were measured between 09:00–12:00 hr in fully expanded and undamaged leaves of mid-height using a portable infrared CO_2 gas analyser (LiCor Li6400XT, Li-Cor, Inc., Lincoln, NE, USA) fitted with a 2 cm^2 leaf cuvette. The settings of the gas analyser were: flow rate at 500 µmol $s,^{-1}$ PAR set at 1,500 µmol photon m^{-2} s^{-1}, [CO_2] to 350 ppm, and block temperature set at 25ºC. Water-use efficiency (WUE) was calculated as the ratio A_{area} $g_{Sarea}{}^{-1}$. After recording A_{area} and g_{Sarea}, six leaves per plant of the same characteristics were cut to estimate leaf relative water content (RWC). RWC was calculated as RWC = ((FW–DW)/(SFW–DW))×100, where FW is fresh leaf weight, DW is dry leaf weight and SFW is saturated fresh leaf weight. After weighing the leaves to obtain FW, were immersed them in deionised water in 90 mm Petri dishes. The leaves were weighed again after 24hr at 20–25 ºC to record SFW. DW was obtained after weighing the oven-dried leaves at 60ºC for 72h.

Statistical analysis

The shoot dry mass production, leaf:stem ratio, SLA, phytovolume, mean leaf area, thick roots dry mass and leaf nutrients content of Lanza® without competition (Control) and with competition (Competition) in both soil types (S and C) were tested by two-way ANOVAs. The effect of soil type and competition on the physiological measurement of Lanza® was also tested by two-way ANOVAs. Total root dry mass, thin root dry mass production and their proportion in the total root dry mass were further investigated for Lanza® in each soil type for Control plants only by one-way ANOVAs. The differences in shoot dry mass, root dry mass, and total dry mass production by pot between Competition (*L. multiflorum* plus Lanza®) and Lanza® grown alone (Control) were also tested by two-way ANOVAs. Variables were log-transformed when necessary to meet normality and homoscedasticity assumptions. When differences were significant (p <0.05), post-hoc Tukey's test at the 0.05 level was carried out to separate homogeneous groups. The differences in phenology between treatments were investigated by cumulative link mixed models (Christensen, 2019a). Cumulative link models, also known as ordinal regressions models (Agresti, 2003), can be used to test the effects on a response variable following an ordered finite set of categories. In this case, the different BBCH stages were considered as the response variable following an ordinal scale, and soil type, level of competition, and date of observation as predictor categorical variables to account for the main effects. The interactions of Soil type x Competition, Soil type x date of evaluation and Competition x date of evaluation were also tested. To account for dependence among observations on different dates over the same plant, the plant identity was considered as a random effect. Models were fitted using the package “ordinal” of R (Christensen, 2019b), following Christensen (2019a) and Mangiafico (2022). The significance of main effects and their interactions were tested by an analysis of deviance (ANODE) with the “Anova.clm” function from “RVAideMemoire” package (Hervé, 2022). All statistical analyses were performed using the software R v. 3.6.1 (R Development Core Team, 2019).

RESULTS

Effect of competition and soil type on the development of Lanza®

Figure 1 schematise the phenology of Lanza® in each treatment. In these conditions of culture in the shade house, none of the Lanza® plants reached reproductive stages (inflorescence emergence which corresponds to stage five in BBCH). Therefore, only the main growth stage corresponding to stem elongation was recorded (from 3.1, meaning that 10% of the final biomass has been reached, to 3.9, where 90% has been reached). All *L. multiflorum* plants reached the reproductive stage of seed ripening (stage 8). The phenology of Lanza® reported significant differences by soil type, level of competition and by the interaction of level of competition and date of evaluation (Table S 1). Lanza® plants reached more advanced phenological stages when grown in S soil compared to plants grown in C soil (df= 1; LR Chisr:15.15; *p* <0.001). The interaction of level of competition by date of evaluation (df= 18; LR Chisr:124.93; *p* <0.001) revealed that plants grown without the competition of *L. multiflorum* also reached higher penological stages and that their growth did not follow the same pattern over the time than the growth of plants under competition. The advance in the phenological stage 3 of Control plants plateaued from 15 July onwards on stage 3.39, while the advance of plants in competition seems to be subjected to the phenology of *L. multiflorum* (Figure 1).

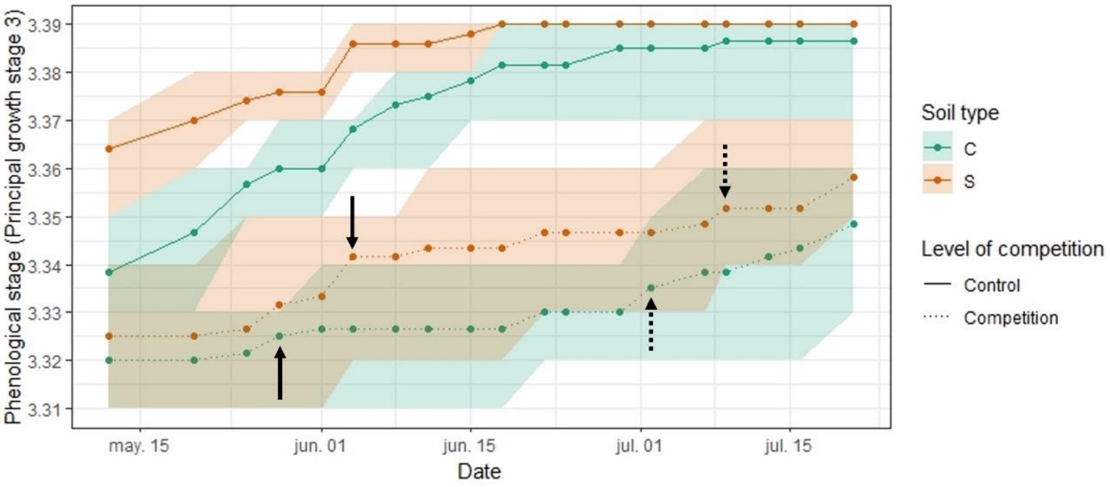

Figure 1. Phenology of Lanza® in each treatment from 12 May 2021 to 21 July 2021 according to the BBCH system. Shaded area indicates range of the phenological stage (from plant with the lowest phenological stage to the plant with the highest). Solid arrows indicate the date at which all *L. multiflorum* plants reached stage 5 (Inflorescence emergence) in each soil type. Dotted arrows indicate the date at which all *L. multiflorum* plants reached stage 8 (Ripening or maturity of fruit and seed) in each soil type.

This is in agreement with the results of the analysis of the phytovolume of Lanza®. Significant differences were found by level of competition (df=1; F= 44.04; $p<0.001$) and soil type (df=1; F=7.71; $p<0.05$) for the phytovolume measured at day 65. Control plants showed significantly higher phytovolume than their counterparts grown under competition (averaged values by level of competition: 9,160 ± 1,003 SE cm^3 in Control and 2,396 ± 607 SE cm^3 in Competition). Plants grown in S soil also had higher phytovolume than plants grown in C soil (averaged values by soil type: 7,193 ± 1,456 SE cm^3 in S and 4,362 ± 984 SE cm^3 in C). At harvesting time at day 135, the differences by level of competition were also significant (df=1; F= 86.04; $p<0.001$), but no significant differences were found by soil type (df=1; F=2.68; $p=0.117$). Figure 2 shows an example of plants at the two dates of measurement in each treatment.

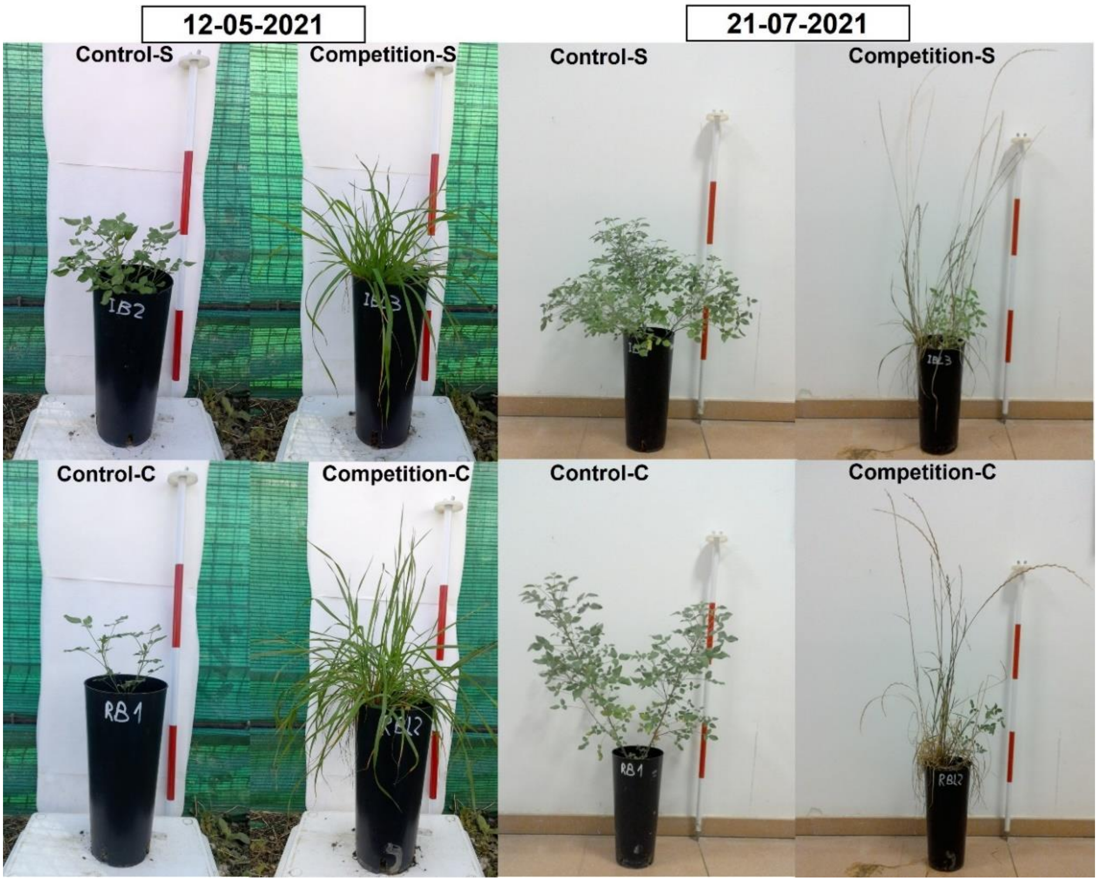

Figure 2. Example of plants of each treatment in two dates, 12 May 2021 and 21 July 2021. Control-S: Lanza® plants grown without competition in loamy sand soil, Control-C: Lanza® plants grown without competition in clay soil, Competition-S: Lanza® plants grown with competition of *L. multiflorum* in loamy sand soil, Competition-C: Lanza® plants grown with competition of *L. multiflorum* in clay soil. Red and white marks on scale bar are 20 cm intervals.

Effects of competition and soil type on dry mass and morphological traits

There was a significant effect (df=1; F=137.6; *p*<0.001) of Competition on shoot dry mass production of Lanza® (Figure 3 A). The shoot dry mass of Lanza® decreased an 86% when grown with competition of *L. multiflorum* (Figure 2 – Figure 3A). Although a slower development of Lanza® in clay soil was observed during the initial stages (Figure 1 and Figure 2), at the end of the experiment only a trend of lower shoot dry mass production was observed (df=1; F=3.97; p=0.060) (Figure 3 A - Table S2).

The leaf:stem ratio was not affected by soil type (Table S2). It significantly increased a 30% (df=1; F=14.46; *p*<0.001) in Lanza® plants grown in Competition with *L. multiflorum* (0.86 ± 0.3 SE) compared to Lanza® plants of Control (0.66 ± 0.3 SE). SLA was not affected by either soil type or competition (Table S2), showing a mean value of 170.3 $cm^2\ g^{-1}$ ± 6 $cm^2\ g^{-1}$. The mean leaf area of Lanza® plants grown in competition (2.6 cm^2 ± 0.3 SE) was a 28% significantly lower (df=1; F=9.53; *p*<0.01) than plants without the competition of *L. multiflorum* (3.6 ± 0.1 SE cm^2) with no significant differences by soil type (Table S2).

When the total shoot dry mass production by pot (*L. multiflorum* plus Lanza®), was compared to the shoot dry mass of Lanza® grown alone, significant differences were found by level of competition (df=1; F=16.78; *p*<0.001) while no differences were found by soil type (df=1; F=0.33; *p*=0.571). The average Lanza® shoot dry mass was 59.6% higher (29.2 ± 2.1 SE) than the average total shoot dry mass production of the mixture of Lanza® and *L. multiflorum* (18.3 ± 1.7 SE) (Figure 3 B). Lanza® contributed with 22.9% of the shoot dry mass of the mixed culture of Lanza® and *L. multiflorum*. There was a trend (df=1; F=4.85; *p*=0.052) to a lower proportion of Lanza® in the total shoot dry mass of the mixture when grown in C soil (15.1% ± 3.8 SE) compared to plants grown in S soil (30.9% ± 6.1 SE).

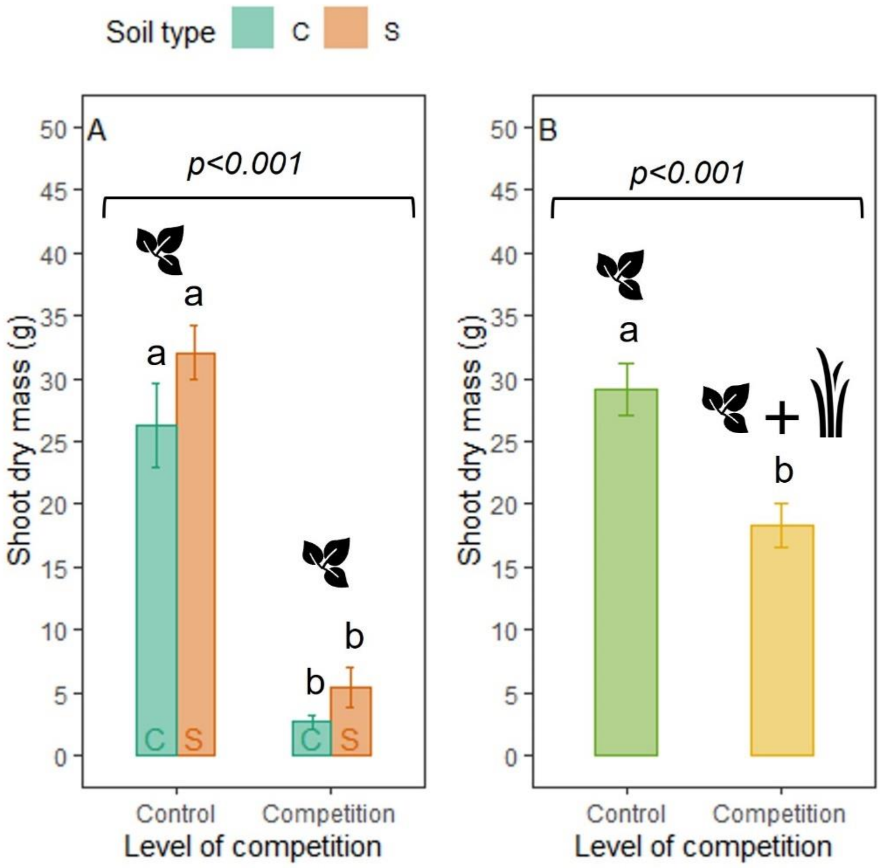

Figure 3. A: Mean shoot dry mass of Lanza® by soil type and level of competition. **B**: Mean shoot dry mass of Lanza® averaged by soil type grown without competition (Control) compared to total shoot dry mass in competition treatment (*L. multiflorum* plus Lanza®). p values indicating the main effect of Competition, the main effect of soil was not significant. Mean values (n = 6) not sharing a common letter differ significantly (p<0.05) according to Tukey's test. Error bars show standard errors.

Table 2 shows mean values of thick and thin roots dry mass and thin roots proportion of Lanza® plants grown in both soil types and level of competition. The dry mass of thick roots showed significant differences according to level of competition (df=1; F=91.22; p<0.001), being 90% lower in plants under competition (Figure S 3). No significant differences in thick roots dry mass were observed between plants grown in different soils (Table 2). However, significant differences by soil type were found for thin roots dry mass and their proportion of the total root dry mass when only control plants were analysed (Table S4). A significant decrease (df=1; F=5.57; p<0.05) of 44% in thin roots dry mass of plants grown in clay soil was observed compared to plants grown in sandy soil. Similarly, the proportion of thin roots in clay soil was also lower than in sandy soil (Table 2). See Figure S4 for an example of the root system of Lanza® in clay and sandy soil. As for the shoot dry mass, when the total root dry mass per pot was analysed (*L. multiflorum* plus Lanza®), there were significant differences by level of competition only (df=1; F=7.35; $p<0.05$). The average Lanza® root dry mass was 13.9 ± 1.5 SE, a 70% higher than the average total root dry mass production of the mixture of Lanza® and *L. multiflorum* (8.2 ± 1.6 SE). All Lanza® plants presented nodules of *Rhizobium* at the moment of harvesting (after 135 days of culture).

Table 2. Effect of level of competition and soil type (sandy and clay) on thick roots, thin roots and thin roots proportion of Lanza®.

Level of competition	Soil type	Thick roots dry mass (g)	Thin roots dry mass (g)	Thin roots proportion (g)
Control	**S**	7.1 ± 0.9 a	9.0 ± 1.5 a	0.55 ± 0.04 a
	C	6.8 ± 1.1 a	5.0 ± 0.8 b	0.42 ± 0.01 b
Competition	**S**	1.0 ± 0.3 b	-	-
	C	0.4 ± 0.1 b	-	-

Note: Mean values (n = 6) not sharing a common letter differ significantly (p <0.05) according to Tukey's test. Standard error is showed. S: Pots with sandy soil; C: Pots with clay soil. Thin root separation was not possible in Competition.

Leaf nutrients content

Lanza® plants under competition had higher N content (df=1; F=7.44; $p<0.05$) than Control plants with 2.20% ± 0.08 SE and 2.72% ± 0.18 SE respectively, while the soil type did not affect N concentration (Table 3). This resulted in a lower $C\ N^{-1}$ ratio in plants under competition (16.90 % ± 0.97 SE) compared to Control plants (20.1 % ± 0.59 SE). P was affected by soil type (df=1; F=66.20; p=<0.001), being higher in plants grown in S (0.37% ± 0.03 SE) soil than in C soil (0.13% ± 0.01 SE). The $N\ P^{-1}$ ratio was lower in plants grown in S soil, as affected by the higher P concentration of these plants. No significant differences were found for K and Ca by level of competition or soil type (Table S5). Mg content showed a significant interaction (df=1; F=10.49; p=<0.01) by soil type and level of competition (Table 3). Control plants grown in C soil had lower Mg content than Control plants grown in S soil and plants under competition in C soil while no differences were found in plants grown in S soil with or without competition. Soil type (df=1; F=4.98; p=<0.05) and competition (df=1; F=18.94; p=<0.001) had a significant effect on the content of S, being higher in plants under competition and in C soil (Table 3).

Table 3. Leaf content (%) of macronutrients, C N^{-1} and N P^{-1} ratios of Lanza® plants

Level of competition	Soil type	*N*	*P*	*C N^{-1}*	*N P^{-1}*
Control	**S**	2.10±0.07 b	0.42±0.03 a	20.7±0.67 a	5.16±0.45 b
Control	**C**	2.30±0.14 b	0.12±0.02 b	19.4±0.95 a	21.4±2.58 a
Competition	**S**	2.58±0.16 a	0.33±0.06 a	17.5±0.97 b	9.03±1.46 b
Competition	**C**	2.89±0.37 a	0.13±0.01 b	16.1±1.86 b	22.8±2.36 a
Level of competition	**Soil type**	***K***	***Ca***	***Mg***	***S***
Control	**S**	2.69±0.22 n.s.	1.29±0.09 n.s.	0.85±0.04 a	0.24±0.01 d
Control	**C**	2.16±0.10 n.s.	1.24±0.17 n.s.	0.52±0.08 b	0.27±0.01 c
Competition	**S**	2.63±0.18 n.s.	1.23±0.13 n.s.	0.68±0.05 ab	0.30±0.02 b
Competition	**C**	2.60±0.18 n.s.	1.19±0.14 n.s.	0.78±0.08 a	0.32±0.01 a

Note: Mean values (n = 6) not sharing a common letter differ significantly (p <0.05) according to Tukey's test. Standard error is showed. S: Pots with sandy soil; C: Pots with clay soil

Physiological response of Lanza® to soil type and competition

There was a significant interaction between soil type and competition in net photosynthesis per area (A_{area}) (df=1; F=4.3; p=0.05) and stomatal conductance per area (g_{Sarea}) (df=1; F=5.4; p<0.05). Plants grown under competition in sandy soils did not differ in A_{area} values from the rest of the treatments (Figure 4 A). However, plants under competition in clay soils showed significantly higher A_{area} than plants in pure culture (in clay and sandy soils). For g_{Sarea}, plants grown in competition in both soil types showed higher g_{Sarea} values than plants in pure culture in clay soils (Figure 4 B). Plants grown under competition in clay soil showed the highest g_{Sarea} values (0.35 mol H_2O m^{-2} s^{-1} ± 0.02 SE), significantly higher than g_{Sarea} of plants without competition in sandy soil also. These interactions were not detected for water use efficiency (WUE) (Table S 3). WUE was affected by competition only, showing a significant decrease (df=1; F=18.9; p<0.001) of 18% in Lanza® plants grown with *L. multiflorum* competition compared to those grown in pure culture (Figure 4 C). The leaf relative water content (RWC) was not affected by either soil type or competition (Table S3) with a mean value of 85.6% ± 0.4 SE.

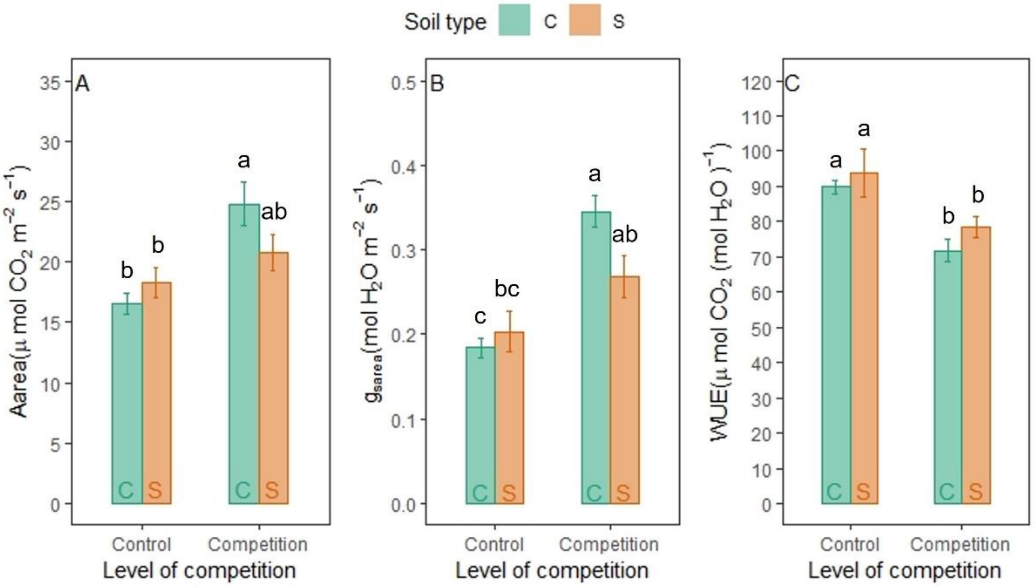

Figure 4. Effect of soil type and competition on net photosynthesis per area (A_{area}) (**A**), stomatal conductance per area (g_{Sarea}) (**B**) and water use efficiency (WUE) (**C**) of Lanza® measured at the end of the experiment. Mean values (n = 6) not sharing a common letter differ significantly (*p* <0.05) according to Tukey's test. Error bars show standard errors.

DISCUSSION

The results previously reported are discussed and interpreted, providing comments on the effects associated with short-term pot experiments, the need for field-scale trials, and identification of research gaps for future studies.

Effect of competition

The first hypothesis, *"The interspecific competition of Lanza® with Lolium multiflorum will reduce Lanza® development"* was confirmed. The results reported in this study showed that Lanza® had low competitive ability. Competition with *L. multiflorum* had a strong effect on the dry mass production of Lanza®. Therefore, this species could not be suitable for use in mixtures with grasses such as *L. multiflorum*, since its production is greatly reduced, and the mixture is less productive than the pure culture of Lanza®. Bell et al., (2012) also reported legume biomass reductions of 80-98% when co-sown with forage cereals, being this reduction more severe in perennial legumes (*Hedysarum coronarium* L. and *Medicago sativa* L.).

The higher A_{area} of plants under competition did not compensate for their also higher g_{Sarea}, leading to a lower WUE (Figure 2) at the same RWC (Table S3), which is in line with the lower biomass production of plants under competition. Thus, the establishment, production, and persistence of Lanza® can be challenged when competing with annual grasses. This might be a major limitation for this species to be introduced in Mediterranean permanent grasslands where annual grasses are abundant (Olea and San Miguel, 2006). It also highlights the importance of avoiding competition in the initial phases of development to achieve a proper establishment in temporary grasslands. The analysis of leaf nutrients content suggests that competition for nutrients was not the reason limiting the development of Lanza® since the only significant differences were the higher *N* and *S* content of plants under competition. K, P and S content was similar to values reported in previous studies for Lanza® (Real et al., 2022b). The higher *N* and *S* content could be related to a dilution effect due to the higher biomass of Control plants. The higher N content of plants in competition can explain the larger A_{area} of plants under competition due to the known relationship between photosynthesis and nitrogen content in leaves (Evans, 1989). Water limitation was neither the reason for competition since soil content water was maintained high. Therefore, the causes for the strong suppression of Lanza® when grown with *L. multiflorum* might be competition for light (shoot competition) and/or competition for space belowground (root competition) (Kiær et al., 2013). Likely, the fibrous and more vigorous root system of *L. multiflorum* might impose strong competition for space belowground with Lanza®. However, the specific effects of root and shoot competition over Lanza® are out of the scope of this study and should be addressed in future research with a specific experimental design for such purpose (Kiær et al., 2013).

Further research is needed to confirm if these results apply to field conditions, and in combination with species of less aggressive development than *L. multiflorum.* For example, it has been demonstrated that the competition effect on herbaceous plants is dependent on the identity of its neighbours, being least suppressed by their frequent neighbours (Lüscher et al., 1992; Semchenko et al., 2013). Also, the sowing density and spatial aggregation are expected to affect competitive ability, for example, Semchenko et al., (2013) showed that weaker competitors tend to form aggregated spatial distribution patterns as a possible strategy to aid their persistence in the community. Multi-species mixtures including Lanza® could increase the ecosystem resistance and resilience to drought, however the effect of interspecific competition must be minimised by targeting at functional complementarity among species (Malisch et al., 2017; Volaire et al., 2014). Malisch et al., (2017) demonstrated that the proportion and persistence of *Onobrychis* *viciifolia* (a forage legume of low competitive ability) in mixtures can improve trough the selection of suitable partner species. Although our experiment clearly proves the low competitive ability of Lanza® with *L. multiflorum*, the above-commented aspect of competitive ability needs to be further investigated in long-term field-scale trials testing different partner species and sowing densities. The competition effect might be higher in field conditions as root and shoot competi-tion tend to be underestimated in pot experiments (Kiær et al., 2013).

These results open a gap for future research on the options to reduce early competition of grasses and forbs to successfully establish Lanza® in monocultures. The overall main alternatives could be i) crop rotations to reduce weed burden, ii) leaving the fields fallow the season previous to sowing to avoid seeds production of forbs and grasses, iii) strategic grazing management , and iv) use of herbicides. Recently, Real et al., (2022c) have provided options for herbicide package for weed control in Lanza®. They found that propyzamide and carbetamide as pre- or post-emergent applications and butroxydim, clethodim, and haloxyfop for post-emergent applications can be recommended to control grasses such as annual ryegrass (*Lolium rigidum*) (Real et al., 2022c). Since Mediterranean permanent grasslands have been labelled as a biodiversity hotspot (Myers et al., 2000), are commonly associated with High Natural Value (HNV) farming systems such as dehesas and montados from the Iberian Peninsula (Plieninger et al., 2021) and are greatly valued for their plant diversity, the use of herbicides could be inappropriate. Therefore, the feasible alternatives point in the direction of the other options or combinations of them. For example, some experiences in Australia have shown that sheep without previous knowledge of *B. bituminosa* avoided this species during the first days of grazing (Real, 2021, personal communication), which may be used to relieve Lanza® from competition. However, this technique could be of difficult practical implementation by farmers, as the beneficial effect of relieve from competition against grazing damage depend on careful grazing management. The sowing time might also play an important role on the competitive ability of Lanza® with weeds. Real, 2022a reported that Lanza® performs best when sown just before or early after the start of the rainy season which might have also a beneficial effect due to a lower competition by grasses and forbs. The option of leaving the fields fallow the season before sowing might be of limited effectiveness in Mediterranean grasslands due to their rich seed bank (Lavorel et al., 1999; Levassor et al, 1990). Therefore, this management option may be more effective if combined with early sowing to favour asynchrony with competing species and to allow Lanza® to develop before winter, when it is less vigorous than grasses.

The high phenological asynchrony between *L. multiflorum* and Lanza® together with the high drought resistance of Lanza® (Foster et al., 2015) might have an interacting effect on the performance of Lanza® under competition. Since *L. multiflorum* shows earlier senescence than Lanza® due to their contrasting strategies to face summer drought (dehydration escape vs avoidance) (see Volaire, 2018), Lanza® could take advantage of the time after *L. multiflorum* senescence to develop without the competition of *L. multiflorum*. This will greatly depend on the previous successful establishment, development, and the biomass produced until this moment which could determine the ability to respond to the cease of competition. It will also depend on the soil water content, although *B. bituminosa* has the advantage of a deep root system and high drought resistance (Foster et al., 2015), being able to maintain shoot biomass under drought (especially var. *albomarginata*) (Martínez-Fernández et al., 2012). In this context, late spring rainfall, after senescence of grasses, could also benefit Lanza® allowing a greater after-competition development. This was supported by the increase in the development in stage 3 of phenology of Lanza® plants under competition (especially in soil C) after maturity of fruit of *L. multiflorum* shown (Figure 2). This study investigated the effect of competition and soil type at high soil water content (between θ_s and 70% of θ_s). However, interspecific competition can vary along environmental gradients (Volaire et al, 2014), therefore studies incorporating water shortage and its possible interaction with the competitive ability of Lanza® are necessary to fully understand the response of Lanza® to competition in different scenarios. Especially during the establishment phase, Lanza® could show higher competitive ability under drought conditions due to its drought resistance even at the seedling stage (Foster et al., 2012). Future research should also investigate the competitive ability of Lanza® plants of different ages, in order to clarify if competition by annual grasses has a limiting effect on Lanza® culture after the first year of establishment.

It is worth mentioning that the shade created by the shade house (nominal shading factor of 60%) where the plants were grown might have affected the phenology of Lanza®, probably delaying the flowering of this species. This effect could be also caused by the shade of trees canopy in agroforestry systems such as dehesas and montados where it could negatively affect the persistence of Lanza®, in fact, the absence of shade-tolerant legumes for agroforestry systems has been pointed out by Hernández-Esteban et al., (2019).

Effect of soil type

The successful inoculation with *Rhizobium* proves that Lanza® roots can be inoculated by *Rhizobium* from natural populations of the Iberian Peninsula. This is crucial to ensure the successful establishment, development, and ecosystem services provision by Lanza® as a new forage for Mediterranean livestock systems.

The second hypothesis, *"Heavy-textured clay soils in conditions of high soil water content could limit the development of the root system of Lanza® due to its water retention capacity, and hence reduce the biomass production of this species"* was rejected. Lanza® established successfully with similar root and aerial dry mass production at harvesting time in both soils with soil water content close to saturation. This proves that *B. bituminosa* is well adapted to a wide range of environmental conditions. In fact, the use of the *crassiuscula* and *albomarginata* varieties to obtain Lanza® could have provided this new variety with a wider aptitude for different soils (Real et al., 2014). However, although it did not translate into reduced shoot dry mass production at harvesting time, the soil type did affect the dry mass and proportion of thin roots (< 2mm), being lower in plants grown in clay soil with soil water content close to saturation (Table 2), which also presented a slower development compared to plants grown in loamy sand soil. Oxygen depletion in heavy clay soil with slow drainage is a well-known phenomenon (Ben-Noah and Friedman, 2016; Drew and Lynch, 1980; Friedman and Naftaliev, 2012) which might induce deficient root function and thin root dry mass reduction (Bhattarai et al., 2004; Irving et al., 2007). C has two-fold the water content of S soil at saturation (Table 1). Fernandez-Habas et al., (2021) pointed out that roots system development of *B. bituminosa var albomarginata* could be negatively affected by high soil water content in soils with elevated soil water retention capacity such as clay/silty soils, which could explain the reduction in thin roots dry mass of plants grown in clay soil. Differences in soil *P* content could also affect roots biomass (Haling et al., 2016; Hill et al., 2006;). Previous studies have reported a positive response of *P* addition to root and shoot growth in *B. bituminosa* reaching a maximum of shoot and root dry mass and root length to an optimum concentration of 12 µg *P* g^{-1} soil followed by a plateau with higher *P* concentrations, although they did not report the extractable *P* of the tested concentration (Pang et al., 2010a; Pang et al., 2010b). Real et al, (2022b) also reported Lanza® reaching peak of productivity in soils at 3-19 mg kg^{-1} *P* Colwell. However, these studies do not analyse the distribution of biomass between fine and thick roots. In this study, C soil had 7 mg kg^{-1} *P* Olsen, which is within the range of *P* Colwell values and *P* concentrations (Edmeades et al., 2006; Moody et al., 2013; Saggar et al., 1999) indicated by these authors at which maximum root biomass and peak of productivity can be achieved. The differences in leaf nutrients in *P*, *Mg* and *S* were in relation to the differences in concentration of these nutrients in both soils. The *P*, *K* and *S* leaf content was above the critical shoot nutrient concentration at which Lanza® reached the 90% of peak biomass in Real et al., (2022b) in both soils with no symptoms of deficiency or toxicity for these and the rest of the nutrients analysed.

This result might indicate preference and suitable adaptation of Lanza® for light sandy soils. This is in agreement with the distribution of *B. bituminosa* in the Canary Islands, where the genetic material to produce Lanza® was collected (Mendez et al., 1991; Real et al., 2014). Mendez et al., (2006) studied the agronomic traits and soils of nineteen populations of *B. bituminosa* from the Canary Islands and the Iberian Peninsula. They found that soils from most of the populations (14 out of 19) were soils with more than 50% of sand with just one population in clay soils (Mendez et al., 2006). The fact that *B. bituminosa* is usually found in poor sandy and rocky soils might be related to its adaptation to this soil type, but also due to a lower competition with plant species that might outcompete *B. bituminosa* in other soil types. In addition to its application as a new forage legume in Mediterranean climates, *B. bituminosa* could also be used for the revegetation and improvement of disturbed and poor soils (low competition environments) where it could increase the fertility of the soil by fixing atmospheric N_2. Previous studies have demonstrated tolerance of *B. bituminosa* to heavy metals (Martínez-Fernández et al., 2011; Walker et al., 2007) and suggested its use as suitable species in the initial stages of revegetation of non-saline soils with pH between 6,5-8,0 and moderate levels of heavy metals contamination (Martínez-Fernández, 2012).

CONCLUSION

This study investigated the effects of contrasting texture soils (loamy sand vs clay) at high soil water and the competition from *Lolium multiflorum* on the development of Lanza®. As expected, Lanza® showed low competitive ability and strong dry mass reduction when grown with *L. multiflorum*. Lanza® developed in both soils, clay, and loamy sand at high soil water content, with no significant effect on shoot and total root dry mass production. However, the lower thin roots dry mass production in clay soil might denote preference and suitable adaptation of Lanza® for light sandy soils. This low competitive ability with annual grasses might compromise the successful establishment and persistence of this novel perennial legume in Mediterranean grasslands. Further research should investigate measures aimed at reducing early competition from grasses to establish Lanza® as monoculture. Functional complementarity among species must be considered and further explored to avoid interspecific competition when designing multi-species mixtures including Lanza®.

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Supplementary material

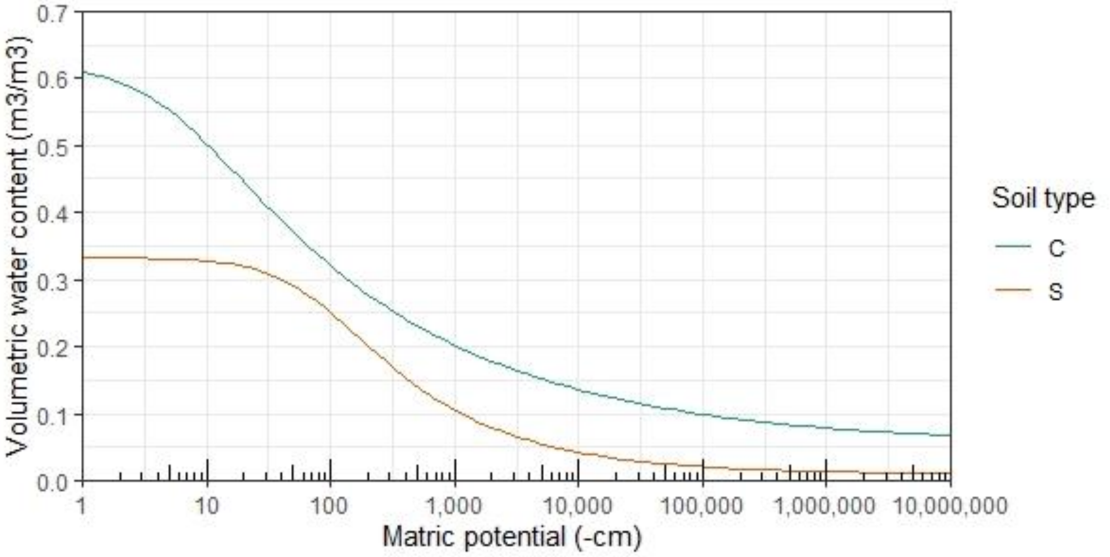

Figure S 1. Water retention curves for loamy sand (S) and clay (C) soils adjusted to Van Genuchten equation. Van Genuchten parameters for S soil: θs= 0.33, θr=0.01, α=0 .013, n=1.47. Van Genuchten parameters for C soil: θs= 0.62, θr=0.05, α=0 .186, n=1.26

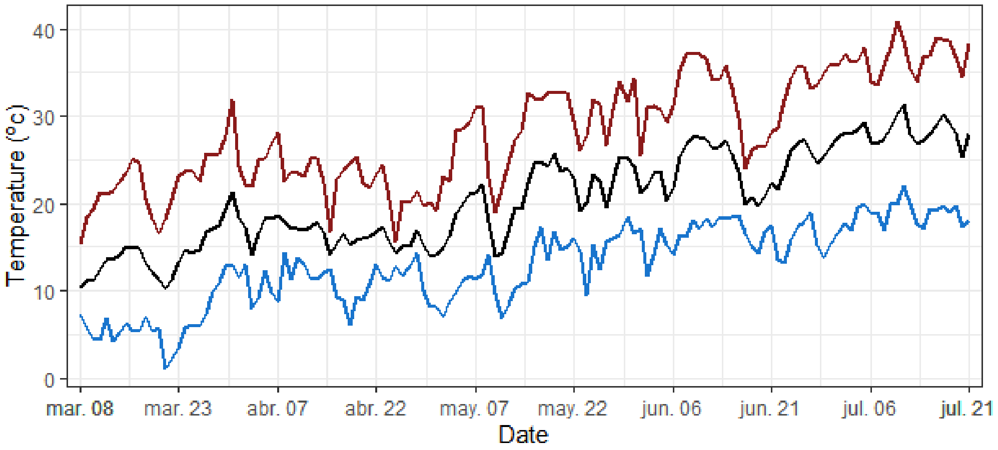

Figure S 2. Daily mean (black), maximum (red) and minimum (blue) temperatures during the experiment.

Figure S 3. Example of roots of Lanza® and L. multiflorum grown in the same pot (Competition).

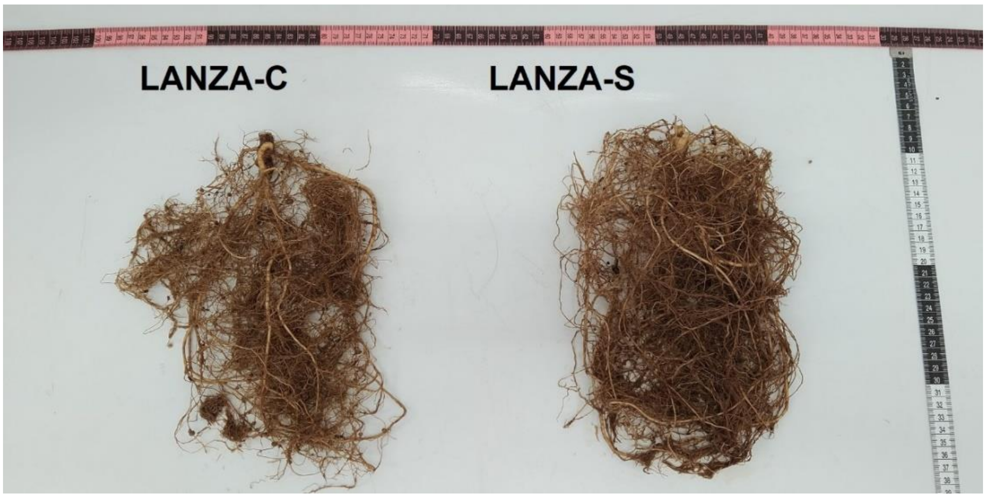

Figure S 4. Example of roots after separation from soil in two plants of Lanza® grown in clay soil (left) and grown in loamy sand soil (right).

Table S 1. Cumulative link mixed model fitted for phenology analysis.

Term	LR Chisq	d.f.	p
Competition (Comp)	45.89	1	<0.01
Soil type (Soil)	15.15	1	<0.001
Date	595.39	18	<0.001
Comp x Soil	3.67	1	0.055
Comp x Date	124.93	18	<0.001
Soil x Date	26.06	18	0.099

d.f. = degrees of freedom, LR Chisq = likelihood ratio chi square. Level of significance $p = 0.05$.

Table S 2. Summary results of the two-way ANOVA evaluating the effects of Competition, soil type and their interaction on shoot mass production and leaf to stem ratio of Lanza®.

Variable	**Source of variation**	**d.f.**	**SS**	**F**	***p***
Phytovolume 12 May	Competition (Comp)	1	$2.4\text{x}10^8$	44.04	<0.001
	Soil type (Soil)	1	$4.8\text{x}10^7$	7.71	<0.05
	Comp x Soil	1	$8.7\text{x}10^6$	1.39	0.253
	Error	20	$1.2\text{x}10^8$		
Phytovolume 21 July*	**Competition (Comp)**	1	5.83	86.04	<0.001
	Soil type (Soil)	1	0.18	2.677	0.117
	Comp x Soil	1	0.06	0.910	0.351
	Error	20	1.36		
Shoot dry mass	**Competition (Comp)**	1	4093.0	137.56	<0.001
	Soil type (Soil)	1	109.24	3.97	0.060
	Comp x Soil	1	13.68	0.50	0.489
	Error	20	550.09		
Leaf:Stem ratio	**Competition (Comp)**	1	0.23	14.46	<0.01
	Soil type (Soil)	1	0.05	3.20	0.089
	Comp x Soil	1	0.06	3.58	0.073
	Error	20	0.32		
SLA	**Competition (Comp)**	1	1107.85	1.18	0.291
	Soil type (Soil)	1	117.15	0.12	0.728
	Comp x Soil	1	73.36	0.08	0.783
	Error	20	18806.89		
Mean leaf area	**Competition (Comp)**	1	5.98	9.53	<0.01
	Soil type (Soil)	1	0.14	0.22	0.641
	Comp x Soil	1	1.09	1.74	0.202
	Error	20	12.56		
Thick roots*	**Competition (Comp)**	1	38.65	91.22	<0.001
	Soil type (Soil)	1	1.12	2.65	0.119
	Comp x Soil	1	0.71	1.68	0.210
	Error	20	8.47		

d.f. = degrees of freedom, SS= sum of squares, MS= Mean of squares. Level of significance p = 0.05. * log-transformed variables.

Table S 3. Summary results of the two-way ANOVA evaluating the effect of soil type and Competition on net photosynthesis per area (A_{area}), stomatal conductance per area (g_{Sarea}), water use efficiency (WUE), specific leaf area (SLA) and mean leaf area of LANZA measured at the end of the experiment

Variable	Source of variation	d.f.	SS	F	*p*
A_{area}	**Competition (Comp)**	1	173.44	14.81	0.001
	Soil type (Soil)	1	7.79	0.67	0.424
	Comp x Soil	1	50.77	4.34	0.050
	Error	20	234.19		
g_{Sarea}	**Competition (Comp)**	1	0.08	29.84	<0.001
	Soil type (Soil)	1	0.01	2.00	0.173
	Comp x Soil	1	0.01	5.43	<0.050
	Error	20	0.05		
WUE*	**Competition (Comp)**	1	0.04	18.91	<0.001
	Soil type (Soil)	1	0.00	1.74	0.202
	Comp x Soil	1	0.00	0.39	0.540
	Error	20	0.05		
RWC	**Competition (Comp)**	1	3.43	0.67	0.423
	Soil type (Soil)	1	2.20	0.43	0.520
	Comp x Soil	1	9.31	1.81	0.193
	Error	20	102.57		

d.f. = degrees of freedom, SS= sum of squares. Level of significance *p* = 0.05. * log-transformed variables.

