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Assessment of the spatial variability of vegetative status in vineyards using non-destructive sensors. Application of remote and proximal sensing technologies in precision viticulture
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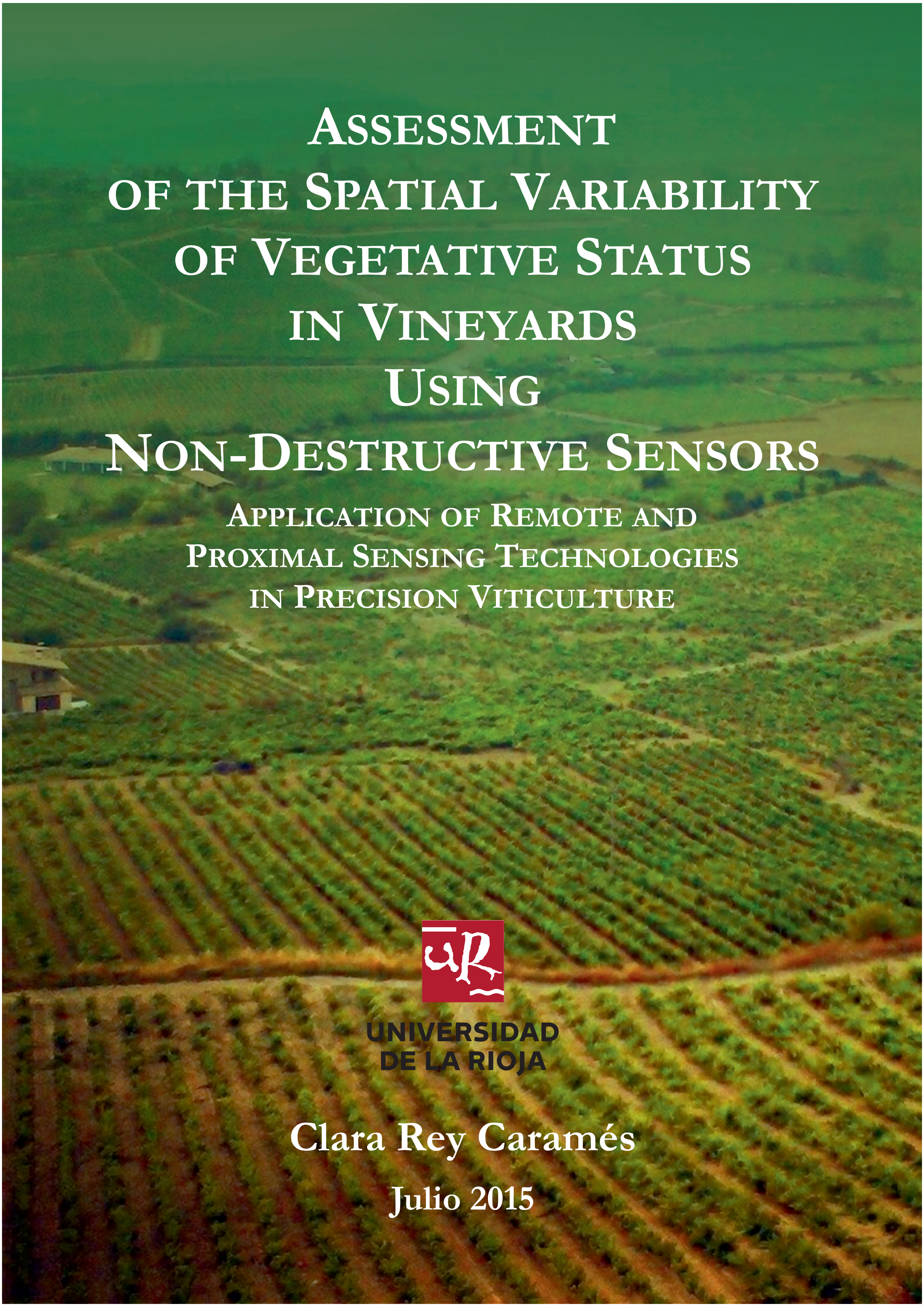

Assessment of the Spatial Variability of Vegetative Status in Vineyards Using Non-Destructive Sensors

Application of Remote and Proximal Sensing Technologies in Precision Viticulture

UNIVERSIDAD DE LA RIOJA

Clara Rey Caramés

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A mis padres

A Samo

PROFESIÓN DE FE

Quizá debiera hoy felicitarme,
recibir mi cordial enhorabuena
por tantos equilibrios, por estar
aquí, sencillamente,
sencillamente pero nada fácil
habitar esta tarde, haberla conquistado
a través de batallas,
caídas, días grises, desamores, olvidos,
pequeños triunfos, muertes
muy pequeñas también,
pero también muy grandes.
Haber llegado aquí, hasta esta luz
que anoto para luego,
para acordarme luego, cuando sea difícil
admitir la existencia de esta tarde
a la que llego solo, disponible,
sano, joven aún, y decidido incluso
a olvidar el cansancio, la experiencia,
convencido de nuevo de que sí,
de que a partir de hoy, acaso, todo
lo que tanto he soñado, todavía,
pudiera sucederme.

Vicente Gallego

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Abstract

Introduction: The vegetative status of grapevines influences yield and grape composition. The assessment of the vineyard spatial and temporal variability in precision viticulture requires a large amount of data. Traditional methods are not suitable, as they are time and labour demanding, making the analysis of a high number of samples at different timings not feasible. For this purpose, remote and proximal sensing techniques could be useful for monitoring the vineyards in a reliable, fast and non-destructive way.

Objectives: The aim pursued by this research was to assess the spatial and temporal variability of the vegetative status of a vineyard using non-destructive sensors. Towards that end, the usefulness of a remotely piloted aerial system (RPAS) multispectral imagery was tested to assess the vegetative growth of a vineyard. A special focus was set on proximal sensing, especially on a fluorescence sensor used either manually and on-the-go, to determine chlorophyll, flavonol and nitrogen content in grapevine leaves.

Materials and methods: A multispectral sensor mounted on a RPAS was employed for monitoring the spatial variability of the vegetative status of a commercial vineyard (*Vitis vinifera* L.) and the assessment of vegetative parameters, such as leaf area, shoot length and pruning weight, leaf chlorophyll content and nitrogen status. Subsequently, a fluorescence sensor, used manually and mounted on a quad, was used for assessing the leaf chlorophyll, epidermal flavonol and nitrogen content and monitoring the spatial variability of the vineyard vegetative and nutritional status.

Results and discussion: The spectral indices derived from RPAS multispectral imagery yielded significant and moderate correlations with pruning weight, secondary shoot length, secondary leaf area, leaf chlorophyll content and nitrogen status. These results indicated its potential to appraise the vineyard vegetative status but also revealed some disadvantages regarding technological and operational factors. Regarding proximal sensing, the hand-held fluorescence sensor demonstrated its capability to properly measure the chlorophyll, epidermal flavonols and nitrogen content in grapevine leaves. The best indicators of these vegetative and nutritional components were found to be the fluorescence indices of the whole leaf (adaxial and abaxial). Thanks to the calibration equations provided, the leaf chlorophyll concentration can be obtained from the fluorescence measurements. Concerning the nitrogen status, among all the possible equations of the nitrogen balance index (NBI), the one calculated as the chlorophyll-to-flavonol ratio yielded the best evaluation of the nitrogen status of the grapevine. The hand-held fluorescence sensor allowed characterising the spatio-temporal variability of leaf chlorophyll content and nitrogen status along the ripening season. While the nitrogen status showed different spatial variability across the season, leaf chlorophyll content spatial behaviour remained stable. Moreover, the fluorescence sensor adapted to be mounted on a vehicle demonstrated its capability to reliably estimate the chlorophyll, epidermal flavonol and nitrogen content on-the-go in grapevine leaves, and to assess their spatial variability within the vineyard.

Conclusions: Remote and proximal sensing have proved to be certainly useful in precision viticulture as they are able to provide a large amount of data in a fast and non-destructive way, overcoming the disadvantages of the classical manual, destructive, laborious methods. Specifically, the fluorescence sensor has shown to be a precise tool to assess

key vegetative and nutritional parameters in the field. Furthermore, its successful adaptation to operate mounted on a vehicle and perform an on-the-go assessment of the vegetative status of the vineyard is a significant step forward in the current process of sensor integration on mobile platforms and the practical implementation of the precision viticulture techniques.

Keywords: Remote piloted aerial systems (RPAS), chlorophyll fluorescence sensor, on-the-go, plant phenotyping, vegetative growth, nitrogen, *Vitis vinifera* L.

Resumen

Introducción: El estado vegetativo del viñedo influye en la producción y en la composición de la uva. La estimación de la variabilidad espacial y temporal en viticultura de precisión requiere de una gran cantidad de datos. Los métodos tradicionales no son adecuados, ya que son muy costosos en términos de tiempo y esfuerzo, lo que hace que el análisis de una gran cantidad de muestras en diferentes momentos no sea factible. Con éste fin, la teledetección y la detección próxima podrían ser útiles para monitorizar el viñedo de forma fiable, rápida y no destructiva.

Objetivos: El objetivo perseguido en este trabajo de investigación fue estimar la variabilidad espacial y temporal del estado vegetativo del viñedo mediante el uso de sensores no destructivos. Con esta finalidad, se analizaron imágenes multiespectrales tomadas por sistemas aéreos pilotados de forma remota (RPAS) para estimar el crecimiento vegetativo del viñedo. Especial atención se puso en los sistemas de detección próxima, especialmente en el uso de un sensor de fluorescencia, bien manualmente o instalado en un vehículo, para determinar el contenido de clorofila, flavonoles y nitrógeno en las hojas de vid.

Materiales y métodos: Se utilizó un sensor multiespectral instalado en un RPAS para monitorizar la variabilidad espacial del estado vegetativo de un viñedo comercial (*Vitis vinifera* L.) y estimar los parámetros vegetativos: superficie foliar, longitud de pámpano, madera de poda y el contenido foliar de clorofila y nitrógeno. Posteriormente, se empleó un sensor de fluorescencia, tanto de forma manual como instalado en un *quad*, para estimar el contenido foliar de clorofila, flavonoles y nitrógeno y monitorizar la variabilidad espacial del estado vegetativo y nutricional del viñedo.

Resultados y discusión: Los índices derivados de las imágenes multiespectrales de RPAS reportaron correlaciones significativas y moderadas con la madera de poda, la longitud de pámpano secundaria, el área foliar secundaria y el contenido foliar de clorofila y nitrógeno. Estos resultados han mostrado su potencial para estimar el estado vegetativo del viñedo, pero también revelaron algunos inconvenientes relacionados con factores tecnológicos y operacionales. Relacionado con la detección próxima, el sensor de fluorescencia manual ha demostrado su capacidad para medir apropiadamente los contenidos foliares de clorofila, flavonol epidérmico y nitrógeno en vides. Los mejores indicadores de los componentes vegetativos y nutricionales fueron los índices de fluorescencia calculados para la hoja completa (haz y envés). Gracias a las ecuaciones de calibración aportadas, es posible obtener la concentración foliar de clorofila a partir de los índices de fluorescencia. En cuanto a los niveles de nitrógeno, entre todas las posibilidades de calcular el índice de balance de nitrógeno (NBI), el calculado como el ratio clorofila entre flavonol ha sido el que ha aportado la mejor estimación de los niveles de nitrógeno de la vid. El sensor de fluorescencia manual ha permitido caracterizar la variabilidad espacio-temporal del contenido foliar de clorofila y de los niveles de nitrógeno a lo largo del período de maduración. Mientras los niveles de nitrógeno mostraron diferencias en la variabilidad espacial a lo largo de la temporada, el comportamiento espacial del contenido foliar de clorofila se mantuvo estable. Asimismo, el sensor de fluorescencia adaptado para ser instalado en un vehículo, demostró su capacidad para estimar en marcha y de forma fiable, el contenido de clorofila, flavonol y nitrógeno en hojas de vid y estimar su variabilidad espacial en el viñedo.

Conclusiones: Las técnicas de teledetección y de detección próxima han demostrado ser muy útiles en viticultura de precisión, dado que son capaces de recopilar un gran número de datos de forma rápida y no destructiva, lo que supone una importante mejora respecto a los métodos manuales clásicos, que resultan destructivos y laboriosos. En especial, el sensor de fluorescencia ha demostrado ser un instrumento muy preciso a la hora de estimar en campo parámetros vegetativos y nutricionales clave. Así mismo, la exitosa adaptación del sensor de fluorescencia para operar instalado en un vehículo en movimiento supone un significativo avance en el actual proceso de integración de sensores en plataformas móviles y la implementación práctica de la viticultura de precisión.

Palabras clave: RPAS (Remotely Piloted Aerial System o, en español, sistemas aéreos pilotados de forma remota), sensor de fluorescencia de la clorofila, en movimiento, fenotipado vegetal, crecimiento vegetativo, nitrógeno, *Vitis vinifera* L.