Table S 4. Summary results of the one-way ANOVA evaluating the effect of soil type on thin roots, thick roots and thin roots proportion of Lanza®.

Variable	Source of variation	d.f.	SS	F	*p*
Thin roots dry mass	**Soil type**	1	48.26	5.57	**<0.05**
	Error	10	86.58		
Thick roots dry mass	**Soil type**	1	0.13	0.02	0.884
	Error	10	56.95		
Thin roots proportion	**Soil type**	1	0.05	8.78	**<0.05**
	Error	10	0.06		

d.f. = degrees of freedom, SS= sum of squares. Level of significance *p* = 0.05.

Table S 5. Summary results of the two-way ANOVA evaluating the effect of soil type and Competition on leaf content of macronutrients and C N^{-1} and N P^{-1} ratios.

Variable	Source of variation	d.f.	SS	F	*p*
N	**Competition (Comp)**	1	1.63	7.44	**<0.05**
	Soil type (Soil)	1	0.38	1.75	0.202
	Comp x Soil	1	0.02	0.09	0.077
	Error	19	4.16		
P*	**Competition (Comp)**	1	0.009	0.430	0.519
	Soil type (Soil)	1	1.301	66.197	**<0.001**
	Comp x Soil	1	0.061	3.104	0.093
	Error	20	0.393		
C N^{-1}	**Competition (Comp)**	1	61.635	8.536	**<0.01**
	Soil type (Soil)	1	11.014	1.525	0.232
	Comp x Soil	1	0.008	0.001	0.974
	Error	19	137.199		
N P^{-1}	**Competition (Comp)**	1	41.65	2.071	0.166
	Soil type (Soil)	1	1307.58	65.033	**<0.001**
	Comp x Soil	1	8.77	0.436	0.517
	Error	19	382.02		
K	**Competition (Comp)**	1	0.228	1.245	0.278
	Soil type (Soil)	1	0.462	2.528	0.128
	Comp x Soil	1	0.371	2.029	0.170
	Error	20	3.659		
Ca	**Competition (Comp)**	1	0.0177	0.162	0.691
	Soil type (Soil)	1	0.0096	0.087	0.770
	Comp x Soil	1	0.0003	0.003	0.959
	Error	20	2.1856		
Mg**	**Competition (Comp)**	1	0.017	0.358	0.557
	Soil type (Soil)	1	0.075	1.609	0.220
	Comp x Soil	1	0.512	11.062	**<0.01**
	Error	19	0.880		
S	**Competition (Comp)**	1	0.0180	18.935	**<0.001**
	Soil type (Soil)	1	0.005	4.979	**<0.05**
	Comp x Soil	1	0.0001	0.109	0.745
	Error	19	0.0181		

d.f. = degrees of freedom, SS= sum of squares. Level of significance *p* = 0.05. * log-transformed variables, **power-transformed variables.

CHAPTER V. Yield, forage quality and phenology of the new forage perennial legume *Bituminaria bituminosa* var. *albomarginata* cv. Lanza® in response to rainfall reduction and competition

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Abstract

The use of drought-resistant perennial legumes is a strategic alternative to lessen the vulnerability of Mediterranean extensive livestock systems to the projected reduction of annual rainfall under climate change scenarios. *Bituminaria bituminosa* is one of the most promising species. This study aimed at testing the response of *Bituminaria bituminosa* cv Lanza® (tedera) to 33% rainfall reduction and competition in terms of yield, forage quality, and phenology. A field experiment was carried out form February 2021 to July 2022 in Cordoba, south Spain. Tedera and alfalfa were grown in monoculture and under competition from annual grasses and forbs with two treatments of rainfall, control and 33% reduced by rainout shelters. Tedera established in monoculture in late winter was as productive as alfalfa in rainfed conditions in the first year (2,700 kg ha^{-1}). During this first year, late spring and summer rainfall allowed tedera to maintain green leaves over the summer season, while alfalfa shed leaves. In the second year, suitable distribution of rainfall until April and a mild winter allowed a high production of tedera (9,000 kg ha^{-1}). Annual rainfall reduction did not affect tedera but reduced alfalfa DM yield by 52% (2,215 kg ha^{-1}in control vs 1,074 kg ha^{-1} under 33% reduced rainfall). During this second year, tedera did not maintain green leaves over the summer, maybe due to high biomass accumulation and drier conditions in May-July. Competition had a strong effect on both species, especially in tedera, leading to failure to be productive (DM yield<250 kg ha^{-1}). Tedera shows suitable forage quality as an out-of-season forage, especially in the leaf fraction (CP>13%). However, uncut two-years old tedera developed thick and lignified stems that together with leaf shedding caused a great decline in shoot forage quality in the second year. Forage quality was more affected by competition than by rainfall reduction, which had little or no impact. Tedera shows early phenology for the reproductive stages from inflorescence emergence to ripening, with long flowering (from early April to mid-May) and important overlaps between flowering, fruit development and ripening. Competition caused a delay of flowering in tedera but not in alfalfa, and both species showed a delayed start of fruit development and maturity in plants under competition. Rainfall reduction only affected tedera, by inducing a more advanced inflorescence emergence and flowering (~10 days), whereas alfalfa showed no differences. In the conditions of this study, tedera showed higher probabilities of reaching reproductive stages than alfalfa, and for both species, competition reduces the probability to reach such stages. Tedera is resistant to a reduction of 33% of annual rainfall, although seasonality of rainfall seems to influence leaf shedding in the two years of experiment, and thus its ability to provide green forage over the summer season. Further research is needed to test the response of tedera to years of contrasting seasonal meteorology in different locations and under different managements to define optimal management strategies to fulfil its potential role as an out-of-season perennial legume in farming systems of the Mediterranean basin.

Keywords: Tedera, Mediterranean systems, dehesas, flowering, climate change

INTRODUCTION

Extensive livestock systems in Mediterranean climates have been the main livelihood for human societies for centuries. Nowadays, these systems are recognised not only for their importance to provide animal products but also as a key element of the landscape to provide multiple ecosystem services of essential importance to human well-being such as biodiversity, wildfire prevention and sustaining of rural population (Porqueddu et al., 2016, 2017; Varela and Robles-Cruz, 2016). In Mediterranean climates, extensive livestock systems rely on rainfed grassland production in combination with browse and fruit production from trees and shrubs, and crop residues to feed livestock in heterogeneous landscapes (Porqueddu et al., 2017; Jouven et al., 2010). These systems are constrained by the characteristics of the Mediterranean climate with low annual rainfall, high seasonality and high temperatures in the summer season. The result is a large variability in the inter- and intra- annual feed offer for livestock in quantity and quality, especially from grasslands (Laidlaw et al., 2006; Moore, Bell, and Revell, 2009). In some areas like the Iberian Peninsula, these grazing systems have shaped the landscape to create ecosystems such as the emblematic open woodlands known as dehesas in Spain and *Montado* in Portugal (Joffre et al., 1988; Moreno and Pulido, 2008; Olea and Miguel-Ayanz, 2006; Pinto-Correia et al., 2011). These ecosystems combine the production of natural grasslands with the provision of browse, acorns, cork and the diversifying and stabilising effect of the tree canopy on pasture production (López-Carrasco et al., 2015). Transhumance has also been traditionally associated with extensive livestock systems in Mediterranean climates to cope with seasonal grassland production (Ruiz and Ruiz, 1986).

However, technological and socioeconomic changes during the second half of the last century have led these systems, in most cases, to change from nomadic to sedentary systems (Joffre et al., 1988; Ruiz and Ruiz, 1986). This change has forced the dehesa farms to become more dependent on external feed resources to feed livestock (Joffre et al., 1988; Moreno and Pulido, 2008). This has important implications for the sustainability of farms at all three components of sustainability, mainly due to a decoupling of production and local resources (Garrett et al., 2020). The recent Ukrainian war has demonstrated the risk of relying on imported resources to feed livestock (Bentley et al., 2022; Galanakis, 2023; Pörtner et al., 2022). In the case of Spain, in the last year, the price of concentrates has increased by over 40% (MAPA, 2023). In addition, climate change is already impacting Mediterranean farming systems through enhanced aridity, more pronounced seasonality and more frequent extreme climatic events such as heat waves and droughts (Cramer et al., 2020; EEA, 2017; Giannakopoulos et al., 2009; Giorgi and Lionello, 2008; IPCC, 2021). According to the Mediterranean Experts on Climate and environmental Change (MedECC) (Cramer et al., 2020) a reduction of annual precipitation in the range of 4 to 22% depending on the scenario at the end of the 21st century is expected in the Mediterranean basin. The reduction could reach a 30% in the south of the Iberian Peninsula under scenario RCP 8.5 according to multi-model ensemble average of regional climate models simulations from the EURO-CORDEX initiative (EURO-CORDEX, 2023; European Environment Agency, 2022). These impacts affect the already variable grassland production and therefore the ability of farms to feed livestock self-sufficiently.

In this context, there is a need for feed resources on-farm that could increase self-sufficiency, resilience and resistance to climate change, and other socioeconomic drivers (Rognli et al., 2021). Research efforts are being directed to find drought-resistant grassland species that could buffer the impact of drought and provide out-of-season forage (Moore et al., 2021; Volaire et al., 2014). Legumes are of special interest because of their high protein content and their ability to improve soil fertility through atmospheric N_2 fixation (Hynes et al., 2008). Deep-rooted perennial legumes in particular, could have the advantage of providing out-of-season forage after the senescence of annual species in natural grasslands and early regrowth in autumn (Annicchiarico et al., 2013; Moore et al., 2021; Moore et al., 2009).

In the research of forage legumes for Mediterranean climates, the new forage perennial legume *Bituminaria bituminosa* is gaining increasing attention (Real et al., 2014; Real et al., 2011). This legume, known by the common name of Tedera, is native to the

Mediterranean basin and the Canary Islands where there are three varieties namely *B. bituminosa* var. *albomarginata, B. bituminosa* var. *crassiuscula* and *B. bituminosa var. bituminosa* (Méndez et al, 1991). In the Canary Islands this species has been traditionally used by local herders to feed livestock (Ventura et al., 2009). Previous research have tested this species (especially var. *albomarginata*) proving its good forage aptitude and drought resistance (Martínez-Fernández et al., 2012; Oldham et al., 2015; Oldham et al., 2013; Pecetti et al., 2007; Real et al., 2014). A long-term breeding programme led by Australian and Spanish scientists (Oldham et al., 2013; Real et al., 2014) have recently developed a new variety registered under the trademark of Lanza® (Real, 2016). Lanza® combines attributes from elite plants of var. *crassiuscula* and var. *albomarginata* to seek ideotypes with cold tolerance and drought resistance (Real et al., 2014). This species has started to be used in some farms in Australia and might have the potential to be used in extensive livestock systems in the Mediterranean basin (Fernández-Habas et al., 2021; Melis et al., 2018; Porqueddu et al., 2016). Lanza® might be used as a monoculture to be grazed at the end of the growing season or included in mixtures to increase the resilience and resistance of the mixture to droughts. Several factors are involved in its successful use in both cases, such as the competitive ability, phenology, forage quality and how these factors and the performance of this species might be affected under future climate change scenarios. Previous research suggests that factors such as cold winters and competition by annual grasses could compromise the successful establishment of this species (Fernández-Habas et al., 2023; Raeside et al., 2012). Recently, Real et al., (2022a; 2022b; 2022c) have provided information on key aspects of agronomic practices and fertilisation. The adoption of new pasture legumes is likely to be hampered by unreliable production in variable environments (Hogg and Davis 2009), therefore more research is required to fully understand this species' potential and the best management strategy for the Mediterranean basin.

In order to provide further information on the potential of this species in Mediterranean farming systems, this study aimed at testing the response of *Bituminaria bituminosa* cv Lanza® to 33% rainfall reduction, competition by grasses and forbs and their interaction effect in terms of yield, forage quality, and phenology. To do so, we established a field experiment with rainout shelters to exclude 33% of annual rainfall and tested the performance of Lanza® in i) monoculture and growing in competition with annual grasses and forbs in ii) both scenarios of rainfall (control and 33% reduced) in comparison with the well-studied forage perennial legume alfalfa. The results provide useful information to advance in the knowledge of the potential of this species to be used in extensive livestock systems in Mediterranean climates.

MATERIAL AND METHODS

Seeds of *Bituminaria bituminosa* cv Lanza® (tedera onwards) were provided courtesy of the Department of Primary Industries and Regional Development (DPIRD), Australia under previous contract with the University of Cordoba for experimental purposes. To ensure germination, seeds were manually scarified by nicking the outer seed coat with a surgical scalpel and then germinated in flat trays with wet filter paper (Carruggio et al., 2020; Castello et al., 2015). When the radicle reached 1 cm, the seeds were planted in seedlings trays until developing three leaves, when were transplanted into the experimental plots. The decision of transplanting tedera seedlings instead of sowing was motivated by the limited amount of seed available. *Medicago sativa* cv. Letizia (Alfalfa onwards) inoculated seeds were directly sown in the experimental plots as described below.

Site description and climate

The experiment was established in the experimental farm of the University of Cordoba on the Campus of Rabanales, Cordoba, Spain (37° 55′ 01″ N and 04° 42′ 57″ W). The field has a gentle slope north-south of <5% and soils are deep Vertisol (Campillo García et al., 1993) with the following soil physicochemical properties: 40.0% silt, 50.2% clay, 9.8% sand, 1.97% oxidable organic matter, 0.13% N (Kjeldahl), 7.8 pH, 7 mg kg^{-1} P (Olsen), 370 mg kg^{-1} K. Previous establishment of the experiment the field was grown with oats and temporary grasslands which were not grazed. The average annual rainfall and mean temperature are 579 mm and 17.7 ºC, respectively (period 2002-2022, data from the meteorological station at the Cordoba Airport, Cordoba 37º 51' N and 04º 48' W) (RIA, 2023). Mean total monthly precipitation, mean, mean of maximum and mean of minimum monthly temperatures of historical data (2002-2022) and period of experiment duration are shown in Figure 1. Meteorological data for the period of the experiment were downloaded from the meteorological station at the Campus of Rabanales, Cordoba, Spain (37º 93′ N and 4º 72′ W). The 20/21 agronomic year, with 550 mm, was similar in annual rainfall to the long-term average, while the 21/22 with 470 mm was 19% drier.

After a dry Mach, the spring of 2021 was wet. In this year the monthly rainfall in June (51 mm) and July (22mm) was considerably higher than the long-term average (Figure 1). The agronomic year of 21/22 started with close-to-normal rainfall in September followed by 46 mm in October, 26 mm in November and a very wet December, with 34% of the total annual rainfall. January and February of 2022 accumulated little rainfall. After a close-to-average rainfall in April of 2022, only 8.4 mm were accumulated from May to the end of August compared to 96 mm in 2021 during the same period. The long-term average for these three months is 57 mm. During these months the temperatures (means, means of maximum and means of minimum) were also higher in 2022 than in 2021 and that of long-term average.

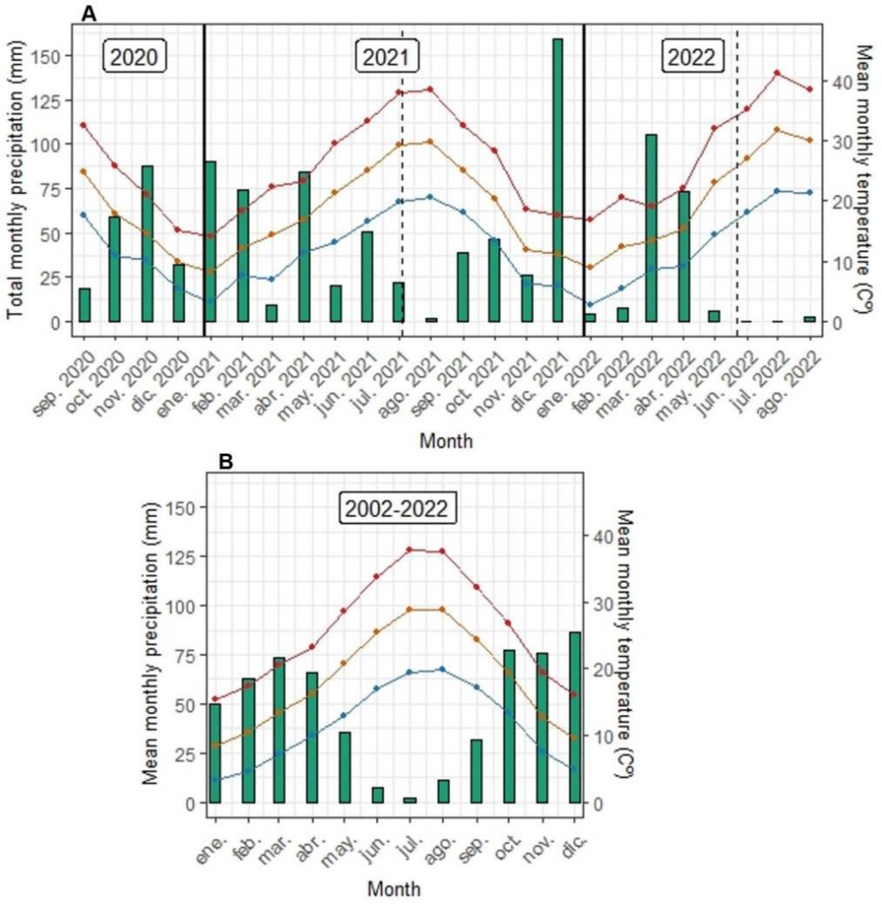

Figure 1. A: Total monthly precipitation (bars), mean monthly temperature of the daily mean (orange line), maximum (red line) and minimum (blue line) temperature for the two growing seasons of the experiment (2020-2021 and 2021-2022. Data from the meteorological station at the Campus of Rabanales, Cordoba, Spain (37º 93′ N and 4º 72′ W). **B**: Historical mean monthly precipitation (bars), mean monthly temperature of the daily mean (orange line), maximum (red line) and minimum (blue line) temperature from the period 2002-2022. Data from the meteorological station at the Cordoba Airport, Cordoba (37º 51' N and 04º 48' W) (RIA, 2023).

Concerning the frost days, the average number of days below 0 ºC in Cordoba according to the historical data is 13. During the agronomic year of 20/21, 13 frost days were registered, with minimum temperatures of -3.2 in early January and seven consecutive days of minimum temperatures below 0ºC, partly due to an unusual winter storm (*"Filomena"*) of historic low temperatures in Spain (RIA, 2023; Smart, 2021; Zschenderlein and Wernli, 2022). In the winter of the agronomic year of 21/22, only 6 frost days were registered, which although consecutive, did not drop below -0.9.

Experimental design and management

The experiment consisted of a Split-plot design with one "main plot" factor, two subplot factors and ten complete replicates. The "main plot" factor was Rainfall, with two treatments, 33% rainfall reduction (Reduction) and no rainfall reduction (Control). The subplot factor Species had two treatments corresponding to the two species tested tedera and alfalfa. The subplot factor Culture had two treatments, Monoculture in which each species was grown alone without interspecific competition, and Competition in which each legume species grew with interspecific competition of volunteer species. The subplots were 2 x 3 m and the treatments were randomized within the main plots being separated 0.5 m among them. Each plot then comprised four subplots (one by treatment of Species and Culture), except for two replicates that included a fifth subplot with bare soil subplots (which was not used for this study). Due to logistic constraints, the Rainfall factor was not randomized within replicates. This design resulted in a total of 80 subplots (4 subplots nested in 2 Rainfall treatments x 10 replicates).

Rainfall reduction was imposed using rainout shelters (4 x 1.90 x 24 m) following a similar design to Yahdjian and Sala, (2002). Polycarbonate gutters of 22 cm width were placed at 52 cm separation. The water intercepted by each gutter was collected by a transversal gutter placed at the lowest side of the shelter that channelled the water to two 1 m^3 storage tanks, one at each side of the shelter. Each shelter covered two plots; therefore, each storage tank collected the intercepted water for one plot (four subplots). An example of the rainout shelter design can be found in Figure S1 of Supplementary material. The 2x3 m subplots under the Rainfall treatment were placed 0.5 m to the inside of the shelter to prevent the effect of lateral rainfall on windy days. Soil preparation consisted of conventional tillage before installation of the experiment plus an extra pass with a rototiller after the installation of the shelters. No fertilisation was applied in line with the expected general practises for tedera.

Tedera and alfalfa were established in rows 20 cm apart and plant spacing of 20 cm and 40 cm in monoculture and competition respectively. This resulted in a plant density of 25 plants/m^2 in monoculture and 13 plants/m^2 under the competition treatment. Plants were first established on 14 and 15 December 2020, however, the low temperatures during the end of December and early January led to failed establishment and death of all plants (see 2.2. Site description and climate). Plants were finally established on 25 and 26 of February 2021. Volunteer species that grew in competition were mainly the annual grasses *Avena barbata, Lolium multiflorum, Phalaris minor* and *Bromus rubens*, and the forbs *Cichorium intybus, Leontodon tuberosus, Sonchus oleraceus, Picris echioides, Hirschfeldia incana, Convolvulus arvensis, Ridolfia segetum, Daucus carota, Anthemis arvensis, Trifolium campestre, Trifolium resupinatum* and *Medicago polymorpha*. In monoculture, weeds were controlled by manual deweeding and herbicide applications of Pulsar plus es-00646 (Imazamox 2.5% p/v). Occasional attacks of *Colaspidema barbarum* in alfalfa were controlled with the insecticide Cibelte es-18.316/11 (cypermethrin 10% p/v). Senescent plant material was removed at the end of July in all subplots but in tedera monoculture for which aerial biomass remained non-senescent over summer.

Phenology measurements

Reproductive phenological stages of tedera and alfalfa were recorded following the general scale of the Biologische Bundesanstalt Bundessortenamt und Chemische Industrie (BBCH) (Bleiholder et al., 2001) from February to July. The phenological stages recorded were: 5 (Inflorescence emergence), 6 (Flowering), 7 (Development of fruit), 8 (Ripening or maturity of fruit) and 9 (Beginning of dormancy and senescence). Phenology was assessed weekly in 2022 at plant level in five plants per subplot in every treatment of five repetitions. In total 25 plants by treatment were monitored for phenology. As the phenology of the year 2021 might be affected by the late establishment, for this year only the beginning of dormancy and senescence was recorded. Each stage was continuously monitored from start to end by plant. Inflorescence emergence was considered to start with the first flower bud visible and end when all buds flowered. Beginning of flowering was recorded when the first fully emerged flower was observed and was considered to finish when all flowers had petals dry or fallen and showed fruit set visible. Start of development of fruit was recorded when the fruit reached 10% of the final size and the end when all fruits reached the final size. Maturity of fruit was considered to start with the beginning of fruit colouration and end of this stage when all fruits showed fully-ripe colour and beginning of fruit abscission. Beginning of dormancy and senescence was recorded as the moment when 50% of the leaves were senescent of shed.

Yield and forage quality measurements

The establishment was evaluated in July 2021 and September 2021 in two rows per subplot for each treatment and the average was expressed as the percentage of plants alive of the total. To assess the potential of tedera to provide out-of-season forage, yield and forage quality measurements were performed once per year (2021 and 2022) at the end of the growing season, after the senescence of natural grasslands. The yield was sampled in each subplot by cutting the biomass in 0.5x0.5 m quadrats at 7 cm stubble height. Leaf, stem and reproductive organs (inflorescence/fruits) were separated, oven-dried at 60ºC for 72h and then weighed for dry matter estimation (DM). In competition treatment, the same procedure was followed the previous separation of target legumes from volunteer species. In monoculture treatment, due to high biomass production, the separation was performed in a subsample of at least 15% of the total DM. Yield was expressed as DM kg ha^{-1}. Leaf to stem ratio was measured as leaf DM/steam DM for both legumes. In competition treatment, the percentage of legumes was calculated as the proportion of target legumes DM in the total DM of the quadrat x100.

After DM measurement, samples were ground to pass through a 1-mm sieve. Crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM) were determined by near-infrared spectroscopy (NIRS) with a portable LabSpec 5,000 spectrometer (350–2,500 nm; ASD Inc., Boulder, Colorado, USA) using previously calibrated equations (Fernández-Habas et al., 2021). For both legumes, this was done in leaf and stem separately. The quality of the whole legume plant was estimated by weighting the quality of leaf and stem by their proportion in the sample. In 2021, due to irregular development after establishment in some subplots of monoculture because of late deweeding, the sample size was reduced to four subplots by treatment.

Statistical analysis

Statistical analyses were performed R v. 3.6.1 (R Development Core Team, 2019). Due to the differences between both years, all target variables were modelled separately for each year. Yield, percentage of legumes in competition, leaf:stem ratio and forage quality variables of the target legume species were modelled by linear mixed effects models as responses of the fixed effects of Species, Rainfall, Culture plus their three two-way interactions and the three-way interaction. The effect of rainfall reduction on the yield of volunteer species was also tested. Plot nested in Replicate were set as random effects.

We followed a top-down strategy for model selection (Zuur et al., 2009). First, we fitted a full model with all main fixed effects and interactions. Then the full model was simplified based on the Akaike Information Criterion (AIC), always fitting meaningful models and using maximum likelihood (ML) when comparing models with different fixed effects (Zuur et al., 2009). The optimal model (lowest AIC) was refitted using restricted maximum likelihood (REML) (Zuur et al., 2009) and visually assessed for homogeneity of variance and normal distribution of residuals (Kozak and Piepho, 2018). In this process, the three-way interaction of Species x Culture x Rainfall was dropped in model selection for all target variables and therefore no further investigated. F and p values were estimated by Wald F tests with Kenward-Roger's approximation of the degrees of freedom. Target variables were log-transformed when necessary to meet model assumptions. "lme4" package was used to fit linear mixed effects models (Bates et al., 2023). The different sampling in 2021 resulted in an incomplete design, and therefore only Plot was set as random effect. In case the models reported singularity issues due to low estimates of the random effects, the random effect was dropped, and linear models were used instead.

We performed Tukey post hoc test to compare marginal means of the factors influencing the target variables using the "emmeans" package (Russell et al., 2023). Factors dropped from the final model or not influencing the target variables were averaged. Estimated marginal means were reported in all cases (back-transformed in case of previous log-transformation).

For the phenological measurements, some plants in the different treatments did not reach the phenological stages recorded. Therefore, there was an excess of zeroes in the count data of the number of days to each stage. We modelled this data using zero-inflated Poisson regression models with the package "glmmTMB" (Magnusson et al., 2023). These regressions allow the modelling of positive counts following a Poisson distribution in a conditional model and the data containing zeroes in a zero-inflated model using a logit link (Brooks et al., 2017). The zero-inflated model describes the probability of observing an extra zero count not generated by the conditional model (Brooks et al., 2017). In the conditional model, Species, Culture and Rainfall were set as fixed effects as well as their three two-way interactions and their three-way interaction. To account for the Split-plot experimental design and repeated measures, subplot nested in plot nested in replicate were set as random effects. For the zero-inflation model, the main effects of Species, Culture and Rainfall were tested. More complex structures in the zero-inflation model failed to achieve model fit and convergence. Two different models were fitted for each phenological stage (four reproductive stages measured), one for the count of days to start of the stage and a second one for count of days to end of the stage. For the stage of beginning of dormancy, no models were fit due to the lack of variance in the data (omnibus test reported non-significance p>0.05) as the time to this stage was highly synchronic, therefore the results were descriptively explained. The full models were simplified based on the AIC. The three-way interaction, the Culture x Rainfall interaction in the conditional models and the fixed effect Rainfall in the zero-inflation model were removed in all the best fit models and therefore were not reported. In all models the three main fixed effects were kept in the conditional model to allow graphical comparability among phenological stages. Residual diagnostics were performed using the “DHARMa” package (Hartig, 2020). Based on the best-fit models, the estimated means of the days to start and end of each stage were reported from the conditional model as well as the estimated probability of an extra zero count from the zero-inflation model using the “emmeans” package (Brooks et al., 2017). A significance level of p=0.05 was chosen, although marginally significant p-values 0.05<p< 0.10 were also interpreted.

RESULTS

Response of yield to rainfall reduction and competition

The establishment of alfalfa measured in July 2021 was high (92% on average), with values equal to or higher than 90% in both culture treatments. Tedera also showed high values of establishment, although lower than alfalfa (84% on average), with average values of 85% and 83% in monoculture and competition, respectively. After the summer, in September 2021, the number of plants of alfalfa remained at 90% in both culture treatments and over 82% for tedera in monoculture but decreased to 48% of tedera under competition.

Table 1 shows the effects of species, culture, rainfall and interactions on DM yield, percentage of legumes and leaf:stem ratio in 2021 and 2022. In 2021, the 33% reduction of rainfall did not affect yield, percentage of legumes or leaf:stem ratio. There was a two-way interaction of species by culture (F=28.1, <0.0001) for DM yield. Both legumes had similar DM yield (about 2,700 kg ha^{-1}) in monoculture. Competition reduced drastically DM yield for both species although alfalfa was five times more productive than tedera, which had a very low DM yield of 41 kg ha^{-1} (Figure 2: A). This was in line with the low percentage of legumes in competition (<7%), which was also significantly higher (F=26.6, p=0.0001) for alfalfa compared tedera (Figure 3).

Table 1. Main effects and interactions of legume species (tedera vs alfalfa), culture (monoculture vs competition) and rainfall (control vs reduced) on legume yield, legume percentage (for competition only) and leaf to stem ratio in 2021 and 2022.

			Effects					
Year	**Target variables**		**Species**	**Culture**	**Rainfall**	**Species x Culture**	**Species x Rainfall**	**Culture x Rainfall**
2021	**Yield legumes (DM kg ha)**	**F**	27.9	552.9	-	28.1	-	-
		P	<0.0001	<0.0001	-	<0.0001	-	-
	Percentage legume (%)	**F**	26.6	--	-	-	-	--
		P	0.0001	--	-	-	-	--
	Leaf to stem ratio g g^{-1}	**F**	93.5	21.5	-	-	-	-
		P	<0.0001	<0.0001	-	-	-	-
2022	**Yield legumes (DM kg ha)**	**F**	32.2	471.1	0.4	68.4	7.1	4.3
		P	<0.0001	<0.0001	0.5512	<0.0001	0.01043	0.04272
	Percentage legume (%)	**F**	8.1	--	1.9	-	3.2	--
		P	0.0107	--	0.2059	-	0.0891	--
	Leaf to stem ratio g g^{-1}	**F**	0.3	4.0	-	10.7	-	-
		P	0.5965	0.0523	-	0.0019	-	-

F- and *p*- values are only provided for the main effect and interactions that were retained in the final model after model selection. “- “ indicates that the effect/interaction was not retained in the final model and “--” indicated that the effect/interaction was not used to fit the model.

In 2022, the DM yield was affected by the interactions of species by culture, species by rainfall and culture by rainfall (Table 1). Figure 2B shows the mean comparisons by species and rainfall treatment within culture treatment. Tedera was very productive (about 9,000 kg ha^{-1}) in monoculture irrespectively of the reduction of rainfall, although with high variability. Alfalfa showed significantly lower production than tedera and a significant 52% reduction under reduced rainfall (Figure 2 B). When grown in competition both species showed similar DM yield which was not affected by rainfall reduction in either alfalfa or tedera. However, in control treatment tedera was significantly less productive than alfalfa (Figure 2 B).

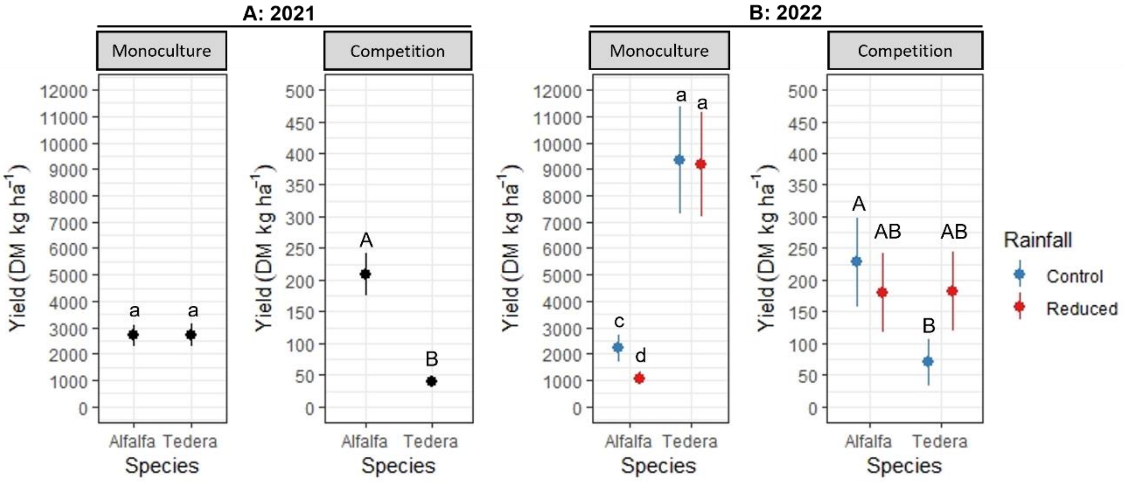

Figure 2. A: Dry matter (DM) yield of alfalfa and tedera in 2021 by culture treatments (averaged over rainfall treatments). B: Yield alfalfa and tedera in 2022 by culture and rainfall treatments. Points represent estimated marginal mean ± standard error. Different lowercase letters indicate significantly different means between species within monoculture treatment for each year ($p < 0.05$). Different uppercase letters indicate significantly different means between species within competition treatment for each year ($p < 0.05$). Note different scale for monoculture and competition treatments.

As in 2021, the percentage of DM of legumes in competition in 2022 was minimal and lower for tedera (Figure 3 B). There was a marginally significant interaction (F=3.2, p=0.0891) of species by rainfall indicating a trend to a lower percentage of tedera in control treatment compared to 33% rainfall reduction while alfalfa showed no differences. The DM yield of volunteer species was lower under reduced rainfall in both years, although in 2022 was only marginally significant (2021: F=10.9, p=0.0122; 2022: F= 8.4, p= 0.0512). In 2021, volunteer species yielded 4,670 kg ha^{-1} in control treatment and 2,983 kg ha^{-1} under reduced rainfall, a 36% lower. In 2022, the DM yield of volunteer species was overall higher than in 2021, with 6,823 kg ha^{-1} in control treatment and 5,101 kg ha^{-1} under reduced rainfall, a 25% lower.

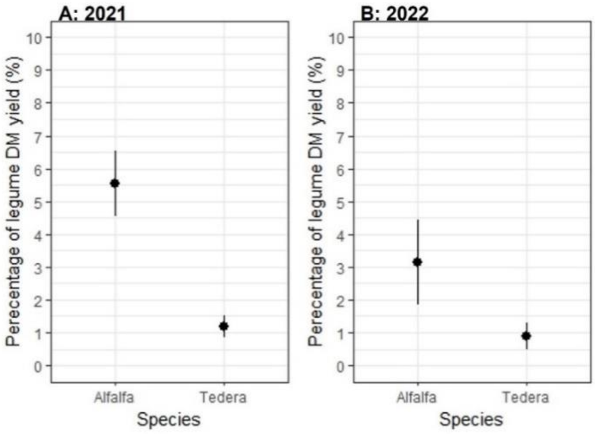

Figure 3. Percentage of dry matter alfalfa and tedera in total dry matter in competition treatment (averaged over rainfall treatments) in 2021 (A) and 2022 (B). Points represent estimated marginal mean ± standard error.

In 2021 tedera had a higher leaf:stem ratio than alfalfa (F=93.5, p<0.0001), and for both species, it decreased in monoculture compared to competition (F=21.5, p<0.0001) (Figure 4 A). In 2022, the leaf:stem ratio was much lower than in 2021, especially for tedera (Figure 4 B). In this year, the leaf:stem ratio was lower for alfalfa in competition while culture did not affect the leaf:stem ratio of tedera (F=10.7, p=0.0019, Figure 4 B). In monoculture, alfalfa had a higher leaf:stem ratio than tedera (Figure 4 B).

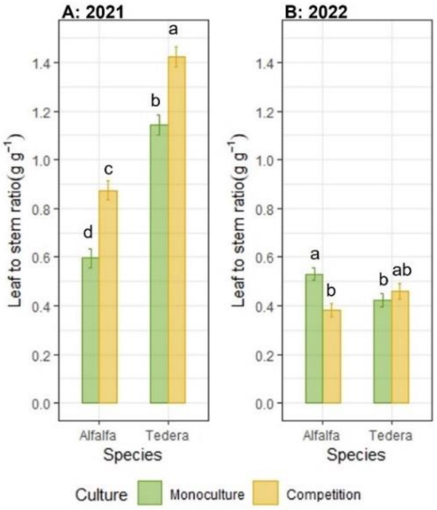

Figure 4. Leaf to stem ratio by culture treatment (averaged over rainfall treatments) for 2021 (**A**) and 2022 (**B**). Bars represent estimated marginal mean ± standard error. Different letters indicate significantly different means (p <0.05).

Response of forage quality to rainfall reduction and competition

Overall, alfalfa showed better forage quality than tedera, although for both species, culture influenced the forage quality of leaf (Table S1) stem (Table S2) and the weighted forage quality (Table S3) while Rainfall had no or limited impact. Both species maintained high forage quality for leaf (CP>13%) (Table 2). However, tedera showed poor forage quality on stem (CP<6%), especially in 2022 (Table 2), which influenced the weighted shoot forage quality (Table 3). In 2021, alfalfa in competition had the highest leaf CP content (21.6±0.5 %), higher than in monoculture while tedera showed higher leaf CP content in monoculture (Table 2). In 2022, both species had lower leaf CP content in competition, although the differences were minimal. Rainfall significantly reduced the CP content of leaf in 2022 (F=8.3, p=0.0189), although the reduction was low, a 3.7% in average across treatments. Overall, leaf of tedera tended to have a lower content of NDF and higher ADF than leaf of alfalfa (Table 2). Despite some differences in EDOM by species and culture, both species showed high EDOM of leaf (EDOM>83%). In 2022, EDOM of stem of tedera in monoculture dropped to very a low value of 55.3±0.9 %.

The CP and EDOM of the stem were overall, higher for alfalfa than tedera and higher for tedera in competition. The differences between monoculture and competition were larger for tedera in 2022 (Table 2). Alfalfa maintained a moderate level of CP in stem (CP>8.7%), with no differences between monoculture and competition in 2022 (Table 2). However, tedera showed very low CP content in stem especially in monoculture and in 2022. EDOM showed a similar pattern to CP. In 2021 alfalfa had higher NDF and ADF than tedera and always lower for competition in both species. In 2022, tedera in monoculture showed the highest values for NDF and ADF, while alfalfa did not differ in fibre content between culture treatments and only in ADF content with tedera in competition (Table 2).

Table 2. Forage quality of leaf and stem for alfalfa and tedera in 2021 and 2022 by culture treatment (averaged over Rainfall). Values are estimated marginal mean ± standard error. Different lowercase letters indicate significantly different means between species and culture treatments for each year in leaf ($p<0.05$). Different uppercase letters indicate significantly different means between species and culture treatments for each year in stem ($p<0.05$).

		Leaf				**Stem**			
		Monoculture		**Competition**		**Monoculture**		**Competition**	
	Target variable	**Alfalfa**	**Tedera**	**Alfalfa**	**Tedera**	**Alfalfa**	**Tedera**	**Alfalfa**	**Tedera**
2021	**CP**	18.3±0.5 b	15.3±0.5 c	21.6±0.5 a	13.1±0.5 d	8.7±0.4 B	2.5±0.4 D	11.5±0.4 A	5.3±0.4 C
	NDF	22.1±0.4 b	20.7±0.4 bc	25.9±0.4 a	19.8±0.4 c	51.1±0.7 A	48.0±0.7 B	44.9±0.7 C	41.8±0.7 D
	ADF	14.2±0.3 b	18.9±0.5 a	13.0±0.3 c	14.5±0.3 b	33.9±0.7 A	32.0±0.7 B	26.8±0.7 C	24.9±0.7 D
	EDOM	87.8±0.6 ab	89.4±0.6 a	86.0±0.6 b	87.7±0.6 ab	69.5±0.5 D	71.6±0.5 C	75.6±0.5 B	77.7±0.5 A
2022	**CP**	21.0±0.3 a	16.9±0.3 c	19.8±0.3 b	15.7±0.3 d	11.0±0.4 A	1.1±0.4 C	11.4±0.4 A	4.5±0.5 B
	NDF	27.8±0.6 a	21.3±0.6 b	27.6±0.7 a	21.1±0.7 b	50.7±1.0 B	59.3±1.0 A	48.4±1.1 B	52.5±1.2 B
	ADF	15.3±0.5 b	18.2±0.4 a	17.0±0.4 a	17.1±0.5 a	36.1±0.8 BC	43.4±0.8 A	35.0±0.9 C	39.0±1.0 B
	EDOM	83.0±0.6 c	85.3±0.6 ab	84.9±0.7 bc	87.2±0.7 a	70.9±0.9 A	55.3±0.9 C	70.5±1.0 A	61.2±1.1 B

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EDOM: Enzyme digestibility of organic matter.

Concerning the forage quality weighted by leaf and stem proportions, in 2021 alfalfa in competition showed the highest CP content (16.4±0.4 %) while alfalfa in monoculture had 12.1±0.4 % and tedera in both, monoculture and competition had values around 9.5% of CP. In 2022, alfalfa also had significantly higher CP content than tedera, which also showed lower content of CP in monoculture compared to competition, with 5.7±0.4 % and 8.8±0.5% respectively. Overall, in 2021 alfalfa showed higher values of NDF and ADF than tedera, and for both species, the content of NDF and ADF decreased in competition compared to monoculture (Table 3). In 2022, tedera in monoculture reported the highest NDF and ADF content, significantly higher than in competition, while alfalfa showed similar values to tedera in competition and no differences between culture treatments (Table 3). EDOM reported the lowest value for alfalfa monoculture in 2021 (76.7±0.6 %) and both species showed differences between culture treatments. In 2022, tedera in monoculture showed the minimum value of EDOM with 64.1±0.9 %, significantly lower than in competition and that for alfalfa, which did not differ in EDOM between culture treatments.

Table 3. Forage quality weighted by leaf and stem proportions for alfalfa and tedera in 2021 and 2022 by culture treatment (averaged over Rainfall). Different lowercase letters indicate significantly different means between species and culture treatments for each year (p<0.05).

		Monoculture		**Competition**	
	Target variable	**Alfalfa**	**Tedera**	**Alfalfa**	**Tedera**
2021	**CP**	12.1±0.4 b	9.4±0.4 c	16.4±0.4 a	9.7±0.4 c
	NDF	40.6±0.6 a	33.6±0.6 b	36.1±0.6 b	29.1±0.6 c
	ADF	27.2±0.6 a	25.1±0.6 b	20.5±0.4 c	18.9± 0.4 d
	EDOM	76.7±0.6 c	80.6±0.6 b	79.9±0.6 b	83.8±0.6 a
2022	**CP**	14.5±0.4 a	5.7±0.4 c	13.9±0.4 a	8.8±0.5 b
	NDF	43.2±1.0 b	47.9±1.0 a	42.5±1.1 b	43.1±1.2 b
	ADF	30.6±0.8 b	36.0±0.8 a	30.1±0.9 b	32.3±1.0 b
	EDOM	75.1±0.9 a	64.1±0.9 c	76.1±1.0 a	70.6±1.1 b

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EDOM: Enzyme digestibility of organic matter.

Response of phenology to rainfall reduction and competition

Figure 5 schematise the phenological development of tedera and alfalfa in every treatment. In the conditions of the year 2022, tedera showed earlier phenology for reproductive stages than alfalfa with inflorescence emergence and flowering starting and being completed earlier. Inflorescence emergence started by the end of February-beginning of March and flowering by the end of March-beginning of April in monoculture. The emergence of inflorescence lasted until mid-end March while flowering ended by mid-May in monoculture. After the start of flowering, tedera did not stop producing new leaves. On average tedera started and finished the stage of inflorescence emergence ~54 and ~36 days, respectively, before alfalfa. Concerning flowering, tedera started and completed this stage ~44 and ~20 days, respectively, before alfalfa. However, there were important differences by treatment in tedera. There was an interaction species by culture for the start and end of inflorescence emergence and the start of flowering (Table S4, conditional models). In competition, tedera showed delayed phenology with later start and end of inflorescence emergence (15 days approximately) and later start of flowering (~10 days), while alfalfa showed no differences among culture treatments (Figure 5). An interaction species by rainfall (Table S4, conditional models) also revealed earlier inflorescence emergence and flowering of tedera under reduced rainfall compared to control (~10 days), whereas alfalfa showed no differences. This interaction was only marginally significant for the end of both phenological stages (Table S4 conditional models).

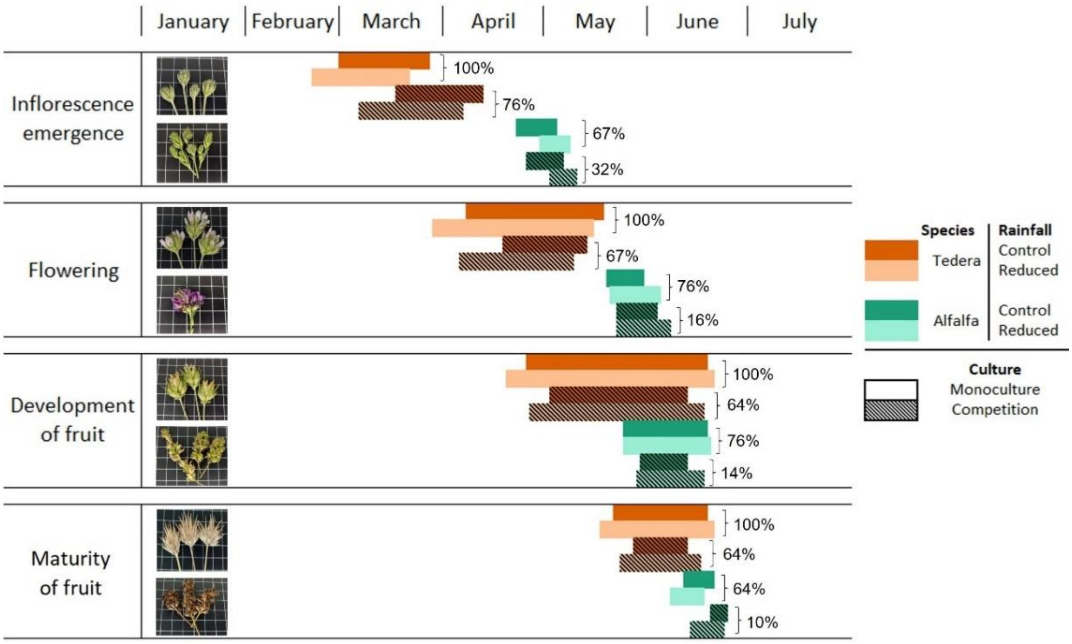

Figure 5. Reproductive phenological stages of alfalfa and tedera for each factor in 2022. Start and end of the bars represent the estimated marginal means of the day of the year to start and end of each stage by treatment. Numbers represent the percentage of plants that reached the phenological stage by species and culture (averaged over rainfall). Rainfall showed no influence on the percentage of plants that reached each stage (Tables S4 and S5).

Concerning the stages of development of fruit and maturity of fruit, both stages started significantly earlier in tedera than in alfalfa (~30 days for development and ~21 days for maturity, averaged over culture and rainfall). Tedera started the development of fruit by mid-end April and fruit maturation by mid-May. Also, for both species, competition delayed the beginning of development and maturity of fruit (~6 days) (Table S5 conditional models, Figure 5). However, there were no differences in the time to completion of both stages which ended by mid-June (Table S5 conditional models, Figure 5). Rainfall showed only a marginally significant effect to earlier start of the development and maturity of fruit under reduced rainfall (Table S5).

As shown by the zero-inflated models (Tables S4 and S5, zero-inflated models), the probability of not reaching the recorded reproductive phenological stages was influenced by the species and culture, while rainfall showed no influence. Tedera showed lower probabilities of not reaching reproductive stages than alfalfa, and for both species, competition increased the probabilities of not reaching the different reproductive stages (see Table S6 for estimated probabilities from zero-inflation models).

Concerning, the stage of beginning of dormancy, there were important differences between years and culture treatments in 2021, while rainfall had little impact. In 2021, 90% of the plants in alfalfa monoculture reached 50% of leaf shedding by the end of July and lost all the leaves over the summer. On the same date, only 5% of tedera plants in monoculture showed 50% of leaf shedding. Tedera maintained green leaves over the rest of the summer (Figure 6: B). In competition, only 16% of the plants of both species showed 50% of leaf shedding at the end of July. However, in 2022 both species reached 50% of leaf shedding synchronically in both culture treatments in early June, about ~52 days earlier than in 2021. By the end of July, both species were in dormancy after complete leaf shedding (Figure 6).

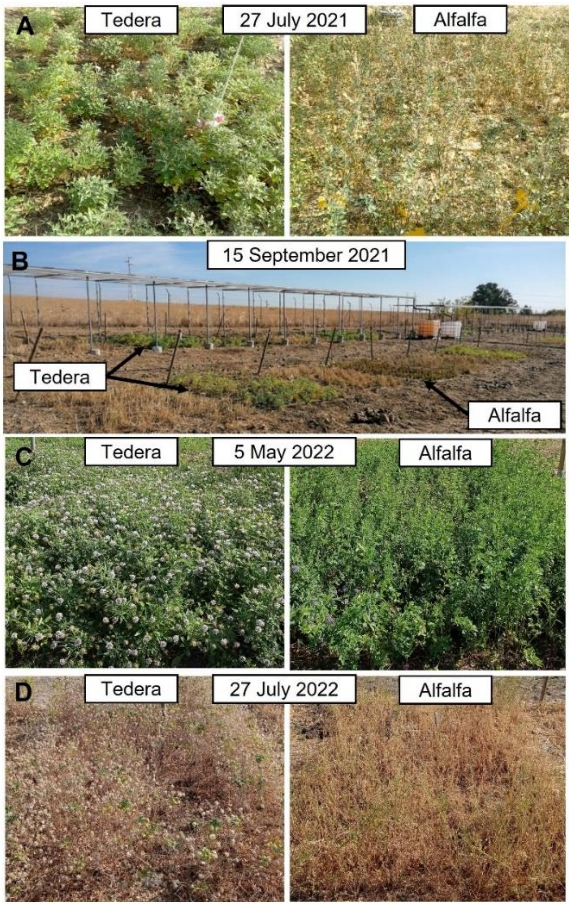

Figure 6. Overview of subplots of tedera and alfalfa in monoculture in July of 2021 (**A**) with alfalfa at 50% leaf shedding and in July of 2022 (**D**) with both species in dormancy after complete leaf shedding. **B**: Overview of the experiment showing subplots of tedera with green leaves after summer. **C**: Shows tedera in early May with flowers comparatively with alfalfa

DISCUSSION

Late sowing in February 2021 resulted in a similar DM yield for both species of 2,700 kg ha^{-1} in monoculture in July 2021, five months after sowing. This production is in agreement with the annual yield of tedera reported in previous studies in Mediterranean environments between 2,000 and 3,000 kg ha^{-1} (Raeside et al., 2012; Real, 2022; Sternberg et al., 2006). This acceptable DM yield and the lack of differences by rainfall treatments could be favoured by the late rainfall in June and July, 43 mm, and 20 mm respectively higher than the long-term average rainfall for these months. The occurrence of late spring and early summer rainfall events, mid-spring droughts and heat weaves reinforces the strategic use of drought-resistant perennial legumes. These species can resist mid-spring heat waves and lack of rainfall to continue producing while annuals advance their phenology to terminate the cycle (Volaire et al., 2014). Summer rainfall events reduce the already low forage quality of senescent annuals (Olea et al., 1989), while summer active perennials such as tedera can benefit from such events to keep or increase green herbage production (Suriyagoda et al., 2013). During this first year of establishment, tedera maintained green leaves over the summer while alfalfa showed leaf shedding by mid-July and lost all aerial biomass over the rest of the summer. The higher leaf to stem ratio of tedera compared to alfalfa in the first year might have been also affected by leaf shedding in alfalfa at the harvesting time.

During the second year, tedera had suitable growing conditions from early autumn to May. There was early rainfall in mid-September and mild temperatures during winter, which allowed undefoliated tedera to start regrowth early in Autumn and maintain growth until May. The fact that tedera maintained green leaves until September may also have allowed early regrowth. In the period from September to May, the least rainy months were January and February, when tedera is expected to reduce development due to cold temperatures. This resulted in high biomass production at the end of the second growing season in June 2022 with 9,000 kg ha^{-1} of tedera. Alfalfa however started regrowth from the crown and showed considerably lower DM yield, which was also due to a sporadic attack of *Colaspidema barbarum* at the end of April. In this second year, although April precipitation was slightly higher than the long-term average, rainfall in May was very low (5.5 mm, 15% of the long-term average) and there was no rainfall in June and July. In these conditions, tedera was not affected by the 33% rainfall reduction while alfalfa reduced its DM yield by 52%. Both species showed earlier leaf shedding than in the previous year. Most of the plants of both species showed 50% of leaf shedding by early June with no differences among them. The high accumulation of biomass of tedera in monoculture was probably highly water-demanding and together with the drier conditions in May 2022 might have caused earlier leaf shedding than in 2021 irrespective of the rainfall treatment. Leaf shedding affected the bottom half of the plants which were the oldest leaves as a common strategy of other perennial species to reduce evapotranspiration under water stress (Begg and Turner, 1976). This might have been aggravated by the higher plant density of tedera in this experiment of 25 plants m^{-2} compared to the plant densities between 8-16 plants m^{-2} recommended by Real, (2022) and Suriyagoda et al., (2013), although annual rainfall in these locations is lower than in the location of this experiment. These factors, together with the development of woody stems over the second year affected the leaf to stem ratio of tedera which decreased from about 1.1 g g^{-1} in 2021 to 0.4 g g^{-1} in 2022. These results reflect the shrub-like growth form of tedera in the absence of cutting. Previous studies on the cutting frequency of tedera showed that higher cutting frequency can increase leaf to stem ratio limiting the proportion of non-edible stem (Suriyagoda et al., 2013). According to previous studies, the effect of frequency and moment of defoliation on tedera seem to be dependent on site and seasonal temperatures and rainfall, especially in summer. Suriyagoda et al., (2013) in an experiment with <375mm of annual rainfall reported three cuts per year, with two in summer–autumn as the most desirable strategy for herbage accumulation and maintenance of a high leaf to stem ratio. In the same study cutting in early summer reduced herbage for use in late summer–autumn. In a different experiment under higher annual rainfall (445 mm) one cut yielded better than two, three and four cuts (Real and Kidd, 2012). Future studies should investigate the effect of intensity and timing of grazing on, yield, leaf proportion and shedding in summer as well as regrowth in early autumn in years of contrasting rainfall in spring-early summer. In a context of increased inter-annually variability of rainfall, this information is essential to plan the strategic use of tedera in summer and autumn depending on the spring-early summer conditions.

The failure to establish tedera in early winter and field observation support the limitations of this species to persist in environments of cold winters with frequent frost days. Raeside et al., (2012) also reported tedera var. *albomarginata* to fail despite excellent production over summer due to frost damage in winter in southern Australia, in a location with 40 frost days on average per year. In the second year of this experiment, minimum temperatures did not drop below -1ºC and the higher biomass accumulation might have allowed tedera to be less damaged by frost. However, during cold days in December 2021 and January 2022, tedera plants showed a characteristic yellowish colour. Based on these observations and results from previous studies, we believe that cold tolerance must be improved in Lanza® tedera to seek ideotypes adapted to cold environments of the south and west of the Iberian Peninsula with frequent frost days. The genetic diversity of tedera offers opportunities to improve cold resistance (Walker et al., 2010), especially based on var. *crassiuscula* which survives under snow in its native environment (Méndez et al., 1991).

The results of yield and percentage of legumes in competition treatment indicate failure of both legumes to be productive in environments with high competition by annual grasses such as *Avena barbata*. Comparatively, tedera was more susceptible than alfalfa to competition which is known to have a better competitive ability than other perennial legumes due to higher vigour and better ability to compete for light (Annicchiarico et al., 2019). These results confirm observations from Raeside et al., (2012), who reported competition by invading weeds as one of the factors leading to failure of tedera to establish. It also reinforces previous results in a pot experiment of the strong reduction of tedera biomass production when grown with *Lolium multiflorum* (Fernández-Habas et al., 2023). The low yield of tedera under competition in the second year was also influenced by mortality over summer. It must be noted that the volunteer species and the high fertile and deep Vertisol soil of this experiment might have generated a more competitive environment than expected in Mediterranean grasslands of sandy soils of lower fertility. However, it seems clear that tedera is unable to be productive when competing with fast-growing and winter-vigorous species. As suggested in previous research (Fernández-Habas et al., 2023), testing suitable partner species for tedera could improve the performance and persistence of this species. Ideal partners should display complementarity in growth patterns and resources used in space and time (Annicchiarico et al., 2019). Tedera might be over-competed when growing with fast-growing species that occupy the soil rapidly and develop erectus and vigorous aerial biomass. This might impair tedera's strategy to develop a deep root system that confers the advantage to access deep soil water under water shortage. This was supported by the 35% decrease in plant survival of tedera during the first summer. Complementary in space could be achieved by seeking divergent rooting systems with partner species displaying shallow-growing root systems (soil niche complementarity) and prostrate aerial biomass development. A prostrate growth habit of partner species might also promote ground cover to avoid invasion of weeds (Frankow-Lindberg, 2012). Suitable alternatives to establish tedera in mixtures may also include the increase of legume proportion and sowing rate and the selection of less fast-growing grasses in mixtures. For example, Malisch et al., (2017) showed that *Festuca pratensis* and *Lolium perenne* performed better than *Lolium multiflorum*, *Phleum pratense* and *Dactylis glomerata* as partner species of sainfoin (*Onobrychis viciifolia* Scop.), a perennial legume of low competitive ability, in terms of weed suppression and suitable proportions of sainfoin. Partner species should also have cold tolerance to ensure winter production and compensate for the low winter vigour of tedera, which could also benefit from the cover of partner species in winter. Previous research have shown that *B. bituminosa* var *bituminosa* tedera is able to maintain relatively stable cover under heavy and moderate grazing, which may even have a positive effect on plant cover due to reduced competition from companion species (Gutman et al., 2000; Sternberg et al., 2006; Sternberg et al., 2000). Moderate grazing (<0.5 LU ha^{-1}) at the end of the winter could also have a beneficial effect to release tedera from competition, as tedera is not preferentially grazed in presence of other more palatable species (Gutman et al., 2000; Sternberg et al., 2006). Suitable partner species for tedera might be legumes such as *Trifolium subterraneum*, *Astragalus pelecinus*, *Ornitopus compresus*, forbs such as *Plantago sp*. and grasses such as *Lolium perenne* and *Poa annua* at low sowing rates. Grasses and forbs could benefit from the N_2 fixation by legumes and extract N from soil to prevent from destabilising effects of eutrophication in grasslands (Carroll et al., 2022). Further research is needed to test suitable partner species for tedera and its use in mixtures.

Results from forage quality in the first summer after late establishment confirms that tedera shows acceptable forage quality, lower than fresh alfalfa and similar to low-quality alfalfa hay (Ventura et al., 2009). Leaf of tedera showed good forage quality with CP content over 13% in both years. These values are far above the CP content below 8% in senescent grasslands of dehesas in summer (Olea and Miguel-Ayanz, 2006; Olea et al., 1989). Maintaining a high leaf to stem ratio is key to guaranteeing high forage quality availability in summer. The forage quality of stem was very low, especially in 2022. This had a strong impact on the weighted shoot forage quality as it decreased drastically for tedera in monoculture, especially in 2022 with values (CP=5.7 %) far below those reported by previous studies (Fernández-Habas et al., 2021; Oldham et al., 2013; Pecetti et al., 2007; Ventura et al., 2009). However, their results might be biased because of the no separation between edible and non-edible stems which may be a large proportion of stem biomass, especially in 2022 when tedera became stemmier. This highlights the importance of suitable grazing management as outlined before to avoid the development of woody stems that would reduce forage quality and intake (García-Favre et al., 2022; Suriyagoda et al., 2013). In the second year, when tedera was grown in competition, it developed less thick stems (with a similar leaf to stem ratio) which had a positive effect on weighted shoot forage quality, showing acceptable CP content, significantly higher EDOM and lower NDF and ADF than in monoculture.

The lack of a clear and consistent effect of rainfall reduction on forage quality is in line with the findings of the meta-analysis by Dumont et al., (2015), who reported drought effects causing changes of small amplitude of N (with sometimes contradictory results) and NDF, and lack of a clear effect on digestibility (with high variation among experiments). In a previous experiment testing two different water regimes in the three varieties of tedera we did not find an effect of water shortage on forage quality of tedera (Fernández-Habas et al., 2021). Catunda et al., (2022) did find an effect of short-term drought on NDF, ADF, digestibility and CP on alfalfa. They reported an increase in NDF, ADF and a decrease in digestibility and CP of 5%. The latter is in line with the overall decrease in CP of 3.7 % in leaf in 2022 (with drier spring) which might be explained by a lower nutrient uptake and higher leaf senescence due to more advanced phenology under drier conditions (Catunda et al., 2022; Küchenmeister et al.,2013). Küchenmeister et al., (2013) also found a tendency to a decrease in CP in perennial forage legumes under strong temporary drought. However, these authors concluded that the effects of drought stress on forage quality are small, and less important than factors such as suitable selection of forage legumes and stand composition (monoculture or mixture) to ensure suitable forage quality under climate change scenarios. We support this conclusion as species and culture factors, and differences between years showed a greater impact on forage quality than the rainfall factor.

In the conditions of year 2022, tedera showed early phenology, significantly earlier than alfalfa for all reproductive stages. Early flowering is a key trait for the persistence of annual legumes in Mediterranean environments of short seasons but is also important for perennials, as it enables suitable fruit production before the onset of summer drought

(Berger and Ludwig, 2014; Real et al., 2012). This was demonstrated by the short flowering of alfalfa, which was terminated in early June, probably due to the strong water stress and terminal drought that imposed the high temperatures and lack of rainfall in May-June 2022. In fact, in monoculture, only 76% of alfalfa plants flowered against the 100% of flowering in tedera plants in monoculture. A disadvantage of early flowering by tedera might be frost damage in inflorescence and flowers in late February and early March in cold environments. Grazing might delay flowering and therefore reduce frost damage risk in reproductive organs, although it might come at the expense of lower seed production (Sternberg et al., 2006). Tedera plants responded to rainfall reduction with an earlier inflorescence emergence and flowering denoting plasticity to rainfall reduction which might play an important role to reduce the effect of an increased irregularity of rainfall (Daryanto et al., 2015). Melis et al., (2018) also evaluated first flower appearance among other traits in *Bituminaria* accessions of *B. bituminosa* and *B. morisana* in Sardinia (Italy). Overall, they reported later first flower emergence than in this study, although the higher spring temperatures in Cordoba might account for these differences. In the same study, they reported differences among accessions in first flower appearance and found a negative correlation between seed yield and days to first flower appearance in support of a positive relationship between early flowering and high seed yield in perennial legumes in Mediterranean rainfed environments (Melis et al., 2018). By mid-April tedera plants were already developing fruits. The development of fruit lasted about two months and overlapped with maturity of fruit. Both stages ended simultaneously. Therefore, tedera plants had a range of fruits in different maturity stages, from immature to ripening. In line with the yield, phenology was also strongly affected by competition in both species by reducing the number of plants that reached reproductive stages. For tedera, competition also delayed the start of inflorescence emergence and flowering, and for both species the fruit development and maturation. This effect might be caused by the shade of the species that dominated in competition treatment in agreement with a delayed flowering of legumes in response to shade (Mauro et al., 2014). Based on previous experiments (Fernández-Habas et al., 2023) and the native environments of the Canarian varieties, we believe that tedera might be susceptible to shade. In the face of its potential use in agroforestry systems, the response of tedera to shade in terms of yield, phenology and seed production prompts further research.

Future research should confirm these trends and test phenological responses in years with springs of contrasting rainfall and temperatures. It is worth noting that results from this study should be interpreted carefully as the response of tedera in yield and phenology to drought might differ in shallower and sandy soils as drought response is highly dependent on soil depth and texture (Daryanto et al., 2015). Sandy soils with lower water-holding capacity might lead to earlier flowering and leaf shedding.

CONCLUSIONS

Results from this study showed that tedera established in monoculture in late winter can be as productive as alfalfa in rainfed conditions, and in case of late spring and occasional summer rainfall, is able to maintain green leaves over the summer season. Tedera can be highly productive, more than alfalfa in rainfed conditions, in years of early autumn rainfall and mild winters, although lack of rainfall in May-July can reduce the offer of green forage during summer due to leaf shedding. However, in these conditions of a 33% reduction of annual rainfall, at the same annual rainfall distribution, tedera can maintain a similar yield, while alfalfa reduces its yield considerably. Thus, tedera seems to be resistant to a reduction of 33% of annual rainfall. However, the yield and contrasting leaf shedding in the two years of experiment reveal important implications of the seasonality of rainfall on the ability of tedera to provide out-of-season forage.

Both species, and especially tedera, fail to be productive in environments of high competition from annual grasses and forbs, independently of the rainfall reduction. Future research should investigate suitable partner species for tedera in mixtures.

Tedera shows suitable forage quality to be used as an out-of-season forage, especially in the leaf fraction. The forage quality of tedera can decrease greatly in case of a reduction leaf to stem ratio due to leaf shedding and the development of lignified stems. Proper management should prevent a decline in leaf to stem ratio to ensure suitable forage quality. A 33% reduction in rainfall has little impact on the forage quality of both species.

Compared to alfalfa, tedera shows early phenology for the reproductive stages from inflorescence emergence to ripening, with long flowering (from early April to mid-May) and important overlaps between flowering, fruit development and ripening. In the studied conditions, rainfall reduction seems to induce earlier inflorescence emergence and flowering in tedera with no effect in alfalfa. Competition delays flowering in tedera but not in alfalfa, and in both species fruit development and maturity are started later by plants under competition. In the conditions of this study, the probability of reaching reproductive stages is higher for tedera, and for both species, competition reduces the probability to reach such stages. The early start and length of the reproductive phenological stages of tedera might play an important role in its persistence and adaptation in Mediterranean environments with early drought onset.

It remains uncertain the ability of Lanza® tedera to persist and be productive during years of colder winters, and also its ability to offer green forage over the summer under different management strategies (defoliation time and frequency). Further research in long-term experiments is needed to confirm these results and test the response of tedera to years of contrasting meteorology in different seasons and locations, and its interaction with management. This would provide essential information to define optimal management strategies under variable meteorological conditions to fulfil its role as an out-of-season perennial legume in farming systems of the Mediterranean basin.

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Supplementary Material

Figure S 1. View of rainout shelter with storage tank and subplots.

Table S 1. Main effects and interactions of legume species (tedera vs alfalfa), culture (monoculture vs competition), rainfall (control vs reduced) on parameters of forage quality in leaf for 2021 and 2022.

Year	Target variables		Effects					
			Species	Culture	Rainfall	Species x Culture	Species x Rainfall	Culture x Rainfall
2021	CP	F	134.3	1.1	-	30.3	-	-
		P	<0.0001	0.3045	-	<0.0001	-	-
	NDF	F	80.8	11.8	-	31.5	-	-
		P	<0.0001	<0.0001	-	<0.0001	-	-
	ADF	F	79.8	60.6	-	14.6	-	-
		P	<0.0001	<0.0001	-	0.0008	-	-
	EDOM	F	6.3	7.1	-	-	-	-
		P	0.0196	0.0137	-	-	-	-
2022	CP	F	272.2	23.9	8.3	-	-	-
		P	<0.0001	<0.0001	0.0189	-	-	-
	NDF	F	99.4	0.1	-	-	-	-
		P	<0.0001	0.7682	-	-	-	-
	ADF	F	12.3	0.5	-	10.5	-	-
		P	0.0010	0.4696	-	0.0023	-	-
	EDOM	F	11.1	6.5	-	-	-	-
		P	0.0018	0.0139	-	-	-	-

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EDOM: Enzyme digestibility of organic matter.

Table S 2. Main effects and interactions of legume species (tedera vs alfalfa), culture (monoculture vs competition), rainfall (control vs reduced) on parameters of forage quality in stem for 2021 and 2022.

			Effects					
Year	**Target Variables**		**Species**	**Culture**	**Rainfall**	**Species x Culture**	**Species x Rainfall**	**Culture x Rainfall**
2021	**CP**	**F**	149.7	29.9	-	-	-	-
		P	<0.0001	<0.0001	-	-	-	-
	NDF	**F**	16.1	64.1	-	-	-	-
		P	0.0004	<0.0001	-	-	-	-
	ADF	**F**	7.7	108.3	-	-	-	-
		P	0.0110	<0.0001	-	-	-	-
	EDOM	**F**	11.1	94.8	0.6	-	7.0	-
		P	0.0025	<0.0001	0.4383	-	0.0131	-
2022	**CP**	**F**	249.8	24.1	-	16.3	-	-
		P	<0.0001	<0.0001	-	0.0002	-	-
	NDF	**F**	38.4	19.9	-	5.0	-	-
		P	<0.0001	<0.0001	-	0.0296	-	-
	ADF	**F**	53.5	12.9	-	4.6	-	-
		P	<0.0001	0.0008	-	0.0370	-	-
	EDOM	**F**	206.8	10.3	-	13.4	-	-
		P	<0.0001	0.0023	-	0.0006	-	-

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EDOM: Enzyme digestibility of organic matter.

Table S 3. Main effects and interactions of legume species (tedera vs alfalfa), culture (monoculture vs competition), rainfall (control vs reduced) on parameters of forage quality weighted by leaf and stem proportion for 2021 and 2022.

Year	**Target variables**		**Effects**					
			Species	**Culture**	**Rainfall**	**Species x Culture**	**Species x Rainfall**	**Culture x Rainfall**
2021	**CP**	**F**	126.6	30.1	-	22.7	-	-
		P	<0.0001	<0.0001	-	<0.0001	-	-
	NDF	**F**	106.0	42.2	-	-	-	-
		P	<0.0001	<0.0001	-	-	-	-
	ADF	**F**	11.3	141.9	-	-	-	-
		P	0.0021	<0.0001	-	-	-	-
	EDOM	**F**	38.3	26.5	-	-	-	-
		P	<0.0001	<0.0001	-	-	-	-
2022	**CP**	**F**	242.7	7.7	-	16.9	-	-
		P	<0.0001	0.0079	-	0.0001	-	-
	NDF	**F**	8.2	8.7	-	5.0	-	-
		P	0.0062	0.0049	-	0.0301	-	-
	ADF	**F**	26.9	8.0	-	5.1	-	-
		P	<0.0001	0.0067	-	0.0291	-	-
	EDOM	**F**	94.7	19.7	-	10.9	-	-
		P	<0.0001	<0.0001	-	0.0018	-	-

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EDOM: Enzyme digestibility of organic matter.

Table S 4. Results of zero-inflated poisson mixed models for the start and end of the phenological stages inflorescence emergence and flowering. Estimates ± standard error, z-values and p-values are shown for the conditional and zero-inflation model. Levels in squared brackets are the reference level related to the estimate shown. Note that in the zero-inflated model, negative estimates indicate lower probabilities of an extra zero count and positive estimates higher probabilities than the not shown level (Species: Alfalfa, Culture: Monoculture). Factors and interactions not shown were not retained in the final model.

	Inflorescence						**Flowering**					
	Start			**End**			**Start**			**End**		
Effect	**Estimate**	**Z**	**P**	**Estimate**	**Z**	**P**	**Estimate**	**Z**	**P**	**Estimate**	**Z**	**P**
Conditional model												
Intercept	4.46±0.02	191.5	<0.0001	4.68±0.02	293.2	<0.0001	4.76±0.01	392.8	<0.0001	4.97±0.01	519.3	<0.0001
Species[Tedera]	-0.31±0.02	-15.2	**<0.0001**	-0.17±0.01	-12.3	**<0.0001**	-0.19±0.01	-18.0	**<0.0001**	-0.07±0.01	-7.3	**<0.0001**
Culture[Competition]	0.07±0.02	3.2	**0.0013**	0.05±0.01	3.4	**0.0007**	0.03±0.01	2.6	**0.0100**	-0.01±0.01	-0.6	0.5599
Rainfall[Reduced]	-0.02±0.02	-1.0	0.3417	-0.01±0.02	-0.6	0.5736	-0.03±0.01	-2.8	**0.0058**	-0.001±0.01	0.2	0.8793
Species x Culture	0.06±0.02	2.7	**0.0069**	0.04±0.01	2.9	**0.0039**	0.02±0.01	2.0	**0.0486**	-0.02±0.01	-1.7	0.0969
Species x Rainfall	-0.05±0.02	-2.5	**0.0117**	-0.02±0.01	-1.8	0.0695	-0.03±0.01	-3.6	**0.0003**	-0.0±0.01	-1.7	0.0814
Zero-inflation model												
Intercept	-1.14±0.21	-5.4	<0.0001	-1.14±0.02	-5.4	<0.0001	-1.03±0.22	-4.7	<0.0001	-1.03±0.22	-4.7	<0.0001
Species[Tedera]	-1.15±0.21	-5.6	**<0.0001**	-1.16±0.02	-5.6	**<0.0001**	-1.31±0.23	-5.6	**<0.0001**	-1.31±0.23	-5.6	**<0.0001**
Culture[Competition]	0.92±0.23	4.6	**<0.0001**	0.92±0.02	4.6	**<0.0001**	1.54±0.24	6.4	**<0.0001**	1.54±0.24	6.4	**<0.0001**

Table S 5. Results of zero-inflated poisson mixed models for the start and end of the phenological stages inflorescence emergence and flowering. Estimates ± standard error, z-values and p-values are shown for the conditional and zero-inflation model. Levels in squared brackets are the reference level related to the estimate shown. Note that in the zero-inflated model, negative estimates indicate lower probabilities of an extra zero count and positive estimates higher probabilities than the not shown level (Species: Alfalfa, Culture: Monoculture). Factors and interactions not shown or with “-“ were not retained in the final model.

	Development of fruit						**Maturity of seed**					
	Start			**End**			**Start**			**End**		
Effect	**Estimate**	**Z**	**P**	**Estimate**	**Z**	**P**	**Estimate**	**Z**	**P**	**Estimate**	**Z**	**P**
Conditional model												
Intercept	4.87±0.01	474.3	<0.0001	5.12±0.01	548.7	<0.0001	5.03±0.01	545.7	<0.0001	5.13±0.01	516.1	<0.0001
Species[Tedera]	-0.12±0.01	-11.3	**<0.0001**	-0.00±0.01	0.3	0.7390	-0.07±0.01	-8.0	**<0.0001**	-0.01±0.01	-0.6	0.5160
Culture[Competition]	0.02±0.01	2.2	**0.0247**	-0.01±0.01	-1.4	0.1560	0.02±0.01	2.2	**0.0300**	-0.01±0.01	-1.2	0.2470
Rainfall[Reduced]	-0.02±0.01	-1.7	0.0811	0.01±0.01	0.7	0.4880	-0.01±0.01	-1.9	0.0585	0.00±0.01	0.3	0.7570
Species x Culture	0.01±0.01	0.6	0.5207	-	-	-	-	-	-	-	-	-
Species x Rainfall	-0.1±0.01	-1.6	0.1079	-	-	-	-	-	-	0.01±0.01	0.7	0.4950
Zero-inflation model												
Intercept	-0.97±0.22	-4.4	<0.0001	-0.97±0.22	-4.4	<0.0001	-0.6974±0.21	-3.4	<0.0001	-0.6974±0.21	-3.4	<0.0001
Species[Tedera]	-1.34±0.24	-5.6	**<0.0001**	-1.34±0.24	-5.6	**<0.0001**	-1.58±0.26	-6.0	**<0.0001**	-1.58±0.26	-6.0	**<0.0001**
Culture[Competition]	1.62±0.25	6.5	**<0.0001**	1.62±0.25	6.5	**<0.0001**	1.58±0.26	6.0	**<0.0001**	-1.58±0.26	-6.0	**<0.0001**

Table S 6. Estimated probability of an extra zero ± standard error (not reaching the phenological stage) from the zero-inflated models fitted for the start of the recorded phenological stages. Probabilities are shown by Species and Culture (averaged over Rainfall), Rainfall was not retained in the zero-inflation models (see Table S 4 and S 5).

Effects		**Stage**			
Species	**Culture**	**Inflorescence**	**Flowering**	**Development of fruit**	**Maturity of fruit**
Tedera	**Monoculture**	0.04±0.017	0.02±0.011	0.02±0.010	0.02±0.012
	Competition	0.20±0.055	0.31±0.066	0.33±0.068	0.33±0.068
Alfalfa	**Monoculture**	0.29±0.063	0.22±0.059	0.22±0.059	0.33±0.068
	Competition	0.72±0.060	0.86±0.046	0.88±0.043	0.92±0.034

CHAPTER VI. Investigating the potential of Sentinel-2 configuration to predict the quality of Mediterranean permanent grasslands in open woodlands

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Abstract

The assessment of pasture quality in permanent grasslands is essential for their conservation and management, as it can contribute to making real-time decisions for livestock management. In this study, we assessed the potential of Sentinel-2 configuration to predict forage quality in high diverse Mediterranean permanent grasslands of open woodlands. We evaluated the performance of Partial Least Squares Regression (PLS) models to predict crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM) by using three different reflectance datasets: (i) laboratory measurements of reflectance of dry and ground pasture samples re-sampled to Sentinel-2 configuration (Spec-lab) (ii) field in-situ measurements of grasslands canopy reflectance resampled to Sentinel-2 configuration (Spec-field); (iii) and Bottom Of Atmosphere Sentinel-2 imagery. For the three reflectance datasets, the models to predict CP content showed moderate performance and predictive ability. Mean R^2_{test}=0.68 were obtained using Spec-lab data, mean R^2_{test} decreased by 0.11 with Spec-field and by 0.18 when Sentinel-2 reflectance was used. Statistics for NDF showed worse predictions than those obtained for CP: predictions produced with Spec-lab showed mean R^2_{test}=0.64 and mean RPD_{test}=1.73. The mean values of R^2_{test}= 0.50 and RPD_{test}=1.54 using Sentinel-2 BOA reflectance were marginally better than the values obtained with Spec-field (mean R^2_{test}=0.48, mean RPD_{test}=1.43). For ADF and EDOM, only predictions made with Spec-lab produced acceptable results. Bands from the red-edge region, especially band 5, and the SWIR regions showed the highest contribution to estimating CP and NDF. Bands 2, blue and 4, red also seem to be important. The implementation of field spectroscopy in combination with Sentinel-2 imagery proved to be feasible to produce forage quality maps and to develop larger datasets. This study contributes to increasing knowledge of the potential and applicability of Sentinel-2 to predict the quality of Mediterranean permanent grasslands in open woodlands.

Keywords: Crude protein, fibre, digestibility, dehesa management, PLS, canopy reflectance

INTRODUCTION

Mediterranean permanent grasslands are presented in South Africa, California, Chile, southern Australia and in the Mediterranean basin itself (Cosentino et al., 2014), being the latter a global biodiversity hotspot due to its high number of endemic plants (Myers et al., 2000). Mediterranean grasslands play a vital role to satisfy the demand for animal products and the provision of ecosystem services such as carbon sequestration, control of soil erosion and wildfires, and biodiversity conservation (Porqueddu et al., 2016; Porqueddu et al., 2017). Permanent grasslands in the Mediterranean basin are especially important in open woodland, which cover about 3.1 million hectares in Spain and Portugal (Moreno and Pulido, 2009). This savanna-like agroforestry system, known as dehesa in Spain and *Montado* in Portugal is recognised as a highly biodiverse and multifunctional ecosystem, being an example of the integration of land-use and biodiversity conservation (Bugalho et al., 2011; Moreno and Pulido, 2009; Plieninger and Wlbrand, 2001). Dehesa and Montado farms are devoted to livestock rearing at low stocking rates whose feed relies mainly on rain-fed permanent grasslands and acorn of evergreen oaks (Plieninger and Wlbrand, 2001).

These grasslands are species-rich communities with high diversity and mainly dominated by annuals (Marañón, 1991; Olea and San Miguel, 2006) with a low yield that is strongly affected by the inter- and intra- annual variability of rainfall (Cosentino et al., 2014; Olea and San Miguel, 2006). Its high diversity together with the low synchrony among species and functional groups (Pérez-Ramos et al., 2020) contribute to increasing the spatial and temporal heterogeneity in pasture production and quality of these grasslands. Under future climate change conditions, the expected reduction of rainfall and the uncertainty on its inter-annual distribution (Giannakopoulos et al., 2009; Giorgi and Lionello, 2008) challenges the productivity of Mediterranean grasslands and hence, their capacity to sustain livestock production and their associated ecosystem services (Ma et al., 2017). In this context, the development of tools for continuous monitoring to provide real-time information for decision-making has become pivotal for the conservation and efficient management of permanent grasslands (Defourny et al., 2019; Gómez-Giráldez et al., 2019; Stumpf et al., 2020; Wolfert et al., 2017). The use of remote-sensing technologies is proving to be a promising tool to support efficient management of permanent grasslands through the provision of information about botanical composition, structure, phenology, quantity and quality (Ali et al., 2016; Fauvel et al., 2020; Gómez-Giráldez et al., 2020; Wachendorf et al., 2018). Quality can be defined as the set of properties inherent to grasslands that allow assessing their value. In this study, we refer to the quality of grasslands as their value to feed livestock. In this context, grasslands quality depends on properties such as nutrients concentration and physical composition that determine the intake, digestibility and partitioning of metabolized products (Dumont et al., 2015). Pasture quality is estimated by chemical analyses that typically report the content of crude protein or nitrogen, ash, fibre (acid detergent fibre and neutral detergent fibre), metabolisable energy and digestibility (Dumont et al., 2015; Pullanagari et al., 2013). There are other parameters relevant to livestock performance such as biomass. In this study, we will focus on quality, determined by the following pasture quality variables: crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM). Based on this definition of pasture quality and the variables studied, a pasture of high quality is characterised by a high content of CP and a low content of fibres which leads to a high EDOM. Assessment of pasture quality is essential for the management of grasslands as it is crucial to make real-time decisions for adjusting stocking rates, carrying capacity and additional feedstuff needs (Raab et al., 2020; Ramoelo and Cho, 2018; Starks et al., 2006). Laboratory chemical methods have been traditionally used to determine the quality of grasslands. Conversely to remote-sensing techniques, laboratory methods are costly, time-consuming and do not provide real-time information or possibilities for grassland quality mapping (Mansour et al, 2012; Starks et al., 2006). The amount of destructive sampling required to obtain representative data, the difficulty to access some sampling sites and the delay between sampling and availability of the results determine the low practicability of laboratory-based methods to assess pasture quality in Mediterranean grasslands (Pullanagari et al., 2013). Remote-sensing-based methods allow timely spatial predictions at a lower cost with the disadvantage of a lowered accuracy of the assessments (Pullanagari et al., 2013). However, the commented advantages might compensate the loss of accuracy and facilitate its implementation in farms of Mediterranean open woodlands. The development in the last decades of new remote-sensing technologies such as unmanned aerial vehicles (UAVs) equipped with hyperspectral cameras and machine learning algorithms enable more accurate predictions of grassland quality (Ali et al., 2016; Gao, 2006). UAVs can provide hyperspectral data at high spatial resolution; however, this technology needs to be operated by specialised companies, which might imply an economic constraint (Askari et al., 2019; Raab et al., 2020). In order to facilitate the applicability to farm management, the technology to implement must be low-cost and easy to use by farmers and grassland managers. Satellite images, although at a coarser resolution, can provide information for evaluating large areas. The Sentinel-2 satellite constellation, launched in 2015, has proven to be a promising tool for permanent grassland monitoring (Punalekar et al., 2018). Sentinel-2 is a sensor system developed by the European Space Agency (ESA) that provide freely available data worldwide with a revisiting time of 5 days and 13 spectral bands: four bands at 10 m, six bands at 20 m and three bands at 60 m spatial resolution (ESA, 2020). The spectral configuration of Sentinel-2, with the availability of three red-edge and two NIR bands has a great potential to study grassland quality due to the known sensibility of these regions of the spectrum to changes in nitrogen, chlorophyll and fibre content of plants (Curran et al.,1992; Frampton et al., 2013; Jacquemoud et al., 1995; Kawamura et al., 2008; Kokaly, 2001). It allows establishing relationships between reflectance at certain Sentinel-2 bands with grassland quality parameters CP, NDF, ADF and digestibility. In the last decades new algorithms of multivariate machine learning have arisen and gained popularity such as of Partial Least Squares Regression (PLS), random forest, support vector machine and artificial neural network. In particular, PLS has become the state-of-the-art method and one the most widespread and efficient techniques to analyse spectroscopy data (Kucheryavskiy, 2018; Wold et al., 2001). In addition to be one of the most studied and robust methods to deal with reflectance and forage data, its popularity is also due to the few hyperparameters that need to be set; only the number of latent variables (PLS components) used to decompose the predictors and responses which can be determined by automatically cross-validation (Kucheryavskiy, 2018).

Previous studies have aimed at establishing relationships between Sentinel-2 bands and grassland quality parameters. Ramoelo et al. (2015) demonstrated the potential of Sentinel-2 to predict leaf nitrogen content in rangelands from South Africa using simulated Sentinel-2 data, reporting R^2 values of 0.90 with high importance of the red-edge and shortwave region bands. Raab et al. (2020) investigated the use of Sentinel-1 and Sentinel-2 data to estimate pasture quantity and quality of semi-natural grasslands in the south-east of Germany and obtained high R^2 values for ADF concentration ($R^2 = 0.79$) and CP ($R^2 = 0.72$) with the bands from the narrow near-infrared and red-edge regions being the most important ones for the predictions. The utility of combining resampled field spectra data and actual satellite images to predict grass foliar nitrogen concentration, CP and NDF have also been explored in previous studies (Lugassi et al., 2019; Mutanga et al., 2015; Ramoelo and Cho, 2018). In some of these works, field spectroscopy has allowed the development of models based on spectroradiometer data resampled to Sentinel-2 configuration that can be used on Sentinel-2 images (Ramoelo and Cho, 2018). The comparison of models for pasture quality estimation using spectral data resampled to Sentinel-2 spectral configuration recorded with both, field spectroradiometers on grassland canopy and with Visible-Near-Infrared (Vis-NIRS) spectrometer on dried and ground pasture samples, can provide useful information. For example, they can inform about the potential of Sentinel-2 to predict pasture quality in high diverse Mediterranean permanent grasslands and be used to investigate the factors affecting the accuracy of models. In particular, field spectroscopy can contribute to developing more robust models. Some of the limiting factors to calibrate robust models with Sentinel-2 data are the labour of intensive sampling collection, the match between the sampled data and the reflectance at pixel level in heterogeneous grasslands, and the mismatch between sampling and Sentinel-2 data (Pullanagari et al., 2013; Ramoelo and Cho, 2018). Field spectroscopy data can be easily collected ensuring a good match between the sampled data and the reflectance recorded. It is more flexible in terms of collecting a wide range of data due to the finer spatial resolution (Pullanagari et al., 2012; Pullanagari et al., 2021). Also, it can help to overcome the constrain of scattered trees in open woodlands to obtain tree-free signal of reflectance to calibrate predictive models. Therefore, the combination of field spectroscopy resampled to Sentinel-2 spectral configuration and Sentinel-2 imagery can be an interesting approach to facilitate the use of Sentinel-2 in the management of grasslands from open woodlands.

Overall, there is a need for information about the potential of Setinel-2 for the management and conservation of high diverse permanent Mediterranean grasslands and the limitations affecting its implementation. The availability of high-quality pasture for grazing livestock has been pointed out as essential by stakeholders of agroforestry systems to ensure the system resilience (Camilli et al., 2018). Extensive systems such as Dehesa, rely mainly on pasture to feed the livestock (Olea and San Miguel, 2006). Therefore, it is of key importance for farmers of open woodlands grasslands to have timely information about the pasture quality. Through targeted management, remote sensing of pasture quality using Sentinel-2 data can contribute to a more efficient and competitive management of open woodlands farms. In the context of conservation, dehesas and montados are considered as habitats to be protected under the European Habitats Directive ("Dehesas with evergreen Quercus spp", code 6310) (Habitats Directive, 1992), which means that the member states are obligated to guarantee the good state of conservation of this habitat. Its conservation relies on the land use by grazing livestock in a human-managed extensive system and can therefore be altered by both overgrazing and abandonment (Moreno and Pulido, 2009). This association between conservation and land use has motivated its acknowledgement as a typical high nature value (HNV) farmland area (Paracchini et al., 2008, Ferraz-de-Oliveira et al., 2016). The continuous monitoring of the pasture quality of these systems using remote sensing can be implemented to facilitate a management compatible with the conservation of this high-interest ecosystem.

There are very few studies focused on monitoring pasture quality in high diverse permanent Mediterranean grasslands using remotely sensed data (Lugassi et al., 2019; Serrano et al., 2018). In this study, the potential and limitations of Sentinel-2 configuration to promote and facilitate the implementation of this technology in Mediterranean permanent grasslands is investigated. In particular, we evaluate the accuracy PLS models to predict CP, NDF, ADF and EDOM in high diverse Mediterranean permanent grasslands based on data from: (i) laboratory measurements of Vis-NIRS reflectance of dry and ground pasture samples resampled to Sentinel-2 configuration (ii) field in-situ measurements of grassland canopy reflectance resampled to Sentinel-2 configuration; (iii) and Bottom Of Atmosphere Sentinel-2 imagery. The contribution of specific Sentinel-2 bands to these predictions and the combination of satellite imagery with field spectroscopy for pasture quality estimation and mapping was also explored. This study will provide further insight into the potential of Sentinel-2 configuration to estimate pasture quality with PLS models and the implications and limitations of this technology for the management of Mediterranean permanent grasslands of open woodlands.