List of abbreviations

_G	excitation by green light
_R	excitation by red light
_UV	excitation by ultraviolet light
AB	abaxial
AD	adaxial
AU	absorbance units
C_0	nugget of the variogram
C_1	sill of the variogram
CHL	chlorophyll optical index
CmbI	cambardella index
CMOS	complementary metal-oxide-semiconductor
CV	coefficient of variation
DGPS	differential global positioning system
DN	digital numbers
DX4	Dualex4™
ELA	exposed leaf area
FLAV	flavonol fluorescence index
FR	fine registration
FRF	far red fluorescence
FRF_G	far red fluorescence evolved from green light excitation
FRF_R	far red fluorescence evolved from red light excitation
FRF_UV	far red fluorescence evolved from uv light excitation
FWHM	full-width at half-maximum
G	greenness index
GIS	geographic information systems
GNSS	global navigation satellite systems
GPS	global positioning system
LAI	leaf area index
LED	light emitting diode

LMA	leaf mass per area
MCARI	modified chlorophyll absorption in reflectance index
ME	mean error
MLA	main leaf area
MSAVI	modified soil adjusted vegetation index
MSE	mean square error
MSL	main shoot length
MSR	modified simple ratio
MSSE	mean square standardised error
$MTVI_1$	modified triangular vegetation index
MX_H	hand-held Multiplex™
MX_M	Multiplex On-the-Go™
NBI	nitrogen balance index
NDVI	normalised difference vegetation index
NIR	near infrared
NVI_1	normalised vegetative index 1
NVI_2	normalised vegetative index 2
OLS	ordinary least squares
OSAVI	optimized soil adjusted vegetation index
PCD	plant cell density
PNOA	Plan Nacional de Ortofotografía Aérea (Spanish national program of aerial ortophotography)
PRI	photochemical reflectance index
PVR-	photosynthetic vigour ratio
PW	pruning weight
PW2	Pixel Wrench 2
r	correlation coefficient
R	reflectance
R^2	determination coefficient
RDVI	renormalised difference vegetation index
RF_G	red fluorescence evolved from green light excitation

RF_R	red fluorescence evolved from red light excitation
RMSE	root mean square error
RPAS	remotely piloted aerial system
RSS	residual sum of squares
RVI	ratio vegetation index
SD	standard deviation
SEPC	standard error of prediction corrected for bias
SFR	simple fluorescence ratio
SLA	secondary leaf area
SPW	shoot pruning weight
SSL	secondary shoot length
T	transmittance
TCARI	transformed chlorophyll absorption in reflectance index
TIF	tagged image format
UAV	unmanned aerial vehicle
UGV	unmanned ground vehicle
US	United States
UV	ultraviolet
VLAI	vertical leaf area index
VRA	variable rate application
VRT	variable rate technology
VSP	vertically shoot-positioned

1 Introduction

1.1. Precision viticulture

1.1.1. Origins of precision viticulture

Crops are influenced by the variability of the land. The interaction between crops and land lead to differential development of the plants across the field and therefore, different management actions are required for each subarea (Whelan and McBratney 2000). The differential management within the crop field and the concept of being spatially variable are covered by a discipline known as precision agriculture (Stafford 2000). Precision agriculture consists on an information and technology-based agricultural management system to improve crop production efficiency by adjusting farming inputs to specific conditions within each field area. Its purpose is to enable crop management to be targeted in a way that recognises that, far from being homogenous, the productivity of agricultural land is inherently variable (Stafford 2000).

Precision agriculture is not a new concept, as subsistence farmers have always carried it out dividing their fields into different subareas and planting the most suitable crops for each of them (Oliver 2010). However, this process began to reverse in the second half of the XIX century, due to the intensive production and mechanisation and the merge of the small fields into larger units, increasing the within-field variability, which was more difficult to manage without the appropriate technologies (Stafford 2000). It was in the 1980s when the advances in technology made possible the implementation of precision agricultural practices, with the introduction of an on-the-go yield meter (Oliver 2010) and an on-the-go fertiliser capable of conducting variable rate application (VRA) based on maps (European Commission Report 2014). At the beginning of the 1990s, the global positioning system (GPS) technology started to be more reliable for precision agriculture applications, especially by mid-1990s when the differential GPS (DGPS) was able to substantially improve its accuracy (Oliver 2010). Since then, new sensing technologies have emerged allowing measuring different variables related to the soil status, weather information and crop physiological conditions in a non-destructive and rapid way (Lee et al. 2010). The availability of these new sensing technologies and the DGPS together with the geographical information systems (GIS) made possible the practical implementation of precision viticulture (Zhang et al. 2002).

Precision viticulture derives from precision agriculture. As did the farmers, grapegrowers have always been familiarised with the vineyard variability but because of the lack of appropriate tools to assess and manage this variability, the vineyards have been mostly managed as a uniform unity (Bramley 2010b). The adoption of the precision agriculture technologies in viticulture is more recent; in fact, it was not until 1999 when the first results of the application of precision agriculture techniques in viticulture were published in Australia by Bramley and Proffitt (1999) and in USA by Wample et al. (1999). The appearance of yield and other monitoring sensors on the market made possible the analysis of the spatial variability and the variable rate application of inputs and selective harvesting (Arnó et al. 2009). This led to increase interest in the investigation of the vineyard spatial variability and the implementation of precision viticulture techniques, which started in Australia with the research works conducted mainly by Bramley, Proffitt, Lamb or Hamilton (Bramley 2001, Bramley and Hamilton 2004, Bramley and Lamb 2003). Since the release of the first yield monitor, the sensing technology developed rapidly, allowing obtaining large amounts of data related to the vegetative status, the yield and grape composition of the vineyard. Proffitt et al. (2006) published a book summarising the application of these new technologies on precision viticulture with very interesting examples of their application. Another comprehensive review regarding the implementation of precision viticulture was published by Arnó et al. (2009). Bramley (2010b) reviewed the research works regarding the spatial variability of vigour, yield, grape and wine quality in vineyards, along with the tools employed to appraise this spatial variability.

Up to now, the investigation in precision viticulture has increased but still the most advanced country implementing precision viticulture is Australia (Bramley 2005, Bramley and Hamilton 2004, Hall et al. 2011, Lamb et al. 2004, Proffitt and Malcolm 2005, Taylor and Bates 2013), followed by USA (Cortell et al. 2007a, Cortell et al. 2007b, Dobrowski et al. 2002, Johnson 2003). Other countries of the "new world" as Chile (Best and León 2007, Ortega and Esser 2005), Canada (Reynolds and Rezaei 2014, Reynolds et al. 2007) or Argentina (Bragachini 2002) have also contributed to the study of precision viticulture. In the African continent, South Africa has carried out interesting research in precision viticulture (Smit et al. 2010, Strever 2007). In Europe, the research in precision viticulture is mainly carried out in France (Cerovic et al. 2009, Goutouly et al. 2006, Tisseyre and Taylor 2008), Spain (Arnó et al. 2012, Baluja et al. 2012b, Zarco-Tejada et al. 2005), Italy (Agati et al. 2007, Barnaba et al. 2014) and Greece (Tagarakis et al. 2013a, Taskos et al. 2015).

1.1.2. The cyclical process of precision viticulture

The precision viticulture approach requires objective and continuous monitoring of key parameters for rational and differentiated agronomical management of vineyards based on the spatio-temporal variability of growth, yield and grape composition within the plot. The implementation of this approach for vineyard management is a continuous cycle (Bramley 2001), consisting on three main steps (figure 1): 1) observation and data collection; 2) data interpretation and evaluation; and 3) implementation of the management plan (Proffitt et al. 2006).

Figure 1. Cyclical process of precision viticulture (Figure adapted from Proffitt et al. 2006).

The first step should be obtaining a large amount of data of the variables or components of the vineyard using various proximal and remote sensors. It is crucial that these data are accurately georeferenced, for which a global navigation satellite systems (GNSS) should be employed (Proffitt et al. 2006).

In a second step, all collected data must be analysed and interpreted. In this framework is where the geographic information systems (GIS) gain relevance, as they allow carrying out the spatial analysis required and the establishment of different management areas within the vineyard (Bramley 2010b).

On a third step, all the information analysed in the second step will be used for vineyard management adopting differentiated strategies for each different subareas of the plot according to its requirements, instead of managing the whole plot as a homogeneous unit. It should be recommendable to study the vineyard for at least a couple of years before implementing changes to have a better understanding of the grapevines performance taking into account the season factor (Proffitt et al. 2006).

Finally, a repetition of the three previous steps that conform the cyclical process of precision viticulture should be conducted (Bramley 2010b). This repetition along with the fact that grapevines are perennial plants, will allow to observe the response of the plants to the management practises applied. Thanks to this repeated observation and analysis of the data, the vine grower may be able to value if the management is effective and to take decisions as to whether this management could be improved in the following seasons. On the other hand, the iterative process of precision viticulture over several seasons yields a useful historical knowledge regarding the vineyard management that could allow carrying out predictions and helping the grapegrower making proactive decisions (Arnó et al. 2009, Proffitt et al. 2006).

1.1.3. Tools and technologies in precision viticulture

In the last decades, a wide range of sensors has been developed to assess a large kind of information related to agricultural crops. Remote and ground sensing have been applied to precision viticulture studies with very remarkable outputs (Arnó et al. 2009). These devices allow recording information related to soils, weather or the crop physiological status at high spatial resolution (Lee et al. 2010). At present, there are commercially available sensors that are being used to measure specific soil properties of the vineyard, canopy growth parameters, as well as grape yield and composition. Thanks to these new technologies and methodologies, grape growers will be able to better understand the vineyard, adopting new managements actions and more efficient experimental designs (Tisseyre et al. 2007).

The data collected in the vineyard must be associated to their geographical coordinates to be able to assess the vineyard spatial variability. The Global Navigation Satellite Systems (GNSS) technology is widely used in many farms, being the global positioning system (GPS) the most commonly known and used (European Commission Report 2014). It can be used either manually, by collecting the specific positioning of the monitored plants, or mounted on a vehicle. It saves the longitude, latitude, altitude and the time.

Geographic information systems (GIS) are an essential element for the analysis of the data recorded by the different sensors, allowing to produce useful maps for the grapegrower that represent the crop physiological condition and soil status (van Leeuwen 2010). A GIS is a complex concept that could be understood as a system that integrates information technology, persons and geographic information and whose most important function is to capture, analyse, store, edit and represent georeferenced data (Olaya 2012). Different layers of information such as altitude, slope, aspect, climatic variables, soil variables, vine vegetative growth, yield or grape composition can be represented, combined and analysed to determine whether there is spatial variability within a given vineyard and to delineate management areas. Nowadays there are numerous GIS software packages available in the market adapted to be used in the computer, tablet or mobile phone. They had become more affordable and there is also free GIS software that are perfectly adequate for most purposes (Green 2012).

The identification of how a vineyard spatially varies enables a precise application of nutrients or pesticides in the proper amount required in each subarea. The technology that allows this spatially adjustable application of inputs is known as variable rate technology (VRT) (Taylor et al. 2005). A lot of research has been carried out making possible the variable application of crop protection products, fertilisers or seeds (Arnó et al. 2009, Zhang et al. 2002), which will lead to environmental benefits such as reducing ground water contamination and costs reductions (European Commission Report 2014).

1.1.4. Geostatistics in precision viticulture

1.1.4.1. Introduction

Geostatistics involve a set of models and techniques developed by Matheron in the 1960s (Matheron 1963) from Krige's empirical observations (Oliver 2010). It is based on the theory of regionalised variables. A regionalised variable is any attribute spatially correlated at some scale, i.e. its value at a particular place depends, in a statistical sense, on those of the neighbours, and this dependence decreases as the distance increases (Webster and Lark 2013). The variance of the regionalised variable depends on the separation in space of any two sites and not on their absolute positions (Oliver and Webster 1986). This implies that local values can be estimated from those surrounding them. Also these values can be interpolated to generate a continuous surface, being able to incorporate the estimated errors of the interpolation (Webster and Lark 2013).

Geostatistics involve techniques to characterize the spatial autocorrelation of a regionalised variable and to perform a local prediction by kriging, based on the spatial behaviour of the variable. For applying kriging, a model of the variable spatial correlation is required. This model will usually derive from the variogram function, which must be estimated from the sample data (Oliver 2010).

The variogram is the key of geostatistics. It provides a description on how the variables are autocorrelated with distance. Its function, defined by (Matheron 1963), is a vector function that quantifies the spatial variability of a variable Z(x). The experimental variogram is calculated as follows:

$$\gamma\,(\mathrm{h}) = \frac{1}{NP(h)} \sum_{i=1}^{NP(h)} \left(Z(x_i + h) - Z(x_i)\right)^2 \quad (1)$$

Where $Z(x_i)$ and $Z(x_i+h)$ are the actual values of the regionalised variable (Z) at places x_i and x_i+h, and NP(h) is the number of paired comparisons at lag h. The quantity γ is known as the semi-variance, and corresponds to half the variance of the difference between values at two sites; so the function γ(h) that relates γ to the lag is the variogram (Oliver and Webster 1986).

The experimental variogram can provide a concise and unbiased description of the scale and pattern of spatial variation (Oliver and Webster 1986). It is estimated from the data collected and then it should be fitted to the most suitable mathematical model. The parameters of the model fitted will then be used for local estimation by kriging. Figure 2 shows an example of an experimental variogram fitted to a spherical model and the parameters that define the variogram.

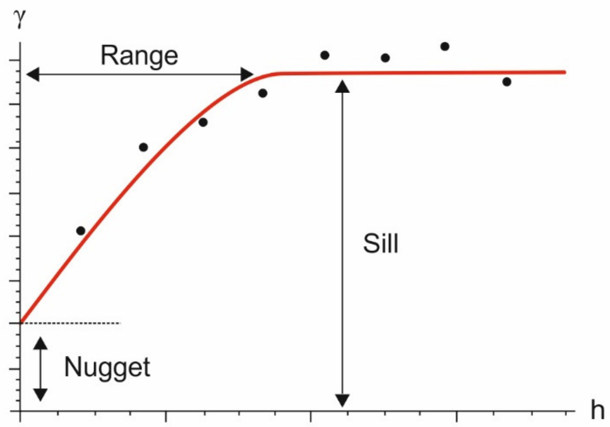

Figure 2. Example of an experimental variogram (dots) fitted to a spherical model (line) showing its components: nugget, sill and range.