MATERIAL AND METHODS

Study area

The study was carried out on eight dehesa farms from the southern Spain region of Andalusia (Figure 1). This region is characterised by a Mediterranean continental climate with hot summers and cool rainy winters. Soils are mainly cambisols, with pH ~5-7, with a loamy-clay and loamy-sandy texture and low fertility (CSIC-IARA, 1989). The general topography is flat or characterised by a sequence of rolling hills and plateaus, with no pronounced slopes. The altitude ranges from 370 m.a.s.l. to 750 m.a.s.l. The mean annual rainfall varies from 516 mm to 620 mm along the farms and the mean annual temperature is around 17 °C (Global Climate Monitor, 2020). Two of the farms are devoted to sheep and Iberian pig breeding and the other six to cattle and Iberian pig breeding. Permanent grasslands of farms included plant communities dominated by annual low-grown herbs and grasses belonging to the *Helianthemetalia guttati*, *Malcomietalia* and *Poetalia bulbosae* alliances (Rodwell et al., 2002). Irrigated grasslands of *Trifolium repens* and *Lolium* spp. and permanent grasslands reseeded with commercial seed mixtures, mainly legumes, were also present on these farms.

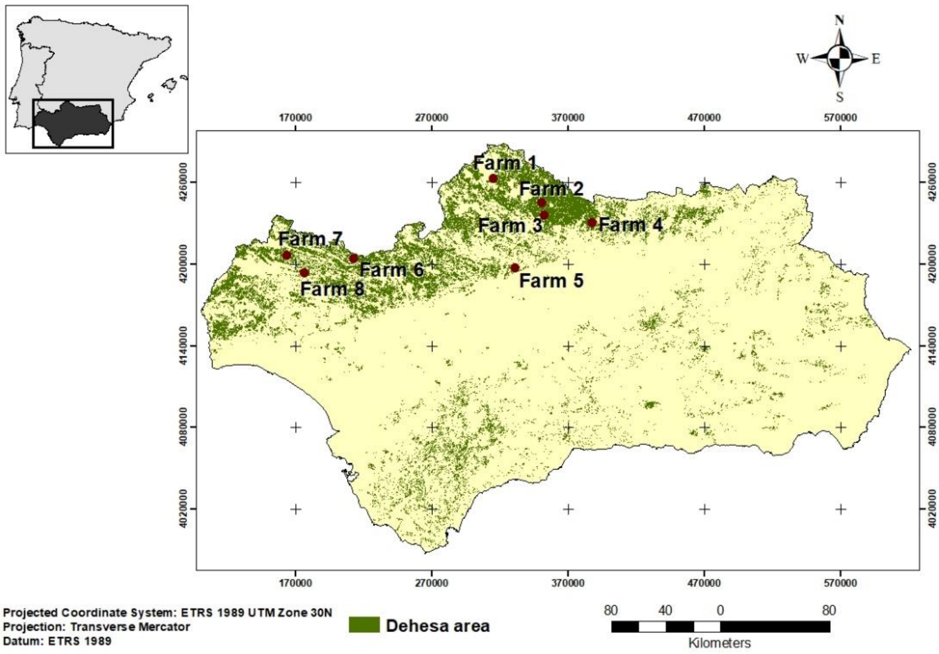

Figure 1. Location of farms in the dehesa area of Andalusia (Spain) where permanent grasslands were sampled. Source: Dehesa area illustrated in green is provided by the WMS of the dehesa systems distribution in Andalusia (REDIAM, 2020).

Grassland sampling and reference measurements

Grassland samplings were conducted during the growing season of 2012-2013 in farms 1-4 and during the growing season of 2018-2019 in farms 5-8. These samplings were designed to cover the different types of grasslands of dehesa farms throughout the growing season. It included permanent natural grasslands, reseeded grasslands with commercial seed mixes and irrigated grasslands. The pasture sampling carried out in 2012-2013 was designed to study the effect of grazing on pasture quality (Fernández et al., 2014).

For that purpose, one grazing exclusion plot of 4 x 8 m was established per farm in permanent natural grasslands from farms 1 to 4. Samples of pasture contained in sampling quadrats (0.4 x 0.4 m) were cut to ground level, four inside and four outside of the exclusion plots. Eight samples were collected per farm (farms 1 to 4) in five dates, January/February, March, April, May and June, which provided with 160 samples. After removing 35 samples from quadrats with extremely low pasture production or partially covering bare ground, 125 were available from the 2012-2013 sampling campaign. The design of the sampling campaign of 2018-2019 was governed by the presence of adjacent trees, which together with the geolocation error of 10 m 95.45% conf. level of Sentinel-2 (Gascon et al., 2017) may affect the reflectance of proximal pixels. 25 tree-free 20 x 20 m Sentinel-2 pixels were identified in total on fields of the farms 5 to 8 (13 in permanent natural grasslands, 9 in reseeded grassland and 3 in irrigated grasslands). Once located the pixels on the ground using a SXBlue II GPS sub-meter receiver (Geneq inc, Montreal, Quebec, Canada) we selected one the 10 x 10 m pixel, from now on referred to as “site”, and we randomly set four sampling quadrats (0.4 x 0.4 m) and the pasture contained in it cut to ground level. This sampling was repeated on three dates: November/December, February and May (Table 1 and Table S1) over the 25 pixels which provided 300 samples from 2018-2019. With both sampling campaigns, 425 samples were available for modelling (Table S1).

Table 1. Sentinel-2 data acquisitions and sampling date of the pasture samples.

Farm	Sampling date	Sentinel-2 data acquisitions	Spacecraft	Tile
Farm 5	2018-11-29	2018-11-30	Sentinel-2A	30SUH
	2019-02-19	2019-02-21	Sentinel-2A	
	2019-05-14	2019-05-14	Sentinel-2B	
Farm 6	2018-12-05	2018-11-28	Sentinel-2B	29SQC
	2019-02-25	2019-02-26	Sentinel-2B	
	2019-05-07	2019-05-07	Sentinel-2B	
Farm 7	2018-12-04	2018-12-06	Sentinel-2A	29SPC
	2019-02-26	2019-02-26	Sentinel-2B	
	2019-05-08	2019-05-05	Sentinel-2A	
Farm 8	2018-12-04	2018-12-08	Sentinel-2B	29SQB
	2019-02-26	2019-02-26	Sentinel-2B	
	2019-05-08	2019-05-07	Sentinel-2B	

The pasture samples were dried in the oven for 48 h at 60ºC and ground to pass through a 1-mm sieve. Then, the ground samples were subjected to chemical analysis for crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM) at the Laboratory of Animal Nutrition of SERIDA (Villaviciosa, Spain).

Spectra measurement and processing

From the 2012-2013 sampling campaign, field in-situ canopy reflectance was available for all samples (125 samples), whereas from the second sampling campaign (2018-2019) field in-situ reflectance was measured in the sampling performed in May (48 samples) on farm 5, which adds up to 173 samples (Spec-field from now on) (Figure 2). Spec-field reflectance spectra of the pasture contained within the sampling quadrats (0.4 x 0.4 m) was measured with an ASD FieldSpec Spectroradiometer (ASD Inc, Boulder, Colorado, USA) before cutting the pasture. Reflectance was measured in the whole range of 350 nm to 2500 nm with an interpolated resolution of 1 nm. This interpolation is done internally by the spectrometer, which has a resolution of 1.4 nm in the 350–1000 nm range (SWIR-1 sensor) and 2 nm in the 1000–2500 nm range (SWIR-2 sensor). The spectra were taken using a fibre optic probe attached to the pistol grip between 10h00 and 15h00 under clear sky conditions from a nadir orientation at 1.20 m height resulting in a 0.22 m^2 recording area. Four reflectance measurements were recorded for each sampling quadrat and white references were taken on a Spectralon panel (Labsphere, NorthSutton, NH) every four samples. The final reflectance measurement representative of each quadrant was the average of the four replicates.

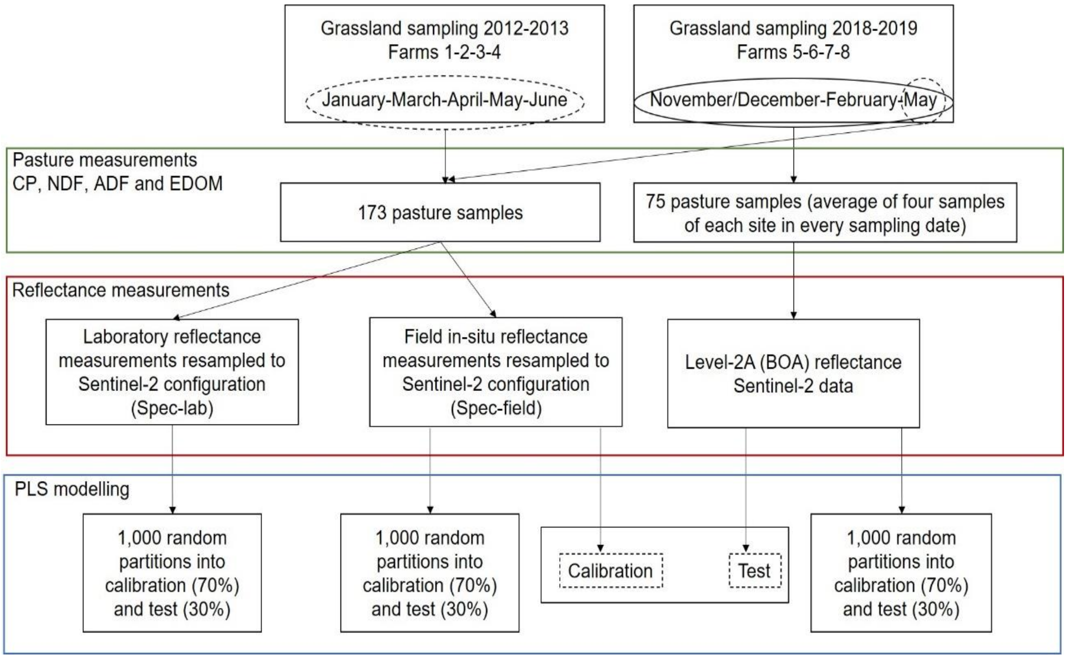

Figure 2. Conceptual framework of the modelling approach followed in this study.

For comparison purposes, the spectrum Vis-NIRS of these 173 pasture samples was recorded in a laboratory after drying and grounding the samples (Spec-lab from now on). Spec-lab measurements of the ground samples were scanned with a portable LabSpec 5,000 spectrometer (ASD Inc., Boulder, Colorado, USA) using IndicoPro 6.0 spectrum acquisition software. The equipment has a nominal spectral resolution of 3 nm at 700 nm (visible and near-infrared region) and 10 nm at 1,400 and 2,100 nm (short-wavelength infrared region). Internal data sampling rate of spectrometer (1.4 nm at 350–1,000 nm and 2.2 nm at 1,001–2,500 nm) is interpolated to 1 nm across the full spectral range (350-2,500 nm). The pasture samples were measured using High-Intensity Muglight, model-A122100, (ASD Inc.) equipped with a sapphire window using an ASDI sampling tray adapter with a quartz window having a 110 mm^2 spot diameter (ASD Inc.). Four replicates of each sample were scanned, two for each tray adapter by rotating it 45º. An average of 50 spectra was collected from each replicate and stored as an average spectrum. White reference scans were taken between every sample scan. The final spectrum was obtained by averaging the four replicates.

Both, Spec-field and Spec-lab measurements were then spectrally resampled to match the spectral specifications of the Sentinel-2 data (Table 2).

Table 2. Spectral and spatial specifications of the Sentinel-2 bands

Band	Band Centre (nm)	Bandwidth (nm)	Spatial resolution (m)
1-Coastal aerosol	443	20	60
2-Blue	490	65	10
3-Green	560	35	10
4-Red	665	30	10
5-Red-edge-1	705	15	20
6-Red-edge-2	740	15	20
7-Red-edge-3	783	20	20
8-Near-infrared (NIR)	842	115	10
8A-Narrow NIR	865	20	20
9-Water vapour	945	20	60
10-Short-wave infrared (SWIR)-cirrus	1375	30	60
11-SWIR-1	1610	90	20
12-SWIR-2	2190	180	20

The spectral resampling was performed using the "*resample2*" function of the *prospectr* package (Stevens and Ramirez-Lopez, 2014) in R v. 3.6.1 (R Development Core Team, 2019). This function resamples the original signal to a lower resolution signal (configuration of Sentinel-2 data in this case) using full-width half maximum (FWHM) values (Stevens and Ramirez-Lopez, 2014; Lugassi et al. 2019). Band 1, band 9 and band 10 were excluded from all the analyses in this study because of their coarser spatial resolution (60 m) as their main use is atmospheric applications. Level-2A Bottom Of Atmosphere (BOA) reflectance Sentinel-2 data (ESA, 2020) of the 25 selected pixels of 20 and 10 m resolution of farms 5 to 8 was extracted on the three different dates (Table 1) from Google Earth Engine platform (Gorelick et al., 2017). Data was extracted ensuring that the images were cloud- and cloud shadow-free over the study area. Bands of 20 m resolution were not resampled to 10 m spatial resolution to prevent the inclusion of tree signal from adjacent pixels. Level-2A product are derived from the associated Level-1C products and are systematically generated by ESA for the Euro-Mediterranean region since

March 2018 (ESA, 2020). Using Sen2Cor processor, the Level 1C input data are atmospheric-, terrain and cirrus corrected to deliver the final Level-2A products (Mueller-Wilm et al., 2017). The differences of the three reflectance datasets, Spec-lab and Spec-field, and their variations related to the pasture quality variables were evaluated. All analyses of this study were performed in R v. 3.6.1 (R Development Core Team, 2019). The K-mean cluster analysis using the variables of pasture quality was performed using the *kmeans* function of the *"factoextra"* package (Kassambara and Mundt, 2017). The number of clusters was defined by the "elbow method", being three the optimal number (Kodinariya and Makwana, 2013). The average reflectance spectra of the clusters obtained was calculated and plotted for the three reflectance datasets.

Modelling approach and statistical analysis

The potential of Sentinel-2 configuration to estimate pasture quality was compared between Spec-lab, Spec-field and Sentinel-2 BOA reflectance. Since Spec-field measurements were available from the 2012-2013 sampling (125 samples) and May from 2018-2019 sampling on farm 5 (48 samples), the same 173 samples were available to compare Spec-lab and Spec-field-based modelling as mentioned above (Figure 2). For Sentinel-2 BOA-based modelling, the pasture quality measurements of the four quadrats at each one of the 25 sites selected were averaged for every sampling, so a representative value of the quality variable at the site could be associated with its corresponding reflectance on each of the three dates (N=75). A table summarising the grassland samplings and data used for each reflectance dataset can be found in Table S1 of Supplementary Material.

When PLS and other machine learning algorithms are used, is key to develop a representative training dataset of the spectral features of the vegetation to calibrate robust models. Outliers in the training data can lead to biased predictions and underfit of the models (Wang et al., 2018). Outliers, which can be defined as samples departing from the bulk of the data can be produced by objects belonging to underrepresented data or samples from another population, laboratory errors or instrument errors (Martens and Naes, 1992; Valderrama et al., 2007). Since these sources of errors are common in Vis-NIRS spectroscopy and remote sensing, detection of outliers is commonly applied (Morellos et al., 2016; Xu et al., 2018; Xu et al., 2014). The novel outlier detection approach based on Projection-Based Modelling implemented in *"mdatools"* was used to exclude outlier samples (Kucheryavskiy 2019; Rodionova and Pomerantsev 2020). This method is based on the calculation of the score distance, orthogonal distance, and Y-residuals which are used to compute a so-called “total distance” of every sample, and an outlier threshold for outlier detection in regression problems (Rodionova and Pomerantsev 2020). The method consists of an iterative process that avoids masking and swamping effects in outlier removal. Further detailed information on the method and examples can be obtained in Rodionova and Pomerantsev (2020) and Kucheryavskiy (2020a).

Relationships between pasture quality variables (CP, NDF, ADF and EDOM) and reflectance were assessed using Partial Least Squares Regression (PLS) models performed with *“mdatools”* package (Kucheryavskiy 2019; Kucheryavskiy 2020b). Pasture quality measurements were log- or squared transformed when necessary to meet normality (Shapiro-Wilk test, $p>0.05$). Although non-normally distributed data can be used to fit PLS models, substantial loss of power of PLS models was observed when small datasets were used (Goodhue et al., 2012). PLS regression is a standard and widely-used tool in chemometrics (Wold et al., 2001) and prediction of pasture quality (Lobos et al., 2013; Parrini et al., 2018). It relates a vector **Y** with the response variable (CP, NDF, ADF or EDOM) and a matrix **X** with the predictor variables (reflectance values at the Sentinel-2 bands) by a lineal multivariate regression model. PLS decomposes **Y** and **X** on *n* orthogonal latent variables (LVs) (PLS-components) that maximise the covariance between response and predictors (Wold 1966; Zhou et al. 2019). By using these LVs, calibration equations can be created to predict the variable of interest **Y**, when a new **X** matrix is used. This method is highly effective in dealing with collinearity and a high number of predictor variables (Wold et al., 2001).

Data were randomly split into 70% for calibration and 30% for the external test. The models built with the calibration set were validated using leave-one-out (LOO) cross-validation. The optimal number of LVs was selected according to the Wold’s R criterion, which is based on the cross-validation. During LOO cross-validation, N-1 models (N being the number of samples) of one LV are built by iteratively withholding each sample (one sample at a time is kept out the calibration and used for prediction). The total Predicted Error Sum of Squares (PRESS) is calculated by summing the PRESS of the N-1 models. This procedure is repeated for *n* LVs until the ratio between PRESS value of the current and the next LV is the unity (Wold 1978; Li et al., 2002). This value denotes that the optimal number of LVs has been reached. The R^2, Root Mean Squared Error (RMSE), bias, Standard Error of Prediction (SEP) and Ratio of Predicted Deviation (RPD) were used to assess and validate the models. The R^2 is a measure of how well the data fit the regression model. The RMSE is used to assess the average accuracy of the prediction. The SEP indicates the precision of the predictions while the bias is the systematic difference between the predicted and the measured values. The RPD is the ratio of the standard deviation of the pasture quality variables from the SEP and is used to estimate the predictive ability of the model. An RPD value of one would mean that the SEP is equal to the standard deviation of the laboratory measurements and therefore, the model would have no use for predictions. Using R^2 and RPD, the prediction ability of the models can be classified following the thresholds proposed by Askari et al. (2015) and used by Askari et al. (2019) to assess PLS predictive models built with Sentinel-2 data: "excellent" ($RPD \geq 2.5$ and $R^2 \geq 0.8$), "good" ($2 \leq RPD < 2.5$ and $R^2 \geq 0.7$), "moderate" ($1.5 \leq RPD < 2$ and $R^2 \geq 0.60$) and "poor" accuracy($RPD < 1.5$ and $R^2 < 0.6$). Viscarra et al. (2006) classified RPD values as: RPD values between 1.4 and 1.8 indicate fair predictive ability, useful only for qualitative assessments and correlations while RPD values over 1.8 can be used for quantitative assessments.

The importance of the bands in the predictive models was assessed by looking at the regression coefficients of the PLS models. The inference of the regression coefficients was obtained by applying a Jack-Knifing approach implemented in *"mdatools"* package (Kucheryavskiy 2020b). Jack-Knifing is a resampling method used to calculate bias and the variance of estimates (Martens and Martens, 2000; Friedl and Stampfer, 2002). The resamples are generated by deleting single cases from the original sample (Friedl and Stampfer, 2002). In this case, Jack-Knifing was used to calculate a p-value of the regression coefficients of the PLS models by setting the Jack-Knifing option and full cross-validation in *"mdatools"* (Kucheryavskiy 2020b). A regression coefficient was considered as significant when the Jack-Knifing inferred p-value was <0.05.

Given the relatively small size of the datasets, the stability (or the robustness) of the models was tested by a bootstrap procedure. Following the approach of Mutanga et al. (2004) and Kawamura et al. (2008), the random partition in calibration (70%) and test (30%) was repeated 1000 times with replacement. A PLS model was built for each partition and the value of R^2, RMSE, RPD and regression coefficients were extracted. Mean and confidence intervals (CI) (2.5 and 97.5 percentiles) of R^2, RMSE, RPD and regression coefficients were reported. The number of LVs was described by the mode of the 1000 models. This procedure improves the methodology of previous studies in which a single value of these statistics is reported using a dataset of similar size (Askari et al., 2019; Lugassi et al., 2019; Ramoelo et al., 2015). The bootstrap procedure allowed determining the certainty of the results reported by each model (Mutanga et al., 2004; Mutanga et al., 2015). For the regression coefficients, the percentage of times that each band resulted as significant ($p<0.05$, according to Jack-Knifing procedure) in the 1000 models was also included in order to assess their stability.

Spatial predictions based on Sentinel-2 imagery using models calibrated with field spectrometry

The use of field spectrometry to calibrate PLS predictive models based on Sentinel-2 configuration was investigated by “combining” the whole Spec-field dataset (N=173) for calibration and cross-validation and the Sentinel-2 BOA dataset (N=75) for test. Since the reflectance spectra of the two datasets were acquired using different sensors, the representativity of both sets was checked by Principal Components Analysis (PCA) based on the covariance matrix, and the Distance of Mahalanobis (DMH). These algorithms helped to evaluate that the calibration set and the test set showed representativity (they are in the same spatial space and have the same variance–covariance) so that the prediction error depended on the model, and not on the differences of the data sets (Jouan-Rimbaud et al., 1998). The reflectance values of both sets were subjected to a PCA and plotted over a score plot to check the overlap of their spatial location. Additionally, the DMHs of each Sentinel-2 sample to the centre of the population was calculated in order to identify extremely different observations of the overall characteristics of the calibration dataset (Spec-field). According to Shenk and Westerhaus, (1996) criterion, sample spectra with DMH > 3.0 are not suitable to be predicted.

After the PLS model was calibrated and cross validated with the Spec-field dataset, the reflectance values of the Sentinel-2 data were used to test the predictive accuracy and precision over CP, NDF, ADF and EDOM. The R^2, RPD and RMSE of calibration, cross-validation and test of the model were reported. Spatial prediction maps at 10 m resolution over fields of irrigated and natural grasslands were computed using the calibration model and Sentinel-2 free cloud images on four different dates (2018-11-13; 2019-04-04; 2019-05-07; 2019-06-28). Pixels with tree influence on the reflectance value were excluded from the mapping. For ease of maps interpretation, CP values were back-transformed to the original scale with the follow adjustment for variance:

$$\hat{y} = 10^{\left(\hat{\Psi} + \frac{\frac{1}{N}\sum_{i=1}^{N}(\hat{\Psi}_i - \Psi_i)^2}{2}\right)},$$

where $\hat{y}$ are the back-transformed values, $\hat{\Psi}$ are the estimated and Ψ the observed values, both on the logarithmic scale. The expression of the numerator in the exponent represents the mean squared error of the test dataset.

RESULTS

Pasture quality values

An overview of the pasture quality values provided by the samplings is summarised in Table 3. Due to the diverse grasslands sampled and mainly to the different dates of sampling throughout the growing season, there was a large variation and wide range of values within all the pasture quality variables. CP showed a range of 24 points in the dataset used in combination with Spec-lab and Spec-field. For the same dataset, NDF showed a range of 46.5 points, while ADF reported a lower range, 29.1 points. The largest range was obtained for EDOM with a value of 47.8 points. The range of variables used for the analysis using Sentinel-2 images was lower. Overall, in both datasets, CP and EDOM values decreased from November to July while the opposite occurred with NDF and ADF (Figure S1). CP was the variable that showed the largest variation, with a coefficient of variation of 46.2% for the dataset used for Spec-lab and Spec-field and 34.3% for the reference measurements used with Sentinel-2 BOA reflectance data. NDF and ADF showed a CV of 21% for the Spec-lab and Spec-field dataset, slightly higher than the CV of EDOM (19%). The CV of the fibres was lower in the Sentinel-2 dataset. The EDOM values used for the Sentinel-2 dataset showed the lowest variation (CV=12%).

Table 3. Descriptive statistics of the pasture quality variables used to fit the PLS models.

	Pasture variables (% DM)	**Minimum**	**Mean**	**Maximum**	**Range**	**SD**	**CV**
Spec-lab and Spec-field (N=173)	CP	3.7	12.2	27.7	24.0	5.7	46.2
	NDF	24.9	51.2	71.3	46.5	10.7	20.9
	ADF	15.7	31.3	44.8	29.1	6.6	21.0
	EDOM	38.5	59.0	86.2	47.8	11.2	19.0
Sentinel-2 (N=75)	CP	5.3	13.4	26.0	20.7	4.6	34.3
	NDF	31.7	45.4	69.6	37.9	8.9	19.6
	ADF	16.8	30.1	39.0	22.2	5.4	17.9
	EDOM	42.7	62.8	80.6	37.9	7.9	12.6

CP-Crude protein; NDF-neutral detergent fibre; ADF-acid detergent fibre; EDOM-enzyme digestibility of organic matter; SD- Standard deviation; CV- coefficient of variation.

Figure 3 A illustrates the groups produced by a K-means cluster analysis performed using the pasture variables. Three clusters of samples can be identified showing clear differences in their pasture quality variables. The higher the CP and EDOM % of the clusters, the lower the NDF and ADF% and vice versa. The pasture quality can be ordered from lower to higher quality as: cluster L<cluster M<cluster H. These differences between clusters can also be observed in some regions of the mean reflectance spectra of Spec-lab, Spec-field and Sentinel-2 BOA of each cluster (Figure 3. b; c; d). Spec-field and Sentinel-2 mean reflectance is very similar, showing the comparable features in the spectra. For the three sets of reflectance datasets, the clusters M and H, with higher CP and EDOM, showed lower reflectance values than the mean reflectance of the cluster L along the visible (490-665 nm) region of the spectra, especially in band 4 (red-665 nm). In the case of Spec-lab, this difference remains in the Red-edge (705-783 nm) and NIR region (Figure 3. b). In Spec-field and Sentinel-2 datasets, the mean reflectance of cluster H was clearly higher in the Red-edge (705-783 nm) and NIR regions (842-865 nm) than the reflectance spectra of clusters L and M. Finally, the mean reflectance values of the three clusters for Spec-field and Sentinel-2 showed some differences in bands 11 and 12 (SWIR region;1610-2190 nm) while for Spec-lab reflectance, these values were identical.

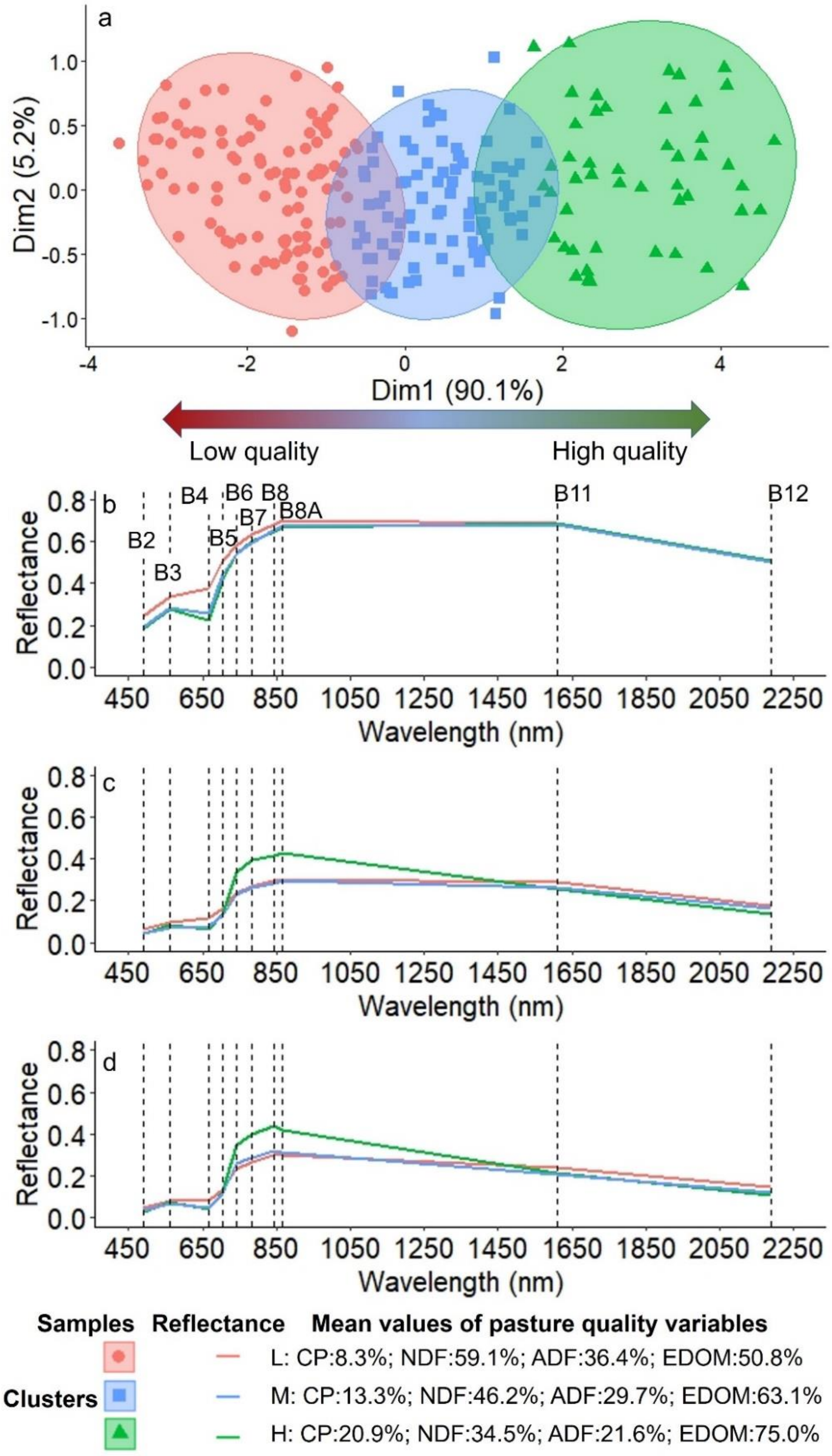

Figure 3. (a) Results of K-means clusters analysis performed using the following pasture variables: Crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and enzyme digestibility of organic matter (EDOM). The ellipse represents the 95% confidence interval. L, Cluster grouping low quality samples; M, Cluster grouping medium quality samples; H, Cluster grouping high quality samples. Mean reflectance of samples

included in each cluster at the centre of Sentinel-2 bands for: Spec-lab dataset (N=173) (b), Spec-field dataset (N=173) (c) and Sentinel-2 BOA dataset (N=75) (d).

Performance of models

Table 4 shows the mean, the confidence intervals of R^2, RMSE and the mode of nLV for calibrations and R^2, RMSE and RPD for cross-validations as summary of the 1000 PLS models built for each reflectance dataset (Spec-lab, Spec-field and Sentinel-2 BOA). To predict CP using Spec-lab reflectance data, the mode of LVs used to build the models was 5. For the rest of the models, the mode of LVs was 2-3 depending on the combinations of variables/reflectance data. Models fitted with Spec-lab data performed better than models based on Spec-field and Sentinel-2 BOA. Overall, means of R^2 and RPD decreased according to the reflectance data used following the order of Spec-lab>Spec-field>Sentinel-2 BOA. The opposite occurred with RMSE and the amplitude of the CI models fitted with Sentinel-2 data reported higher RMSE values and wider CI than models fitted with Spec-field and Spec-lab respectively. The statistics of models built with Spec-lab data reflect acceptable calibration models with R^2_{cv} and RPD_{cv} mean values over 0.60 and 1.60 respectively (Table 4).

Acceptable calibration models were obtained to predict CP with Spec-lab (mean R^2_{cv}= 0.69 and mean RPD_{cv}=1.80) and Spec-field (mean R^2_{cv}= 0.61 and mean RPD_{cv}=1.60). In models built with Sentinel-2 data for CP prediction, mean R^2_{cv} and mean RPD_{cv} were lower, 0.52 and 1.47 respectively, although the upper limit of CI of the 1000 models laid over 0.60 in R^2_{cv} and over 1.60 in RPD_{cv} (Table 4). Moderate results were obtained by models calibrated for NDF with Spec-lab (mean R^2_{cv}= 0.66 and mean RPD_{cv}=1.72) and poor with Spec-field (mean R^2_{cv}= 0.46 and mean RPD_{cv}=1.36). The statistics obtained when Sentinel-2 data was used to predict NDF (mean R^2_{cv}= 0.53 and mean RPD_{cv}=1.48) were slightly better than in models run with Spec-field, with values of the upper limits of the CI of 0.64 in R^2_{cv} and 1.67 in RPD_{cv} indicating that some models of the 1000 runs had moderate prediction ability. The calibration model statistics obtained for ADF were very poor for models fitted with Spec-field and Sentinel-2. The best statistics were obtained for EDOM prediction models that showed a mean R^2 value of 0.70 and RPD of 1.85. However, these statistics dropped to very low values when the models were built with Spec-field and Sentinel-2 reflectance data.

Table 4. Summary statistics of calibration models for Spec-lab, Spec-field and Sentinel-2 datasets. Mean and confidence intervals (95%) of R^2, RMSE, RPD and mode of nLV calculated from N=1000 random partitions of the datasets.

Spectral data	Variable	n	Mean	nLV	R^2 cal	RMSE cal	R^2 cv	RMSE cv	RPD cv
Spec-lab	**Log (CP) %**	124	1.04 [0.86]	5	0.73 (0.68-0.78)	0.10 (0.09-0.11)	0.69 (0.63-0.74)	0.11 (0.10-0.12)	1.80 (1.65-1.97)
	NDF^{2}g/10g	124	27.38 [44.12]	3	0.69 (0.64-0.75)	5.81 (5.17-6.26)	0.66 (0.61-0.72)	6.11 (5.55-6.53)	1.72 (1.60-1.89)
	ADF^{2}g/10g	124	10.24 [17.10]	2	0.66 (0.62-0.69)	2.31 (2.14-2.47)	0.63 (0.59-0.67)	2.39 (2.21-2.54)	1.66 (1.58-1.76)
	Log (EDOM) %	124	1.76 [0.35]	2	0.73 (0.67-0.79)	0.043 (0.037-0.047)	0.70 (0.65-0.76)	0.045 (0.040-0.049)	1.85 (1.69-2.04)
Spec-field	**Log (CP) %**	124	1.04 [0.86]	3	0.64 (0.58-0.70)	0.12 (0.11-0.13)	0.61 (0.54-0.67)	0.12 (0.12-0.13)	1.60 (1.48-1.74)
	NDF^{2}g/10g	124	27.38 [44.12]	3	0.49 (0.41-0.58)	7.39 (6.85-7.83)	0.46 (0.37-0.55)	7.66 (7.07-8.12)	1.36 (1.27-1.49)
	ADF^{2}g/10g	124	10.24 [17.10]	3	0.48 (0.41-0.57)	2.84 (2.56-3.04)	0.45 (0.37-0.54)	2.94 (2.65-3.15)	1.35 (1.27-1.47)
	Log (EDOM) %	124	1.76 [0.35]	3	0.53 (0.43-0.62)	0.056 (0.050-0.064)	0.49 (0.39-0.60)	0.058 (0.052-0.064)	1.41 (1.28-1.58)
Sentinel-2	**Log (CP) %**	55	1.10 [0.67]	3	0.62 (0.54-0.71)	0.10 (0.08-0.11)	0.52 (0.43-0.62)	0.11 (0.09-0.12)	1.47 (1.33-1.64)
	Log (NDF) %	55	1.65 [0.33]	3	0.61 (0.52-0.71)	0.05 (0.04-0.06)	0.53 (0.43-0.64)	0.06 (0.06-0.05)	1.48 (1.34-1.67)
	ADF^{2}g/10g	55	9.38 [12.10]	2	0.40 (0.29-0.51)	2.39 (2.14-2.64)	0.33 (0.22-0.43)	2.54 (2.28-2.77)	1.23 (1.14-1.34)
	EDOM %	55	62.7 [35.2]	2	0.40 (0.25-0.55)	6.01 (4.93-6.86)	0.32 (0.19-0.44)	6.43 (5.48-7.13)	1.23 (1.12-1.35)

CP-Crude protein; NDF-neutral detergent fibre; ADF-acid detergent fibre; EDOM-enzyme digestibility of organic matter; Mean - average of pasture variable measured with range of the observed values in squared brackets; nLV- mode of the number of latent variables; RMSE- root mean square error; RPD-ratio of predicted deviation; cal- calibration statistics; cv-cross-validation statistics. Values in brackets correspond to the confidence interval (2.5 and 97.5 percentiles). A different transformation was applied to NDF and EDOM for Sentinel-2 dataset.

Results of the predictions on the external test samples are presented in Figure 4 (a) (Spec-lab and Spec-field) and Figure 4 (b) (Sentinel-2). The figures show the density distribution of the 1000 values of R^2_{test}, $RMSE_{test}$ and RPD_{test} calculated by the bootstrap procedure, indicating the mean and the CI (2.5 and 97.5 percentiles). As for the calibration, the test statistics showed an overall decreasing performance, stability and certainty of the predictions following the order Spec-lab>Spec-field>Sentinel-2 BOA. This was denoted by lower R^2_{test} and RPD_{test}, higher $RMSE_{test}$ and wider CI. MeanR^2_{test}, RPD_{test} and $RMSE_{test}$ values were similar to their respective mean values of $RMSE_{cv}$ and $RMSE_{cal}$ for all variables and models built with the different reflectance datasets (Table 4). The mean systematic error given by the bias was negligible for all predictions. Therefore, the precision of the predictions (SEP_{test}) (data not shown) was very similar to their accuracy ($RMSE_{test}$).

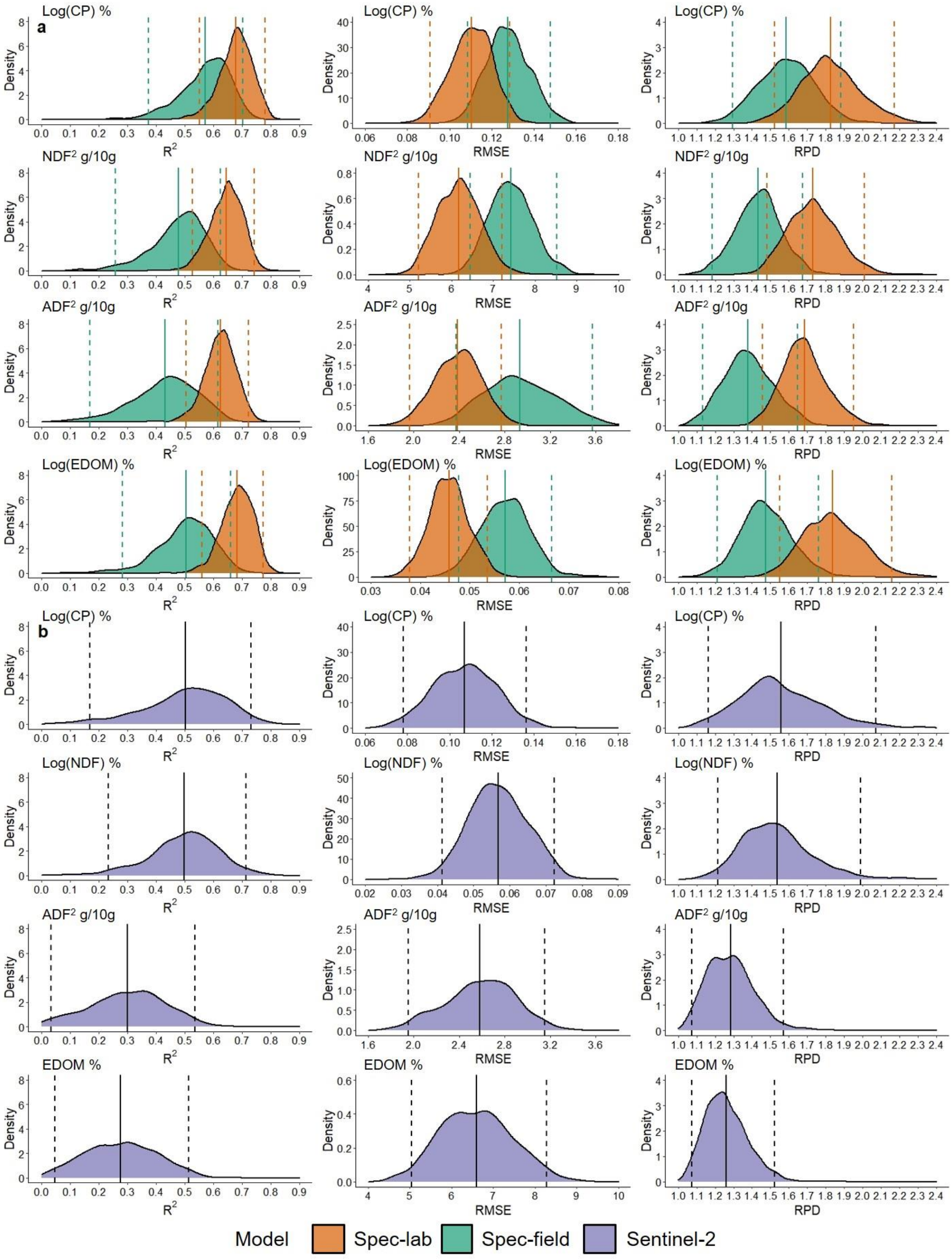

Figure 4. Density distribution of R2, root mean square error (RMSE) and ratio of predicted deviation (RPD) of predictions over Spec-lab and Spec-field test data (N=49) (a) and Sentinel-2 test data (N=20) (b). Calculated from N=1000 random partitions of the dataset. The predicted parameters are; crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and enzyme digestibility of organic matter (EDOM). Solid lines show the mean and dashed lines show the confidence intervals (2.5 and 97.5 percentiles). A different transformation was applied to NDF and EDOM for Sentinel-2 dataset.

For all reflectance datasets, the prediction ability of CP content was relatively moderate, with RPD_{test} mean values always over 1.50. Compared to the values obtained with Spec-lab reflectance (mean R^2_{test}=0.68), the mean R^2_{test} decreased by 0.11 with Spec-field and by 0.18 when Sentinel-2 BOA reflectance was used. The difference of the R^2_{test} mean value between models fitted with Spec-field reflectance (R^2_{test}=0.57) and models built with Sentinel-2 BOA reflectance (R^2_{test}=0.50) was marginal. However, Figure 4.(b) shows that the density distribution of R^2_{test} and RPD_{test} for CP predictions using Sentinel-2 BOA are specially flattened, presenting a wide CI, which indicates lower stability and certainty of the predictions. The average accuracy of the prediction, denoted by the mean $RMSE_{test}$ were 0.11, 0.13 and 0.11 (log-transformed values) for models built with Spec-lab, Spec-field and Sentinel-2 BOA reflectance respectively. Note that although the mean $RMSE_{test}$ of the models fitted with Spec-field was higher than the mean $RMSE_{test}$ of Sentinel-2 BOA models, the range of the data used has to be considered when comparing both models, being 0.86 for values used with Spec-lab and Spec-field vs 0.67 for reference data used with Sentinel-2 BOA reflectance (Table 4).

R^2_{test} and RPD_{test} statistics for NDF were worse than those obtained for CP using Spec-lab and Spec-field and very similar when Sentinel-2 BOA reflectance was used. Predictions made with Spec-lab reported a moderate predictive ability with mean R^2_{test}=0.64 and mean RPD_{test}=1.73. The mean values of R^2_{test} and RPD_{test} using Sentinel-2 BOA reflectance (mean R^2_{test}= 0.50 and mean RPD_{test}=1.54) were marginally better than the values obtained for predictions made with Spec-field (mean R^2_{test}=0.48 and mean RPD_{test}=1.43). However, it can be observed in Figure 4(b) that the distribution of R^2_{test} and RPD_{test} for Sentinel-2 BOA data is more flattened and has wider CIs, indicating lower stability and certainty of the predictions than those made with Spec-field.

For the rest of the variables, only predictions made with Spec-lab produced acceptable results. In the case of EDOM, the mean values obtained for R^2_{test} (0.68) and RPD_{test} (1.84) were similar to those obtained for CP predictions. Weak predictions were obtained for ADF and EDOM with Spec-field reflectance and especially with Sentinel-2 BOA data, showing very low predictive ability with mean values of R^2_{test}=0.30 and RPD_{test}=1.30.

Sentinel-2 bands contribution to pasture quality PLS models

The importance of the Sentinel-2 bands using Spec-lab, Spec-field and Sentinel-2 reflectance to predict CP, NDF, ADF and EDOM are presented in Figure 5 and Figure 6. Main attention will be focused on those models that reported better predictive accuracy and precision. To assess CP using Spec-lab, the bands of the Red-edge region, band 7 (783 nm), band 6 (740 nm), and especially band 5 (705 nm) showed, together with band 12 (SWIR-2, 2190 nm), the greatest coefficients and therefore impact on the predictions. All these bands were significant in most of the 1000 PLS models built for this dataset with the bootstrap procedure. The bands 4 (red, 665 nm) and 8a (narrow NIR, 865 nm) also showed influence on the PLS predictions, although the coefficients were inferior and the percentage of models in which these bands resulted significant were lower: 75.7% and 73.8% respectively. For the prediction of CP with Spec-field, the red-edge region and the band 4 (red, 665 nm) also showed a great influence on the prediction. As well as with Spec-lab, the band 5 (red-edge-1, 705 nm) had the greatest coefficient for CP prediction. The main difference between models built with Spec-lab and models built with Spec-field was in the SWIR region (bands 11 and 12). While for most of the PLS models of Spec-lab the band 11 (SWIR-1, 1610 nm) was not significant, for models built with Spec-field this band was significant in 74% of the models. The opposite occurred with the band 12 (SWIR-2, 2190 nm). In the case of the models fitted with Sentinel-2 BOA reflectance data for CP prediction, the most important bands for the predictions, as well as in models of Spec-field (Figure 5), were bands 4 (red, 665 nm), 5 (red-edge-1, 705 nm) and 11 (SWIR-1, 1610 nm) (Figure 6).