The nugget variance (c_0) usually refers to the measurement error and variation that occurs over distances shorter than the sampling interval. Some variograms reach a maximum of the increase of the semivariance with the distance, known as the sill variance (c_0 + c), which comprises any nugget variance (c_0) and the spatially correlated variance (c). The distance at which the sill is reached, is known as the range (a), i.e. it is the distance from which there is no spatial correlation, places further apart than the range are spatially independent (Oliver 2010).

Finally, a spatial estimation can be carried out allowing to know the values of the variable where it has not been sampled within the boundaries of the field studied. The geostatistical method to accomplish this is known as Kriging interpolation. This method takes into account the way a property varies in space through the variogram function and provides not only predictions but also the kriging variances or errors, which are a guide to the reliability of the estimates (Oliver 2010).

1.1.4.2. Applications in precision viticulture

Since the late 1980s, geostatistics have been applied in precision agriculture to analyse the spatial and temporal variability of experimental data, allowing the production of maps and the delineation of the managements zones within the plots (Oliver 2010). The first application of geostatistics in agriculture is dated in the early 1980s on soil data (Burgess and Webster 1980). The aim was to quantify the spatial structure in the variation with the variogram and based on it, to produce a map of the spatial variability of soil properties by kriging. But the first authors applying geostatistics directly in precision agriculture were Mulla and Hammond (1988). They mapped patterns in soil P and K, determining its nature and extent and the sampling frequency necessary to identify the major patterns in the soil. Since then, geostatistics have been gradually applied in precision agriculture to assess the spatial variability of the crop characteristics and delineate differentiate management areas.

In precision viticulture, geostatistics have been applied by Morari et al. (2009) to estimate the spatial variability of soil properties and their relationships with apparent electrical conductivity by factorial kriging analysis. Bramley and Hamilton (2004) leant on the geostatistical interpolation method, kriging, to study the yield spatial variability within a vineyard and its temporal stability. Later, Bramley (2005) carried out the same study but for grape quality. The PhD thesis of Taylor (2004) applied geostatistics to the study of the spatial variability of grape composition and yield. Baluja (2012) also wrote a PhD Thesis in which geostatistics had a great prominence in studying the spatial and temporal variability of the grape composition within a vineyard by the analysis of the variograms and the generation of maps by ordinary kriging. Taylor and Bates (2012) compared different sampling methods by a variographic analysis to determine the best one for monitoring the pruning weight within a vineyard.

1.1.5. Benefits and implementation of precision viticulture

Through the application of precision agriculture, the farmer would be able to optimize crop production and profitability. Part of that profitability will come from reducing the inputs, such as machinery, labour, chemicals, water, energy, etc. It will also contribute to diminish the environmental impact of pesticides, nutrients leaching and fossil fuels, among others (Tey and Brindal 2012). Regarding the economic profitability, a review of 210 studies published from 1988 to 2004 revealed that the implementation of some sort of precision agriculture technology was found to be profitable in 68% of the cases (Griffin et al. 2004). But while the benefits of a higher profitability will be seen in a short term, the environmental benefits will not only manifest themselves at a farm level, but also in the surrounding environment: flora, fauna, soil, streams, aquifers, etc. and they may take years to reveal. Some studies have shown that the environmental impact is reduced when applying precision agricultural methods due to a more efficient use of the fuel resulting in reducing the carbon footprints; or the variable rate application (VRA), reducing groundwater pollution (European Commission Report 2014).

Holland et al. (2013) reviewed the use of precision technologies over time in the United States of America. Their study revealed that precision services and the manual control guidance systems were the most used (figure 3). The autosteer guidance system experimented a rapid adoption. Remote sensing services and soil electrical conductivity mapping have also increased their acceptance and application. Nevertheless, the implementation of these technologies has mainly increased through the years and it is expected to continue raising. However, some obstacles to the adoption of precision agriculture by farmers have been identified. These impediments involve a lack of technical expertise and knowledge, lack of infrastructures and institutional restrictions as long as high investment costs (European Commission Report 2014).

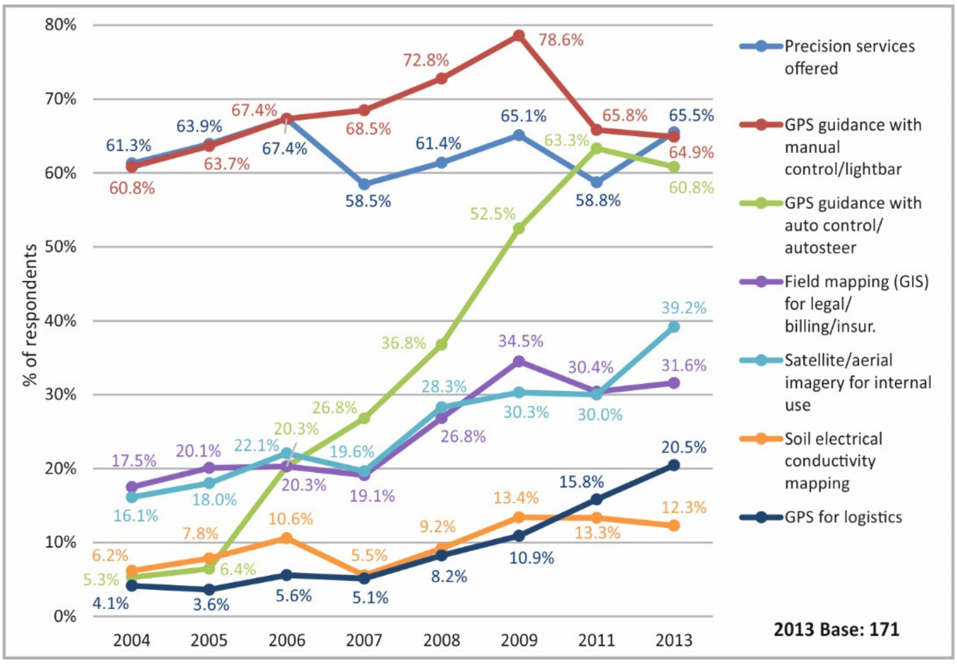

Figure 3. Use of technology in precision agriculture over time (Holland et al. 2013).

A very interesting report from the European Commission has been recently issued regarding the role of precision agriculture in the European Union (European Commission Report 2014). This study shows that precision agriculture is applied mostly on arable farms, above all those with large fields and business orientation approach located in the mayor grain productive areas of Europe.

More recent is the adoption of this technologies in viticulture, but due to the high economic value of the crop and its quality, a lot of research has already been developed in this field in wine producing regions (European Commission Report 2014). Some of this research has been focused on the development of new sensors (Ben Ghozlen et al. 2010a) and their evaluation to be used in precision viticulture (Agati et al. 2013b, Baluja et al. 2012c). Other researchs have focused on the develoment of propper tecniques to analysed the data obtained by theses new technologies (Smit et al. 2010, Zarco-Tejada et al. 2005). And most of them applied these new technologies for assessing the spatial variability of vine vigour (Dobrowski et al. 2002, Goutouly et al. 2006, Johnson 2003, Taylor and Bates 2013), yield (Bramley and Hamilton 2004), berry composition (Bramley 2005, Cerovic et al. 2009) or water status (Baluja et al. 2012a, Grant et al. 2007) within the vineyard.

1.2. Grapevine vegetative status

1.2.1. Vegetative growth and development

Grapevine is a perennial ligneous plant. Its annual growth cycle encompasses a vegetative and a reproductive cycle (Mullins et al. 1992, Pearce and Coombe 2004). The vegetative cycle begins in spring when budburst occur. The initial growth of the shoots depends on the carbohydrate reserves stored in the vine. Later on, as the leaves start to develop, the energy and carbohydrates are provided by the photosynthesis, which will also feed the fruits, once their growth begins. The shoot lengthening increases as the temperature rises until, usually, the onset of fruit ripening when they are transformed into canes by lignification. However, if there is enough water and nitrogen, the growth may continue until harvest. In summer it is also when the latent buds, responsible of the next season shoots, enter in a dormant stage. The number of leaves increases as the shoots grow, and both parameters are intrinsically related. Grapevine leaves first separate from the shoot tip and begin to open until they are fully expanded. The vegetative cycle ends in autumn with the fall of the leaves after senescence, produced by frost or water stress events. The plant remains dormant during the winter.

1.2.2. Vegetative growth parameters

The grapevine canopy is the part of the plant that is above soil. It involves the trunk, shoots (leaves, petioles, shoot stems, shoot tips, lateral shoots and tendrils), fruits and cordons or canes. The structure of the canopy is determinant for the development of the grapevine. It induces specific light exposure, humidity and temperature conditions inside or around it, which is known as microclimate (Jackson 2008, Smart and Robinson 1991). Temperature, light, humidity, wind speed and evaporation will be different outside or inside the canopy and these differences will also depend on whether the canopy is open or dense. Leaves of a dense canopy will receive sunlight only if they are exposed leaves, in outer layers. The ones located inside the canopy will receive only the amount of radiation that was not absorbed or reflected by the external leaves. Therefore, internal leaves will not have enough light to achieve a high efficiency in photosynthesis and they will act as resource sinks, competing with clusters. Leaf temperature is also regulated by transpiration, wind speed and evaporation. Transpiration prevents the leaves from reaching too high temperatures, especially in exposed leaves or open canopies, unless the plant suffers from water stress. Open canopies will also be more affected by the wind while dense canopies remain more protected because leaves slow it down. Finally, the evaporation of dew or rain water will occur mainly in open canopies where there is sunlight, higher temperatures, wind and low humidity, preventing fungal infections.

The length and the amount of shoots and leaves determine whether the canopy is open or dense (Smart and Robinson 1991). Leaf area is an important factor, being responsible for creation of shade. Leaves are also the source of sugars, essential for vegetative growth and fruit ripening. Leaf area is closely linked to shoot vigour, since high vigour shoots elongate rapidly and produce large leaves. Therefore, shoot and leaf measurements are important elements to describe the canopy and its development. Traditionally, shoot and leaf measurements are carried out manually and, especially leaf area measurements are destructive and time-demanding (Smart and Robinson 1991). The most indicative measurements of vine vigour are the number of canes and the pruning weight, the latter is also a good indicator of the vegetative growth of the previous season and proportional to the total leaf area. Shoot length, shoot number and leaf area should be managed in order to achieve a grapevine balance and a canopy structure that better fits each terroir (Jackson 2014). For that purpose, vegetative management is necessary to ensure an appropriate development of the vegetative and reproductive cycles. Vegetative management involves several techniques to design a correct canopy and an appropriate microclimate such as winter or summer pruning, leaf removal, shoot thinning, etc. These actions will produce positive outcomes such as yield regulation, improvement of berry composition together with reducing disease incidence and production costs.

1.2.3. Nitrogen: a key factor on vegetative growth

A very important factor in grapevine metabolism and in biomass production is the nitrogen (N) content, being crucial for vine development and fruit yield (Roubelakis-Angelakis and Kliewer 1992). Its critical role in chlorophyll production and therefore in the photosynthesis process makes the N an essential nutrient for plants. Furthermore, N is involved in various enzymatic proteins that catalyse and regulate plant-growth processes. Also N fertilization deeply influences crop yield and biomass (Tremblay et al. 2011). In grapevines, an excess in N can cause more damage than its deficiency because vines would be more prone to diseases and insect infestations due to an increase in the density of the canopy or clusters with more berries (Dordas 2009). Moreover, over-fertilization usually produces lower quality grapes (Keller 2010) and plants become more susceptible to flowering abortion and reduced fruit set (Vasconcelos et al. 2009). Therefore, a good estimation of the N content at the time of potential application is crucial, especially in precision viticulture, where spatial variability is taken into account (Bramley 2010b).

Grapevine N fertilization is a key factor in vineyard management and has been widely studied by many authors around the world (Tremblay et al. 2012). Not only maintaining the appropriate balance of the plant is important but also environment protection needs to be taken into account. Nitrate leaching, soil denitrification, and volatilization are the main impacts produced by the loss of an excess of N fertilization (Zebarth et al. 2009). The importance of N fertilization has also been demonstrated as most of the VRT technologies have been focused on nitrogen-fertilizer applicators (Zhang et al. 2002).

There are different ways of assessing the N status of plants. Among the non-destructive techniques, chlorophyll-meters have been used to estimate the N status in several crops (Tremblay et al. 2012) because large quantity of leaf N is allocated to chlorophyll molecules (Evans 1989). But some studies reported a low accuracy in assessing leaf N by a chlorophyll-meter (Brunetto et al. 2012). The reason might be that surface-based chlorophyll is compared with mass-based N, so there is a need to transform surface-based measurements into mass-based measurements, for which leaf mass per area is needed (Poni et al. 1994). Light produces an increase in leaf mass per area and also an increase in chlorophyll content (Posada et al. 2009) but N remains constant. Meyer et al. (2006) demonstrated that flavonols can act as a surrogate of the leaf mass per area, compensating for different leaf light exposure. Moreover, Cartelat et al. (2005) have shown that the flavonoid and chlorophyll contents are both important for the assessment of the N status of the plant. This ratio is known as the nitrogen balance index (NBI = chlorophyll / flavonols) and its relation with the N status has also been reported by other authors for different species (Tremblay et al. 2012) and very recently in grapevine (Cerovic et al. 2015).

1.2.4. Influence of vegetative growth on yield and grape quality

Grape quality and yield are conditioned by canopy structure and microclimate. Vegetative management should be carried out to achieve a balanced canopy and ensure optimal results in the vineyard (Jackson 2014).

The canopy structure and microclimate affects yield (Bramley 2010b, Smart and Robinson 1991). The principal factor is the light exposure of the buds, which will produce shoots and clusters in the following season. If these buds are shaded, they may not develop in the next season. Furthermore, the shade may also block the budburst (Pratt 1974). It will also produce a reduction of the fruit set and the berry growth due to restricted photosynthesis, which will delay the sugar accumulation in the berries, hence harvest date. Finally, yield may also be reduced by fungal infections, which are more likely to occur in dense and shaded canopies.

By the photosynthesis, grapevine leaves produce sugars. Those sugars will translocate to the berries, being crucial for berry ripening. High temperatures usually promote sugar accumulation, but if temperature arises above 32 or 35ºC, it may result in a disruption of photosynthesis (Kriedemann 1968). High temperatures also increase respiration, which may lead to an increase in sugar concentration due to the loss of water (Jackson 2008). Therefore, the exposure of leaves to sunlight is critical to ensure an appropriate sugar content of the berries. But not only sugar content is determined by the exposure to light, there are other berry compounds that will vary depending on the exposure to light and so on the canopy microclimate. Sunlight exposure influences the anthocyanin and phenol contents in red grapes, as well as to boost the levels of tartaric acid and monoterpene flavour compounds while promoting the degradation of the malic acid (Crippen and Morrison 1986, Reynolds et al. 1986, Zoecklein et al.

1998). It has also been proved that the exposure of the clusters diminish the incidence of *Botrytis* bunch rot (Reynolds et al. 1986). Hence, proper canopy management is crucial to increase grape and wine quality.

1.2.5. Spatial variability of the vegetative status

For the aim of precision viticulture, it is important to assess the spatial variability of the vegetative growth. The spatial variability of the vegetative status has been demonstrated. Moreover, its influence on yield and grape quality have also been established, along with the repercussions on the profitability of the vineyard and on the environment. Vegetative growth is determined mainly by the availability of the plant to water and nutrients (Mullins et al. 1992). Under no restrictions, shoots can elongate, so the laterals and the number of leaves will also increase. But scarcity of nutrients or water supply may affect the vegetative growth leading to low vigour grapevines, which, in some cases, can also impact the yield. Water and nutrients supply depend mainly on the synergy between the root system and the soil. Another important factor for vine development is the mesoclimate. This climate at a parcel level will vary according to the existence of differences in elevation, slope or aspect leading to differences in temperature, wind speed, solar radiation or humidity (van Leeuwen 2010). So the spatial variability of soil and mesoclimatic characteristics will lead to different vegetative growth conditions depending on where grapevines are planted and on the different vegetative management actions carried out.

The pruning weight has been one of the most studied vegetative vigour variables of vineyards. Tisseyre et al. (2008) investigated the spatial variability of shoot pruning weight of a Syrah vineyard along seven years, finding a significant temporal stability. The temporal stability of the spatial behaviour was also confirmed for the pruning mass of Concord grapes studied through three years (Taylor and Bates 2013). Another variable related to pruning weight were found to have a stable spatial behaviour too, canopy size was reported to be temporally stable over a three year-study in a Syrah vineyard (Tisseyre et al. 2008). Concerning only the spatial variability, Baluja et al. (2012b) assessed the spatial variability of grapevine pruning weight within a Tempranillo vineyard, along with the spatial variability of shoot length (total and mean shoot length), another important indicator of the vegetative status and vigour of the vineyard. They also analysed the correlation of these variables with spectral indices, reporting good relationships with normalised difference vegetation index (NDVI) and plant cell density (PCD) measured at veraison, in agreement with the results reported by Sepulcre-Cantó et al. (2009). It has also been shown that vegetative expression and the resulting leaf area showed significant within-field variability in vineyards with different red and white varieties (Johnson et al. 2003). The spatial variability of vine vigour has been studied by Bramley et al. (2011b), who assessed it through the multispectral index PCD. Vine vigour has been shown to vary spatially in accordance with land spatial variations (Bramley et al. 2011c). As regards intra-seasonal variability, the spatial variability of canopy descriptors within the season was studied by Hall et al. (2011) and Mathews (2014).

The spatial variability of nutrients and physiological components has also been attempted. Davenport and Bramley (2007) studied the spatial variability of nutrients in plant tissues and its relation with the spatial variability of these nutrients in the soil. Likewise, the spatial variability of the nitrogen status of a Champagne vineyard was addressed by Garcia et al. (2012). Moreover, hyperspectral images were used to assess the spatial variability of chlorophyll (Zarco-Tejada et al. 2005) and carotenoid content in Tempranillo vineyards from remote platforms (Zarco-Tejada et al. 2013).

1.3. Sensing technologies in precision viticulture

1.3.1. Non-destructive technologies in precision viticulture

Traditionally, the monitoring of the vineyards and other crops has been carried out by methods, which are too laborious and expensive to be used operationally by grapegrowers (Smart and Robinson 1991). The assessment of soil characteristics and their spatial variability have been investigated by destructive methods like soils pits (Rossi et al. 2013). Likewise, the assessment of the vegetative growth parameters consists mainly on manually measuring the length of the shoots, the pruning weight and the leaf area (Smart and Robinson 1991). Furthermore, the classical methods for assessing the content of key components like chlorophyll, nitrogen, flavonol, among others in grapevine leaves or berries are based on wet chemistry analysis (Lashbrooke et al. 2010, Muñoz-Huerta et al. 2013, Stalikas 2007). Nevertheless, a large number of samples is needed for the analysis of the spatial variability and the implementation of precision viticulture techniques. Therefore, the use of rapid, non-destructive sensors is valuable and necessary to assess the spatial variability of the vegetative growth and leaf components within the vineyard.

Several different new sensors have been developed in the last years for the assessment of the crop status, as shown by Lee et al. (2010) in a very interesting review. Sensors for the detection and characterisation of crop biomass, weed, soil properties, nutrients, crop water status and micro or mesoclimate conditions are being gradually implemented in agricultural practices. These technologies have also been applied in the last years in viticulture (Arnó et al. 2009).

These sensors are used either remotely, on the ground or both. Remote sensing has been proved as an effective way to assess spatial information about a vineyard (Hall et al. 2002) but it requires a complex processing of the images, which provide information only from a top view of a limited portion of the canopy. In this way, ground sensors have evolved to be more adapted to the needs of vineyard management regarding the capability to obtain data of the grapevine sides, the speed to collect data, more friendly interfaces, cheaper prices and more ergonomic designs (Green 2012).

1.3.2. Remote sensing in precision viticulture

Remote sensing involves the observation and measurement of the characteristics of an object from a distance, based on the energy reflected or emitted by this object (Chuvieco 2010). In its origins, early 1960s, the term "remote sensing" was mainly applied to aerial images, as it was the principal sensor at that time. In that same decade, the first satellite images were taken. Its development continues up to now with the launch of numerous satellites specialised in natural resources and the development of new platforms as the unmanned aerial vehicles (UAV), also known as drones, recently renamed as remotely piloted aerial systems (RPAS).

Remote sensing, therefore, involves a sensor mounted on satellites or airborne platforms (figure 4). The latest include the manned airborne vehicles and the RPAS. Each of these platforms has advantages and disadvantages related to technological, operational an economic factors (Matese et al. 2015).

Satellite images capture large areas but at a too coarse resolution for precision viticulture application (Matese et al. 2015). Even though the spatial resolution provided has been increased to 0.5 m, this is not always enough and, besides, this improvement came with higher costs of the images.

Furthermore, the temporal resolution is either too low for precision viticulture or the images result too expensive if the revisit time is short. The aerial platforms allow obtaining images with very-high spatial resolution (2 to 10 cm) in comparison to satellite platforms. In addition, the temporal resolution of airborne platforms is higher than that of the satellites, allowing also to obtain the images at the moment of interest.

In the last years, RPAS have arisen as an alternative to the manned airborne vehicles allowing to obtain the same spatial and spectral resolution but at reduced costs. Furthermore, this new technology also provide the grapegrower with more independence to perform the measurements when needed. It has been proved that the results obtained with a low cost RPAS systems in agriculture can generate similar estimations of leaf area index (LAI), chlorophyll and water stress to those obtained with the traditional manned airborne vehicles (Berni et al. 2009).

Figure 4. Different platforms in remote sensing. On top-left, the Landsat 8 satellite (NASA); bottom-left, an airborne platform (NERC); and on the right, a remotely piloted aerial system (RPAS).

Remote sensing has been widely and successfully used in precision viticulture to assess the within field spatial and temporal variability (Hall et al. 2002) of water status (Acevedo-Opazo et al. 2008, Baluja et al. 2012a), chlorophyll and carotenoid contents (Martin et al. 2007, Zarco-Tejada et al. 2013) as well as vineyard canopy structure (Hall et al. 2008, Mathews and Jensen 2013). The use of aerial images, visible and infrared, for crop monitoring was established in Australia at the beginning of 1970s (Harris and Haney 1973). Since then, it has been spread out to other grape producing countries, such as Chile (Ortega and Esser 2005), South Africa (Strever 2007), USA (Johnson 2003), France (Tisseyre et al. 2007) and Spain (Zarco-Tejada et al. 2005), but the use of remote sensing in commercial vineyards is still more spread in Australia than in other countries (Bramley 2010b).

1.3.3. Ground-based sensing in precision viticulture

In cool or template climates, including Europe, Northern North America, Southern Australia and Southern South America, the vertical trellising (ie. VSP), the most spread in worldwide viticulture, gives rise to narrow canopies of 30-40 cm width. This vineyard architecture causes problems with aerial and satellite sensing due to the large percentage of background noise in the image (Taylor et al. 2005) produced by mixed pixels containing soil, grass and vine canopy (Tisseyre and Taylor 2008). In order to cope with these issues, ground-based monitoring systems have been developed to assess and map canopy properties (Tisseyre and Taylor 2008) and they have emerged as a successful alternative to retrieve physiological and vegetative growth-related data in precision viticulture (Baluja et al. 2012c, Bramley 2010a, Proffitt et al. 2006). Ground-based sensing involves proximal sensing, which includes all detecting technologies that provide information from an object when the distance between the sensor and the object is less than, or comparable to, some of the dimensions of the sensor, but unlike ground-based sensing, without making contact with the object.

These systems involve different sensing technologies. Some of these systems are based on digital imaging, which provides the measurement of several parameters such as canopy height and canopy porosity (Praat et al. 2004, Tisseyre et al. 1999). Others systems are based on ground-based NDVI measurement which have provided information strongly related to VLAI (Vertical Leaf Area Index) and canopy porosity (Goutouly et al. 2006). Chlorophyll fluorescence has also been implemented in proximal sensors used to assess leaf chlorophyll content, epidermal flavonols and N content of the vine (Cerovic et al. 2012, Tremblay 2013, Tremblay et al. 2012).

In ground sensing, the sensors can be either hand-held or embedded onto a motor driven platform, both allowing the acquisition of data in a non-destructive way (Tisseyre 2013). The differences between hand-held and on-the-go solutions may fall on the spatial resolution of the data recorded, i.e. the number of measurements per surface. While the hand-held sensors need from an operator to carry them and carry out the measurements, the on-the-go sensors are mounted onto some kind of vehicle, as a tractor or a quad, and measure automatically (in motion) depending on the sample protocol design. Therefore, the spatial resolution can be increased with the on-the-go sensors, and more data can be recorded in less time (Tisseyre and Taylor 2008). In all these cases the use of a global positioning system (GPS) is required so the data points obtained can be georeferenced and interpolated to generate maps of the vineyard status (Tremblay 2013).

Tractor-based mapping would be extremely useful, as in vineyard management the tractors frequently move within the rows for several agricultural operations so sensors could be mounted on these tractors retrieving information with no additional time-cost and at different stages of vine development (Taylor et al. 2005). The development of sensors to be used on-the-go in precision viticulture started in the last years for assessing the spatial variability of different vegetative components such as the vine vigour (Debuisson et al. 2010), leaf area (Drissi et al. 2009) and nitrogen (Garcia et al. 2012) among others. Currently, some projects are focusing on the construction of agricultural robots, for instance the ongoing European project named VineRobot, which aims at developing an unmanned ground vehicle (UGV), equipped with several non-destructive sensing technologies to monitor parameters, such as grape yield, vegetative growth, vineyard water status and grape composition (VineRobot Project 2013).

1.3.4. Non-destructive sensors

Due to the necessity of assessing a large amount of data of the vineyard, new non-destructive sensing technologies have emerged in the last years in the field of precision viticulture. Most of them are mainly related with the interaction between the light and the plant. The plant, either its leaves, shoots or fruits, responds to incident light depending on the plant physiology status (Tremblay 2013). The radiation at different wavelengths will be absorbed, reflected or transmitted by different constituents of the leaves (figure 5). It will also be emitted as heat or chlorophyll fluorescence. The measurement of these different responses to incident light will enable to assess the content of different leaf components as chlorophyll, carotenoids, flavonols, N or the plant water status among others.