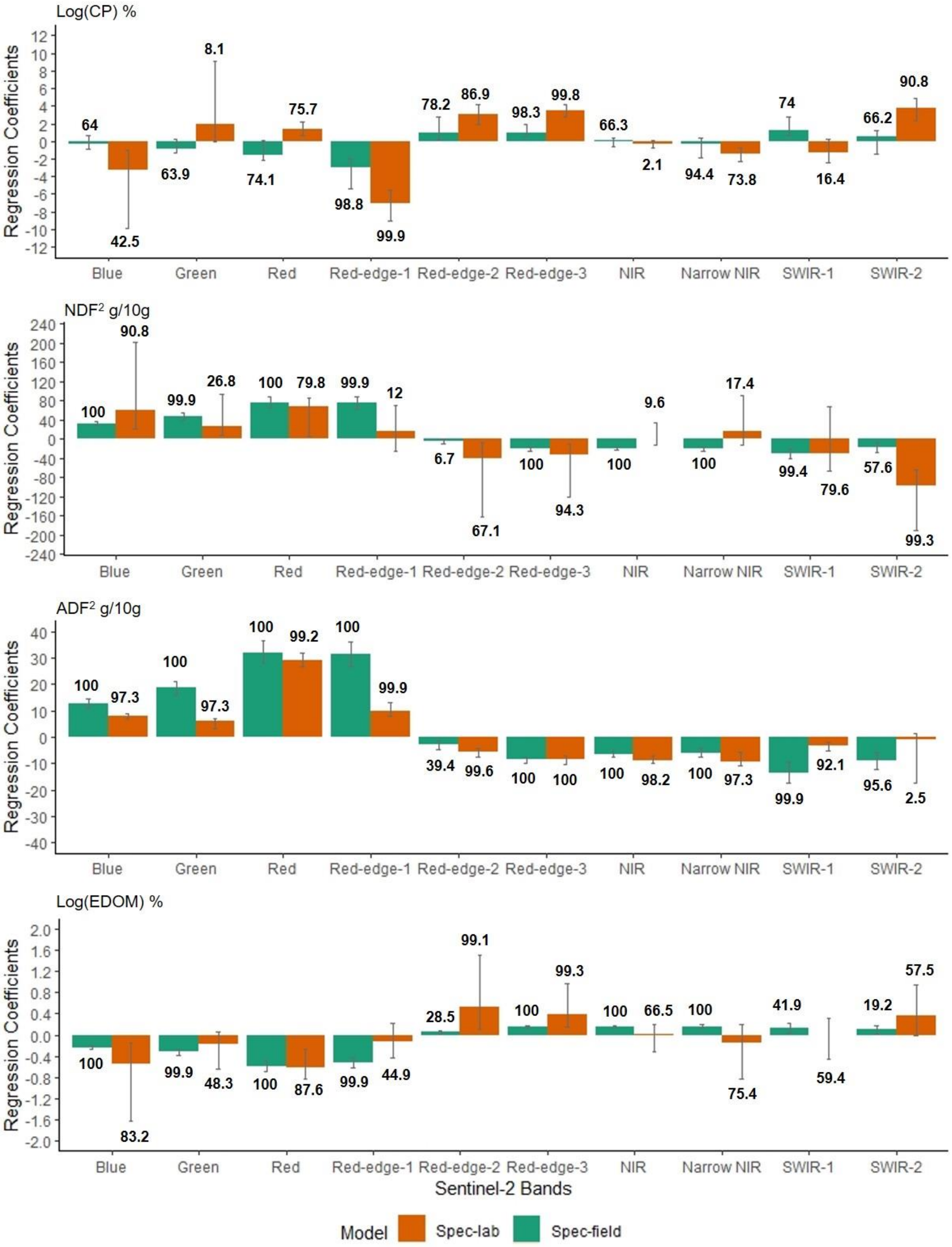

Figure 5. Regression coefficients averaged over N=1000 PLS models fitted with Spec-lab and Spec-field data. CP- Crude protein; NDF- neutral detergent fibre; ADF- acid detergent fibre; EDOM- enzyme digestibility of organic matter. Error bars indicate the confidence intervals (2.5 and 97.5 percentiles). Numbers indicate the percentage of PLS models in which this band resulted as significant based on jack-knifing procedure.

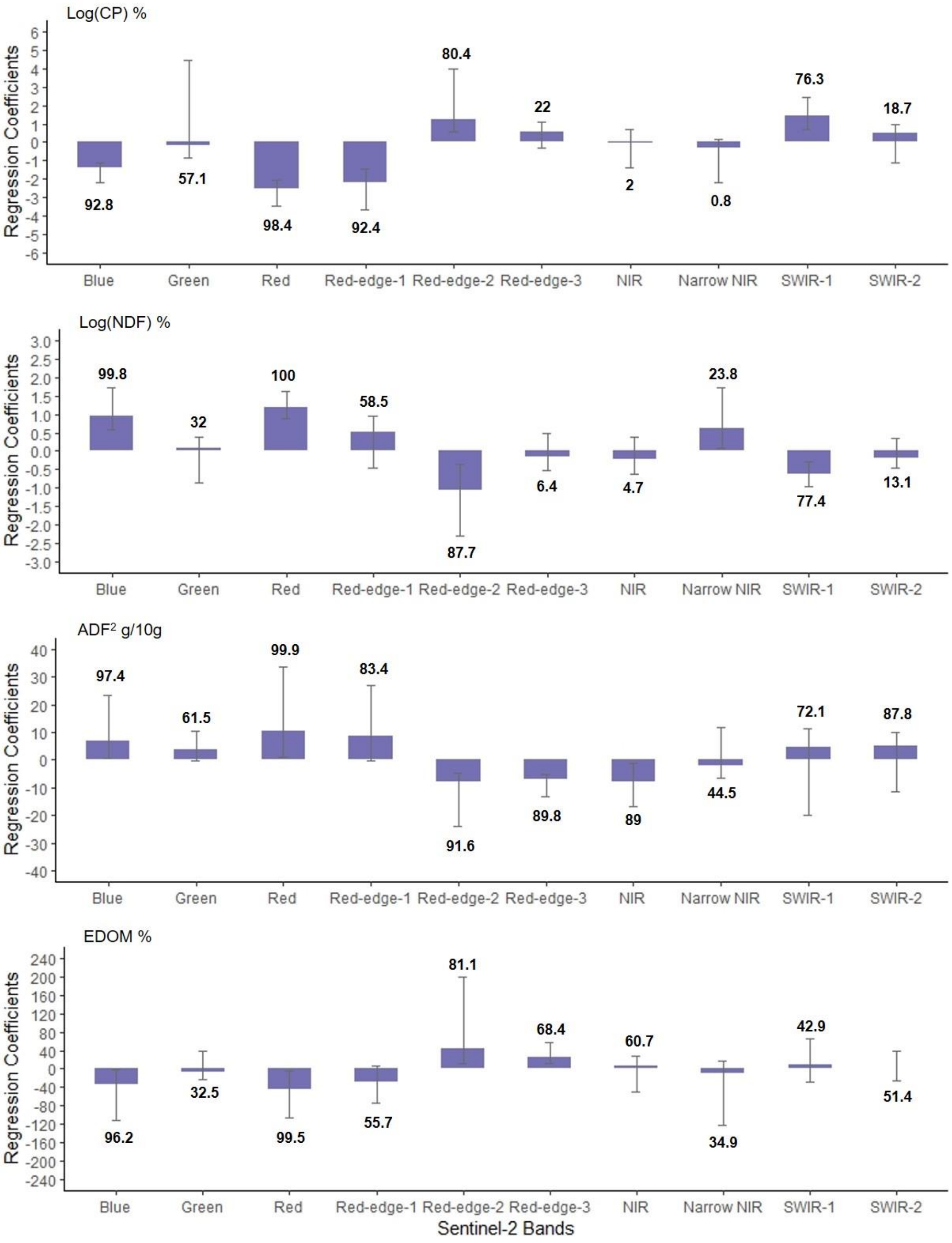

Figure 6. Regression coefficients averaged over N=1000 PLS models fitted with Sentinel-2 data. CP- Crude protein; NDF- neutral detergent fibre; ADF- acid detergent fibre; EDOM- enzyme digestibility of organic matter. Error bars indicate the confidence intervals (2.5 and 97.5 percentiles). Numbers indicate the percentage of PLS models in which this band resulted as significant based on jack-knifing procedure.

Bands 2 (blue, 490 nm), 4 (red, 665 nm) and 12 (SWIR-2, 2190 nm) were the most relevant for NDF prediction in models built with Spec-lab. Bands 7 (red-edge-3, 783 nm), and 11 (SWIR-1, 1610 nm) were also important. However, the CI of the bands was considerably wide. For the predictions of the fibres with Spec-field, all bands but the bands 6 (red-edge-2, 740 nm) and 12 (SWIR-2, 2190 nm) for NDF and the band 6 for ADF reported high coefficients, being significant in almost all the 1000 PLS models built. The most important bands were located at the visible region (bands 2, 3 and 4) and the band 5 (red-edge-1, 705 nm). The bands 2 and 4 from the visible region together with band 6 (red-edge-2, 740 nm) and 11(SWIR-1, 1610 nm) had the maximum importance on the PLS models developed for NDF with Sentinel-2 BOA data.

Regarding the predictive model of EDOM, only models run with Spec-lab reported acceptable statistics, the bands 2 (blue, 490 nm) and 4 (red, 665 nm) from the visible region and 6 and 7 from the red-edge region being the most important bands in the models.

Calibration of Sentinel-2 model based on field spectrometry

The result of the PCA performed with canopy reflectance spectra recorded in situ (Spec-field) and Sentinel-2 BOA from satellite images are presented in Figure S2. Both datasets showed overlap, which indicates that they are in the same spatial location. The three first principal components were used to calculate the DMH as they explained 99% of the variance. The DMH to the centre of the population of calibration (Spec-field) was always below 3.

Calibration and cross-validation statistics of the models built with the Spec-field reflectance dataset are presented in Table 5. Moderate performance was obtained for the model calibrated for CP with R^2=0.60 and RPD=1.59 of cross-validation. Weak models were produced for the rest of the variables.

Table 5. Summary statistic of calibration models fitted with Spec-field data to predict over Sentinel-2 data.

Spectral data	Variable	n	Mean	nLV	R2 cal	RMSE cal	R2 cv	RMSE cv	RPD cv
Spec-field	Log (CP) %	173	1.04 [0.87]	3	0.62	0.12	0.60	0.13	1.59
	NDF^2g/10g	173	27.45 [44.72]	3	0.49	7.45	0.46	7.64	1.37
	ADF^2g/10g	173	10.24 [17.59]	3	0.48	2.86	0.45	2.93	1.36
	Log (EDOM) %	173	1.76 [0.351]	3	0.51	0.057	0.49	0.059	1.40

CP-Crude protein; NDF-neutral detergent fibre; ADF-acid detergent fibre; EDOM-enzyme digestibility of organic matter; Mean - average of measurements with range in squared brackets; NLV- number of latent variables; RMSE- root mean square error; RPD-ratio of predicted deviation; cal- calibration statistics; cv-cross-validation statistics.

Figure 7 shows the predicted vs measured plots for each pasture quality variable (CP, NDF, ADF and EDOM) of the test set corresponding to the Sentinel-2 BOA reflectance data. As for the cross-validation, only predictions of CP reported statistics with values close to “moderate” predictive ability with RPD value over 1.50 and R^2 of 0.54. The rest of the models for NDF, ADF and EDOM showed poor predictive ability with RPD values below 1.40 and high RMSE values that indicate low prediction accuracy.

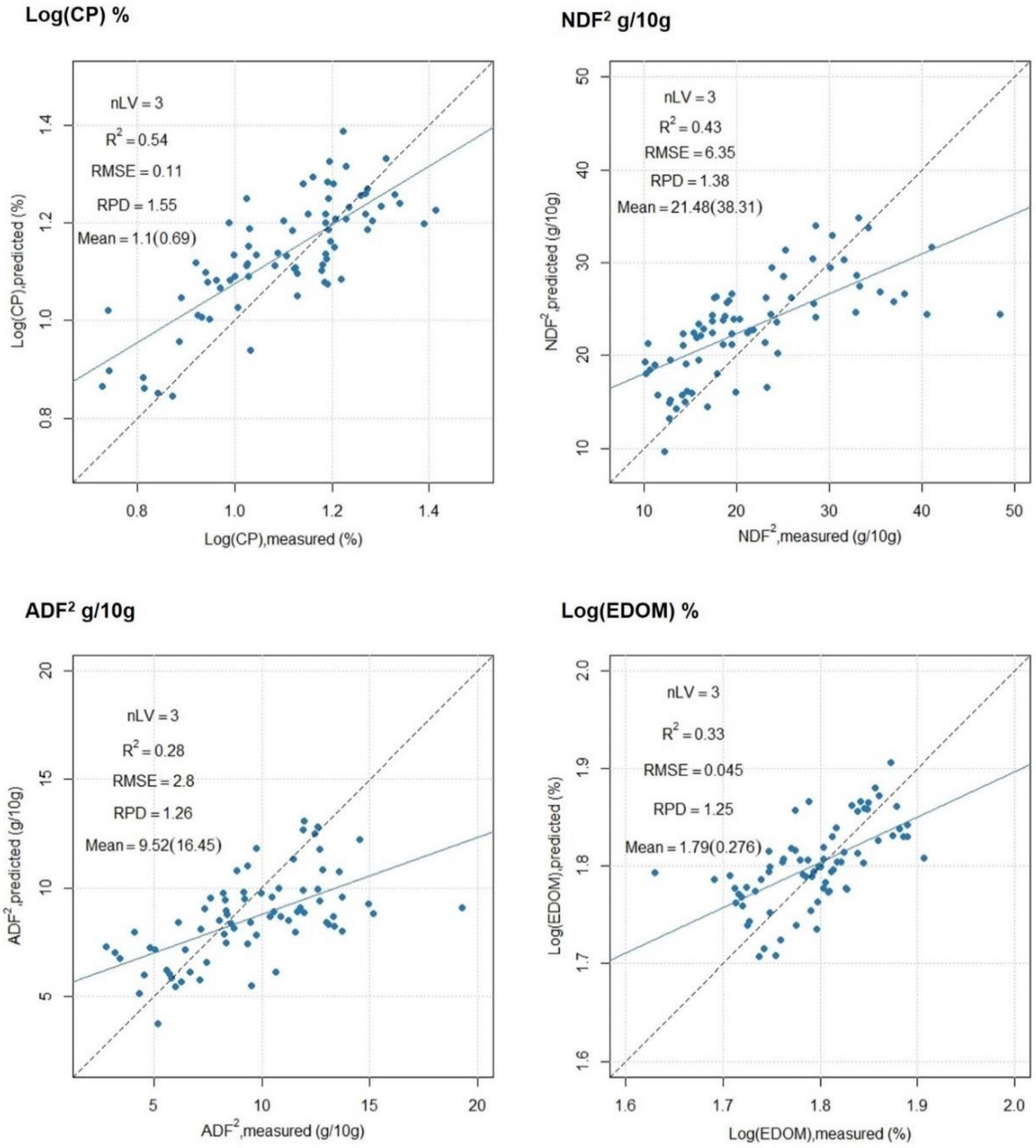

Figure 7. Predictions of CP-Crude protein; NDF- neutral detergent fibre; ADF- acid detergent fibre; EDOM- enzyme digestibility of organic matter using PLS models fitted with Spec-field data (Table 4) and Sentinel-2 data for prediction (N=75). Mean - average of measurements with range in brackets; nLV- number of latent variables; RMSE- root mean square error; RPD- ratio of predicted deviation. Dashed line represents the 1:1.

According to the thresholds to classify spatial prediction models previously explained (Askari et al., 2019), being “moderate”=1.5 ≤ RPD<2 and $R^2 \geq 0.60$, and the results obtained for the different variables (Table 5), only maps for CP were generated. Spatial predictions of CP based on Sentinel-2 images in fields of irrigated and natural grasslands from farm 5 on different dates are presented in Figure 8. Differences over time and between fields can be observed. In November (2018-11-13), at the beginning of the growing season, natural grasslands had higher CP content (18-14%) than irrigated grasslands (14-10%). On the next date, in April (2019-04-04) when pastures were at the peak of growth, both fields showed similar CP content (20-16%). From that date, the CP content in the field of natural grasslands drops to values below 6% in June (2019-06-28) whereas the irrigated field keeps CP values over 12%. It can also be observed that the CP content in the irrigated field is more homogeneous than in natural grasslands (Figure 8).

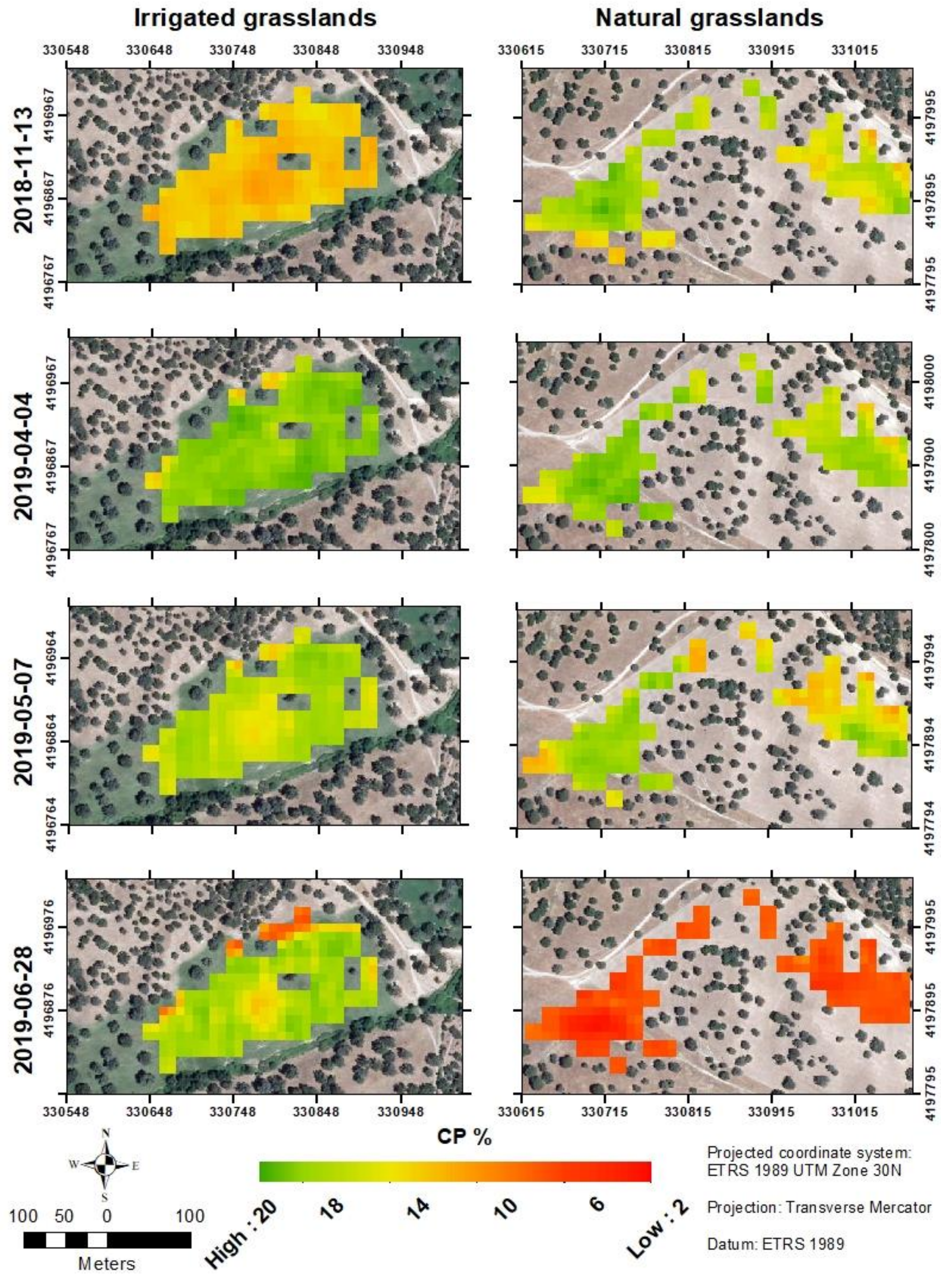

Figure 8. Spatial predictions of crude protein (CP) for four different dates in fields of irrigated and natural grasslands in farm 5. Predictions made using a PLS model fitted with Spec-field data (Table 5) and Sentinel-2 images for prediction. Source: Background image; aerial orthophotography at 0.5 m resolution from July of 2016 (Linea, 2020).

DISCUSSION

Potential of Sentinel-2 configuration for estimating pasture quality and modelling approach used.

This study explored the potential of Sentinel-2 configuration to assess pasture quality in high-diversity grasslands of dehesa systems using PLS models. The values of the pasture quality variables analysed (Table 3) fall within the usual range of pasture quality parameters of Mediterranean permanent grassland in dehesa systems (Perez Corona et al. 1998; Vázquez-De-Aldana et al., 2008). We used three different reflectance datasets to investigate the capabilities of Sentinel-2 configuration to predict pasture quality. Since Vis-NIRS techniques with the whole spectral range (350-2500 nm) are used in laboratories for the chemical analysis of the variables studied here, the use of Spec-lab reflectance allowed to identify the maximum potential to establish a relationship between pasture quality variables and reflectance for Sentinel-2 bands. This reflectance data is free of factors such as water content or soil and atmospheric influences that affect field in-situ canopy reflectance and satellite-based predictions (Mansour et al., 2012). Therefore, the results obtained using Spec-lab reflectance help to inform the maximum accuracy that might be achieved with Sentinel-2 configuration for predictions of the quality variables for the studied pastures. According to Viscarra Rossel et al. (2006), models with RPD values between 1.4 and 1.8 could be used for assessment and correlations while values over 1.8 may indicate that quantitative predictions are possible. The mean RPD_{test} values obtained with Spec-lab were 1.84 for EDOM and 1.82 for CP. Lower values were obtained for the fibres, with 1.73 for NDF and 1.68 for ADF. These results illustrate that the potential of Sentinel-2 configuration to predict pasture quality in Mediterranean permanent grasslands may be limited to moderate prediction models that could allow qualitative assessments. The mean R^2_{test} values ranged between 0.68-0.64, which is considerably lower than R^2 values obtained for predictions of pasture quality variables using the whole spectral range (García-Ciudad et al., 1993). Although Sentinel-2 configuration has improved the spectral and temporal characteristics compared with Landsat and SPOT to assess biophysical variables in vegetation (Frampton et al., 2013), the reduced spectral resolution is a basic limiting factor to obtain precise quantitative predictions. This limitation needs to be considered when planning the tool to be used for pasture quality assessment in order to adjust the specifications to the precision required. For example, Askari et al. (2019) reported better predictions of CP in mixtures of clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne*) using hyperspectral imagery (R^2_{cv}=0.82 and RPD_{cv}=2.51) compared to the predictions made with Sentinel-2 imagery (R^2_{cv}=0.62 and RPD_{cv}=1.60). However, as the same authors stated, the cost of acquiring hyperspectral images is a major limiting factor for the evaluation of grassland (Askari et al., 2019; Mansour et al., 2012) and it might not compensate for the increment of precision. The high-priority candidate mission of the European Space Agency: Copernicus Hyperspectral Imaging Mission for the Environment (CHIME) (Nieke and Rast, 2018) is expected to deliver a new-generation imaging spectrometer. That hyperspectral spectrometer will cover the full spectral range between 400 and 2500 nm with contiguous narrow bands at a spatial resolution similar to Sentinel-2 (30–60 m) (Nieke and Rast, 2018). This new generation of hyperspectral satellites could overcome the constrain of the spectral coverage of multispectral satellites and allow the improvement of grasslands quality assessments from qualitative to quantitative (Rast et al., 2019). Another constraint for the remote sensing of permanent grasslands in open woodland as dehesa and montado is the presence of scattered trees. The spatial resolution of Sentinel-2 forces to seek open pasture areas where there is no influence of trees on the pixel reflectance. This tool could have no possibility of application in dehesa and *Montado* farms with high tree cover and no open areas. A feasible option might be the analysis of pixels as representative observations in sampling areas without tree cover that could characterise the status of the rest of the grassland. However, the information provided might not be representative of the pasture below the canopy since its phenology and quality might differ from the pasture beyond the tree canopy (López-Carrasco et al., 2015). Other satellite constellations with finer spatial resolution such as WorldView-2 (Adjorlolo et al., 2015) or the use of aircraft vehicles (Askari et al., 2019; Lu and He, 2017) could partially overcome this limitation and provide more data although at a higher cost. The development of pasture quality maps using Sentinel-2 imagery is restricted to areas without trees (see 4.3. Use of field spectrometry for Sentinel-2 model calibration and pasture quality mapping). Further research is needed to isolate the influence of tree canopy reflectance in pixels partially covered by trees.

One of the factors affecting the prediction accuracy and model performance is the high species diversity of the permanent grasslands analysed in this study. Heterogeneous pastures with multiple functional groups and different phenological stages might produce

confounding effects on the relationship between pasture quality variables and reflectance (Fava et al., 2009;
Kattenborn et al., 2019; Tong and He, 2017; Zhou et al., 2019). This factor is expected to be especially important when using canopy reflectance and satellite imagery. Differing leaf area index of grassland communities with multiple species may affect the canopy reflectance and therefore the potential to estimate plant components (Kattenborn et al., 2019; Pellissier et al, 2015). In fact, one of the main limitations for remote-sensing of foliar chemistry is that the chemical composition can be confounded by phenology or canopy geometry (Curran, 1989). Contrasting results have been obtained when developing predictive models for specific pastures and/or development status. Biewer et al. (2009) reported improved estimates of CP by using legume-specific calibrations. Zhou et al. (2019) did not find any influence of mixture types and developmental stages on CP predictions, although the pastures studied were less heterogeneous. Zeng and Chen (2018) obtained greatly improved estimates of CP, NDF, and ADF in wheatgrass communities when data from different growth stages were included in the models compared to those developed with data from individual growth stages. However, the differences may be attributed to the wider range of the dataset. Our study provides an overview of the potential of generalised models to predict forage quality in Mediterranean permanent grasslands using samples from high-diversity and different phenological stages. Further research is needed to explore the possibilities to improve predictions using separate prediction models for specific phenological stages or mixtures in high-diversity Mediterranean grasslands. The differences in model performance of Spec-lab-based models and models built with Spec-field and Sentinel-2 BOA seem also to point to the influence of water content, leaf area index and soil background. Although these factors were not explicitly investigated in this study, canopy reflectance spectra is clearly affected by the presence of bare soil over the target areas (Atzberger and Richter, 2012; Jacquemoud et al., 2009; Mansour et al., 2012; Yue et al., 2020). Soil moisture and plant water content can also be a source of noise in the reflectance spectra of Spec-field and Sentinel-2 (Kokaly, 2001; Ramoelo et al., 2011). Water absorbs in similar regions of the spectra than other organic compounds, which results in a combined or masked effect on the reflectance (Kokaly, 2001). For example, the O--H is a common absorption bond for water and lignin (Curran, 1989). The controlled conditions in which Spec-lab reflectance data was acquired and the use of ground dried samples prevents it from introducing this

noise. Overall, the differences in mean R^2_{test} and mean RPD_{test} values between models built with Spec-field and models constructed with Sentinel-2 were marginal. This might indicate a low influence of the atmospheric interference in Sentinel-2 BOA data. However, the CIs of the predictions made with Sentinel-2 denote a higher uncertainty of these models. The differences between models run with Sentinel-2 data and Spec-field can be also affected by the different number of samples. The higher number of samples of Spec-field dataset (173) compared to Sentinel-2 BOA dataset (75) may account for the narrower CIs in models built with Spec-field, which allowed the development of more robust models. Another factor influencing the accuracy and prediction ability of models constructed with Sentinel-BOA data might be the sample collection. Since the reference measurements used are a mean value of four samples, a standard error is associated to this value. Thus, the selection of homogeneous areas with common management and a suitable sampling design are essential to reduce the degree of uncertainty (Raab et al., 2020).

Calibrations built to predict NDF and especially CP showed promising test results that suggest the possibility of performing qualitative assessments based on Sentinel-2 imagery. The mean R^2_{test} obtained for CP predictions using Spec-field reflectance was similar to previously reported values. Ramoelo et al. (2015) obtained values of R^2=0.46 in cross-validations of PLS models of 5 LV to predict leaf nitrogen in rangelands using field in-situ reflectance resampled to Sentinel-2 configuration. Adjorlolo et al. (2015) obtained R^2=0.50 in cross-validation of PLS models fitted with field canopy reflectance resampled to WorldView-2 band settings to predict CP in grassland communities of *Festuca costata*, *Themeda triandra* and *Rendlia altera*. Also, similar values of R^2 (0.60) in cross-validations were reported by Kawamura et al. (2017) in PLS models with 7 LVs using the whole spectral range (400-2350 nm) and band selection procedures to predict CP in mixed sown pastures. Askari et al. (2019) obtained slightly better cross-validation statistics (R^2=0.62 and RPD=1.60) using Sentinel-2 imagery and PLS models to predict CP compared to the test results of our study (mean R^2_{test}= 0.50 and mean RPD_{test}=1.56), although the homogeneity of the studied pasture (a mixture of clover and perennial ryegrass) may explain the improved results.

Few studies have investigated the potential of Sentinel-2 configuration to assess fibre content in pastures. We obtained mean R^2_{test}=0.48 and mean RPD_{test}=1.43 using Spec-field to predict NDF. Lower statistics (bootstrapped mean R^2=0.31) in PLS models to predict NDF were obtained by Kawamura et al. (2017) using the whole spectral range (400-2350 nm, resampled at 5 nm) and band selection, whereas Zeng and Chen (2018) reported improved R^2 values of 0.77 and 0.80 for NDF and ADF using data of three different growth stages. Acceptable values were reported by Adjorlolo et al. (2015) (R^2=0.52) with field spectroscopy and WorldView-2 configuration to predict NDF. We obtained similar goodness to fit using Sentinel-2 BOA imagery (mean R^2_{test}=0.50). The poor results obtained for ADF predictions contrast with the results of the studies mentioned above. Raab et al. (2020) reported high R^2 values (0.79) from ADF predictions using Sentinel-2 and Sentinel-1 data and random forests regressions algorithms. The inclusion of radar data from Sentinel-1 could contribute to estimating ADF since it provides information of the height of the pasture, which is directly related to the cellulose and lignin content. The same study also obtained good predictive models for CP (R^2=0.72). The authors state that Sentinel-2 data might be sufficient to predict forage quality (Raab et al., 2020), thus the improved results could respond to the use of the random forest algorithm along with the higher homogeneity of the grasslands studied and the dense temporal component of their dataset. They also demonstrated that the inclusion of indices and simples ratios combined with variable selection techniques can significantly improve the prediction accuracy of the models (Raab et al., 2020). Although the addition of several indices and ratios together with single bands can lead to multicollineality problems, variable selection techniques can overcome this problem while improving the prediction ability of the models (Belgiu and Drăguţ, 2016; Frenich et al., 1995; Kawamura et al., 2008; Raab et al., 2020; Santos-Rufo et a., 2020). Ramoelo et al. (2015) also obtained better results predicting nitrogen content by using random forest (R^2=0.90 and RMSE=0.04) than with PLS regression (R^2=0.46 and RMSE=0.14). We decided to use PLS regression for the sake of simplicity and to ease the interpretation of results. The PLS models were easy to calibrate and the results were also simple to understand. The similar results between cross-validation and test indicated that there was no overfitting of the models. Machine-learning techniques such as random forest or support vector machine could be an asset to detect the non-linear relationship between pasture quality and canopy reflectance (Zhou et al., 2019). However, these techniques can be sensitive to complex modelling approaches (Igne et al., 2010) while techniques such as PLS regression are simpler to implement and compute (Askari et al., 2019), which might facilitate its use by consultancies and grasslands managers. Studies comparing

PLS, random forest and support vector machine could be helpful to find the optimal model to implement with Sentinel-2 data in terms of accuracy and ease of use. Finally, although EDOM of pasture is positively correlated to CP and negatively to NDF and these two variables were acceptably predicted, weak results were obtained for EDOM. The reasoning behind this could be that EDOM is not a variable directly related to a nutrient of the plants as is the case of CP with nitrogen, but rather it is a result of the fibre and CP content. This, together with the differences in digestibility of the multiple species/ functional groups and their phenology in the sampled grasslands could account for the bad predictions of this variable using Spec-field and Sentinel-2 BOA.

The wide range of the data acquired from 8 different farms allowed us to capture the variability of dehesas grasslands and provide a representative modelling of this ecosystem. We highly recommend the implementation of a bootstrap approach to assess the stability and certainty of the predictive models (Mutanga et al., 2004; Mutanga et al., 2015). Relatively small sample sizes are used in this kind of study, typically around 100 samples (Askari et al., 2019; Kawamura et al., 2008; Mutanga et al., 2004; Raab et al., 2020). As can be observed in Figure 4, the wide confidence intervals denote high variability in the results depending on the corresponding partition, especially for Sentinel-2 BOA reflectance data, whose sample size was 75 compared to the 173-sample size of Spec-lab and Spec field. Reporting a single value of RMSE, R^2 and RPD could lead to biased conclusions since it does not provide information about the certainty of the results (Mutanga et al., 2004). We obtained values of R^2_{test} ranging from 0.1 to 0.8 for CP using Sentinel-2 BOA reflectance. Kawamura et al. (2008) and Mutanga et al. (2004) reported similar bootstrapped values of R^2 from CP and nitrogen content predictions with canopy spectral measurements. We extended this approach to the analysis of the regression coefficients which allowed us to delimitate the certainty of the values of these coefficients as well as the stability of the bands, and therefore permitted a more reliable interpretation of the bands' importance.

Using large and representative datasets that include a wide range of variation of the studied variable is key to reducing the uncertainty and improving the accuracy of the predictive models (Norris and Barnes, 1976). However, the data collection and reference measurement determination can be challenging. Given the growing interest in predictive models of forage quality in grasslands using remote sensing applications, the development of public reflectance spectral libraries from different studies could make available large datasets to improve calibrations. The use of national libraries has been successfully applied for soil property characterisation using Vis-NIRS spectroscopy (Knadel et al., 2012; Liu et al., 2018; Shepherd and Walsh, 2002).

Sentinel-2 bands importance on pasture quality assessment

The mean regression coefficients (average of 1000 values) were used to assess the bands' importance on the predictions of pasture quality variables (CP, NDF, ADF and EDOM) using PLS regression. For the prediction of CP, band 4, the red-edge and the SWIR regions had the greatest impact on the predictions. This was common for all of the three reflectance datasets. Band 5 (705 nm) proved to be especially important for CP prediction. These results are in accordance with previous studies that highlighted the importance of the red-edge region (700-775 nm) and wavelength reflectance between 1200-2400 to estimate CP using ground-based canopy reflectance (Adjorlolo et al., 2015; Kawamura et al., 2008; Kokaly, 2001; Ramoelo and Cho, 2018). Ramoelo et al. (2015), using Sentinel-2 simulated data to predict nitrogen content in rangelands, also noted the importance of bands 4 (665 nm), 5 (705 nm) in the red and red-edge region and bands 11 (1610 nm) and 12 (2190 nm) in the SWIR region. Raab et al. (2020) reported simple ratios based on the red-edge region as particularly important to predict CP. The importance of the red-edge region relies on the correlation between CP (closely related to nitrogen content) and chlorophyll concentration (Curran, 1989; Pellissier et al., 2015; Raab et al., 2020; Tong and He, 2017), whereas the importance of the SWIR region respond to absorption due to C−H, N−H, O−H and C-O bonds (Adjorlolo et al., 2015; Curran, 1989; Curran et al, 1992; Kawamura et al., 2008; Kokaly, 2001). Other studies using Sentinel-2 data have also informed of bands from the visible region (band 3 and band 4) and narrow-NIR (band 8a) as useful bands for CP prediction (Askari et al., 2019; Lugassi et al., 2019).

The differences in the importance of band 12 (SWIR-2, 2190 nm) between models built with Spec-lab and models constructed with Spec-field and Sentinel-2 BOA reflectance could be attributed to the plant water content and soil background effect (Mansour et al., 2012). SWIR absorption is affected by the reflectance of leaf water content, masking reflectance features of other biochemicals, which influence the accuracy of the predictions (Ramoelo et al., 2011). Ripple (1986) pointed out that the spectra reflectance region 2080-2350 nm shows sensitivity to changes in both soil background and relative water content of leaves. Since the Spec-lab data had no noise from water content or soil, the reflectance at band 12 had a great impact on the prediction of CP and NDF. The reflectance of Spec-field and Sentinel-2 could be affected by these two factors resulting in a confounding effect to predict CP or NDF that produced a low regression coefficient. Adjorlolo et al. (2015) informed that the most highly-ranked waveband for predicting CP using in situ canopy reflectance and PLS regression was centred at 2280 nm. Differences between studies might lie in the effect of soil background reflectance. The extent of the influence of soil reflectance on pasture quality prediction might be worth investigating in further research since it seems to be one of the main factors affecting the performance of the predictions.

Concerning the importance of bands for NDF prediction using Spec-lab data, the selection of bands of the visible region (bands 2, blue and 4, red) together with the SWIR region and to a lesser extent the red-edge band 7 is in accordance with previous studies (Kawamura et al., 2008). The models built with Sentinel-2 BOA reflectance also presented high regression coefficients in bands 2 and 4 and the red-edge band 6 coinciding with results obtained for Spec-lab. However, band 12 as well as for CP had lower coefficients, possibly due to the effect of water of soil background as discussed above. Bands 2 and 4 impact on fibre prediction might be related to the detection of pigments in different phenological stages of the pasture (Mansour et al., 2012; Ustin et al., 2009). Bands of the SWIR region have been widely selected for fibre determination (García-Ciudad et al., 1993), due to the overtones C-H, C-N and N-H, closely related to fibre components (Clark and Lamb, 1991). The regression coefficients of models fitted with Spec-field also showed great impact on the visible region and band 5 (705 nm) although the weaker calibrations could have affected the regression coefficients as happened for ADF and EDOM using Spec-field and Sentinel-2 BOA reflectance.

Use of field spectrometry for Sentinel-2 model calibration and pasture quality mapping

As discussed previously, the creation of suitable datasets of pasture quality samples and reflectance libraries will be key to developing effective and efficient Sentinel-2 based predictive models. However, the collection and analysis of samples is tedious and expensive (Starks et al., 2006). Here, we explored the option of using field canopy reflectance resampled to the Sentinel-2 configuration to calibrate models that could be used in combination with Sentinel-2 imagery. This would allow the use of samples and reflectance measurements already collected to develop robust calibrations that could then be applied to Sentinel-2 BOA images.

The PCA analysis (Figure S2) illustrated that both datasets, Spec-field and Sentinel-2 BOA were in the same spatial location and showed representativity. All the Sentinel-2 samples showed a DMH lower than 3 to the centre of the population of calibration (Spec-field) (Figure S2). Since in Vis-NIRS spectroscopy, DMH=3 is generally the maximum DMH acceptable to predict new samples using a calibrated model (Shenk and Westerhaus, 1996; Williams and Sobering, 1996), these results confirm that these two datasets can be combined to perform PLS calibrations and predictions. Previous studies have used field canopy reflectance and combined it with Sentinel-2 images (Lugassi et al., 2019; Ramoelo and Cho, 2018). However, to our knowledge, the representativity of these two different datasets of reflectance has never been checked.

Only the prediction made for CP produced acceptable results that allow its use for qualitative assessments of CP (Figure 7). To illustrate the potential application of these models, the calibrated model for CP was used to perform spatial predictions of CP (Figure 8) showing clear differences between fields associated with their management. The reason for irrigated grasslands having lower CP content than natural grasslands (Figure 8) is the presence of grazing cattle during the summer season in the irrigated field. By the first date of mapping, in November, at the beginning of growing, the irrigated pastures were mostly consumed with mainly stems remaining (with lower CP content than leaves). Thus, the quality is clearly lower than in natural grasslands which are starting to grow and have been reserved from grazing and therefore have higher CP content at this date. The CP maps allow identifying differences between both inter fields and intra fields. Overall, the CP content of natural grasslands is more heterogeneous. Lugassi et al. (2019), using laboratory and field spectral measurements at Sentinel-2 configuration, also found spatial heterogeneity in Mediterranean grasslands responding mainly to changes in topography.

Implications for management of open woodlands and future studies

The present study has demonstrated that qualitative assessments of CP and NDF using free-available Sentinel-2 imagery can be successfully implemented for high-diversity Mediterranean permanent grasslands. The availability of real-time data of CP and NDF content provided by Sentinel-2 imagery can be used to assess the carrying capacity of pastures, adjust stocking rates and plan the spatio-temporal livestock grazing on dehesa farms (Ramoelo and Cho, 2018; Starks et al., 2006). The managers can also decide in real-time, based on the estimation of CP of the pastures, if supplementary feeding is necessary (Raab et al., 2020). It is also an inexpensive way to evaluate interventions such as grassland improvements with legume mixes or fertilisation without the need for laborious fieldwork and expensive laboratory analyses which would need to be done repeatedly. CP maps produced with Sentinel-2 imagery could help to identify patches where overseeding with legumes might be needed to improve the CP offer of dehesa permanent grasslands, improving in this way the efficiency of this intervention. The possibility of performing spatial and real-time temporal monitoring is especially important on large farms where the status of pastures cannot be inspected visually by managers regularly (CSIRO, 2020).

From the point of view of policies, the Common Agricultural Policy is giving increasing importance to the provision of ecosystem services and sustainable management of European grasslands via agri-environment schemes (Harlio et al., 2019; Simoncini et al., 2019). The high revisiting time (5 days) of Sentinel-2 allows the establishment of monitoring systems based on qualitative assessments of pasture quality to provide spatial data very timely. This is especially relevant in Mediterranean permanent grasslands because of the high intra- annual changes that characterise these communities. Therefore, Sentinel-2 reveals as a promising tool to evaluate the effectiveness of the agri-environment schemes since it contributes to monitoring the conservation status, the sustainability of farming management as well as the provision of ecosystem services in Mediterranean grassland communities.

Based on the insights provided here, future studies are needed to go into detail about the remote sensing of Mediterranean permanent grasslands using Sentinel-2. In this study, we used different sources and samplings to explore the feasibility of this technique. As Raab et al., 2020 suggested, the formula by Justice and Townshend (1981) could be used to calculate the required plot size for the grassland samplings: $S = P(1 + 2L)$, where S is the length of the plot, P is the spatial resolution of the pixel and L the geolocation error. However, the plot size provided by this formula can be difficult to use in open woodlands landscapes without including a tree signal. A feasible setup could consist of calibrating robust models based on field spectroscopy and then used them to make spatial predictions with Sentinel-2 imagery. This approach can improve the match between the reflectance data and the pasture quality variables in heterogeneous grasslands while overcoming the spatial constrain of scattered trees. It also would help to reduce the uncertainty of the models by targeting the acquisition of field spectroscopy at capturing the variability of the grasslands, which might be difficult with samplings at the pixel level. Increasing the temporal domain of the dataset will be key to improve the predictions and reducing their uncertainty.

Another research gap for future studies is the comparison of different models such as PLS, random forest, support vector machine and artificial neural network and their optimisation through feature elimination methods. This could also allow the inclusion of multiple ratios and indices that might enhance the relationship between the Sentinel-2 derived data and the predicted variable. Alternative methods to PLS such as convolutional neural networks could capture strong nonlinear spectral-chemical relationships (Pullanagari et al., 2021). However, the prediction of pasture quality using Sentinel-2 data in heterogeneous permanent grasslands could be limited to a certain level regardless of the methodology implemented, and possibly the improvement compared to the results delivered by Spec-lab models would be marginal. Therefore, new sensors with finer spatial and spectral resolution could be necessary to overcome this limitation in Mediterranean permanent grasslands. Future research should aim at simulating hyperspectral data, for example, based on the specifications of the high-priority candidate mission of the European Space Agency; CHIME (Nieke and Rast, 2018), with different machine learning techniques to elucidate the level of accuracy that could be achieved.

CONCLUSION

The results obtained for models built with Spec-lab reflectance show that the potential of Sentinel-2 configuration to predict pasture quality in Mediterranean permanent grasslands using PLS models may be limited to moderate prediction ability for assessment of CP, NDF, ADF and EDOM ($1.5 \leq RPD < 2$ and $0.6 \leq R^2 < 0.5$) that could allow performing qualitative assessments. Models built with Sentinel-2 imagery show test results from predictions of NDF and especially CP suggesting the possibility of qualitative assessments of these parameters in Mediterranean permanent grasslands of open woodlands.

The differences in accuracy obtained for Spec-lab and those reported for both, Spec-field and Sentinel-2 BOA images point at the effects of soil background, water content of vegetation and effects of heterogeneous canopies of diverse grasslands on reflectance. Additionally, intrinsic factors associated with the structure of open woodlands such as the presence of scattered trees restrict the use of Sentinel-2 images to the management of Mediterranean permanent grasslands. Further research is needed to tackle these limitations.

In accordance with previous studies, the red-edge (especially band 5) and the SWIR regions show the highest potential for estimating CP and NDF. Bands 2, blue and 4, red also seem to be important for the prediction of NDF.

The development of large and representative datasets with a wide range of variation of pasture quality is pivotal to reducing the uncertainty and improving the accuracy of the predictive models. Using field spectroscopy in combination with Sentinel-2 imagery can contribute to developing larger datasets and more robust models.

The qualitative assessment of CP and NDF content in permanent grasslands of open woodland farms using Sentinel-2-based models is a powerful tool to improve the efficiency and sustainability of management through more informed and effective decision-making.

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Supplementary material

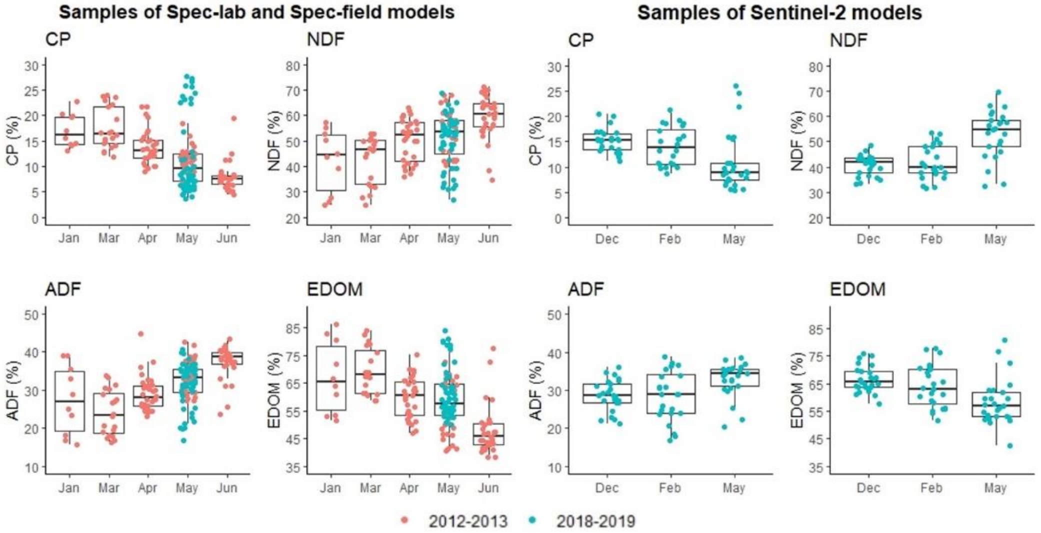

Figure S 1. Pasture quality data used for Spec-lab and Spec-field models (N=173) and for Sentinel-2 models (N=75) by sampling date (note that pasture quality data of Sentinel-2 models is an average of four sampling quadrats, see 2.4. Modelling approach and statistical analysis). Coloured by sampling campaign (2012-2013 and 2018-2019). Black centre line, median; box, interquartile range; box limits, lower and upper quartiles; whiskers, 1.5× interquartile range.

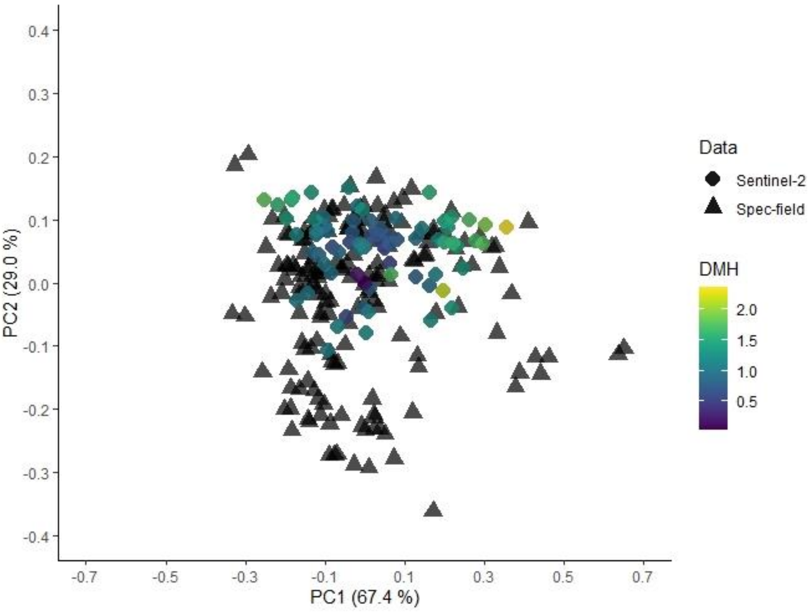

Figure S 2. Scatter plots of Principal component analysis (first two principal components) of Spec-field (N=173) and Sentinel-2 (N=73) spectra samples. In colour, distance of Mahalanobis of samples of Sentinel-2 (N=73) to the centre of the population of calibration (Spec-field dataset).

Table S 1. Summary table of the grassland sampling.

Farm	Sampling campaign	Dates	Permanent grassland type	Sampling plot	Sampling size	Spec-lab	Spec-field	Sentinel-2 imagery
1-4	2012-2013	January/ February March April May June	Permanent natural grasslands	1 4x8 m grazing exclusion plot/farm 4 quadrats inside and 4 quadrats outside	N=125[1]	X	X	
5	2018-2019	November/December February May	Permanent natural grasslands	3 10x10 m plots 4 quadrats randomly set inside	N=36	X(May)[2]	X(May)[2]	X[3]
			Reseeded grasslands	6 10x10 m plots 4 quadrats randomly set inside	N=72	X(May)[2]	X(May)[2]	X[3]
			Irrigated grasslands	3 10x10 m plots 4 quadrats randomly set inside	N=36	X(May)[2]	X(May)[2]	X[3]
6	2018-2019	November/December February May	Permanent natural grasslands	6 10x10 m plots 4 quadrats randomly set inside	N=72			X[3]
			Reseeded grassland	2 10x10 m plots 4 quadrats randomly set inside	N=24			X[3]
7	2018-2019	November/ December February May	Permanent natural grasslands	2 10x10 m plots 4 quadrats randomly set inside	N=24			X[3]
			Reseeded grassland	1 10x10 m plots 4 quadrats randomly set inside	N=12			X[3]
8	2018-2019	November/December February May	Permanent natural grasslands	2 10x10 m plots 4 quadrats randomly set inside	N=24			X[3]
				Total samples	425	173[2]	173[2]	75[3]

[1] 35 samples with extremely low pasture production or partially covering bare ground were removed

[2] Sampling performed only in May 2019.

[3] The pasture quality measurements of the four quadrats at each one of the 25 sites selected were averaged for every sampling, so a representative value of the quality variable at the site could be associated with its corresponding reflectance on each of the three dates (N=75).

CHAPTER VII. Estimating pasture quality of Mediterranean grasslands using hyperspectral narrow bands from field spectroscopy by Random Forest and PLS regressions

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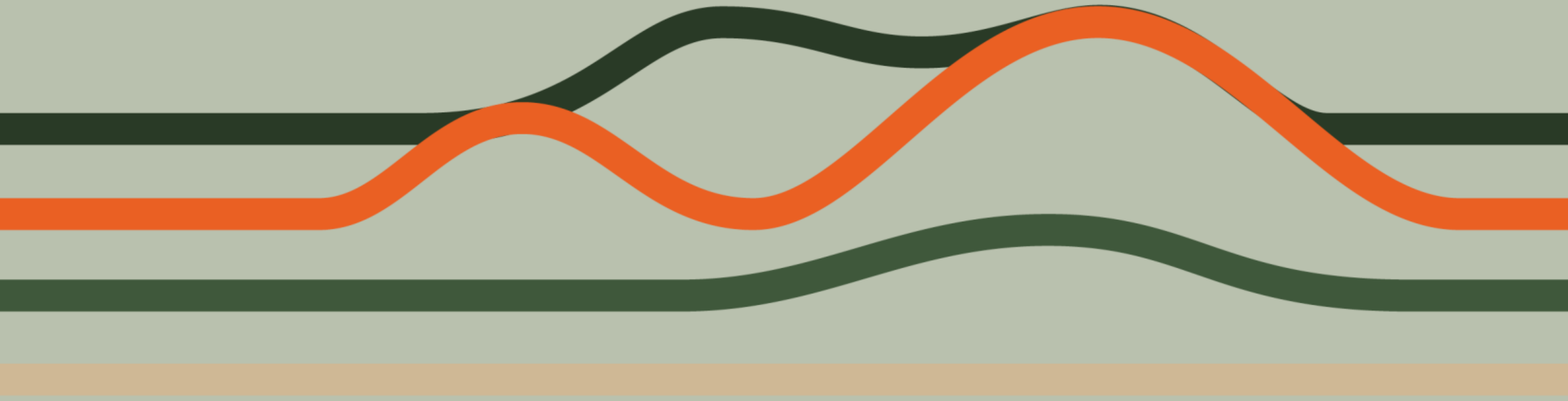

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Abstract

Mediterranean grasslands are a cornerstone ecosystem to provide ecosystem services and sustain human societies. The sustainability and provision of ecosystem services by these systems rely on their management. One of the main attributes to perform sustainable and effective management is pasture quality, which is crucial for animal performance in rain-fed extensive systems. Remote sensing of grasslands can be an effective tool to inform the management of grasslands. The forthcoming high-priority mission candidate of the European Space Agency, Copernicus Hyperspectral Imaging Mission for the Environment (CHIME) with continuous narrow bands of ≥10 nm spectral resolution could be an asset to provide accurate information on the pasture quality of high-diverse and heterogeneous grasslands. In this study, we investigated the potential of CHIME-like field spectroscopy data at 10 nm resolution to assess the quality of Mediterranean permanent grasslands. The pasture quality indicators used were: crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM). To do so, two machine learning methods commonly used in remote sensing were implemented: Partial Least Squares (PLS) regression and Random Forest (RF) regression. The results using all bands in the 400-2300 nm spectral range and the results obtained by Backward Feature Elimination (BFE) were also compared. Finally, using importance measures of PLS and RF and the BFE approach, the importance and stability of the bands to assess the pasture quality indicators were explored. The results showed that field spectroscopy CHIME-like data at 10 nm of spectral resolution show potential to predict CP at “good” accuracy and NDF at “moderate” accuracy level in Mediterranean permanent grasslands. PLS outperformed RF to predict CP and NDF in terms of accuracy and certainty of the predictions. The BFE approach increased the accuracy of the predictions, especially in PLS, for which a ΔRMSE= -12.5 was achieved in cross-validation to predict CP. The models built by BFE approach to predict CP using PLS provided a mean R^2 value of 0.82 and a range of 0.68-0.90 in bootstrapped predictions. The RMSE was low (mean RMSE=2.23%) and the mean RPD=2.47 with values ranging from 1.81 to 3.23. RF models to predict CP produced mean R^2 value of 0.68, mean RMSE=3.00% and mean RPD=1.82. ADF and EDOM were predicted with poor accuracy and similarly by both, PLS and RF. The bands located in the red-edge and NIR region showed high importance and stability to assess the best-predicted variables. Bands centred at 700, 710, 1160, 1170 and 1180 are highly stable and important to predict CP. The bands from the

SWIR region had lower stability. This study provides insightful results on the use of hyperspectral data and future satellite missions such as CHIME to assess the pasture quality of Mediterranean grasslands that can be crucial to inform the management and monitoring of Mediterranean permanent grasslands.

Keywords: Crude protein, Band selection, Backward feature elimination, CHIME, Band importance, Heterogeneity

INTRODUCTION

The high diversity of vascular plants, strongly related to the management practices and characteristics of the Mediterranean climate, make the Mediterranean Basin a global biodiversity hotspot (Cosentino et al., 2014; Myers et al., 2000). Grasslands of the Mediterranean-climate zones contribute substantially to the biodiversity of the Mediterranean Basin and have traditionally played a major role to sustain human livelihood (Jouven et al., 2010; Porqueddu et al., 2016). Mediterranean grasslands are mainly annual high-diverse communities of grasses, legumes and forbs, of low biomass production due to the low rainfall and its high intra- and inter-annual variability which together with the grazing and occasional cropping are responsible for the strong heterogeneity of this ecosystem (Cosentino et al., 2014; Olea and San Miguel-Ayanz, 2006; Porqueddu et al., 2016). In the last decades, increasing attention has been directed to the potential of Mediterranean grasslands ecosystems to provide multiple ecosystem services highly appreciated by the society and of crucial importance for the global environment such as biodiversity conservation, wildfires control and rural population sustain (D’Ottavio et al., 2018; Porqueddu et al., 2016; Porqueddu et al., 2017).

Mediterranean grasslands are associated with extensive livestock grazing by small ruminants and beef cattle (Cosentino et al., 2014) that act as a major driver determining stability, sustainability, and potential of ecosystem services provision (D’Ottavio et al., 2018; Porqueddu et al., 2017; Sollenberger et al., 2019). The increasing effects of climate change challenge the stability and functions of Mediterranean grasslands compromising their resilience (Giannakopoulos et al., 2009; Giorgi and Lionello, 2008; Chang et al., 2017; Ma et al., 2017, Carpintero et al., 2020). In this context, it is crucial to have accurate and routine information on the attributes of grasslands to i) improve the economic, environmental sustainability and efficiency of grassland management at farm level, and ii) monitor their dynamics and conservation status at a larger scale. One of the most important attributes of grasslands concerning their management for livestock rearing is the pasture quality. Pasture's quality can be understood in many ways, but in the context of animal feeding, it usually refers to proximate nutritional principles (Dumont et al., 2015) such as crude protein and fibre, ether extract, minerals, ash, or the energy provided (Pullanagari et al., 2013).

The main methods to assess pasture quality are: i) laboratory-based methods, ii) proximal remote sensing, iii) aerial remote sensing (aircraft and UAVs) and iv) space-based remote sensing (Pullanagari et al., 2013). The accuracy of the estimations is reduced following the order in with these methods have been listed (Pullanagari et al., 2013). Laboratory-based methods are the standard and commonly used to assess pasture quality. However, through the previous calibration using reference data determined by laboratory methods, indirect methods based on the remote sensing of the pasture reflectance are gaining importance in pasture quality determination. Concerning the cost, laboratory-based methods are the most expensive methods due to laborious manual sampling collection and analysis compared to the sensing methods (Starks et al., 2004). Within the sensing methods, satellite technology is especially interesting because of the large-scale coverage and regular data provision. In particular, Sentinel-2 satellites have demonstrated a great potential to monitor grasslands ecosystems (Askari et al., 2019; Fernández-Habas et al., 2021; Raab et al., 2020; Ramoelo et al., 2014; Sibanda et al., 2015) due to the free provision of multispectral data at worldwide level with a frequency of 5 days (ESA, 2021). However, because of the intrinsic heterogeneity of Mediterranean grasslands, multispectral data might have limited potential to provide accurate information on quality grasslands attributes (Fernández-Habas et al., 2021). The European Space Agency has a new high-priority mission candidate Copernicus Hyperspectral Imaging Mission for the Environment (CHIME) (Nieke and Rast, 2018; Rast et al., 2019). The objective of this mission is: *"To provide routine hyperspectral observations through the Copernicus Programme in support of EU- and related policies for the management of natural resources, assets and benefits"*. According to the Mission Requirements Document, this imaging spectrometer will measure in the 400-2500 nm spectral range with continuous narrow bands of ≥10 nm spectral resolution, spatial resolution of 20-30 m and revisiting time of 10 to 12.5 days (Nieke and Rast, 2018; Rast et al., 2019).

In addition to satellite sensors, field spectroscopy has also demonstrated great potential and applicability to in-field pasture quality assessments (Pullanagari et al., 2012). Field spectroscopy has also been used to upscale models to satellite data and to simulate the applicability of different satellite spectral resolutions (Fernández-Habas et al., 2021; Lugassi et al., 2019; Mutanga et al., 2015; Ramoelo and Cho, 2018; Sibanda et al., 2015). In this study, we apply this approach to investigate the potential of the CHIME mission to assess the quality of high-diverse Mediterranean permanent grasslands.

Machine learning algorithms have a great potential to exploit hyperspectral data and to retrieve grasslands attributes (Verrelst et al., 2015). The number of variables is usually larger than the number of samples and, on the other hand, these data tend to suffer from multicollinearity (Adjorlolo et al., 2013; Rivera-Caicedo et al., 2017). The redundancy and correlation between variables in hyperspectral data lead to the 'Hughes phenomenon' where the accuracy of the classification/predictions increases gradually with an increasing number of spectral bands or dimensions to a certain number of bands when it decreases dramatically (Hughes, 1968; Ma et al., 2013). Therefore, the algorithms used have to be efficient in dealing with these issues to avoid the 'Hughes phenomenon', also known as 'curse of dimensionality' (Rivera-Caicedo et al., 2017; Verrelst et al., 2015).. Two methods are commonly used in remote sensing and chemometrics to analyse hyperspectral data: Partial Least Squares (PLS) regression and Random Forest (RF) regression. These methods have been extensively implemented in remote sensing demonstrating their robustness and reliability (Verrelst et al., 2015). PLS is the state-of-the-art non-parametric method for analysing spectroscopic data (Kucheryavskiy, 2018; Wold et al., 2001) and vegetation properties mapping (Biewer, et al., 2009b; Verrelst et al., 2015). RF is an ensemble classification and regression algorithm consisting of an evolution Classification and Regression Trees (CARTs) developed by Breiman (2001) that combines bagging and bootstrapping approaches. It has become popular for remote sensing applications due to its high accuracy, flexibility to be used with complex datasets and few hyperparameters to be set (Belgiu and Drăgu, 2016).

Previous studies have provided insightful information about the potential of field spectroscopy at different spectral resolutions (Pullanagari et al., 2012). For example, Zhou et al. (2019) demonstrated the feasibility of the Yara N-sensor spectrometer at 10 nm spectral resolution to predict the yield and quality of legume and grass mixtures. The recently published research by Pullanagari et al. (2021) provided conclusive results about the prediction of canopy nitrogen concentration in temperate grasslands by a convolutional neural network, PLS and gaussian process regression using field spectroscopy.

Although PLS and RF can deal with multicollinearity, feature selection approaches are highly recommended when using hyperspectral data due to the issues mentioned above (Belgiu and Drăgu, 2016). Several studies have demonstrated that predictions using both PLS and RF can benefit from data reduction by feature selection in hyperspectral data (Mansour et al., 2012; Belgiu and Drăgu, 2016; Kawamura et al., 2008). Another important application of this approach is the identification of important bands or the removal of redundant information. PLS and RF can also provide estimates of the bands importance (Belgiu and Drăgu, 2016; Mehmood et al., 2012; Santos-Rufo et al., 2020). This information has relevant implications to: i) inform the use of hyperspectral data ii) optimise the models and data used and iii) inform the design of hyperspectral-based devices (Chan and Paelinckx, 2008; Pullanagari et al., 2012). These approaches of band selection and band importance identification have direct application to the use of the data provided by the forthcoming CHIME mission whose spectrometer is expected to be equipped with 210 bands (Nieke and Rast, 2018). In fact, the Mission Requirements Document by Rast et al. (2019) pointed out that one of the following analyses of the Mission would be to *"confirm the spectral sampling requirements (10 nm at FWHM) for the target applications and related products, incl. support to product quality specification"*. To the best of our knowledge, the applicability of hyperspectral narrow bands to assess the pasture quality of Mediterranean grasslands has received poor attention. Given the particularities (high heterogeneity and variability) and interest of these ecosystems outlined before, further research focused on this type of grasslands is required to advance in the use of sensing methods in their management and monitoring.

In this context, the overall objective of this study was to assess the potential of hyperspectral data CHIME-like at 10nm spectral resolution to estimate pasture quality in Mediterranean permanent grasslands using field spectroscopy. To achieve this goal, we established the following specific objectives:

i) Evaluate and compare the performance and prediction accuracy of RF and PLS regressions to assess crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM).

ii) Test the implementation of backward feature elimination techniques (BFE) to optimise the predictive models and to identify the most important narrow bands to predict the pasture quality indicators.

iii) Interpret the implications of the outcomes for the management and monitoring of Mediterranean permanent grasslands.

MATERIAL AND METHODS

Pasture sampling

Pasture samples were taken in five dehesa farms from the Cordoba province, in the north of Andalusia region (Spain) during the growing season of 2012-2013 (farms 1-4) and 2018-2019 (farm 5) (Figure 1).

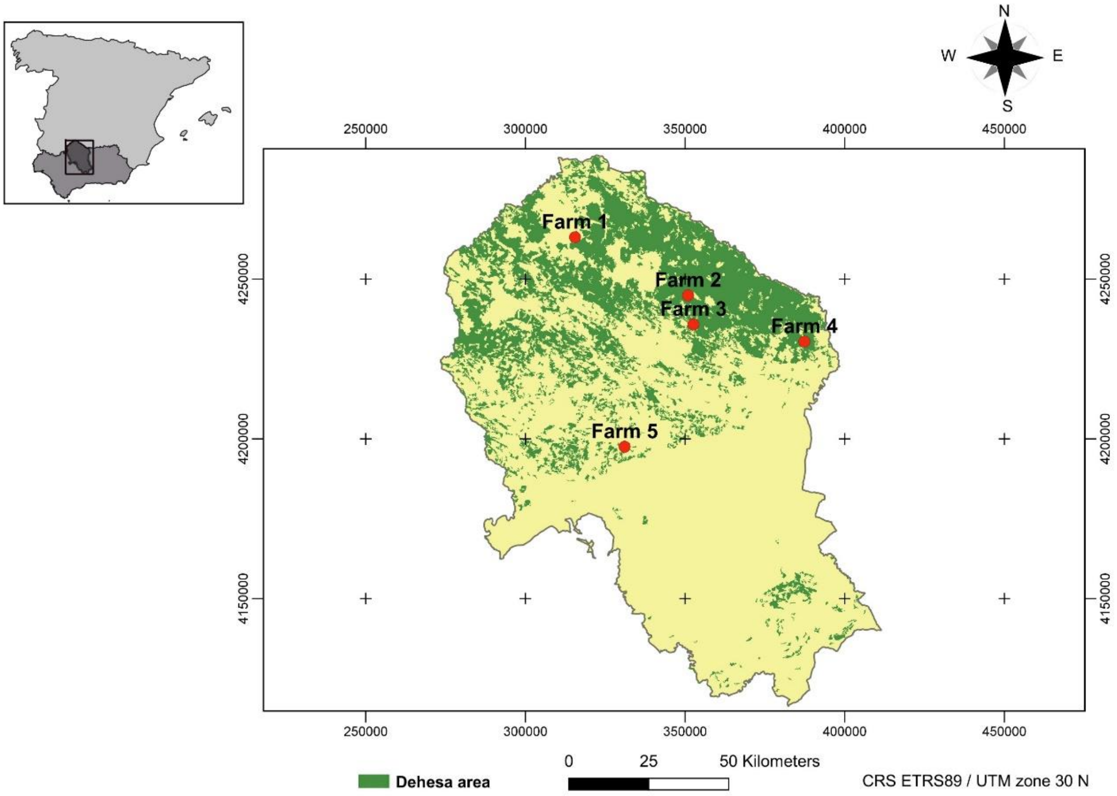

Figure 1. Location of farms where the grasslands samplings were performed. Farms are located within the dehesa area of Cordoba province, in the north of Andalusia region (Spain). Dehesa area layer, coloured in green, is provided by the WMS of the dehesa systems distribution in Andalusia (REDIAM, 2020).

The pasture sampling conducted in 2012-2013 was aimed at studying the grazing effect on pasture quality of natural permanent grasslands of dehesas in a previous study (Fernández et al., 2014). Four sampling quadrats of 0.4 x 0.4 m were randomly set within grazing exclusion plots and four outside of them. This sampling was repeated on five dates; January/February, March, April, May and June which provided 125 samples (Table 1) after removal of those with extremely low pasture biomass for laboratory analysis. Locations of 0.4 x 0.4 m quadrats sampled on previous dates were avoided. In farm 5, pasture samples were collected in May of 2019. Plots of 10x10 m were located in irrigated grasslands (3 plots) natural grasslands (3 plots) and improved grasslands with commercial seed mixtures (6 plots). Within each 10 x 10 m plot, four sampling quadrats were randomly set, providing 48 pasture samples. The pasture contained within the quadrats was clipped to ground level, dried in the oven for 48 h at 60ºC and ground to pass through a 1-mm sieve. In total, 173 samples (Table 1) were analysed at the Laboratory of Animal Nutrition of SERIDA (Villaviciosa, Asturias, Spain) to determine the percentage of crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and enzyme digestibility of organic matter (EDOM). The grasslands sampled consisted of communities mainly dominated by annuals with species such as *Avena* spp., *Astragalus pelecinus, Bromus* spp., *Diplotaxis* spp., *Erodium* spp., *Hordeum* spp., *Lolium* spp., *Ornithopus compressus, Plantago* spp., *Trifolium subterraneum*, *T. cherleri*, *T. tomentosum*, *T. glomeratum*, and *Vulpia* spp in natural grasslands, *T. repens* and *Lolium* spp., in the irrigated field and *T. vesiculosum*, *T. michelianum*, *T. resupinatum*, *O. compressus* and *L. multiflorum* in improved grasslands from farm 5.

Table 1. Grassland type, number of samples and date of sampling of the farms used in the study.

Farm	Coordinates*	Grassland type	Sampling date		Number of samples
1	x= 315534.34 y= 4263109.14	Permanent natural grasslands	2012/2013	January/February March April May June	28
2	x= 350946.02 y= 4244905.06	Permanent natural grasslands	2012/2013	January/February March April May June	33
3	x= 352598.98 y= 4235836.66	Permanent natural grasslands	2012/2013	January/February March April May June	33
4	x= 387377.62 y= 4230454.12	Permanent natural grasslands	2012/2013	January/February March April May June	31
5	x= 331065.33 y= 4197542.60	Permanent natural grasslands	2018/2019	May	12
		Reseeded grasslands			24
		Irrigated grasslands			12
				Total samples	173

*Projected coordinate system: ETRS 1989 UTM Zone 30N

Canopy reflectance measurement and preprocessing

Before pasture clipping, the reflectance of the pasture contained in the 0.4x0.4 m quadrats was recorded using an ASD FieldSpec Spectroradiometer (ASD Inc, Boulder, Colorado, USA). The measurements were taken under clear sky between 10:00 and 15:00. The spectroradiometer records reflectance at a spectral resolution of 1.4 nm within the 350-1000 nm range and 2 nm within the 1000-2500 nm range. The output data is an interpolated reflectance at 1 nm spectral resolution in the whole range of 350-2500 nm. The device is equipped with a fibre optic probe assembled to a pistol grip that is held at 1.20 m height resulting in a 0.22 m^2 measurement area. Four replicated were recorded per quadrat and averaged to provide a unique representative reflectance measurement of the quadrat. Calibrations on white references were done on a Spectralon panel (Labsphere, NorthSutton, NH) every four samples.

The spectra were smoothed applying the Savitzky-Golay (Savitzky and Golay, 1964) filter using a width of filter window of three and second-order of polynomial. Those regions of the spectra displaying noise due to instrumental noise (350-395 nm and 2300-2500 nm), atmospheric noise (1370-1410 nm and 1816-1941 nm) or detector change (1000-1005 nm) were removed. In order to match the spectral specifications of the high-priority candidate mission of the European Space Agency: Copernicus Hyperspectral Imaging Mission for the Environment (CHIME) (Nieke and Rast, 2018), the spectra were resampled to 10 nm spectral resolution using the *"resample2"* function of the CRAN-package "*prospectr*" (Stevens and Ramirez-Lopez, 2015) resulting in 168 hyperspectral bands of 10 nm resolution. Spectral outliers were identified by principal component analysis (PCA) (Morellos et al., 2016; Xu et al., 2018). All analyses, preprocessing and modelling were performed in R v. 3.6.1 (R Development Core Team, 2019).

Partial Least Squares

PLS consist of a lineal multivariate regression model that relates a *Y* matrix of response variables (CP, NDF, ADF or EDOM) with an *X* matrix of predictor variables (168 hyperspectral bands) by decomposing both *Y* and *X* in n-orthogonal Latent Variables (LV) to maximise their covariance. PLS models were calibrated by Leave-One-Out cross-validation (LOOcv). The only parameter to be adjusted in PLS, the optimal number of LV, was selected based on the first local minimum of the root mean squared error (RMSE) of the cross-validated predictions. In this study we describe the basic functioning and characteristics of PLS, further information can be found on Geladi and Kowalski (1986), De Jong (1993) and Wold et al. (2001). PLS models were implemented using CRAN-package *"mdatools"* (Kucheryavskiy 2019; Kucheryavskiy 2020).

Random Forest

The RF regression is a machine learning technique that uses the ensemble of a set of Classification and Regression Trees (CARTs) to make predictions (Breiman, 2001). By bagging approach, RF uses two-thirds of the samples (*in-bag* samples) to create *n* user-defined unpruned and independent trees (*ntrees*). The remaining third of the samples, the so-called *out-of-bag* (OOB) samples, are used to estimate the Mean Squared Error (MSE), known as the OOB error. The OOB error is considered an accurate estimate of the performance of the model (Grimm et al.,2008; Liaw and Wiener, 2002; Mutanga et al., 2012). At each node of the regression trees, instead of choosing the best split among the predictors as in CARTs, RF randomly selects a user-defined number of predictors (*mtry*) (Liaw and Wiener, 2002). The final predicted value is obtained by averaging the predictions of the *ntrees*. The RF algorithm was implemented with CRAN-package*"randomForest"* (Liaw and Wiener, 2002). As explained above, RF has two main hyperparameters, the number of trees to grow (*ntree*) and the number of predictors to select at each node (*mtry*). The default values of *"randomForest"* were used for *ntree* (500 trees) and *mtry* (1/3 of the total number of predictors) since they have shown to be acceptable values and the most common recommendation (Belgiu and Drăgu, 2016; Díaz-Uriarte and Alvarez de Andrés, 2006). To ensure the right choice of these parameters, RMSE was calculated with the default of *mtry*, half of the default, and twice the default as suggested by Liaw and Wiener (2002).

Band importance and selection by backward feature elimination in PLS and RF

The modelling approach followed in this study is schematised in Figure 2. Band importance in PLS models was measured based on the absolute value of the regression coefficients which is a *"single measure of association between each variable and the response"* (Mehmood et al., 2012). Bands with a large absolute magnitude of their associated regression coefficients are expected to have a high impact on the models while small absolute values of regression coefficients indicate that these bands are unimportant or redundant (Garrido Frenich et al., 1995; Kawamura et al., 2008). This technique has shown to be a robust method in variable selection with PLS (Garrido Frenich et al., 1995; Palermo et al., 2009).

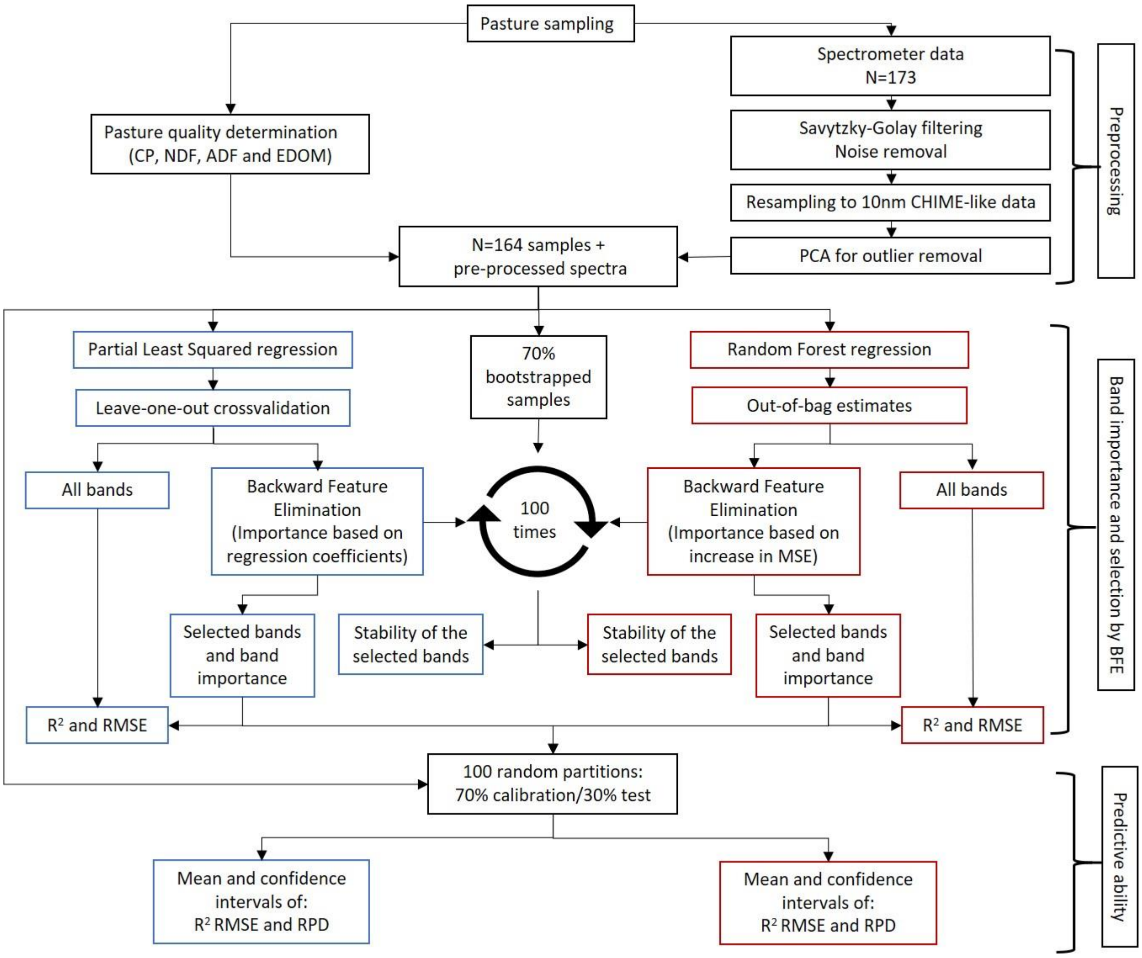

Figure 2. Modelling approach of the study.

The most reliable method for variable importance estimation in RF is the so-called “permutation importance”. The rationale of this method consists of randomly permuting a predictor variable X_j (band in this case) and calculating the MSE of the prediction of the OOB set with the remaining predictors. The difference between the MSE when X_j is permuted and the baseline MSE calculated with all predictors (measured as the percentage increase of MSE) is a measure of the variable importance (Strobl et al., 2007). This process is repeated over all predictors. If the predictor X_j is strongly associated with the response, its exclusion from the predictors produces a substantial increase in the MSE.

The most important bands for the prediction of the studied pasture quality variables were selected based on backward feature elimination (BFE). The BFE in PLS was carried out by means of the filter method based on removing at each iteration the band with the smallest absolute value of its regression coefficient (Mehmood et al., 2012). After removing the least important band, a LOOcv is performed to select the optimal number of LV and the new regression coefficients recalculated. This process was repeated until only two bands were left. At each step, the coefficient of determination (R^2) and the RMSE of the LOOcv are calculated. A similar method was applied to RF. The least important band (based on the lowest increase in MSE) was removed at each step until only two bands were left (Adam et al., 2014; Díaz-Uriarte and Alvarez de Andrés, 2006; Odindi, 2014). The R^2 and the RMSE of the OOB estimation were also calculated at each step. The selection of the most important set of bands was determined by selecting the model that yielded the highest R^2 and the lowest RMSE of LOOcv and OOB estimates in PLS and RF respectively in the BFE process.

To study the effect of the dataset and the stability of the selected bands, the BFE process was repeated n=100 times over 70% of samples selected by bootstrap. The percentage of times that the bands were selected in the 100 repetitions of the BFE was used as an estimate of their stability.

Assessment of performance and predictive ability of PLS and RF models

Following Kawamura et al. (2008), Mutanga et al. (2004) and Mutanga et al. (2015) a bootstrap approach was applied to test the performance, robustness and predictive ability of the models built with the selected bands by BFE. The original dataset (n=164) was randomly split into 70% for calibration and 30% for independent test. This random split was repeated 100 times. For both, PLS and RF, models were built with the calibration set (70% bootstrapped samples) to predict over the remaining 30%. The R^2, RMSE and Ratio of Performance to Deviation (RPD) of the test predictions were recorded. Mean and confidence intervals (CI) (2.5 and 97.5 percentiles) of R^2, RMSE and RPD were calculated and reported. Following Askari et al. (2015), the performance and predictive ability of the models were assessed considering the thresholds: “poor” accuracy ($RPD < 1.5$ and $R^2 < 0.6$), “moderate” ($1.5 \leq RPD < 2$ and $R^2 \geq 0.60$), “good” ($2 \leq RPD < 2.5$ and $R^2 \geq 0.7$) and “excellent” ($RPD \geq 2.5$ and $R^2 \geq 0.8$).

RESULTS

Statistics of pasture quality variables

Table 2 shows the descriptive statistics of CP, NDF, ADF and EDOM. There was a wide range of data and large variability for all variables. CP had the largest CV with 45.4 % while the rest of the variables had a CV close to 19%. These variables also showed high variability across the different dates of sampling (Figure S1).

Table 2. Descriptive statistics of the pasture quality variables used to fit the models.

Pasture variables (% of DM)	Minimum	Mean	Maximum	Range	SD	CV
CP	3.7	11.9	27.7	24.0	5.4	45.4
NDF	24.9	52.0	71.3	46.5	10.1	19.4
ADF	15.7	31.8	44.8	29.1	6.2	19.4
EDOM	38.5	58.4	86.2	47.8	10.8	18.4

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EDOM: enzyme digestibility of organic matter; SD: standard deviation; CV: coefficient of variation.

Performance of PLS and RF models with all bands and with bands selected by backward feature elimination

The PCA of the spectral data revealed nine points laying outside the 95% confidence ellipse (Figure 3) that were omitted from the dataset used in the analysis.

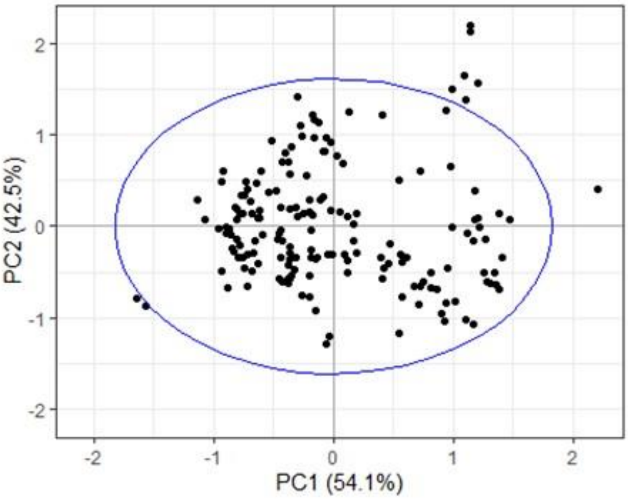

Figure 3. Detection of outliers after principal component analysis (PCA) of the pasture samples (n=173). Blue line represents 95% confidence ellipse.

Overall, the best models were obtained for CP, with R^2 values over 0.70 using all bands and the selected bands with both PLS and RF, having also the smallest RMSE values. R^2 values for NDF were in the range of 0.52-0.67 and between 0.47-0.59 for EDOM. ADF was the parameter that showed the worst statistics with R^2 always below 0.50. PLS outperformed RF in both cases, with all bands and with selected bands for all variables (Table 3). The backward feature elimination improved the performance of the models for all pasture quality variables and for both regression methods, PLS and RF. The ΔRMSE denotes that the improvement was different depending on the variable and always higher for PLS (Table 3). The greatest improvement (-12.5 ΔRMSE) with selected bands was obtained for CP with PLS regression, which was the model that showed the best performance with R^2=0.84 and RMSE=2.17 using 21 bands.

Table 3. Performance of PLS and RF models with all bands and with selected bands. Coefficient of determination R^2 and root mean square error (RMSE) correspond to leave-one-out and out-of-bag estimations for PLS and RF respectively.

		All bands			Selected bands					
Pasture variables (% of DM)	**Model**	**NLV**	**R^2**	**RMSE**	**NLV**	**R^2**	**RMSE**	**NBS**	**%BS**	**ΔRMSE**
CP	PLS	11	0.79	2.48	11	0.84	2.17	21	12.5	-12.5
	RF	-	0.70	2.95	-	0.71	2.89	55	32.7	-2.0
NDF	PLS	11	0.60	6.34	10	0.67	5.77	26	15.5	-9.0
	RF	-	0.52	6.98	-	0.53	6.86	82	48.8	-1.7
ADF	PLS	3	0.40	4.74	6	0.46	4.52	7	4.2	-4.6
	RF	-	0.42	4.68	-	0.45	4.55	53	31.5	-2.8
EDOM	PLS	9	0.53	7.35	6	0.59	6.89	17	10.1	-6.3
	RF	-	0.47	7.83	-	0.51	7.52	34	20.2	-4.0

N=164; NBS: number of bands selected by backward feature elimination; %BS: percentage of bands selected from the original dataset (n=168); ΔRMSE: decrease in root mean squared error from model with all bands to models with selected bands. CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; EDOM: enzyme digestibility of organic matter

Figure 4 illustrates the changes of R^2 and RMSE in backward feature elimination in PLS and RF regressions. In both R^2 and RMSE, the changes in PLS models are more evident than in RF, in which the changes are steadier. In the same line, in PLS both parameters R^2 and RMSE show abrupt changes just after the optimal number of bands selected (Figure 4). However, in RF after this point, there is a steady interval until the values drop rapidly. RF showed the best results or negligible variations with the default value of mtry=1/3 and stabilisation of RMSE before the 500 trees are grown (Figure S5 and Figure

S6) (Belgiu and Drăgu, 2016; Díaz-Uriarte and Alvarez de Andrés, 2006; Liaw and Wiener, 2002).

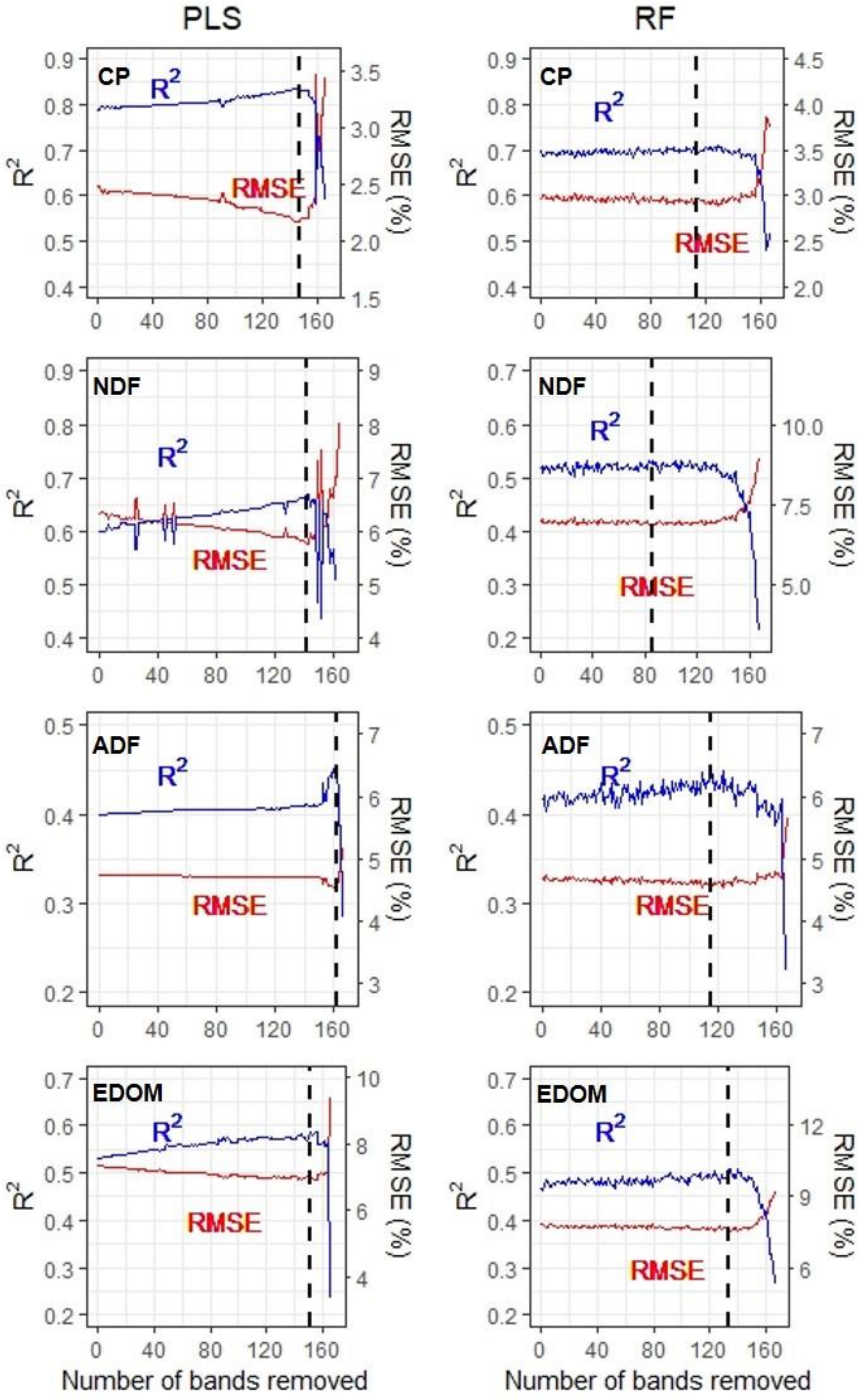

Figure 4. Changes of R^2 and RMSE in backward feature elimination of redundant bands using PLS (leave-one-out estimations) and RF (out-of-bag estimations) regressions for crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and enzyme digestibility of organic matter (EDOM) (n=164). Dashed lines indicate the minimum RMSE value and maximum R^2 at which the optimal number of bands is reached.