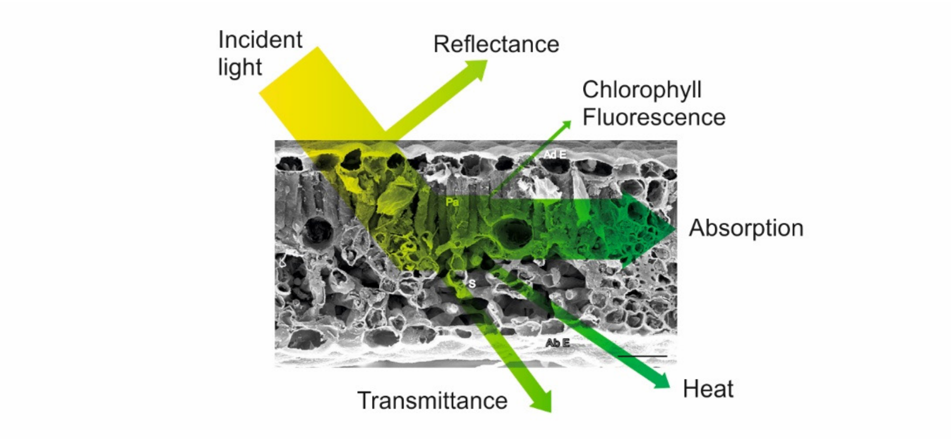

Figure 5. Interactions of the incident light with the leaf on a scanning electron micrograph of a cross-section of a grapevine leaf (Base image of the leaf from Bondada 2011).

The reflected energy can be originated by a natural source as the sun, in which case the sensors that received it are known as passive sensors; or it can be artificial light emitted by the sensor, this being an active sensor. All objects reflect or emit energetic fluxes as radiation. This energy is defined by its wavelength and frequency and it has been organised in bands with similar behaviour to facilitate its study (Chuvieco 2010). These bands are distributed along the electromagnetic spectrum (figure 6). The number of bands and their width recorded by a sensor define the spectral resolution, which describes the ability of a sensor to characterise the different surfaces (Chuvieco 2010).

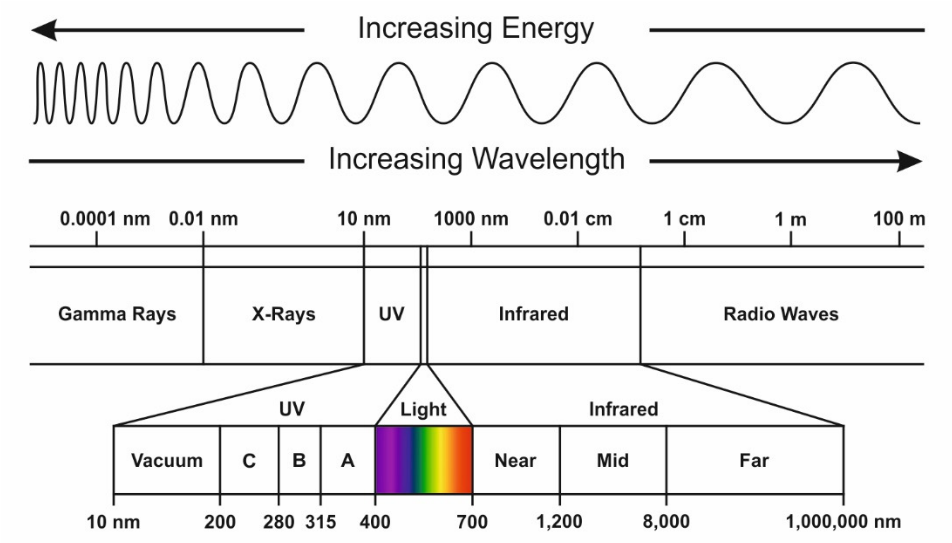

Figure 6. The electromagnetic spectrum.

From the standpoint of agriculture, the spectral bands most commonly used fall within the visible region (from 400 to 700nm) and are mostly related to the leaf pigments; in the near-infrared region (from 700 to 1200 nm), cell structure phenomena are the driving force; and in the mid wave infrared region (from 1200 to 2500 nm), water content is the most determinant factor.

The relative variation of the energy reflected or emitted according to the wavelength constitutes the spectral signature, which is characteristic of every surface and allows identifying them or the changes in their status (Chuvieco 2010). Specifically in vineyards there are three main elements that will be captured by an aerial image: leaves, stems and soil. These elements can be differentiated by its spectral signature (figure 7).

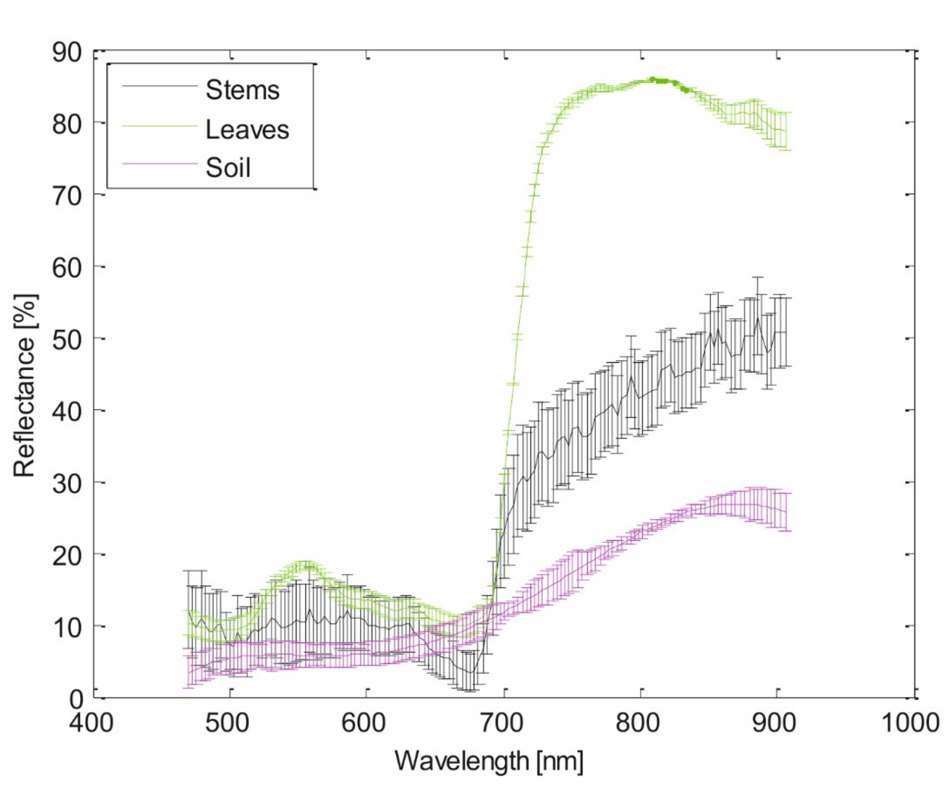

Figure 7. Spectral signatures of the three main vineyard components: stems, leaves and soil (Fernández et al. 2013).

In viticultural applications, current aerial imagery is dominated by multispectral cameras (figure 8) due to cost and camera operability (Taylor et al. 2005). Nevertheless, multispectral cameras can be also used at shorter distances, as mounted on a vehicle (Tremblay 2013). These cameras record the light reflected by an object in a few spectral bands, less than 10. The most usually employed bands fall within the visible and the near-infrared regions. Therefore, one pixel will have information from different wavelengths that can be analysed individually or combined in vegetative indices when required (Proffitt et al. 2006). The images recorded by multispectral cameras will need to be processed before their data can be analysed. The pre-processing of these data is a critical step and involves geometric and radiometric corrections, georeferencing and band-to-band alignment. The geometric corrections are important to solve the distortions produced in the images by the different movements that the aerial platforms can experience. Radiometric corrections allow to reduce the influence of the atmosphere, and band-to-band alignment is only necessary when the cameras have different sensors for the different bands. These very complex procedures can determine future results, so an expert is required to perform these corrections. Aerial multispectral images have been widely applied in viticulture to assess the spatial variability of the vegetative growth of the vineyard. Hall et al. (2003) used multispectral images to assess the canopy size, while Johnson et al. (2003) were able to assess the leaf area variability of a vineyard with a determination coefficient (R^2) of 0.72. Lately, Hall et al. (2011) studied how the correlations between multispectral images and vegetative variables, as pruning weight and petiole N, varied along the season.

RGB images have also been applied in the last years to the assessment, through image classification algorithms, of various key parameters of the plant, such as exposed leaf area, porosity of the canopy, number of exposed clusters, the ratio of senescence leaves or the number of flowers in a grapevine inflorescence (Diago et al. 2012, Diago et al. 2014). In these cases, the RGB camera has been used as a hand-held sensor, but recent research projects are investigating to assess these variables with the sensor mounted on a vehicle.

Hyperspectral images have also been applied in agriculture and, specifically in viticulture, in the last years. Unlike multispectral sensors, hyperspectral sensors (figure 8) record the light reflected by an object in hundreds of narrow spectral bands and provide information that multispectral data have missed (Lee et al. 2010). They have been mainly used from aerial platforms, either aircrafts or RPAS, to assess leaf components and water status of the vineyards (Rodríguez-Pérez et al. 2007, Zarco-Tejada et al. 2013). Regarding ground sensing, its application in viticulture has been limited due to the importance of light conditions and the high costs of the sensors, so they have been mainly used in the laboratory.

The near infrared region (NIR) is the part of the electromagnetic spectrum related with the presence of water and to the water status of the plants. Based on NIR spectroscopy, some portable sensors (figure 8) have been developed and applied to measure, in the field, the grapevine water status (De Bei et al. 2011) and grape composition (Barnaba et al. 2014).

Part of the infrared spectrum (from 8.000 to 14.000 nm) is known as thermal and this is the region where the heat emitted by the vegetation can be detected (Chuvieco 2010). The thermal infrared radiation is related to the temperature of the plant and therefore, it is very helpful to achieve the water status of the plant or any stress that induced the stomatal closure, which increases the leaf temperature (Möller et al. 2007). Thermal imagery has been widely applied in the last years to study the grapevine water status and stablish irrigation strategies in the vineyard (Grant et al. 2007, Möller et al. 2007).

Figure 8. Six types of field optical sensors. Top-left: multispectral camera Mini MCA-6 (Tetracam Inc.); Top-right: hyperspectral camera Micro-HyperspecVNIR™ (Headwall Photonics); Middle left: Thermal camera ThermaCAM® P640 (FLIR Systems); Middle-right: NIR spectrometer Microphazir™ (Thermo Fisher Scientific Inc.); Bottom-left: leaf-clip optical sensor Dualex4™ (Force-A); Bottom-right: hand-held chlorophyll fluorescence sensor Multiplex™ (Force-A).

The leaves not only respond by reflecting light. Part of the incident radiation is absorbed and another fraction is transmitted through the leaf, where it can be collected from the other side to assess the "transmittance" (figure 5). In this transmittance are based most of the common sensing devices denoted as chlorophyll-meters (Tremblay 2013). One of these sensors is a hand-held leaf-clip sensor, called Dualex™ that records light transmittance through the leaf at wavelengths 850 and 710 nm (Cerovic et al. 2012). The ratio of the transmittance values at 710 and 850 nm was found to strongly correlate with the leaf chlorophyll content in wild grapes (Carter and Spiering 2002). The measured value is calibrated against a reference method to yield the leaf chlorophyll concentration, which is often used for N fertilization management (Cerovic et al. 2012, Tremblay et al. 2012).

This sensor is also able to assess the phenolic composition by the chlorophyll fluorescence screening method. The incident light excites the chlorophyll molecule leading to the emission of fluorescence. Based on this, some sensors have been developed to assess phenolic composition (Cerovic et al. 2012) of various crops leaves and fruits. To assess the epidermal flavonols, UV light is emitted; it reaches the chlorophyll molecule and the light is re-emitted as fluorescence in the red to far-red part of the light spectrum (figure 9). But when flavonols are present, they act as a screen and do not allow the UV light to get the mesophyll, where chlorophyll is (Goulas et al. 2004). Comparing the fluorescence from the excitation of UV and red light, an accurate measurement of the absorbance of flavonols is provided (Cerovic et al. 2012). This method is also used to assess the anthocyanin content, but with green light excitation instead of UV (Ben Ghozlen et al. 2010a). Thanks to the assessing of chlorophyll and flavonols by the same instrument, this sensor automatically calculate the Nitrogen Balance Index (Cartelat et al. 2005) that allow to assess the N status of the plant (Cerovic et al. 2015).

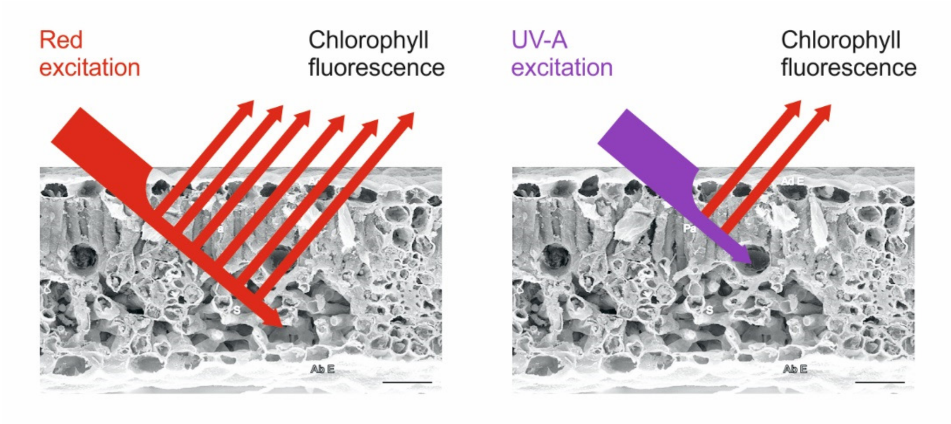

Figure 9. Cross-section of a leaf showing the chlorophyll fluorescence response to red (left) and UV-A (right) excitations (Base image of the leaf from Bondada 2011).

The chlorophyll fluorescence screening method has also been implemented in another hand-held sensor called Multiplex™ (Ben Ghozlen et al. 2010a). This sensor measures the chlorophyll fluorescence emission and has proved to be a very powerful, rapid and efficient phenotyping tool to determine the chlorophyll content (Tremblay et al. 2012), leaf epidermal flavonols (Agati et al. 2011b) and the N status (Agati et al. 2013c, Tremblay et al. 2012, Zhang et al. 2012) in leaves of different species. In grapevines, it has been mainly used to assess berry composition (Baluja et al. 2012c, Ben Ghozlen et al. 2010a) and the control of fungal diseases in plants (Agati et al. 2008, Bellow et al. 2012, Latouche et al. 2013), but few research has been conducted to assess the vegetative and nutritional status of the vineyards (Serrano et al. 2010), therefore further research is needed on this topic.

Lately, this sensor has also been modified to be mounted on a vehicle to take measurements on-the-go, but still little research work using this mounted fluorescence sensor in precision viticulture has been reported. Bramley et al. (2011a) mounted this sensor on a harvester to assess the berry anthocyanin content on-the-go. Regarding the vineyard vegetative status, this was barely explored using this type of fluorescence sensor on the go, and only the nitrogen status (Garcia et al. 2012) and vine vigour spatial variability (Debuisson et al. 2010) were tentatively appraised in a Champagne vineyard. It is still necessary to investigate the capability of this sensor to properly assess the vegetative and nutritional status of the vineyard on-the-go.

1.3.5. Indices in precision viticulture

1.3.5.1. Vegetative spectral indices

The calculation of spectral vegetation indices benefit from the contrast that exists between vine biomass when measured in different wavebands (Chuvieco 2010). They are calculated as a mathematical combination of the reflectance values at different wavelengths, thus reducing the number of bands to a unique numeric value per pixel (Hall et al. 2002). These indices allow to distinguish between the elements recorded or the changes in their status. Furthermore, spectral indices also attenuate other influences as those from the soil, the atmosphere or the illumination (Tucker 1979). Particularly, the differences in the near infrared band make possible the differentiation between a vigorous and healthy vine and that of a poor vigour or stressed one (Hall et al. 2002).

One of the indices most commonly used in viticulture is the normalized difference vegetation index (NDVI) (Rouse et al. 1974). NDVI is based on the relative difference between reflectance of sunlight in the red (R) and infrared (IR) bands (table 1). The NDVI has been extensively used to assess the vegetative growth (Goutouly et al. 2006), shoot length (Baluja et al. 2012b), leaf area index (Johnson et al. 2003) as well as to delineate management areas in vineyards (Tagarakis et al. 2013b). There are other indices applied in precision viticulture such as the plant cell density (PCD), which has been used as an indicator of the variability in vine vigour and as a tool to apply targeted management strategies such as selective harvesting (Bramley et al. 2005) or differential pruning (Proffitt and Malcolm 2005). Both NDVI and PCD are indicators of the photosynthetically active biomass and are therefore correlated with the size and health or absence of stress of vine canopies (Bramley 2010b). Other vegetative indices (table 1) have also been applied in literature, such as the photosynthetic vigour ratio (PVR) or narrow-band hyperspectral vegetation indices sensitive to chlorophyll, carotenoid content or water status (Martin et al. 2007, Rodríguez-Pérez et al. 2007, Zarco-Tejada et al. 2005, Zarco-Tejada et al. 2013).

Table 1. Spectral vegetation indices commonly used in viticulture studies.

Spectral index	Reference
Normalized Difference Vegetation Index (NDVI) $$NDVI = \frac{R_{NIR} - R_{RED}}{R_{NIR} + R_{RED}}$$	(Rouse and Center 1974)
Modified Simple Ratio (MSR) $$MSR = \frac{\left(\frac{R_{NIR}}{R_{RED}} - 1\right)}{\left[\left(\frac{R_{NIR}}{R_{RED}}\right)^{0.5} + 1\right]}$$	(Chen 1996)
Modified Triangular Vegetation Index ($MTVI_1$) $$MTVI1 = 1.2 * [1.2 * (R_{800} - R_{550}) - 2.5 * (R_{670} - R_{550})]$$	(Haboudane et al. 2004)
Renormalized Difference Vegetation Index (RDVI) $$RDVI = \frac{R_{NIR} - R_{VIS}}{\sqrt{R_{NIR} + R_{VIS}}}$$	(Roujean and Breon 1995)
Greenness Index (G) $$G = \frac{R_{GREEN}}{R_{RED}}$$	-
Modified SAVI (MSAVI) $$MSAVI = \frac{1}{2}\left[2 * R_{800} + 1 - \sqrt{(2 * R_{800} + 1)^2 - 8 * (R_{800} + R_{670})}\right]$$	(Qi et al. 1994)
Optimized Soil Adjusted Vegetation Index (OSAVI) $$OSAVI = \frac{(1 + 0.16)(R_{NIR} - R_{RED})}{(R_{NIR} + R_{RED} + 0.16)}$$	(Rondeaux et al. 1996)
Modified C_{ab} Absorption in Reflectance Index (MCARI) $$MCARI = [(R_{700} + R_{670}) - 0.2 * (R_{700} - R_{550})] * \left(\frac{R_{700}}{R_{670}}\right)$$	(Daughtry et al. 2000)
Transformed CARI (TCARI) $$TCARI = 3 * [(R_{700} - R_{670}) - 0.2 * (R_{700} - R_{550}) * \left(\frac{R_{700}}{R_{670}}\right)]$$	(Haboudane et al. 2002)

Spectral index	Reference
Photochemical Reflectance Index (PRI) $$PRI = \frac{(R_{531} - R_{570})}{(R_{531} + R_{570})}$$	(Gamon et al. 1992)
TCARI/OSAVI $$TCARI/OSAVI = \frac{3*[(R_{700}-R_{670}) - 0.2*(R_{700}-R_{550})*(R_{700}/R_{670})]}{(1+0.16)*\frac{R_{800}-R_{670}}{R_{800}+R_{670}+0.16}}$$	(Haboudane et al. 2002)
Plant Cell Density Index (PCD) or Ratio Vegetation Index (RVI) $$PCD = RVI = \frac{R_{NIR}}{R_{RED}}$$	(Jordan 1969)
Photosynthetic Vigour Ratio (PVR) $$PVR = \frac{R_{GREEN}}{R_{RED}}$$	-
Normalised difference water index (NDWI) $$NDWI = \frac{R_{860} - R_{1240}}{R_{860} + R_{1240}}$$	(Gao 1996)

1.3.5.2. Chlorophyll fluorescence-based indices

The signals recorded by the hand-held fluorescence sensor are combined into a set of indices that allow assessing the leaf chlorophyll content, epidermal flavonols and N content (table 2). The simple fluorescence ratio (SFR) index is a chlorophyll fluorescence emission ratio linked to the leaf chlorophyll content (Gitelson et al. 1999, Tremblay et al. 2012). It is a ratio of far-red emission (735 nm) divided by red emission (685 nm) under red excitation or green excitation. Due to the overlap of the chlorophyll absorption and emission spectrum, re-absorption occurs at shorter wavelengths but not at longer wavelengths (Gitelson et al. 1999, Pedrós et al. 2010). Therefore, SFR increases with increasing sample chlorophyll content. The epidermal flavonols index (FLAV) compares the chlorophyll fluorescence intensity emitted as far-red fluorescence under ultraviolet (UV) and red excitation, which represents a differential absorption measurement (in accordance with the Beer–Lambert's law) that is proportional to the flavonols content (Agati et al. 2011b, Ounis et al. 2001). As mentioned before, the Nitrogen Balance Index (NBI) (Cerovic et al. 2008) is related to the N status of the plant and proportional to the chlorophyll-to- flavonols ratio proposed by Cartelat et al. (2005). When increasing N application, chlorophyll content increases while flavonols content decreases, so NBI increases with N fertilization. For the Multiplex™ sensor, it is the ratio of far-red emission under UV excitation and red emission under red excitation or green excitation.

Table 2. Fluorescence indices related with leaf chlorophyll content, epidermal flavonols and N status of the plant provided by the hand-held sensor Multiplex™.

Fluorescence index	Equation	Reference
Simple fluorescence ratio under red excitation (SFR_R)	$SFR\_R = \frac{FRF\_R}{RF\_R}$	(Cerovic et al. 2009, Gitelson et al. 1999)
Simple fluorescence ratio under green excitation (SFR_G)	$SFR\_G = \frac{FRF\_G}{RF\_G}$	
Epidermal flavonols index (FLAV)	$FLAV = \log\left(\frac{FRF\_R}{FRF\_UV}\right)$	(Cerovic et al. 2009, Goulas et al. 2004)
Nitrogen balance index under red excitation	$NBI\_R = \frac{FRF\_UV}{RF\_R}$	(Cerovic et al. 2008)
Nitrogen balance index under green excitation	$NBI\_G = \frac{FRF\_UV}{RF\_G}$	

*FRF: far-red emission under red excitation (FRF_R), under green excitation (FRF_G) or under ultraviolet excitation (FRF_UV). RF: red emission under red excitation (RF_R) or green excitation (RF_G).

These fluorescence indices provided by the fluorescence sensor Multiplex™ have already shown good results in assessing the spatial variability of the nitrogen content in turfgrasses (Agati et al. 2013c) or of grapevine quality (Baluja et al. 2012b, Cerovic et al. 2009). Regarding the vegetative status of the vineyards, few studies are found in literature (Garcia et al. 2012, Serrano et al. 2010). More effort is needed in this area to establish the suitability of the fluorescence sensor and its derived fluorescence indices to study the vegetative status of the vineyards and its spatial variability.

2 Objectives

The main objective pursued in this PhD thesis was to assess the spatial variability of the vegetative status of vineyards (*Vitis vinifera* L.) using non-destructive sensors.

Along with this main goal, other specific objectives have been:

- To assess the vegetative growth and its spatial variability within a vineyard using multispectral imagery acquired by a RPAS and the derived spectral indices.
- To calibrate and evaluate the performance of a hand-held fluorescence proximal sensor and its indices for the assessment of chlorophyll, flavonol and nitrogen content in grapevine leaves under field conditions.
- To appraise the spatial and temporal variability of grapevine leaf chlorophyll content and nitrogen status using a hand-held fluorescence proximal sensor and their relationships with the vegetative growth within a vineyard.
- To assess the leaf chlorophyll, flavonol and nitrogen content on-the-go within a vineyard using a fluorescence sensor. Mapping of the spatial variability of these leaf components.

3 Results and Discussion

3.1. Chapter 1

Using RPAS multi-spectral imagery to characterise vigour and leaf area variability within a vineyard

ABSTRACT

Background and aims: The implementation of remote sensing in the wine industry requires high spatial resolution images and temporal flexibility of acquisitions. The vigour and the leaf development of grapevines influence yield and grape composition. The aim of the present work was to show the capability of multispectral imagery acquired from a remotely piloted aerial system (RPAS), and the derived spectral indices to assess vigour and leaf area in a vineyard (*Vitis vinifera* L.) and to assess and map their spatial variability.

Methods and results: In this work, multi-spectral imagery of 17 cm spatial resolution was acquired 20 days post-veraison using a RPAS. Image pre-processing included band alignment, radiometric calibration, georeferencing and mosaicking. Classical spectral indices and two newly defined normalised indices, $NVI_1 = (R_{802}-R_{531})/(R_{802}+R_{531})$ and $NVI_2 = (R_{802}-R_{570})/(R_{802}+R_{570})$, were computed. Their spatial distribution and relationships with grapevine vigour, yield parameters and fluorescence-based indices were studied. The fine registration applied for the correction of the misalignment among the spectral bands of the images was able to improve the alignment while maintaining the radiometric values unchanged. Most of the spectral indices and field variables studied varied spatially within the vineyard, as NDVI, MSR pruning weight and main leaf area among others, showed through the variogram parameters and the computed maps. Moderate and significant correlations were observed between the spectral indices retrieved from remote imagery with vegetative growth and fluorescence-based indicators, which showed similar spatial patterns. NVI_1 and NVI_2 showed the best correlation coefficients. No relationship was observed between multispectral indices and yield, which was found to be mostly driven by the number of clusters per vine.

Conclusions: The results showed the potential and current limitations of using single-date high resolution multi-spectral imagery acquired from a RPAS to describe the vineyard variability for management decision support.

Keywords: precision viticulture, remote sensing, remotely piloted aerial system, spectral indices, fluorescence indices, band misalignment.

INTRODUCTION

Modern and sustainable viticulture requires objective and continuous monitoring of key parameters for rational and differentiated agronomic management of vineyards based on the spatio-temporal variability of growth, yield and grape composition within the plot. Traditional monitoring of vineyard involves discrete spatial and temporal sampling, but these methods are too laborious and expensive to be used operationally by grapegrowers (Fuentes et al. 2013).

One of the tools that has demonstrated its capability to help in crop management, and more specifically, in studying the within field variability to assess spatial and temporal changes in soil moisture, canopy growth and plant water status, is remote sensing. Several works have shown the suitability of remote sensing for precision viticulture purposes during the last two decades (Hall et al. 2002). Hence, this technique has been used to appraise the vineyard spatial variability of water status (Acevedo-Opazo et al. 2008, Baluja et al. 2012a), chlorophyll and carotenoid content (Martin et al. 2007, Zarco-Tejada et al. 2013), vineyard canopy structure (Hall et al. 2008, Mathews and Jensen 2013), grape colour and phenols content (Lamb et al. 2004), as well as grape quality (Martin et al. 2007). Remote sensing involves the acquisition of spectral data from several platforms, such as satellites, aircrafts, and remotely piloted aerial systems (RPAS, UAV or drones).