Bands selected by backward feature elimination and importance in PLS and RF

The number and proportion of bands selected by backward feature elimination for each pasture quality variable are shown in Table 3. Several differences can be observed between models. PLS tended to select fewer bands than RF in all variables. The variable with a higher proportion of bands selected using backward feature elimination was NDF, for which 15.5% and 48.8% of the bands were selected with PLS and RF respectively (Table 3). Only 7 bands were selected for ADF with PLS while 53 bands were selected using RF. For CP and EDOM, 12.5% and 10.1% were selected with PLS and 32.7% and 20.2% using RF.

The position of these selected bands in the spectral range of 400-2300 nm is illustrated in Figure 5. This figure also illustrates the reflectance curve depending on the content of the pasture quality variable. It can be observed how samples with high values of CP and EDOM and low fibre content show higher reflectance values in the Near Infra-Red region (NIR) (800-1300 nm). Again, some differences can be observed between models. Especially concerning the visible region, while in RF the bands located in this region are mostly selected, in PLS these bands are almost absent. The same happened in the region between 800 nm and 900 nm, in which just band 880 was selected for NDF in PLS, whereas in RF several bands were selected in this spectral region.

Bands from the red-edge region (680-750 nm) were commonly selected for all variables using both PLS and RF (Figure 5). Especially the band centered at 700 nm was selected for all models but ADF using PLS. This band also showed high importance and stability in the predictions (Figure 6). For example, for the predictions of CP with PLS and RF, this band was the second and the most important band respectively, having also the highest value of stability (Figure 6).

Bands from the NIR (800-1300 nm), especially from 900 nm onwards in PLS, and the shortwave infrared region (SWIR) from 1300-2300 were also intensively selected in most of the variables using both, PLS and RF except for ADF using PLS. Bands 960 and 1160 for example were selected with PLS in CP (Figure 6), and NDF (Figure S2.). Band 1960 was selected in all models for CP and NDF.

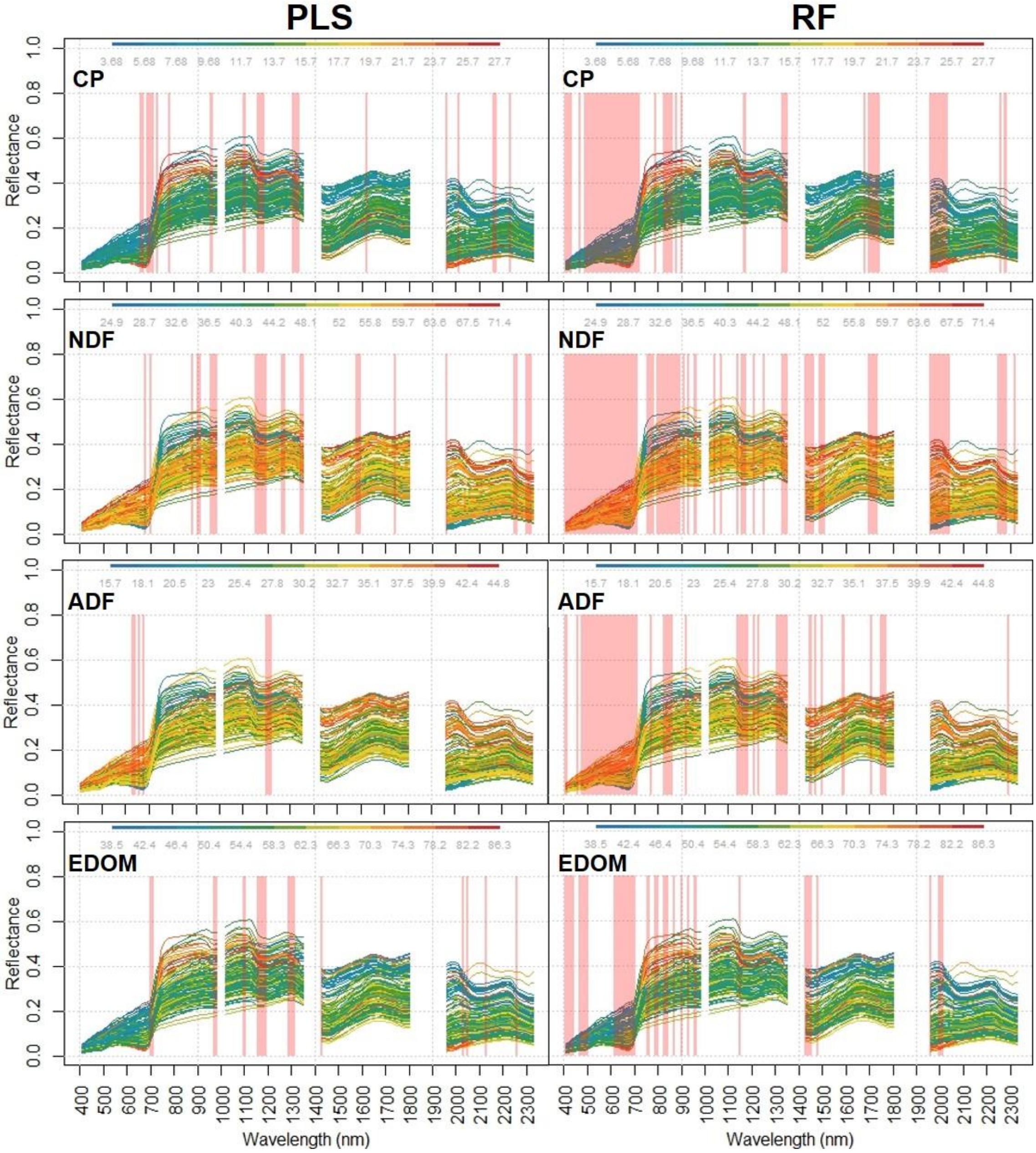

Figure 5. Canopy reflectance of the pasture samples (n=164) coloured by the content of the respective pasture quality variable: crude protein (CP); neutral detergent fibre (NDF); acid detergent fibre (ADF); and enzyme digestibility of organic matter (EDOM). Vertical red lines indicate the selected bands by backward stepwise feature elimination using PLS (left) and RF (right).

Figure 6 shows the importance of the bands selected with PLS and RF for CP prediction. The importance for the rest of the variables can be consulted on Supplementary Material (Figure S2-Figure S4). Overall, bands belonging to sections 1100-1300 nm of the NIR and 2100-2300 of the SWIR regions were rated as the most important bands in PLS. For CP, the red-edge region (680-750 nm) was especially important (Figure 6). In RF models, the most important bands were located at the visible (400-680 nm), especially those belonging to the green and red sections, and red-edge regions.

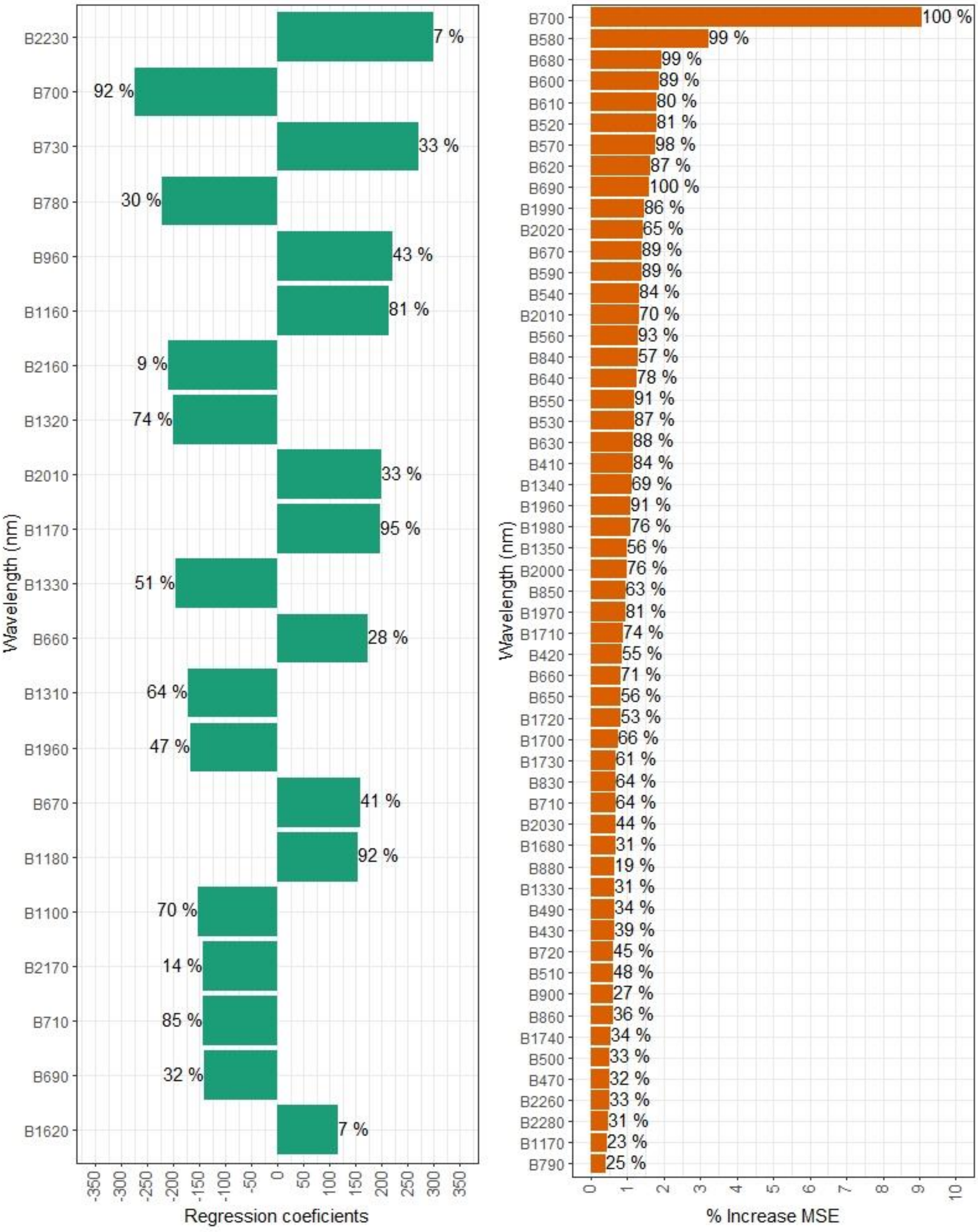

Figure 6. Importance and stability of selected bands for crude protein (CP) by backward stepwise feature elimination with PLS (21 bands) and RF (55 bands). Importance is measured in absolute value of the regression coefficients of selected bands in PLs and in % increase of mean squared error (MSE) in RF. Stability is indicated as % times the bands were selected in 100 repetitions of the backward feature elimination with using 70% of the samples each time selected by bootstrap with replacement.

Important differences can be observed in the stability of the variables. In PLS models, some variables highly ranked showed low values of stability. That is the case of band 2230, which was the most important band for predicting CP and was selected in only 7% of the times that the backward feature elimination was repeated with bootstrapped data (Figure 6). On the contrary, band 710, with a lower regression coefficient had a stability value of 85%. For CP using PLS, bands 700 and 710 from red-edge and bands 1160, 1170 and 1180 from NIR were highly stable (Figure 6). In RF the stability of the top-ranked bands is, overall, more in line with their importance value.

Predictive ability and robustness of PLS and RF models

CP was the variable with the most accurate and stable predictions with both, PLS and RF regressions (Figure 7). PLS outperformed the predictive ability and robustness of RF for CP and NDF, being the predictive statistics of both methods very similar for ADF and EDOM (Figure 7). The prediction of CP using PLS showed “good” accuracy ($2 \leq$ RPD$<$ 2.5 and $R^2 \geq 0.7$) with a mean R^2 value of 0.82 and a range of 0.68-0.90. The mean RMSE=2.23% was low and the mean RPD=2.47 with values ranging from 1.81 to 3.23. These statistics were considerably worse when RF was used. RF models to predict CP produced mean R^2 value of 0.68, mean RMSE=3.00% and mean RPD=1.82, indicating “moderate” accuracy ($1.5 \leq$ RPD<2 and $R^2 \geq 0.60$). For NDF, the PLS models had a “moderate” accuracy with mean values of R^2 and RPD 0.62 and 1.71 respectively and mean RMSE=6.05%. However, the accuracy of NDF models dropped to “poor” when the predictions were made with RF, reporting a mean $R^2=0.47$, mean RPD= 1.41, and mean RMSE=7.20%. For ADF and EDOM, “poor” accuracy was obtained using both PLS and RF since the RMSE was high and mean values of $R^2 < 0.6$ and RPD < 1.5. Only for EDOM predictions with PLS accuracy close to “moderate” was obtained, with mean values of $R^2=0.54$ and RPD=1.55.

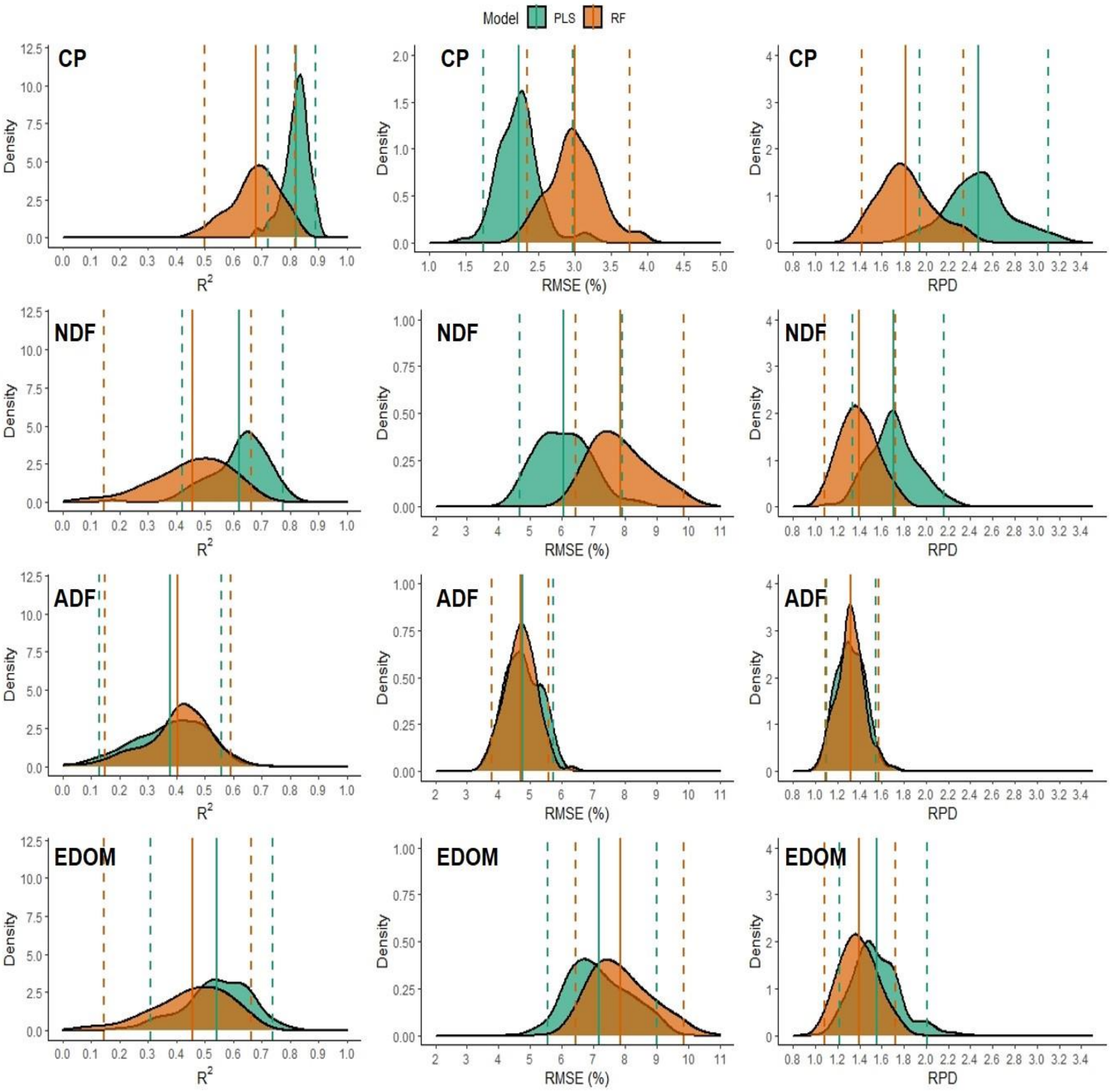

Figure 7. Density distribution of values of R^2, root mean square error (RMSE %) and ratio of predicted deviation (RPD) from predictions over 30% of bootstrapped samples using PLS and RF models. Calculated from n=100 random partitions of the dataset (n=164) into 70% for calibration and 30% for test with replacement. The predicted parameters are; crude protein (CP); neutral detergent fibre (NDF); acid detergent fibre (ADF); and enzyme digestibility of organic matter (EDOM). Solid lines show the mean and dashed lines show the confidence intervals (2.5 and 97.5 percentiles).

DISCUSSION

Performance of PLS and RF, prediction ability, certainty and backward feature elimination

This study compared two machine learning algorithms widely used in remote sensing, PLS and RF. The results showed that PLS outperformed RF in terms of prediction accuracy and certainty of the predictions of CP and NDF (Figure 7). This result differs from several studies reporting higher performance of non-linear algorithms such as Support Vector Machine (SVM), RF or Convolutional neural network (CNN) using hyperspectral data due to their capability to explain complex non-linear relationships in contrast to conventional PLS regression (Pullanagari et al., 2021; Pullanagari et al., 2016; Pullanagari et al., 2018; Ramoelo et al., 2013; Verrelst et al., 2015; Wijesingha et al., 2020; Yao et al., 2015; Zhou et al., 2019). Wijesingha et al. (2020) reported RF outperforming PLS to predict CP and ADF from 194 samples in mountain hay meadows and *Nardus stricta* grasslands using hyperspectral data from UAVs (118 bands of 5 nm spectral resolution, 482–950 nm). However, as Pullanagari et al. (2021) demonstrated using similar data with CNN and PLS, there is a trade off between the number of samples and the performance of the models. In this study, they found that PLS needs a minimum of 200 samples to stabilise the calibration while at least 1500 samples are required for CNN calibration. Little research has been found comparing RF and PLS to predict pasture variables using similar grasslands and comparable hyperspectral canopy reflectance. Further research is needed to explore if the trade off mentioned above between the number of samples and the performance exists comparing RF and PLS regressions.

The results reported on the predictive ability of the models indicate that quantitative predictions of “good” accuracy for CP ($2 \leq \text{RPD} < 2.5$ and $R^2 \geq 0.7$) of Mediterranean permanent grasslands can be achieved using data at a spectral resolution of 10nm. The accuracy drops to “moderate” ($1.5 \leq \text{RPD} < 2$ and $R^2 \geq 0.60$) for NDF. Fernández-Habas et al. (2021), also obtained better calibrations for CP and NDF than for ADF and EDOM using Sentinel-2 data to predict pasture quality in Mediterranean permanent grasslands. Therefore, pasture quality maps in Mediterranean grasslands might be based on CP and NDF predictions.

The performance of the models was comparable or even better than results reported from previous studies using similar data. For example, Biewer et al. (2009b) obtained R^2_{CV} =

0.83 and RPD=2.4 to predict CP in pure swards and binary legume-grass mixtures using field spectroscopy of spectral resolution 3 nm and 30 nm in the 350-1000 nm region and 1000-2500 nm region respectively. However, the accuracy obtained for ADF (R^2_{CV} = 0.75 and RPD=2) was considerably better than in our case. Safari et al. (2016) obtained worse calibrations for CP than Biewer et al. (2009b), despite using higher spectral resolution which was attributed to the heterogeneity of the grasslands and the reduced spectral range up to 1700 nm. The effect of multiple species composition of grasslands in lower regression accuracy was also pointed out by Kawamura et al. (2008) who reported worse mean R^2=0.62 but lower mean RMSE=1.27 to predict CP. Their results for ADF were slightly better than in this study and worse in the case of NDF. Zhou et al. (2019) reported similar statistics of validation (R^2=0.84) to predict CP in legume and grass mixtures using the 10nm spectral resolution Yara-N sensor by Support Vector Machine, while worse results were obtained by PLS (R^2=0.64). There is still a considerable variation in the accuracy of the results of studies using field spectroscopy to assess pasture quality. The main reasons might be related to variations in sample size and differences in the grasslands assessed (Pullanagari et al., 2012). A key factor that enabled high accuracy to predict CP and NDF in this study is the wide range of the data used to calibrate the models (Table 2), promoted by the heterogeneity and inter-annual variability of Mediterranean grasslands. The growth stage of the grasslands is another factor affecting the canopy reflectance (Zeng and Chen, 2018), and thus the accuracy of the models. In this study, the models have been calibrated using samples from different growth stages and managements to test the accuracy of general models rather than the accuracy of specific models for different growth stages or compositions. Previous studies have investigated the effect of different growth stages and stand mixtures on the estimation of biomass and nutrient contents (Biewer et al., 2009a; Biewer et al., 2009b; Zeng and Chen, 2018; Zhou et al., 2019). Zeng and Chen (2018) showed differences in reflectance of samples from boot stage, peak growth, and dormancy. However, the PLS models showed improved R^2 from cross-validation and predictions when samples from all three growth stages were combined. Although the reduced number of samples used for the specific growth stages models might have affected the results. They concluded that is feasible to use a model to predict nutrient contents from vegetative to dormancy stages. Biewer et al. (2009a) and Biewer et al. (2009b) reported improved accuracy of predictions of yield and CP by legume-specific calibrations. On the contrary, Zhou et al. (2019) did not find an influence of sites, developmental stage, and species mixtures on the performance of PLS models. Pullanagari et al. (2021) also reported better performance of models using samples from all seasons combined due to a better cover of the variability compared to the season specific models. In agreement with Zhou et al. (2019), we consider that models developed with samples representing different grow stages, managements (grazed or non-grazed) and sites are more generalisable and useful than models calibrated for specific situations. This is especially important in Mediterranean grasslands due to the high heterogeneity promoted by the high species and functional diversity, management, and differing synchrony of growth stages. Thus, specific models might be of limited application in Mediterranean grasslands. As highlighted by Zeng and Chen (2018), the sample diversification of the calibration dataset covering a wide range of situations (phenological stages, sites, management and species composition) is crucial to improve the estimative ability of the models.

Compared to results reported using Sentinel-2 by Fernández-Habas et al. (2021) to predict CP and NDF, the accuracy was improved considerably. This demonstrates that future high-priority mission candidate CHIME (Nieke and Rast, 2018), could improve the quality of the predictions and the retrieval of information from grasslands canopy compared to currently operating multispectral sensors (Berger et al., 2020; Obermeier et al., 2019; Rast et al., 2019; Thenkabail et al., 2000). This improvement in the quality of the predictions is especially important in Mediterranean ecosystems due to the higher heterogeneity of the grasslands (Fava et al., 2009), which demands finer spectral resolution to provide accurate information on the grassland's attributes. However, it has to be considered that the spatial resolution of CHIME (20-30 m) will also play a major role in its potential to monitor grasslands ecosystems (Meier et al., 2020). Here we only tested the spectral resolution, further research involving the spatial resolution is required to get a complete picture of the potential of this promising sensor (Casa et al., 2020). These studies should aim at including the spatial resolution of 20-30 m of the CHIME data in the sampling approach, which together with the results provided in this study could additionally contribute to defining the sources of error and uncertainty of models developed with true CHIME data in the future. Lastly, although the simulation of the spectral resolution of satellites from field spectroscopy has been extensively used in previous research (Adjorlolo et al., 2015; Lugassi et al., 2019; Mutanga et al., 2015; Ramoelo and Cho, 2018; Sibanda et al., 2015), the results obtained from this data must be treated as an approximation to the potential of the future satellite, not as the actual performance of it.

Figure 7 illustrates the importance of implementing bootstrap approaches to test the performance and predictive ability of the models. Pullanagari et al. (2021) highlighted the relevance of quantifying and reporting the uncertainty of the predictions as well as using an appropriate sample size. The variation of the models' performance statistics associated with the data partition (Figure 7) reveals an inherent uncertainty of the dataset. Reporting information of a single model without testing the certainty of the predictions can lead to biased information (Verrelst et al., 2015). In this study, the interpretation of the model performance was associated with its corresponding uncertainty. This is also relevant when implementing this technology in the management and monitoring of grasslands. It is therefore advisable when reporting information of the predictions, supporting it with the corresponding confidence intervals.

The improvement achieved by the BFE using both algorithms, PLS and RF, is also consistent with previous literature (Mutanga, 2004; Adam et al., 2014; Díaz-Uriarte and Alvarez de Andrés, 2006; Odindi, 2014; Belgiu and Drăgu, 2016; Kawamura et al., 2017; Santos-Rufo et al., 2020). For example, Kawamura et al. (2008) also reported an important decrease of RMSE in cross-validation for CP, NDF and ADF using PLS and 5 nm of spectral resolution of field spectroscopy. The same authors also compared the performance of models using canopy reflectance of the pasture and first derivative reflectance (Kawamura et al., 2008). They found some differences in performance and band selection of models fitted with first derivative reflectance. The spectral preprocessing of the spectra is an interesting topic for future research that, to our knowledge, has not been investigated in deep for pasture quality estimation using field spectroscopy. For example, Dotto et al. (2018) performed a systematic study on 63 spectral preprocessing and multivariate prediction models of soil organic carbon by Vis-NIR spectra using a FieldSpec 3 Spectroradiometer (ASD Inc.). These studies could support choices of spectral preprocessing to improve the prediction capability of the models.

Importance and stability of bands to predict pasture quality variables

Most of the bands of known absorption features (see Adjorlolo et al. (2013) and Kawamura et al. (2008) for review) or those close to them were selected and highly ranked for the prediction of the corresponding compounds. The results of the bands importance analysis align with results from previous studies highlighting the role of the red-edge region to assess pasture quality due to its relationship to the chlorophyll content of the vegetation (Adjorlolo et al., 2015; Horler, 1983; Kawamura et al., 2008; Ramoelo et al., 2011; Ramoelo and Cho, 2018). Our results showed that this region was commonly selected for all pasture quality variables in both models (Figure 5), being also some bands such as band centred at 700 nm highly stable (Figure 6). In this study, the 700 nm centred band, ranked second and first in PLS and RF models respectively to predict CP. Adjorlolo et al. (2015) also found the 700 nm waveband as the most important according to the PLS' variable importance projection (VIP) to predict nitrogen content in C3 and C4 grass species. They also found a strong relationship between the 720 nm waveband and CP. This demonstrates the reliability and importance of the red-edge region to assess pasture quality. Bands from NIR and SWIR regions were also commonly selected in PLS and RF for CP and NDF (the best-predicted variables). The selection of bands in these regions lies in the well-known absorption features of cellulose, protein, nitrogen, and starch due to C–H, C–N, N–H, and O–H bonds (Carter, 1994; Clark and Lamb,1991; Curran, 1989; Kawamura et al., 2008; Kokaly, 2001). These results show that a target-oriented selection of bands in these regions can lead to accurate predictions of pasture quality with few bands (Adjorlolo et al., 2015; Kawamura et al., 2008).

The main difference from previous studies is the contrasting selection of bands in the visible region and their importance in RF models compared to PLS models (Figure 5, Figure 6, and Figure S2). The visible region is related to the content of the pigment of vegetation (Blackburn, 1998; Ustin et al., 2009). The pigment content is strongly related to the CP and fibre content, and it is subjected to changes of the phenological stages during the growing season. However, as pointed out by Kattenborn et al. (2019), the upscaling of pigment concentration to the canopy scale is challenging. Although this trend should be carefully interpreted due to the tendency of RF to select a higher number of bands than PLS by backward feature elimination, one possible explanation for that could lie in the fundamental differences between PLS and RF to model the relationship between predictors and the dependent variable. Since PLS is less suitable for deriving strong non-linear relationships than non-linear models (Pullanagari et al., 2021; Verrelst et al., 2015), RF could better capture the relationships between pigments and canopy reflectance in this region of the spectra. This non-linear relationship between reflectance in the visible range and leaf chlorophyll content has been pointed out in previous research (Blackburn, 1998; Gitelson et al., 2003). For example, Qin (2011) attributed an improved pigments content estimation in grape leaves, using hyperspectral data in the 400-750 nm spectrum, to a non-linear modelling by SVM.

Some of the selected bands showed low stability to the variation of the dataset. This outcome highlights again the importance of testing the uncertainty of the results. The information on the most important bands in these types of studies should be tied to a stability analysis to be more informative. Because of the confounding effect on the reflectance of canopy structure, leaf inclination, plant diversity, plant water content, or different phenological stages (Curran, 1989; Fava et al., 2009; Kattenborn et al., 2019; Pullanagari et al., 2021; Tong and He, 2017; Zhou et al., 2019), the response of the stability of the selected bands in relation to changes in the dataset has important implications to select stable and reliable bands to perform predictions. The stability of band 700 and 710 and 1160-1180 in PLS to predict CP could indicate a strong CP content-reflectance relationship despite the possible cofounding effects mentioned above. However, the bands selected in the region of the spectra from 2000 to 2300 nm reported low stability. This might be caused by the water content of leaves and soil background since Ripple (1986) found the 2080-2350 nm region to be sensitive to both factors. Ramoelo et al. (2011) also highlighted the water effects in the SWIR region for the retrieval of grass nitrogen. In this study, some samples taken in May and June were senescent. The reflectance of senescent grasslands can distinctively show absorption features in the 2006-2196 region of the spectra that otherwise would be masked by the water content in non-senescent grasslands (Mutanga et al., 2004). This can be appreciated in Figure 5 where the reflectance of samples with higher fibre content and lower CP content (senescent conditions), clearly show absorption features in the SWIR region compared to the reflectance of samples with lower fibre content. In RF, the stability was higher, although the considerable number of bands selected might influence that stability measure. Nevertheless, it can be also observed that bands from the SWIR region tend to show lower stability compared to those from the red-edge region. The mix of senescent and non-senescent samples could lead to the lower stability of the SWIR bands in both models. Independent calibration models for different stages could improve the stability of these bands. However, this would reduce the range of the dataset and the generalization of the models since the mix of senescent and non-senescent grasslands is common in Mediterranean grasslands and the transition between both stages is also an important moment to have information about the pasture quality.

Implication for the management and monitoring of Mediterranean permanent grasslands

PLS models calibrated with the selected bands (from the red-edge and NIR regions) by BFE showed good accuracy in the predictions, with high R^2=0.82 and low mean RMSE=2.23%. These results demonstrate that future sensors at this spectral resolution can provide useful information for the management and monitoring of Mediterranean permanent grasslands.

CP content is a crucial attribute of the pasture to inform the management of grasslands and livestock. Having accurate predictions on the content of CP can help the farmers to perform more efficient grazing of Mediterranean grasslands which are subject to high interannual variations of CP. If the predictions can be performed at quantitative level, the utility of the information compared to qualitative predictions increase considerably since it might allow more precise calculation of information such as the carrying capacity of the grasslands or the need and type for supplementary feedstuff for the livestock (Pullanagari et al., 2013; Ramoelo and Cho, 2018; Raab et al., 2020; Starks et al., 2006). If this level of accuracy is achieved with future operational sensors such as CHIME (Nieke and Rast, 2018), this technology might substitute labour manual collection of samples to determine pasture quality (Starks et al., 2004). The difference of precision can be assumed in benefit for spatial predictions acquired on a regular basis in nearly real-time (Pullanagari et al., 2013; Starks et al., 2004). Indeed, pasture quality determination is not frequently performed in farms of Mediterranean permanent grasslands, where the information provided by these determinations might not compensate for the cost of the analysis. Additionally, the delay between manual sampling collection and the reception of the data limits its usefulness since the quality and phenology of Mediterranean grasslands can change rapidly (Pérez-Ramos et al., 2020, Gómez-Giráldez et al. 2020). Therefore, the availability of hyperspectral data can mean a step forward in the adoption of smart farming in Mediterranean grasslands-based farms. However, it has to be considered that a high proportion of Mediterranean grasslands are devoted to traditional small farming (Lowder et al., 2016) for which the implementation of remote sensing technologies might be of limited application and low interest for smallholders.

The analysis of the bands importance and stability to predict the pasture quality indicators has important implications to inform the design of devices aimed at optimising the range of the spectra and the spectral resolution used to assess in-field pasture quality. For example, in the case of CP, it has been demonstrated that field spectrometers with a spectral resolution of 10 nm and 19 target-oriented bands can be sufficient to achieve good accuracy of the predictions.

Finally, the enhanced accuracy provided by forthcoming CHIME in combination with currently operating services such as Sentinel-2 opens new opportunities to monitor Mediterranean grasslands ecosystems in the context of the next renewal of the Common Agricultural Policy (CAP) (Rast et al., 2019). This technology can be used to assess the compliance of the CAP and Natura 2000 regulations and the conservation status of grasslands ecosystems at the national scale (Griffiths et al., 2020).

CONCLUSION

Concerning the objectives of the study the conclusions are:

i) Hyperspectral narrow bands from field spectroscopy at 10 nm of spectral resolution CHIME-like show potential to predict CP at good accuracy and NDF at moderate accuracy level in Mediterranean permanent grasslands. ADF and EDOM were predicted with poor accuracy.

ii) PLS outperformed RF to predict CP and NDF in terms of accuracy and certainty of the predictions.

iii) BFE can considerably reduce the number of bands used in the predictions while improving the accuracy of the models, especially in PLS regressions.

iv) Bands from the red-edge and NIR regions show high importance and stability to assess the best-predicted variables. Bands centred at 700, 710, 1160, 1170 and 1180 are highly stable and important to predict CP. The bands belonging to the SWIR region show lower stability.

v) These results prove the potential of hyperspectral data and future satellite missions such as CHIME to inform the management and monitoring of Mediterranean permanent grasslands.

Further research needs to be carried out to advance towards the applicability of the results here reported to practical farming in Mediterranean permanent grasslands.

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Supplementary material

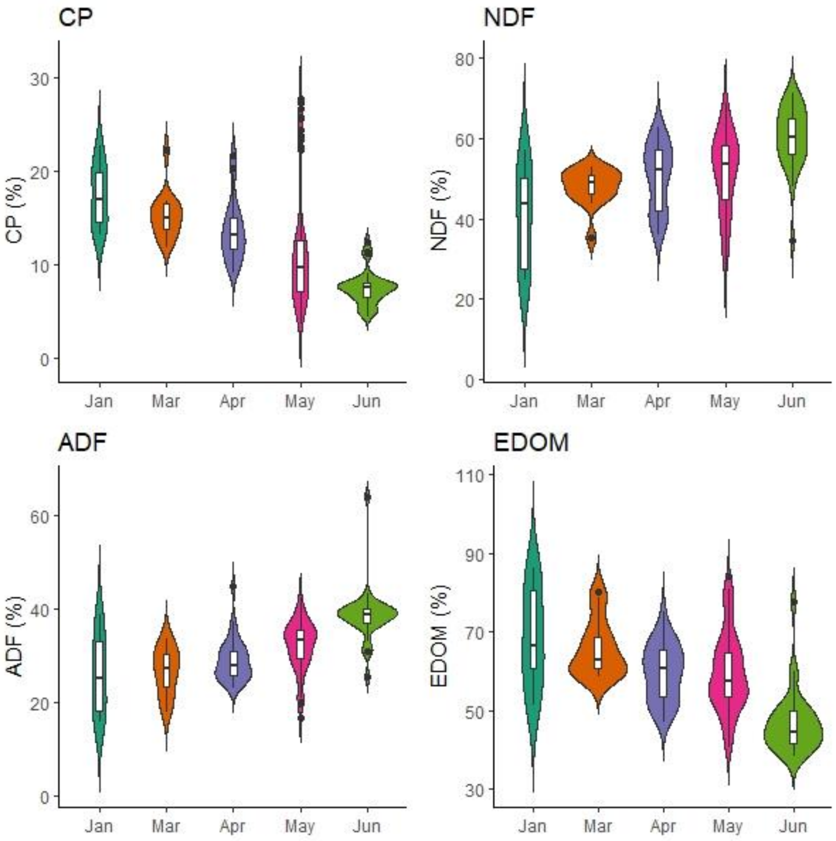


Figure S 1. Variation of pasture quality variables used to fit the models (N=164) by month. Black centre line, median; box, interquartile range; box limits, lower and upper quartiles; whiskers, 1.5× interquartile range; points, outliers. Coloured area indicates the sample distribution. *May includes samples from 2013 and 2019. CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; and EDOM: enzyme digestibility of organic matter.

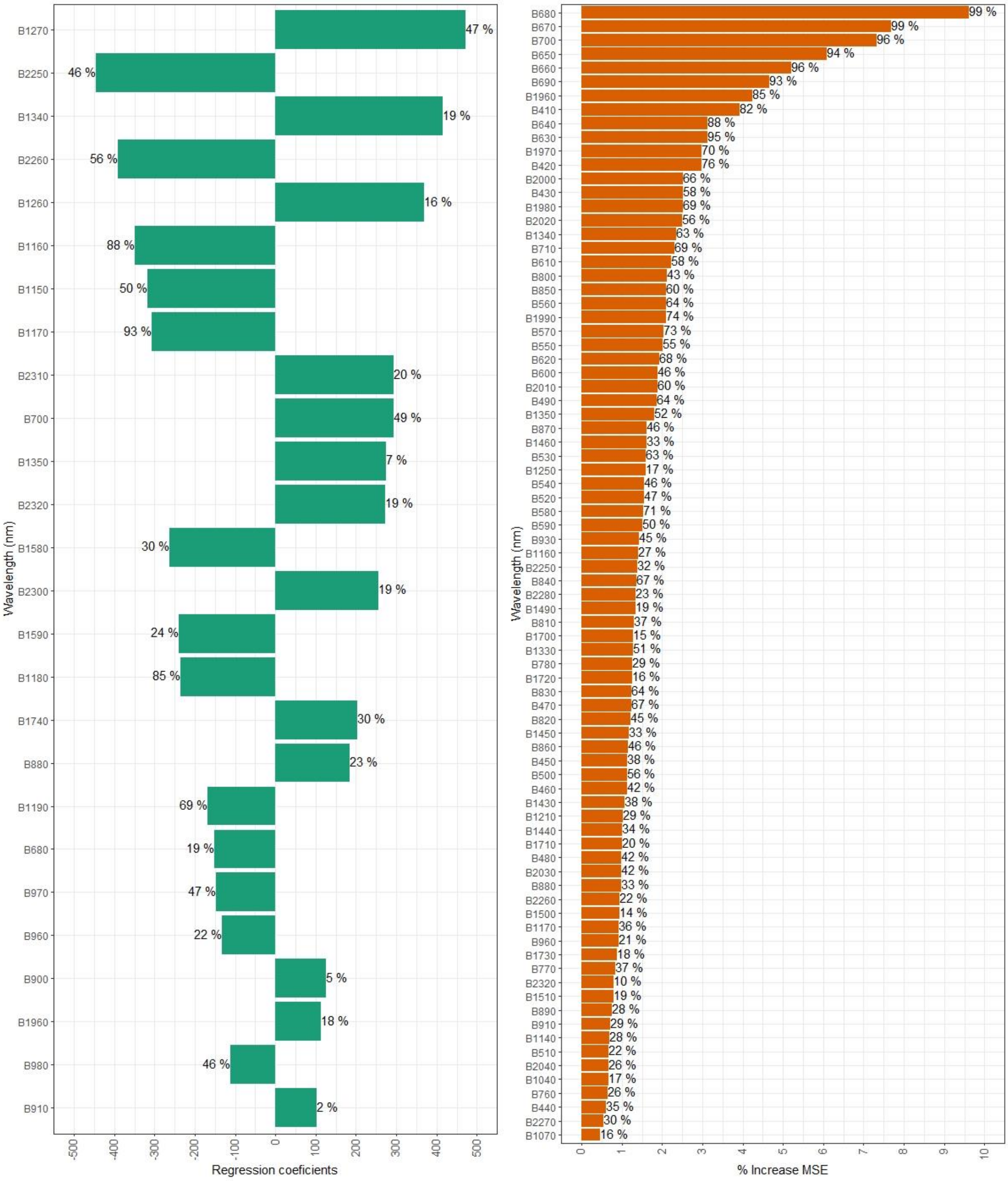


Figure S 2. Importance and stability of selected bands for neutral detergent fibre (NDF) by backward stepwise feature elimination with PLS (21 bands) and RF (55 bands). Importance is measured in the absolute value of the regression coefficients of selected bands in PLs and in % increase of mean squared error (MSE) in RF. Stability is indicated as % of times the bands were selected in 100 repetitions of the backward feature elimination with using 70% of the samples each time selected by bootstrap with replacement.

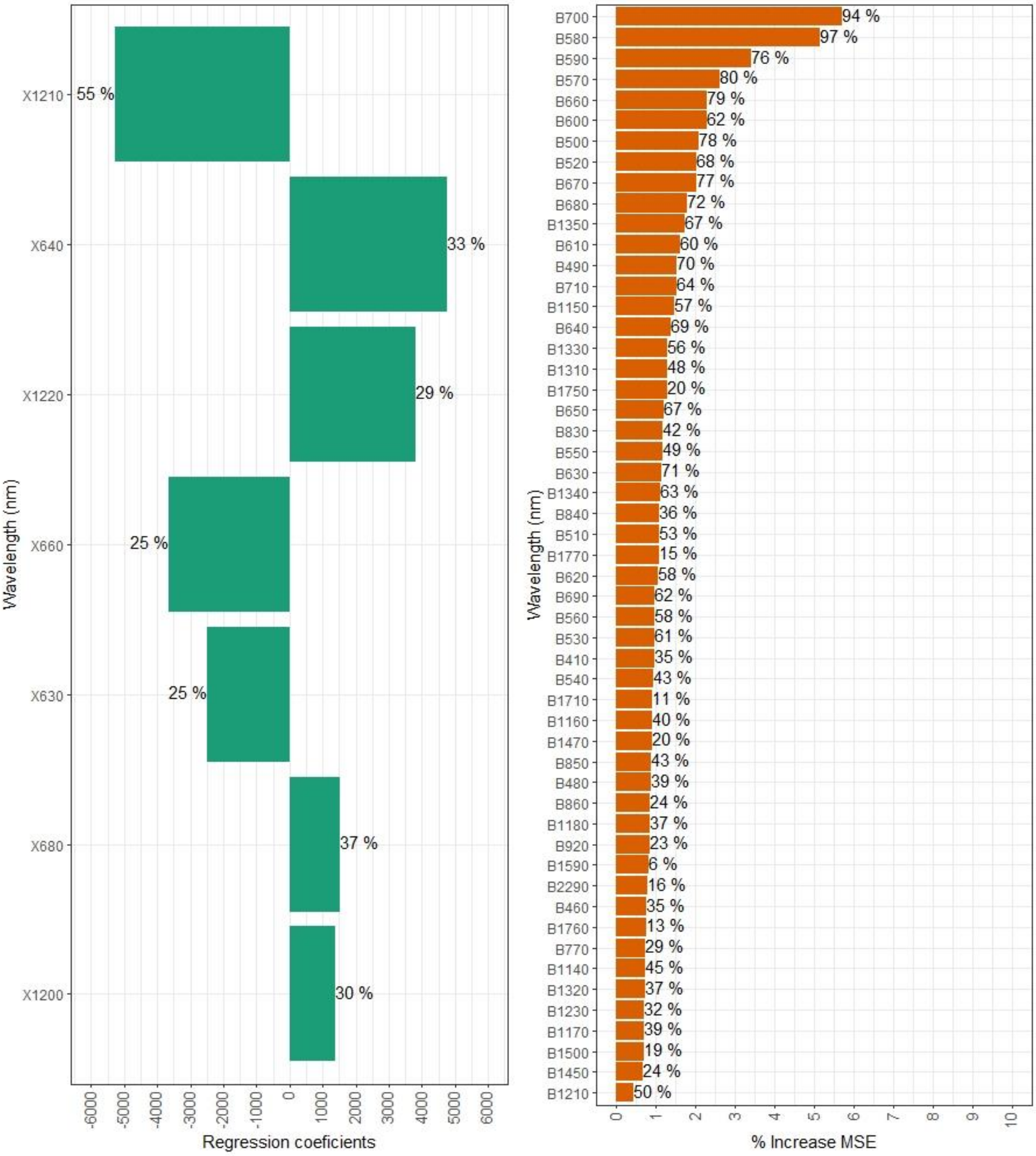


Figure S 3. Importance and stability of selected bands for acid detergent fibre (ADF) by backward stepwise feature elimination with PLS (21 bands) and RF (55 bands). Importance is measured in the absolute value of the regression coefficients of selected bands in PLs and in % increase of mean squared error (MSE) in RF. Stability is indicated as % of times the bands were selected in 100 repetitions of the backward feature elimination with using 70% of the samples each time selected by bootstrap with replacement.