Vineyards are not a continuous crop, unlike cereals, and data from satellite sensors do not allow to accurately differentiate between individual vines and row soil background (Turner et al. 2011). Even for very high resolution spaceborne sensors such as Pleiades Constellation (http://smsc.cnes.fr/PLEIADES/), the highest spatial resolution available is 50 cm/pixel which still could be too coarse to monitor some common vine training systems where crop canopies are very narrow (i.e., 40 - 60 cm in the case of vertical shoot positioned vineyards) and a mixed signal of leaves, shoots, shadow and soil is commonly found in most pixels. Sensors on board of manned airborne vehicles allow overcoming these limitations by acquiring imagery of increased spectral, spatial and temporal resolution than those mounted on satellites, although their operational costs are very high.

In the last years, RPAS have emerged as an alternative, as they allow acquiring imagery of very high spatial resolution, often at a sub-decimetre resolution, at much lower cost than traditional airborne remote sensing. Furthermore, it has been proved that results obtained from a RPAS for agricultural application may yield similar estimations in terms of accuracy and precision than those derived from traditional airborne platforms (Berni et al. 2009). These facts, together with their lower cost and higher temporal flexibility, explain the increased use of RPAS for agricultural purposes (Zhang and Kovacs 2012). As a matter of fact, a very recent report on the economic impact of unmanned aircrafts in the United States (US) (Jenkins and Vasigh 2013) revealed that the use of RPAS in agriculture in 2015 in the US would yield 2,096 million dollars and expected job creation of 21,565 employments. Among the applications of RPAS in viticulture, the assessment of the within-vineyard variability stands out (Sepulcre-Cantó et al. 2009), as the spatial and temporal resolutions are key attributes when the goal of the study is monitoring and managing the vineyard (Hall et al. 2002).

Most of the remote sensing studies in agriculture are based in the use of spectral indices. These are arithmetical combinations of the original spectral bands captured by the sensor, which reduce the complexity of the dataset (Hall et al. 2002) and facilitate the analysis of various vegetation parameters which are directly related to their spectral response at given wavelengths. Vegetation indices, like the normalised difference vegetation index (NDVI), have been extensively used to monitor crop status by relating visible and near infrared spectral data with bio-geophysical properties of the vines such as vigour and leaf area (Sepulcre-Cantó et al. 2009). Other spectral indices like the photochemical reflectance index (PRI) combine information from two narrow bands in the visible region to monitor vegetation stress (Gamon et al. 1992).

In addition to remote sensing, proximal sensing is being widely used in precision agriculture and a large number of sensors and techniques were developed. Among the range of technologies used in proximal sensing, the chlorophyll fluorescence has been introduced in the last years as a powerful and efficient proximal technique for plant monitoring (Bilger et al. 2001, Gitelson et al. 1999, Pedrós et al. 2010). This chlorophyll fluorescence-based technology has been implemented in a new, commercially available non-invasive fluorescence sensor, which measures the chlorophyll fluorescence emission and provides indices related to the chlorophyll, flavonol and nitrogen status of leaves (Ben Ghozlen et al. 2010a, Tremblay et al. 2012).

The present work aims at showing the capability of multispectral imagery acquired from a drone, and the derived spectral indices to assess vigour and leaf area in a vineyard *(Vitis vinifera* L.), and map their spatial variability.

MATERIALS AND METHODS

Study area

The study was conducted in a 3 ha Tempranillo (*Vitis vinifera* L.) vineyard located in Navarra, Spain (figure 10). Grapevines were planted in 2004 in a sandy-clay soil (5% slope towards North), using a 41B rootstock at 2.4m x 1.6m (inter- and intra-row) and row orientation was north-south. Vines were trained to a vertically shoot-positioned (VSP), spur-pruned cordon retaining 16 nodes per linear meter/vine. Vines were uniformly irrigated twice across the season.

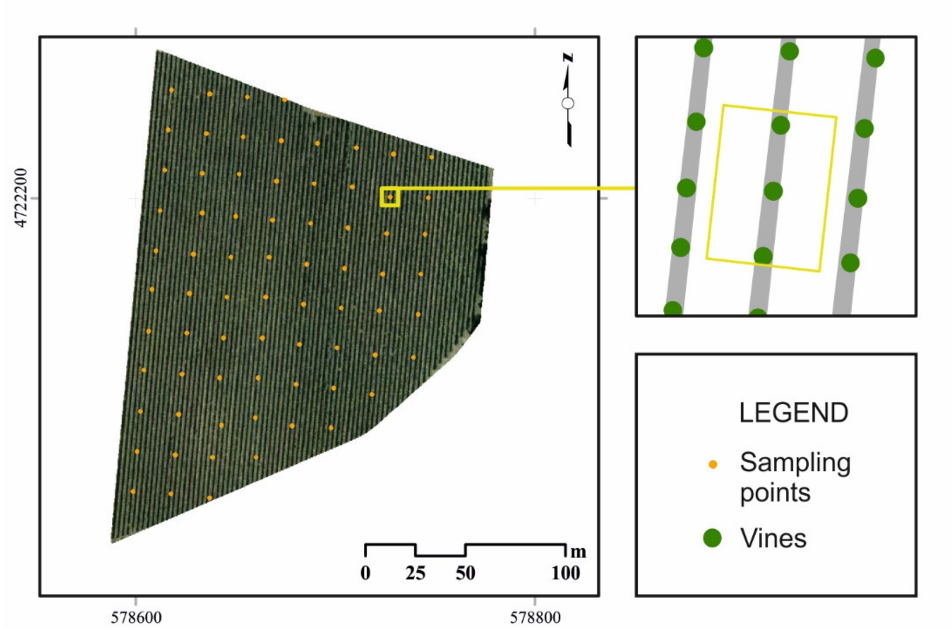

Figure 10. Experimental Tempranillo vineyard plot with the 72 sampling points. Schematic describing the position of the three adjacent vines comprising a sampling point.

Vigour, leaf area and yield components

A regular grid of 72 sampling points at 20 m intervals was generated using a Leica Zeno 10 GPS (Heerbrug, St. Gallen, Switzerland), with real time kinematic correction, working at <0.3 m precision. Each sampling point consisted of three adjacent vines (figure 10). Vigour, leaf area and yield-related data were taken from all sampling points. As indicators of vine vigour, main and secondary shoot length (MSL and SSL) and pruning weight (PW) were measured. Regarding leaf area, main and secondary leaf area per shoot (MLA and SLA) were determined. Shoot length and leaf area per shoot were measured for the three vines included in each sampling point one week prior to the RPAS flight, on the 13 September 2011. For each vine, two shoots were randomly chosen and their main shoot length as well as those for all laterals of more than three leaves, were measured using a meter tape (Martínez de Toda et al. 2007). Main and secondary leaf area per shoot were estimated by defoliating each shoot and weighing the leaves with a portable scale of precision ±2 g (Kern & Sohn GmbH, Balingen-Frommern, Germany), using the disc-leaf method by Smart and Robinson (1991). The pruning weight of each vine was manually determined in the field using a hanging scale on 20 November 2011, prior to vine pruning. Additionally, non-destructive measurement of photosynthetic pigments in plants were taken using fluorescence-based indices related to the chlorophyll content in leaves (SFR_R_{AD}) and the nitrogen balance index (NBI_G_{AD}). Fluorescence measurements were taken on the 13 September 2011 with a handheld, non-destructive fluorescence-based proximal sensor, called Multiplex™ (Multiplex 3.0, Force-A, Orsay, France). The Multiplex™ sensor used for this study was a modified version of the device recently described by Ben Ghozlen et al. (2010b). Multiplex™ measurements were performed on three main leaves (adaxial side) per vine. From these measurements, the fluorescence indices were calculated as follows:

$$SFR\_R_{AD} = \frac{FRF\_R_{AD}}{RF\_R_{AD}} \quad (2)$$

$$NBI\_G_{AD} = \frac{FRF\_UV_{AD}}{RF\_G_{AD}} \quad (3)$$

where FRF_R, FRF_G and FRF_UV refer to the far red fluorescence evolved from red excitation, green excitation and UV excitation, respectively, and RF_R and RF_G refer to red fluorescence excited by red light and green light, respectively (Ben Ghozlen et al. 2010a). The subscript AD stands for the adaxial side of the leaf.

At harvest time (17 October 2011), main yield components, such as cluster number per vine, yield per vine, cluster weight and berry weight were determined for each vine. Each plant was manually harvested, their clusters counted and weighed in the vineyard using a hanging scale, and the average cluster weight calculated. Two clusters per vine were then labelled, kept apart and taken to the laboratory of the University of La Rioja in portable refrigerators, where they were manually destemmed. Berries form each cluster were weighed (TE3102S, Sartorius, Goettingen, Germany) and counted, and the average berry weight calculated.

For all data collected in the field, the values of the three vines per sampling point were averaged and a mean value was then assigned to each sampling point of the grid.

RPAS multispectral images

Multispectral imagery was acquired with a multi-spectral camera, a mini multiple camera array (MCA 6, Tetracam inc, Chatsworth, USA) mounted on a RPAS Md4-1000 (Microdrones GmbH, Siegen, Germany) on the 20 September 2011 (figure 11). The RPAS Md4-1000 is a quadrotor able to carry 1.2 kg of payload mass and to fly up to 88 minutes, with vertical take-off and landing. The camera consisted of six independent image sensors and optics with user-configurable filters. Image resolution was 1280 × 1024 pixels with 10-bit radiometric resolution and optics focal length of 9.6 mm. For this study, the camera was equipped with six 25-mm-diameter bandpass filters of 10-nm full-width at half-maximum (FWHM) (Andover Corporation, NH, U.S.), with centre wavelengths at 531, 551, 570, 672, 701, and 802 nm, and bandwidth of ± 9.31, 10.13, 9.29, 9.82, 9.47 and 10.11 nm respectively.

Figure 11. Multispectral camera mounted on the RPAS Md4-1000 and detail of the multiple camera array.

The RPAS flew at 250 m height and allowed capturing images with a spatial resolution of 17 cm. Images were taken at noon, between 11:15 and 12:15, under stable weather conditions and clear sky (mean wind velocity of 1.39 m/s). At this time, grape berries were at half-way in their ripening process. A total of 6 scenes were acquired (with an overlapping area of 60%) in order to monitor the whole vineyard plot lengthwise.

Image processing

From the 6 images obtained with the multi-spectral camera, two scenes that covered the whole plot were selected for the present project. An initial pre-processing of the images acquired with the Mini MCA camera was carried out using its companion application Pixel Wrench 2 (PW2) written and copyrighted by Tetracam Inc. to produce multi-page Tagged Image Format (TIF) files. As the multi-spectral camera uses six different lenses (one for each spectral band), PW2 processing included an inter-band alignment by applying an affine transformation based on a device-specific matrix.

Band alignment is important to ensure consistent spectral information but when this correction is carried out by PW2, it turns out insufficient according to Laliberte et al. (2011). In agreement with these findings, the results of the inter-band alignment performed with PW2 showed substantial errors in this study and, therefore, it was decided to skip the alignment processed included in PW2 by setting a null aligning matrix and carry out a specific correction. Band alignment was performed by applying the Fine Registration algorithm of the Orfeo Toolbox (Inglada and Christophe 2009) between each band and a reference band (band 2). This process calculates the local shifts in x and y directions that would most improve the local correlation between the band to be processed and the reference band, and creates a deformation field that is subsequently applied to the band to be processed. After a set of tests with a range of radii, the local windows to explore and calculate correlations were set to 5 x 5 pixels for bands 1, 3 and 4 and 7 x 7 pixels for bands 5 and 6. In addition, as the displacement with respect to the reference band was larger for bands 5 and 6, a rigid translation was applied in both x and y directions previous to the fine registration processing for these two bands. In order to evaluate the improved co-registration, the correlation coefficients between each band and the reference band before and after the processing were calculated.

Once the band alignment corrections were completed, the images were georeferenced to be able to correlate image data with field measurements. This was done by using cartographic co-ordinates from 8 reference targets (white discs of 30 cm of diameter) located at the boundaries of the vineyard plot which had been georeferenced in the field during image acquisition using a Leica Zeno 10 Global Positioning System (GPS) (Heerbrug, St. Gallen, Switzerland) . At the same time, an orthophoto of the area of study provided by the Spanish National Program of Aerial Ortophotography (PNOA) with 25 cm spatial resolution was also used to identify 45 ground control points in each scene. These points were evenly distributed over the image area including the borders, where displacement errors were higher because of the larger distance from the photo-center. A second degree polynomial function was applied using a bilinear resampling method to render the geometrically corrected image. Image georeferencing was carried out with an accuracy of 0.62 and 0.35 m (root mean squared error). Interactive location of ground control points, polynomial fit and interpolation were performed with ENVI 4.8 (Exelis, McLean, VA, USA).

Radiometric calibration was performed using an image of a white calibration panel that was acquired from the ground with the mini MCA (at a distance about 1.5 m) prior to the flight. A reflectance spectrum of the same panel was measured at the Environmental Remote Sensing and Spectroscopy Laboratory (SpecLab) using an ASDFieldSpec 3 spectroradiometer (ASD, Boulder, USA). Image DN (Digital Numbers) from the calibration panel were averaged for each band to calculate the observed DN spectrum. The reflectance spectrum was integrated to calculate the theoretical reflectance values of the panel for each mini MCA band using the transmission curves of the filters (www.andovercorp.com). A vector of factors was computed to transform the observed DN spectrum of the image into the modelled reflectance spectrum. These factors were then applied to each band of the image in order to obtain calibrated reflectance for each image pixel. Images were calibrated to apparent reflectance, i.e. reflectance as if the camera had been operated at 1.5 m. Atmospheric correction has not been performed, but as the area is small and the atmospheric conditions very clear, the uniform atmospheric effect eventually present in the indices is taken into account by the linear models between indices and field data. The radiometric calibration operations were conducted by means of specific functions written in R Core Team (2012) and using packages rgdal (Keitt et al. 2012) and raster (Hijmans and Van Etten 2012).