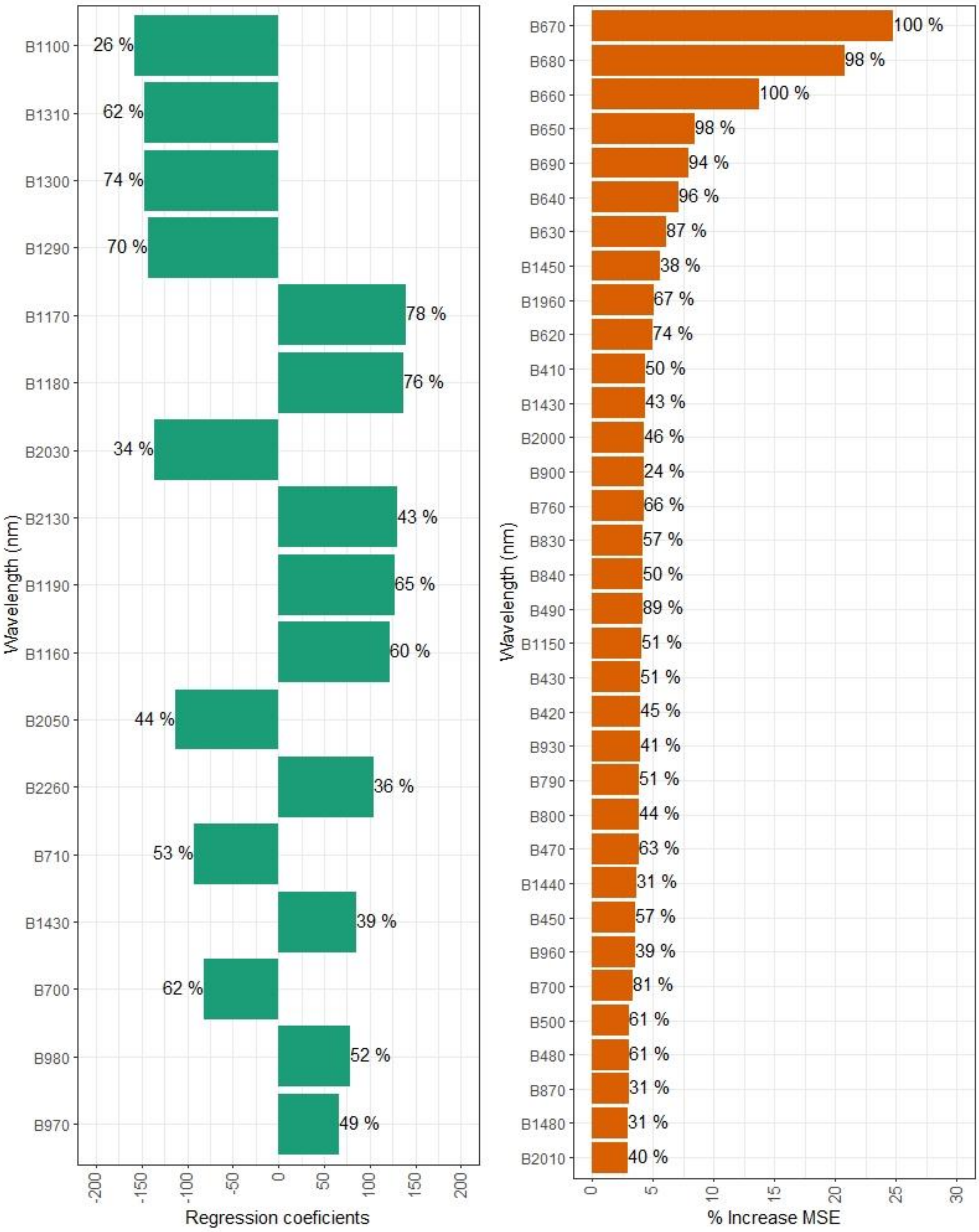


Figure S 4. Importance and stability of selected bands for enzyme digestibility of organic matter (EDOM) by backward stepwise feature elimination with PLS (21 bands) and RF (55 bands). Importance is measured in the absolute value of the regression coefficients of selected bands in PLs and in % increase of mean squared error (MSE) in RF. Stability is indicated as % of times the bands were selected in 100 repetitions of the backward feature elimination with using 70% of the samples each time selected by bootstrap with replacement.

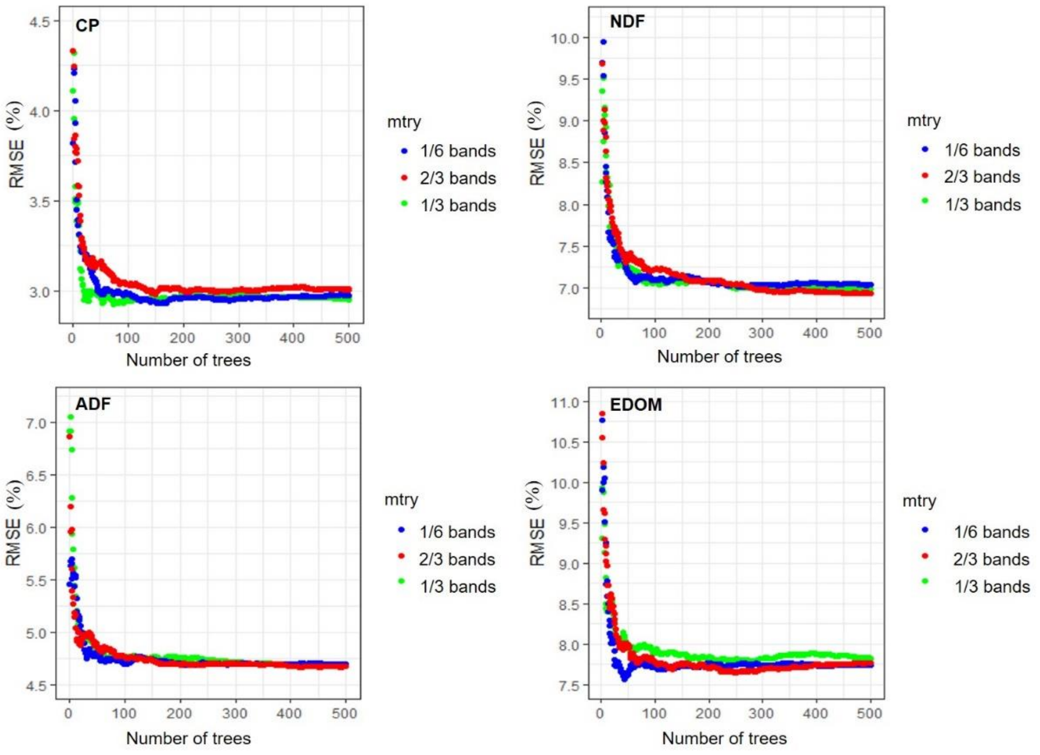


Figure S 5. Changes in RMSE of RF models using all bands for each pasture quality variable with different mtry and ntree values. Default settings are mtry=1/3 and ntree=500. CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; and EDOM: enzyme digestibility of organic matter.

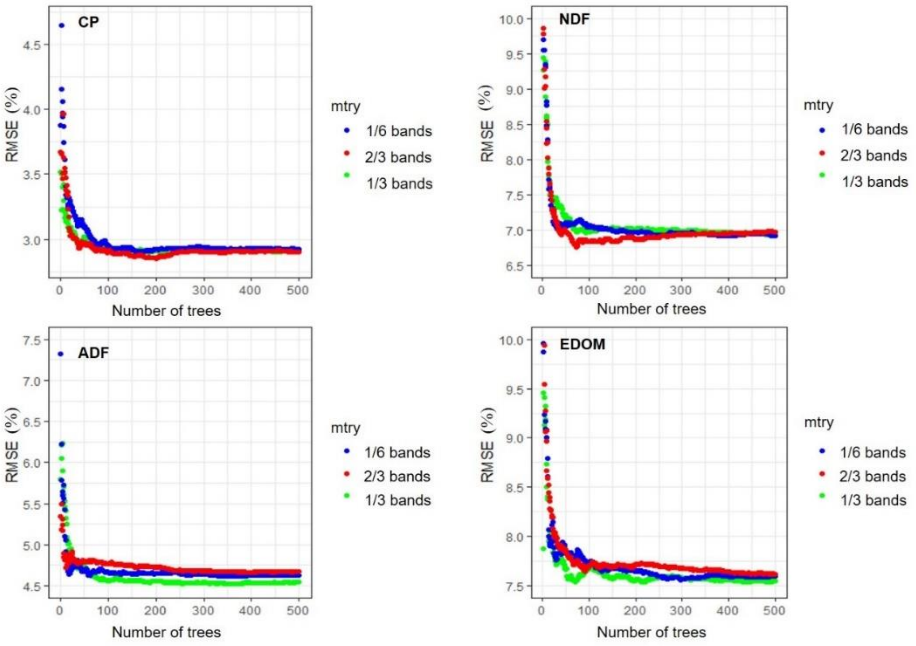


Figure S 6. Changes in RMSE of RF models using the selected bands for each pasture quality variable with different mtry and ntree values. Default settings are mtry=1/3 and ntree=500. CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; and EDOM: enzyme digestibility of organic matter.

CHAPTER VIII. GENERAL DISCUSSION AND FUTURE RESEARCH

This PhD Thesis has studied two innovations with potential to be applied to the management of herbaceous grasslands of dehesa systems: remote sensing of pasture quality and the new perennial forage legume *Bituminaria bituminosa*. This section provides a synthesis and overall discussion of the potential and limitations of both innovations in the management of grasslands of dehesa systems. The discussion seeks to emphasise the implications for management and policy-making in the context of the threat of global change and opportunities for innovations. Research needs arising from this PhD Thesis are also identified and outlined to provide a contextualisation to future studies.

On the relevance of the studied innovations in dehesas systems

The use of remote sensing in agriculture and livestock systems has drawn increasing attention during the last two decades, with an exponential expansion under the so-called “agriculture 4.0”. These tools bring opportunities to improve the efficiency of management by providing spatial information in real-time at farm level (Horn and Isselstein, 2022). However, they have limited potential to solve problems such as the impact of droughts and the dependency on concentrates to feed livestock during feed gaps. They must be used jointly with other agronomic practices that could address these issues such as grasslands improvements or forage crops. In addition, the expected impacts on ecosystem services and environmental sustainability may be indirect, through improved and informed management. Based on the results from **Chapter II** it is evident that farmers do not consider remote sensing and high-tech innovations as highly relevant innovations for the management of dehesa farms. Policymakers and researchers must understand the role of this type of innovations in dehesa systems to avoid overestimation of their potential impact (techno-optimism) and prevent overlooking other innovations that could have a higher impact on the sustainability of dehesa farms. The other innovation studied in this PhD Thesis, the use of the perennial legume *Bituminaria bituminosa* to provide out-of-season forage, seems to have higher relevance for farmers as it directly addresses one of the main threats to dehesas systems; the regular feed gaps of the summer season and irregular feed gaps caused by droughts such as the ongoing 2023 drought in Spain. In a context of technological and digitalisation development, agronomic innovations such as the use of a new perennial legume or the development of legume-rich mixtures might be overlooked. This should be avoided when allocating funding to the development of innovations in extensive livestock systems by understanding the complementary role that each innovation plays in these systems. One threat of the digitalisation development is that it could be biased towards intensive agricultural and livestock systems and thus increasing the unbalance between intensive and extensive systems (Eastwood et al., 2021). The application of remote sensing to provide information for management and monitoring could contribute to increasing the efficiency of dehesa systems, however, it will never achieve the level of control on the system of intensive systems. Public policies should ensure that extensive systems have the same opportunities of access to relevant innovations adapted to their needs as intensive systems to avoid increasing the unbalance between them. In many cases, the low profitability of dehesa systems (Escribano et al., 2018) may hinder the investment and adoption of innovations of any kind. Therefore, public policies should also contribute to creating a suitable environment for an innovation process in dehesa systems. One of the most urgent and necessary measures to create this suitable environment and to improve the sustainability of dehesas is to internalise their environmental positive externalities by payment for ecosystem services and suitable eco-schemes within the Common Agricultural Policy, and raise awareness of the added value of its products among consumers (Bugalho et al., 2011; Gaspar et al., 2016; Rolo et al., 2020; Villanueva et al., 2021). Finally, the integration of innovations in the management of dehesas will require training and transference to be effective. Especially technological innovations entail a considerable learning load for farmers and it might be difficult for them to keep up with (Eastwood et al., 2021). In this regard, public–private coordination could be favoured and facilitated by extension services. This public figure, now missing or privatised in more profitable systems, might play a crucial role in innovation processes in dehesa systems (Labarthe, 2009). Cooperatives could also have an important function in creating suitable environments for the adoption and transference of innovations among farmers.

Potential uses of *Bituminaria bituminosa* and research needs to include it as a new forage perennial legume in dehesas.

This PhD Thesis has investigated the response of *B. bituminosa* to different gradients of water stress, its forage quality, phenology, competitive ability, and adaptation to contrasting soil textures.

Chapter III showed important differences between the three varieties of *B. bituminosa* from the Canary Islands. Var. a*lbomarginata* and var. *crassiuscula* were found to have better forage aptitude than var. *bituminosa*. However, the poor development and lower thin roots proportion of var. *albomarginata* denoted a possible susceptibility of this variety to high soil water conditions in heavy textured soils. In **Chapter IV** the newly developed variety Lanza® was tested in two soils of contrasting texture at high soil water content. The results did not show a detrimental effect of heavy texted soils on total root and aerial biomass, although the slower development, lower thin roots dry mass production and a trend of a lower competitive ability in clay soils indicate that this variety and overall this species is best suited to light textured soils. **Chapters IV** and **V** also demonstrated that this species might have low competitive ability. **Chapters III** and **V** showed that this species has good forage quality to improve dehesa grasslands and to provide high-quality forage during the summer season. Finally, **Chapter V** showed that *B. bituminosa* var. Lanza® can be highly productive and resistant to the forecasted rainfall reductions under climate change scenarios although trade-offs between defoliation and performance over the summer in terms of forage quality and leaf shedding could be expected. This Chapter also found early phenology and plasticity suitable for Mediterranean conditions. Based on these results, field observations and previous research, the following paragraphs outline and discuss the research needs and potential to use this species in dehesa systems.

The fact that as described in **Chapter V,** cv. Lanza® maintained green leaves over the summer of 2021 demonstrates that this species could provide out-of-season forage in Mediterranean grasslands of the south of Spain in agreement with studies in Western Australia (Raeside et al., 2012; Real et al., 2017). Further research is required to clarify if the observed leaf shedding in the summer of 2022 was caused by a drier summer, or the phenology and biomass production during this year. Based on previous studies (Real and Kidd, 2012; Suriyagoda et a.,2013), it seems clear that the defoliation timing and intensity play an important role in the performance of Lanza® in summer and its ability to provide high-quality forage. This should be further investigated to inform the suitable management of this species.

Two major limitations to the use of Lanza® in dehesa systems are its low competitive ability and low cold tolerance. As discussed in **Chapters IV** and **V** future studies should test the competitive ability of Lanza® in less competitive environments and with less aggressive competitors to inform the use of this species in multispecies mixtures. To advance in this line it is essential to clarify what are the reasons for the low competitive ability of this species. When establishing Lanza® in monocultures, the strategy to avoid invasion by weed could be early or late sowing to seek asynchrony with annual species. Also letting the fields fallow previous sowing might reduce the emergence of annuals.

Although cold tolerance has not been specifically studied in this PhD Thesis, field observations indicate that it could perform poorly in cold dehesas with frequent frost days in winter in agreement with Raeside et al., (2012). Therefore ongoing breeding programs to develop new varieties should put special emphasis on improving the cold tolerance of this species as its wide distribution over the Mediterranean basin offers opportunities for improving this characteristic (see Walker et al., 2010). This should be further investigated in studies testing the interaction genotype x environment (Paolo Annicchiarico, 2002). An unexplored aspect of *B. bituminosa* is its tolerance and response to shade which deserves investigation in the face of its use in agroforestry systems (Hernández-Esteban et a., 2019).

B. bituminosa could be used strategically in monoculture to be grazed in two key moments of the year (based on unpublished information by D. Real):

1) After the senescence of annuals to extend the green feed-season for livestock. This could also allow resting annuals during senescence to favour seed production, ensuring seed bank and therefore persistence.

2) At the end of summer-begging of autumn when natural grasslands have low quality and monocultures of *B. bituminosa* could reduce the need for supplementary feeding. This would depend on the ability of *B. bituminosa* to maintain green forage over the

summer. That use could also allow resting natural grasslands when annual species initiate emergence, which would ensure proper establishment and winter production. This could be also possible if good productions of *B. bituminosa* are achieved by early regrowth after the first autumn rains.

B. bituminosa could also have a useful integration in multispecies mixtures if inter-specific competition is avoided through a suitable selection of partner species. The common management package to establish multispecies mixtures in dehesas consists of sowing after phosphorous fertilisation, grazing in early spring and deferred grazing after senescence (stockpiling) (García-Moreno et al., 2016). This management ensures suitable seed production of the annual species established, however, it comes at the cost of lower pasture quality at the moment of grazing. *B. bituminosa*, due to its higher drought resistance and later dormancy, could retain green leaves and thus improve the forage quality of the mixture at the moment of grazing (Porqueddu et al., 2005) if suitable proportion is achieved and interspecific competition is minimised.

An important factor, not studied in this PhD Thesis but essential to address in order to inform the use of *B. bituminosa* in dehesas is its effect on oak trees. Being able to remain green and photosynthetic active under drought conditions *B. bituminosa* could increase the transpiration and thus water depletion in soils during summer (Dupraz et al., 1999; Garba et al., 2022; Li and Huang, 2008). This might have a detrimental effect on oak trees in dehesas which are already suffering from drought stress with the aggravation of the root rot disease caused by *Phytophthora cinnamomi* (Braisier, 1996; Peñuelas and Sardans, 2021). Recent studies have reported negative effects of shrub invasion on the water status and Photosynthetic capacity of Mediterranean oak trees (Haberstroh et al., 2021; Lecomte et al., 2022; Lobo-do-Vale et al., 2023). Dupraz et al., (1999) found perennial leguminous intercrops to be competitive for soil water resources with young walnuts in a Mediterranean climate and suggested that this effect might be compensated by an improved nitrogen status of the trees. The same authors stated that compensating effect was observed during a rather rainy year and therefore the impact of leguminous intercrops on tree growth might depend on the frequency of dry and wet years (Dupraz et al., 1999; Haberstroh et al., 2021). These negative effects, if existing, could be higher if *B. bituminosa* is established as a monoculture than if it is used in multispecies mixtures. Future research should clarify this potential negative effect. However, if used as monoculture *B. bituminosa* could be established in fields of low tree density to minimise competition with trees for water. Commonly in dehesa farms, there are fields of low or null tree cover, close to the livestock facilities that have been traditionally used to crop cereal or winter forages. These could be target locations to establish monocultures of *B. bituminosa* that could be used strategically (Figure 2).

It is still to be determined the impact that *B. bituminosa* will have on ecosystem services such as protection against soil erosion, soil organic carbon storage, and N fixation. Several beneficial effects might be expected as a perennial culture would reduce soil disturbance as well as the cost of management (Annicchiarico et al., 2013; Ergon et al., 2018). This information is essential in the face of the increasing importance of the impact of agronomic practices on the provision of ecosystem services as demonstrated by the last CAP. In the context of the newly introduced eco-schemes, *B. bituminosa* could be potentially included in the eco-scheme of crop rotation introduced in Spain in the CAP 23-27 (MAPA, 2023), which could mean an extra incentive for farmers to establish this species. The impact of *B. bituminosa* on ecosystem services and the research questions raised above are being addressed in a new project that derives from this PhD Thesis: *"Nuevos pastos de leguminosas perennes para la* dehesa*: Provisión de Servicios Ecosistémicos en condiciones de mayor aridez (Pastos-SEcos)"*.

Ultimately, if *B. bituminosa* consolidates as a new forage perennial legume suitable for dehesa systems of the Iberian Peninsula, it would have to reach the market at an acceptable cost for farmers. Despite the diversity of suitable legumes to be used in Mediterranean farming systems, there are few commercial alternatives for farmers, which ironically are commonly developed and produced in Australia. The case of *B. bituminosa* illustrates the potential of the Mediterranean and Macaronesian genetic diversity of legumes that could contribute to increasing the self-sufficiency and resistance of extensive livestock systems to a future drier climate. In the face of the rise of prices of concentrates and increasing droughts, public policies should promote and facilitate research and investment on the use of such resources as it will be key to guarantee the future of extensive livestock systems.

Towards satellite remote sensing of pasture quality to support management and decision making in dehesas

Together with the need for drought-resistant species that could increase the resistance of dehesa systems to summer feed gaps and droughts, it is necessary to increase the information available for a more informed and efficient management of grasslands. **Chapters VI** and **VII** of this thesis have investigated the feasibility of satellite remote sensing to provide information on pasture quality in Mediterranean grasslands as an essential attribute for animal nutrition.

Chapter VI demonstrated that Sentinel-2 configuration could provide qualitative information on pasture quality based on CP estimates, but it is limited to provide more accurate information due to insufficient spectral and spatial resolution. However, there are several potential improvements in the assessment of pasture quality in Mediterranean grasslands that need to be explored. In **Chapters VI** and **VII** empirical models have been used to investigate the potential of Sentinel-2 and CHIME to assess pasture quality parameters. The combination of empirical and physically-based models such as PROSAIL has proven to be able to improve the estimation of N in heterogeneous grasslands (Dehghan-Shoar et al., 2023). This is an interesting field of research to improve the estimates of pasture quality and reduce uncertainty. A major limitation to the application of physically-based such as PROSAIL is the setting of the variables required to apply PROSAIL inversion in highly diverse and heterogeneous grasslands such as grasslands of dehesas. Compared to grasslands sampled in previous studies, dehesa grasslands are much more diverse and heterogeneous, comprising a mix of up to 45 species/m^2 (Marañon, 1985), different functional groups and asynchronous phenological stages. Although challenging, a physical-based or hybrid approximation might help to disentangle the sources of uncertainty in the predictions observed in **Chapter VI**.

Additional to the application of hybrid models, another potential improvement is the use of machine learning and deep learning algorithms (Pullanagari et al., 2021). The limited dataset used in this PhD prevented from using deep learning approaches, which require large datasets. An interesting and flexible machine learning algorithm is Gaussian process regression (Belda et al., 2020; Verrelst et al., 2013) as it allows mapping of the uncertainty associated with the predictions. This is essential as decision-making should be guided not only by differences among values but also by the uncertainty associated with these predictions.

Data collection is labour-intensive and expensive but essential to improve the robustness of models. This applies not only to pasture quality parameters but to biomass and other biophysical variables. Therefore, the creation of open libraries to store data collected by different institutions will be essential to tackle the challenges discussed above (Bahlo et al., 2019). For example, the "Servicio de Información de los Alimentos" (SIA) (SIA, 2023, see: https://www.uco.es/sia/) supported by the University of Cordoba and the Spanish Society for the Study of Grasslands (SEP) provides information on parameters of pasture quality of grasslands, forages and crops collected from different institutions. Similar platforms could provide information not only on these parameters but also on the associated spectral reflectance data from field spectroscopy, drones or satellites and corresponding metadata in a standardised way that could allow transferability and comparability.

As demonstrated in **Chapter VI**, the multispectral bands of Sentinel-2 cover important regions to estimate parameters of pasture quality such as CP, especially Band 5 (705nm) in the red-edge region proved to be especially important. However, there are characteristic absorption features of the electromagnetic spectrum to derive forage quality that remain uncovered by Sentinel-2 multispectral bands as shown by Bareth, (2021) in Figure 1.

Chapter VII addressed this issue with a special focus on the upcoming hyperspectral satellite mission of the European Space Agency Copernicus Hyperspectral Imaging Mission for the Environment (CHIME) (ESA, 2023a; Rast et al., 2019). The CHIME satellite will carry a hyperspectral instrument that will cover the wavelength range from 400 nm - 2500 nm of the spectrum, at a spectral bandwidth equal to or less than 10 nm (ESA, 2023a; Rast et al., 2019). As demonstrated in **Chapter VII** this enhanced spectral resolution could considerably improve the prediction of pasture quality in Mediterranean grasslands.

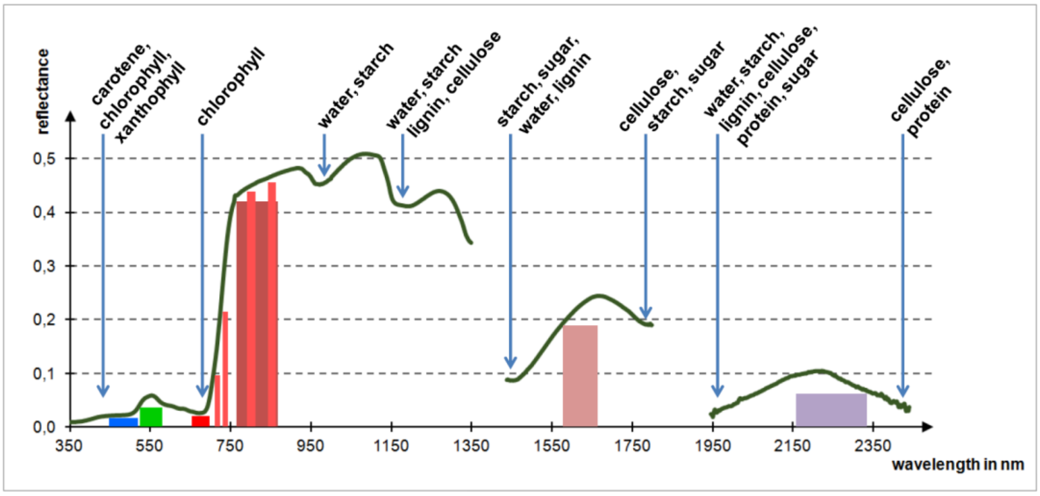


Figure 1. Grassland canopy reflectance recorded with an ASDI field-portable spectroradiometer (FieldSpec-3) with characteristic absorption features for pigments and components relevant to pasture quality (Kumar et al., 2006; Roberts., 2019). Bars represent bands of Sentinel-2. Source: Bareth, (2021).

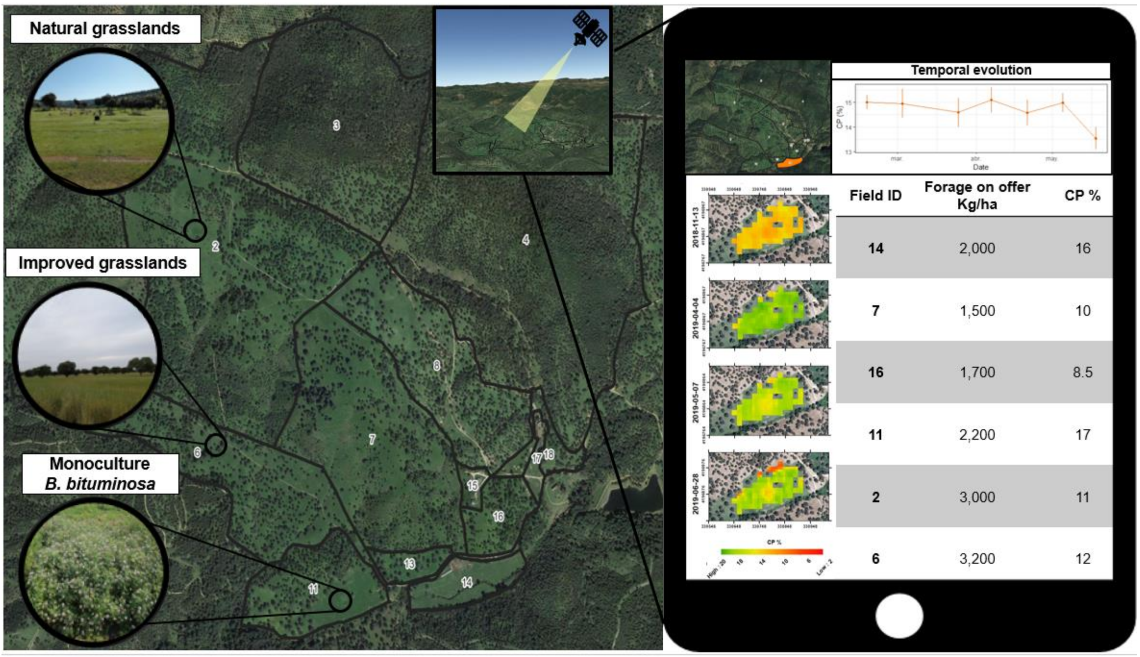
A major problem still to be solved is the spatial resolution of open-source satellites to assess pasture quality and other biophysical parameters. There are hyperspectral satellites that could provide information at higher spatial resolution such as WorldView-3 and Pleidades Neo (ESA, 2023d, 2023b), however, they do not provide open data and acquiring the images is expensive, which prevents from its use in extensive systems. As stated by Gareth, (2021) a minimum spatial resolution of 10 m is required to capture the spatial heterogeneity of grasslands. Considering the heterogeneity of Mediterranean grasslands this resolution could even be too coarse. The most useful bands of Sentinel-2 have a spatial resolution of 10 or 20 m (ESA, 2023c) and CHIME is planned to have a spatial resolution of 20-30 m (ESA, 2023a; Rast et al., 2019). This spatial resolution poses several limitations. At this resolution, the variability of pasture quality within the spatial cover of a pixel can still be high. This becomes even more problematic when pasture does not cover 100% of the pixel and the spectra represent a mixture of covers leading to spectral mixture. This could be frequent if pasture production is low, and a large proportion of the surface is bare soil. In this case, a joint assessment of pasture production and quality is recommendable to ensure suitable interpretation. This issue becomes more limiting when assessing pasture attributes in dehesas and other open woodlands ecosystems as the tree cover restricts the number of pure pixels of grass cover that can be retrieved. Before assessments of the grasslands ‘attributes, it is necessary to perform a classification of the different covers to isolate the effect of non-target canopies. In this step, artificial intelligence coped with high-resolution images could facilitate the mapping of tree cover for this task (see Brandt et al., 2020). Spectral mixture models could also address the problem of tree-grass mixtures at the pixel level (Vermeulen et al., 2021; Yang et al., 2012) and deserve further research.

Finally, one might ask how useful and applicable these models are. The models studied in this PhD Thesis report and RMSE between 2 and 3 % for CP (the best-predicted attribute). This means that if the model predicts a CP content of 16% and the RMSE is 2%, then there is a 95% chance that the true CP content of that sample lies between 12 and 20%. At this accuracy, the usefulness depends on the application. If the intention is to apply them in homogeneous grasslands at a certain moment is likely that the prediction error is higher than the variability of the parameter estimated and therefore it would have no use. However, in heterogeneous Mediterranean grasslands there are large variations of pasture quality in space and time at the farm level (García-Moreno et al., 2016; Olea et al., 1989) and even at moderate prediction errors, model predictions might provide useful information to differentiate among areas of different pasture quality. This information could be used to apply more informed and rational decisions in the management at the strategic or operational level in dehesa farms (Isselstein, 2021).

At this moment this is an incipient technology in Mediterranean grasslands that is far from being used in the management of grasslands of dehesas. After the required steps of confirming the potential to assess pasture quality by addressing the challenges outlined before, the next step would be implementation at the farm level. To ensure an effective application of this innovation in dehesa systems, it must be supported with platforms and user-friendly tools that allow easy use and interpretation by the final users. Good examples of such tools are Pastures from Space and PastureBase Ireland (DPIRD and CSIRO, 2022; Hanrahan et al., 2017; Horn and Isselstein, 2022; Teagasc, 2021). Arising from this PhD Thesis the project "*Plataforma de apoyo a la gestión de los pastos mediterráneos mediante sensores próximos y remotos (GrasSEN)*" aims at developing a demonstrative tool for providing satellite-based information of grasslands of dehesas (pasture quality and productivity) to support their management.

Integration of innovations in the management and use of grasslands of dehesas

Dehesas have witnessed and survived socioeconomic and environmental changes over centuries. New threats hang over this emblematic system, namely climate change and socioeconomic changes with profound impacts on traditional agricultural and livestock systems (Garrett et al., 2020). Grasslands play a central role in the future of this system as they are the main resource to feed livestock and therefore their management will be key to ensure the persistence of dehesas. In order to tackle these threats, innovative solutions need to be explored. As stated by Johannes, (2021) inefficiencies in grassland management and unsatisfactory provision of ecosystem services in complex systems such as dehesas are related to information deficits. Satellite remote can contribute to reducing these information gaps leading to more rational and informed management. This PhD Thesis has focused on investigating the potential of remote sensing of pasture quality to cover this research gap. The combined use of the different applications of remote sensing that are being developed in the context of dehesas (Carpintero et al., 2020; Fernández-Habas et al., 2021; Gómez-Giráldez et al., 2019) could provide valuable information, to farmers, and public authorities to meet the standards for sustainable dehesa systems. At the same time, agronomic innovations such as the use of drought-resistant perennial legumes can increase the self-sufficiency of dehesa systems and buffer their vulnerability to a future drier climate and crisis of global markets. In this line, the use of *B. bituminosa* as a forage perennial legume could be framed within the context of sustainable intensification of dehesa systems. It could consist of a “retro innovation” to recouple crop and livestock systems (Garrett et al., 2020) in the search for higher self-sufficiency and resistance in a context of a drier climate. In summary, an integrated application of the innovations studied in this PhD Thesis (Figure 2) could tackle two key elements in the management of grasslands of dehesas; the lack of information on the spatial and temporal variation of pasture quality and the occurrence of feed gaps, and thus contribute to ensuring the future of this multifunctional system.



Field ID	Forage on offer Kg/ha	CP %
14	2,000	16
7	1,500	10
16	1,700	8.5
11	2,200	17
2	3,000	11
6	3,200	12

Figure 2. Overview of the integration of satellite remote sensing and *B. bituminosa* in the management of grasslands of dehesas.

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CHAPTER IX. CONCLUSIONS

- Innovations aimed at increasing the performance of grasslands such as the use of new seed mixtures and new forage drought-resistant species are considered highly relevant by dehesa farmers.
- High-tech innovations such as GPS collars and remote sensing were given lower relevance, which might denote low applicability in dehesas, the existence of barriers hindering the adoption and the need for further development and better information on their potential.
- Varieties of *B. bituminosa* var. *albomarginata* and var. *crassiuscula* show important adaptations to arid environments and have better forage aptitude than var. *bituminosa*.
- *B. bituminosa* cv. Lanza® can develop in soils of clay and loamy sand texture but shows preference and suitable adaptation to light sandy soils.
- *B. bituminosa* cv. Lanza® shows low competitive ability with annual grasses such as *L. multiflorum* which can drastically reduce its production and persistence.
- *B. bituminosa* cv. Lanza® shows to be resistant to future annual rainfall reductions under climate change scenarios. It can provide green forage long after senescence of annual grasslands, of quality lower than fresh alfalfa but similar to low-medium-quality alfalfa hay.
- In case of late spring and summer rainfall after senescence of annual species, *B. bituminosa* cv. Lanza® can maintain green leaves over the summer thanks to its drought resistance and water use efficiency. However, in very dry conditions from May onwards after high biomass accumulation, it shows generalised leaf shedding in early summer, which reduces the offer and quality of forage during this season.
- *B. bituminosa* cv. Lanza® shows early phenology for the reproductive stages from inflorescence emergence to ripening. This might be an advantageous trait for the persistence of this species in environments prone to early drought.
- This species might fail to persist in environments of frequent frost days which prompts further research to improve its cold tolerance and thus its use in Mediterranean environments with cold winters.

- Further studies should investigate measures aimed at reducing early competition to establish *B. bituminosa* cv. Lanza® in monocultures and suitable partner species to include it in multispecies mixtures. It is also necessary to continue testing this species in long-term experiments under contrasting meteorological conditions to better understand its potential as an out-of-season forage and to define suitable management strategies in Mediterranean farming systems.
- Regarding the use of satellite remote sensing of pasture quality, the potential of Sentinel-2 configuration might be limited to predictions at moderate accuracy of CP, which could allow qualitative assessments of pasture quality in dehesas.
- Future hyperspectral satellites such as CHIME, could improve the assessment of pasture quality of Mediterranean grasslands allowing quantitative estimations of CP.
- Bands from the red-edge show the highest importance to assess pasture quality parameters in Mediterranean grasslands.
- It is essential to develop large and diverse datasets to improve the accuracy of the models, reduce the uncertainty of the predictions and to fully explore the potential of satellite remote sensing of pasture quality. The use of deep learning algorithms, hyperspectral data and integration of physical-based methods could improve the predictions of pasture quality. The spatial resolution of non-commercial satellites remains a limitation to assess biophysical variables in highly diverse Mediterranean grasslands, especially in heterogeneous landscapes such as dehesas.
- The integrated application of satellite remote sensing of pasture quality and the use of *B. bituminosa* in the management of grasslands of dehesas could ameliorate two important limitations; the lack of information on the spatiotemporal variations of pasture quality and the feed gaps, and thus contribute to the persistence of this multifunctional system.

OTHER SCENTIFIC OUTCOMES

International stays

- Temporal stay of three months at the University of Göttingen (Germany) 06/09/2021-05/12/2021.
- Temporal stay of three months at the University of Lisbon (Portugal). 11/01/2023-10/04/2023.

Collaborations

Publications in peer-reviewed scientific journals

- **Fernández-Habas, J**., Fernández-Rebollo, P., Casado, M. R., Moreno, A. M. G., and Abellanas, B. (2019). **Spatio-temporal analysis of oak decline process in open woodlands: A case study in SW Spain**. Journal of environmental management, 248, 109308. Metrics 2019: IF: 5.647; JIF QUARTILE: Q1; JIF RANK: 33/265 (Environmental sciences); Citations Google Scholar: 14.
- Reyna-Bowen, L., Fernandez-Rebollo, P., **Fernández-Habas, J**., and Gomez, J. A. (2020). **The influence of tree and soil management on soil organic carbon stock and pools in dehesa systems**. Catena, 190, 104511. Metrics 2020: IF: 5.198; JIF QUARTILE: Q1; JIF RANK: 22/200 (Geosciences, Multidisciplinary); Citations Google Scholar: 21.
- Navarro-Rosales, F., **Fernández-Habas, J**., Reyna-Bowen, L., Gómez, J. A., and Fernández-Rebollo, P. (2023). **Subsoiling for planting trees in dehesa system: long-term effects on soil organic carbon**. Agroforestry Systems, 97, 699-710. Metrics 2021: IF: 2.419; JIF QUARTILE: Q2; JIF RANK: 28/69 (Forestry); Citations Google Scholar: 0.

Contributions to national and international congresses

- **Fernández-Habas, J**., Hidalgo-Fernández, M. T., Leal-Murillo, J. R., García-Moreno, A. M., Fernández-Rebollo, P. **Using legume-grass commercial seed mixes to improve grasslands of dehesas farms: production, persistence**

and diversity. Poster communication. 28TH General Meeting of European Grassland Federation. Helsinki (Finland) 18/02/2020-19/02/2020.

- Schils, R., Newell Price, P., Klaus, V., Tonn, B., Hejduk, S., Stypinski, P., Hiron, M., Fernández, P., Ravetto Enri, S., Lellei-Kovacs, E., Annett, N., Markovic, B., Lively, F., ten Berge, H., Smith, K., Forster-Brown, C., Jones, M., Buchmann, N., Janicka, M., **Fernandez Habas, J**., Rankin, J., McConnell, D., Aubry, A., Korevaar, H. **European permanent grasslands mainly threatened by abandonment, heat and drought, and conversion to temporary grassland**. Poster communication. 28TH General Meeting of European Grassland Federation. Helsinki (Finland) 18/02/2020-19/02/2020.
- **Fernández-Habas, J**., Komainda, M., Schmitz, A., Fernández-Rebollo, P., Isselstein, J. **Can grasslands' vegetation be estimated from smartphone pictures collected by citizen scientists?** Poster communication. 29TH General Meeting of European Grassland Federation. Caen (France) 26/06/2023-30/06/2023.

Participation in research projects

- Developing SUstainable PERmanent Grassland systems and policies. SUPER-G. H2020-SFS-2017-2 (ID 774124). European Union Horizon 2020 research and innovation programme, grant agreement 774124. PI: Paul Newell-Price
- "Mejora de la producción y comercialización de vacuno ecológico en dehesa. GOP2I-HU-16-0018". European Union and Junta de Andalucía, European Agricultural Fund for Rural Development (EAFRD). PI: Pilar Fernández-Rebollo.
- "Plataforma de apoyo a la gestión de los pastos mediterráneos mediante sensores próximos y remotos (GrasSEN)". G14814354. European Union and Junta de Andalucía, European Agricultural Fund for Rural Development (EAFRD). PI: Pilar Fernández-Rebollo.
- "Nuevos pastos de leguminosas perennes para la dehesa: Provisión de Servicios Ecosistémicos en condiciones de mayor aridez (Pastos-SEcos)". Proy-Excel 00465 PASTOS-SECOS. PI: Pilar Fernández-Rebollo.

Teaching

180 hours from 2019 to 2023 in the degree of "Grado en Ingeniería Forestal" at the University of Cordoba in the subjects:

- "Selvicultura Mediterranea"
- "Pascicultura y Sistemas Agrosilvopastorales"
- "La Dehesa y Otros Sistemas Agrosilvopastorales"
- "Gestión Avanzada de Sistemas Forestales"

Bachelor's thesis supervised:

- "Empleo de dispositivos de posicionamiento del ganado para estimar el gasto energético de locomoción en dehesa". Student: José Antonio Rascón Sánchez. 2019.
- "Estudio del comportamiento del ganado vacuno en pastoreo mediante dispositivos de posicionamiento". Student: Julia Torres Fernández. 2020.
- "Modelización de la calidad nutritiva de los pastos mediterráneos y su relación con la selección en pastoreo". Student: Francisco Javier García Peñas. 2021.
- "Modelos de predicción de calidad de los pastos mediterráneos mediante radiometría de campo y su optimización y herramientas de machine learning". Student: Mónica Carriere Cañada. 2021.

Seminars and dissemination talks

- Measuring grassland utilisation and analysing animal behaviour at grassland. SUPER-G series of internal webinars in 2020-21. 17/12/2020.
- Exploring the use of satellite images (Sentinel-2) to assess pasture production, utilisation and quality at farm level. SUPER-G series of internal webinars in 2020-21. 26/01/2021.
- GPS collars: Measuring grassland utilisation and analysing animal behaviour at grasslands of Dehesa farms. SUPER-G series of internal webinars in 2021-22. 01/02/2022.
- Testing drought tolerant grasslands species for the Mediterranean region. Identification of functional traits that could contribute to drought resistance

and ecosystem services provision. SUPER-G series of internal webinars in 2021-22. 03/02/2022.

- Exploring the use of satellite images (Sentinel-2) to assess pasture production, utilisation and quality at farm level. SUPER-G series of internal webinars in 2021-22. 03/02/2022.
- Testing a citizen science approach to determine plant diversity in grasslands. SUPER-G series of internal webinars in 2021-22. 09/02/2022.
- La Dehesa, origen, características y retos de futuro. Talk on the World Environment Day in Pozoblanco, organised by “Ventana Avierta”. 01/06/2022
- Especies resistentes a la sequía para el montado y la dehesa: El caso de *Bituminaria bituminosa* (Tedera). SUPER-G Stakeholder Webinar Mediterranean BR. 29/03/2023.

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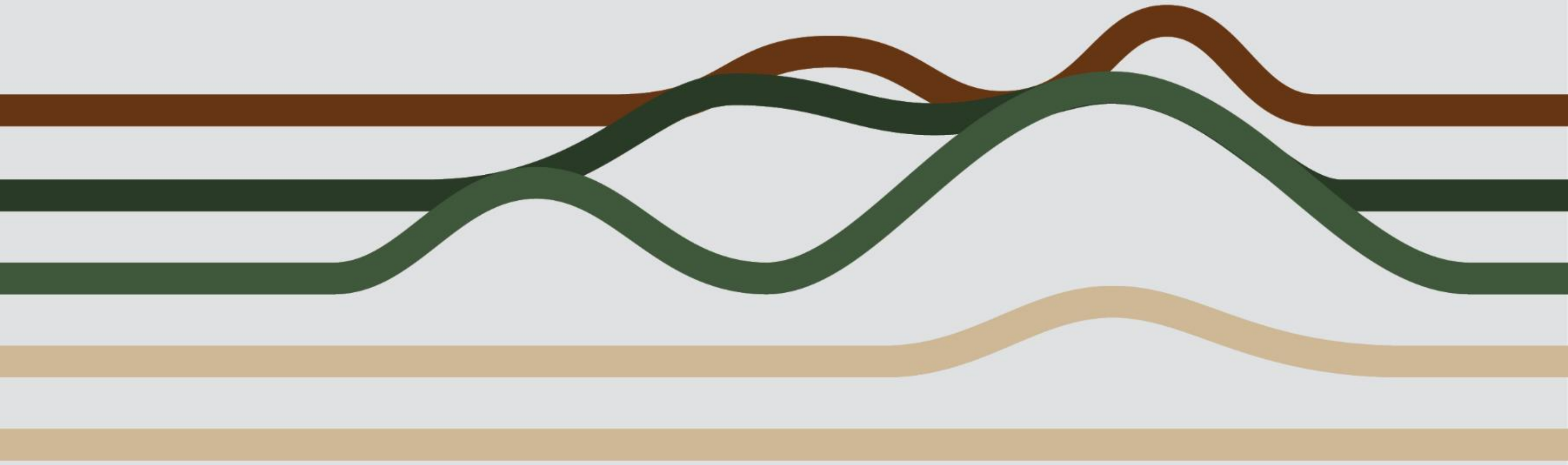

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