The next step was the spatial assembly of the images to build the final mosaic, so the whole vineyard plot fitted within a single image file. The mosaicking process was performed using ENVI software by combining the two scenes that included the whole vineyard. We assigned, as base image, the scene that covered most of the plot, so the colour balance in the second image was carried out to match the base image, by using the histogram matching. A distance of 40 pixels was set to blend the seams between the two images.

Using the corrected image mosaic, a total of 12 spectral indices, selected from the literature as being the most used to characterise vegetation status were calculated. These indices have been proposed to estimate a wide variety of vegetation properties including, pigment contents, leaf area index, plant health status, nutrient stress, etc. and some of them have been widely used in precision viticulture. Some indices specially designed to minimize the effects of soil background on the vegetation signal were also included in the analysis. The spectral indices calculated using the RPAS images were the Normalised Difference Vegetation Index –NDVI-(Rouse et al. 1974), Modified Simple Ratio –MSR-(Chen 1996), Modified Triangular Vegetation Index -$MTVI_1$-

(Haboudane et al. 2004), Renormalised Difference Vegetation Index –RDVI- (Roujean and Breon 1995), Greenness Index -G-, Modified SAVI -MSAVI- (Qi et al. 1994), Optimized Soil Adjusted Vegetation Index –OSAVI- (Rondeaux et al. 1996), Modified C_{ab} Absorption in Reflectance Index –MCARI-(Daughtry et al. 2000), Transformed CARI –TCARI-(Haboudane et al. 2002), TCARI/OSAVI (Haboudane et al. 2002) and Plant Cell Density Index –PCD-(Dobrowski et al. 2003) (table 1).

In addition to these indices, a set of normalised indices were calculated as all possible-combinations between every image band, following the equation:

$$Normalised\ index = \frac{Band1 - Band2}{Band1 + Band2} \quad (4)$$

Statistical analysis

With the aim of establishing a relationship between image data and field measurements, those pixels corresponding to the 72 sampling points were identified. For each sampling point, the two most centred pixels (6 pixels per point) were selected. Image values were extracted from those pixels and averaged per sampling point for each band and index. Prior to the statistical analysis, the data were pre-processed in order to detect potential outliers.

Descriptive statistics were computed for all variables, as well as the spread (eq. 5), expressed in percentage, as an indicator of the variability in the sample (Bramley 2005).

$$Spread = \frac{(maximum - minimum)}{median} \quad (5)$$

To characterise the spatial variability of the various parameters within the vineyard, variograms for the variables were calculated by the R package "gstat" (Pebesma 2004).

The correlations between these values and the field data were analysed using Statistica 9 (Stat Soft Inc., Tulsa, USA). Pearson correlation coefficient and its significance were calculated to determine which pair of variables was correlated at 99 % confidence level. Finally, the parameters of the variograms were also used to interpolate all the variables, using kriging techniques by means of QGIS (QGIS Development Team, 2013).

RESULTS AND DISCUSSION

Image processing

Date presented in table 3 show the results obtained with the improved band-to-band registration method. Correlation coefficients (r) between each band and the reference band are shown. In the original set, "r" was very low (r values from 0.28 to 0.84), even considering the effect of the different responses at different wavelengths. Correlation coefficients increased in all cases after fine registration, indicating improved alignment as radiometric values remained virtually unchanged. It should be considered that the use of a multiple camera array imaging system implies the existence of horizontal and vertical translations between bands. Additionally, a RPAS is a lightweight platform, which is slightly affected by wind, even when it is not very strong. This is a key issue, as the spatial distribution of the grapevines along 40 cm width rows implies that one pixel relative translation between bands may result in comparing a soil pixel in some bands with a vegetated one in others. Therefore, the proper correction of this misalignment is critical to ensure the quality of spectral indices and its relationship with field data.

Table 3. Comparison of the correlation coefficients (r) of each band to the referenced band (band 2) for the original scenes and those obtained after applying the alignment correction operated by Fine Registration (FR) algorithm of the Orfeo Toolbox (OTB, Inglada and Christophe, 2009).

Image scene	Band number	Original	Optimized (Fine Registration)
Scene 1	1	0.59	0.97
	2	1.00	1.00
	3	0.74	0.98
	4	0.61	0.95
	5	0.28	0.96
	6	0.38	0.74
Scene 2	1	0.77	0.99
	2	1.00	1.00
	3	0.83	0.99
	4	0.84	0.97
	5	0.64	0.96
	6	0.41	0.70

Vegetative spectral indices

A set of new normalised spectral indices was computed as all the possible combination of every-two bands of the multispectral images. The indices obtained are shown in table 4, except for the equation that corresponded to the NDVI index.

Table 4. Normalised spectral indices computed as a combination of all the bands of the multispectral images.

	531 nm	551 nm	570 nm	672 nm	701 nm	802 nm
531 nm		$\frac{R_{531}-R_{551}}{R_{531}+R_{551}}$	$\frac{R_{531}-R_{570}}{R_{531}+R_{570}}$	$\frac{R_{531}-R_{672}}{R_{531}+R_{672}}$	$\frac{R_{531}-R_{701}}{R_{531}+R_{701}}$	$\frac{R_{531}-R_{802}}{R_{531}+R_{802}}$
551 nm	$\frac{R_{551}-R_{531}}{R_{551}+R_{531}}$		$\frac{R_{551}-R_{570}}{R_{551}+R_{570}}$	$\frac{R_{551}-R_{672}}{R_{551}+R_{672}}$	$\frac{R_{551}-R_{701}}{R_{551}+R_{701}}$	$\frac{R_{551}-R_{802}}{R_{551}+R_{802}}$
570 nm	$\frac{R_{570}-R_{531}}{R_{570}+R_{531}}$	$\frac{R_{570}-R_{551}}{R_{570}+R_{551}}$		$\frac{R_{570}-R_{672}}{R_{570}+R_{672}}$	$\frac{R_{570}-R_{701}}{R_{570}+R_{701}}$	$\frac{R_{570}-R_{802}}{R_{570}+R_{802}}$
672 nm	$\frac{R_{672}-R_{531}}{R_{672}+R_{531}}$	$NDVI$	$\frac{R_{672}-R_{570}}{R_{672}+R_{570}}$		$\frac{R_{672}-R_{701}}{R_{672}+R_{701}}$	$\frac{R_{672}-R_{802}}{R_{672}+R_{802}}$
701 nm	$\frac{R_{701}-R_{531}}{R_{701}+R_{531}}$	$\frac{R_{701}-R_{551}}{R_{701}+R_{551}}$	$\frac{R_{701}-R_{570}}{R_{701}+R_{570}}$	$\frac{R_{701}-R_{672}}{R_{701}+R_{672}}$		$\frac{R_{701}-R_{802}}{R_{701}+R_{802}}$
802 nm	$\frac{R_{802}-R_{531}}{R_{802}+R_{531}}$	$\frac{R_{802}-R_{551}}{R_{802}+R_{551}}$	$\frac{R_{802}-R_{570}}{R_{802}+R_{570}}$	$\frac{R_{802}-R_{672}}{R_{802}+R_{672}}$	$\frac{R_{802}-R_{701}}{R_{802}+R_{701}}$	

Statistical analysis

Spectral indices selected from literature and the normalised indices calculated in this work were correlated with field data in order to exclude from further analysis those that exhibited weak or not significant correlations. As a result, spectral indices with 'r' values smaller than 0.35 or bigger than – 0.35 for most of the relationships were discarded. The indices finally chosen to correlate with field data were NDVI, MSR, G, PCD. Two indices from the group of normalised indices were also selected, which were named as Normalised Vegetation Index 1, NVI_1 (R_{802}-R_{531}/R_{802}+R_{531}) and Normalised Vegetation

Index 2, NVI_2 (R_{802}-R_{570}/R_{802}+R_{570}) (table 4) both resulting from the combination between NIR and green reflectance values, similar to the Green NDVI proposed by Gitelson et al. (1996).

The summary statistics of the derived spectral indices, in-field vigour, leaf area and yield measurements and fluorescence-based indices are reported in table 5. The results indicate that most of the studied variables exhibited considerable spatial variation within the vineyard. On the basis of coefficient of variation (CV) and Spread parameters, secondary shoot length and secondary leaf area were, by far, the variables showing the largest variation (CV = 79%, Spread ≈ 400 %), followed by yield per vine (CV = 52 %, spread = 250 %) and number of clusters per vine (CV = 56.4 %, Spread = 266 %). These values were similar to those observed by Baluja et al. (2012b), Bramley and Lamb (2003) and Bramley and Hamilton (2004) in Australia, in other varieties. The fluorescence index indicative of leaf chlorophyll content (SFR_R_{AD}) and the normalised indices NVI_1 and NVI_2 showed a CV smaller than 10 %. NBI has been described as a good indicator of the nitrogen status of grapevine leaves (Cartelat et al. 2005, Cerovic et al. 2015, Tremblay et al. 2012). Its calculation depends on the concentration of flavonoids and chlorophyll in the leaf, so its variability must be higher than that of chlorophyll and flavonoids alone (Cartelat et al. 2005). Furthermore, the presence of water deficit due to very warm temperatures (the average of the month mean temperatures exceeded between 1.5 and 3.2 ºC the value of the average of the historical series mean temperatures) and dryness (total rainfall from July to September was 30.9% of average historical rainfall in this period) during season 2011 (Navarra Government's meteorological station of Estella), may have induced a weak mineralization of soil nitrogen, which in turn led to nitrogen deficiency on the leaves of the vines in some areas of the vineyard, hence contributing to the large variability observed for the NBI_G_{AD} index.

Table 5. Descriptive statistics and variogram components for spectral indices (NDVI, MSR, G, PCD, NVI_1 and NVI_2), vigour and vegetative parameters (pruning weight –PW-, main shoot length –MSL-, secondary shoot length –SSL-, main leaf area per shoot –MLA- and secondary leaf area per shoot –SLA-), fluorescence-based indices of nitrogen balance index (NBI_G_{AD}) and chlorophyll content in leaves (SFR_R_{AD}) and for yield parameters (cluster number per vine, cluster weight, yield per vine and berry weight) in a Tempranillo (*Vitis vinifera* L.) vineyard.

	Mean	Min.	Max.	SD	CV (%)	Spread (%)	% of Nugget	Range
				Spectral indices				
NDVI	0.65	0.51	0.76	0.06	10.01	38.11	29	111.41
MSR	1.18	0.68	1.70	0.26	22.13	87.68	25	130.43
G	1.31	1.00	1.66	0.15	11.62	50.69	28	202.01
PCD	4.82	2.83	7.27	1.14	23.72	95.47	25	123.21
NVI_1	0.63	0.51	0.72	0.05	7.42	32.90	7	59.15
NVI_2	0.67	0.57	0.74	0.04	6.45	25.82	13	57.73
				Vegetative variables				
PW (kg)	0.79	0.45	1.32	0.19	24.23	114.41	38	43.4
MSL (cm)	123.11	95.83	172.17	15.28	12.41	61.81	36	129.02
SSL (cm)	63.25	6.67	189.83	49.98	79.02	399.64	26	123.35
MLA (m^2)	0.25	0.20	0.35	0.03	12.33	60.00	36	129.53
SLA (m^2)	0.13	0.01	0.39	0.10	79.03	422.22	25	126.75
				Fluorescence indices				
NBI_G_{AD}	0.16	0.09	0.29	0.04	28.71	1.33	30	174.94
SFR_R_{AD}	1.70	1.41	2.06	0.14	8.09	0.39	37	61.24
				Yield variables				
Cluster number/vine	5.99	1.50	14.00	3.13	52.21	250.00	55	88.23
Cluster weight (g)	284.40	147.50	520	72.25	25.40	133.15	35	179.90
Yield/vine (kg)	1.68	0.37	4.28	0.95	56.36	266.59	---	---
Berry weight (g)	2.30	1.84	2.91	0.22	9.68	47.08	---	---

SD: standard deviation; CV: coefficient of variation
% Nugget = (nugget / (sill + nugget))*100

To quantify the existence of spatial variation and characterise the structure of the different studied parameters within the vineyard, variograms were constructed. All the experimental variograms of the variables studied were fitted to a spherical model, with the exception of yield per vine and berry weight, which showed an absence of spatial structure as did for Cerovic et al. (2009). The range of the variograms is the distance at which the sill is achieved and indicates whether the samples are spatially dependent (samples separated by distances closer than the range) or spatially independent (samples separated by distances larger than the range) (Oliver 2010). The range value was variable for all these parameters studied. Most of the vegetative variables and two spectral indices, MSR and PCD, showed a range around 125 m. The spectral index G, the NBI_G_{AD} and the cluster weight were the variables with the higher range (more than 170 m); the lower values of the range were found for the fluorescence index related to leaf chlorophyll content, for the vegetative variable PW and for the two normalised spectral indices NVI_1 and NVI_2, with values from 43 to 76 m. Specifically, the range obtained for the PW agrees with the range reported by Taylor and Bates (2012) for the case of 3-vines-aggregation. Furthermore, the nugget effect, which is the discontinuity at the origin of the variogram (unexplained variance), yielded, in general, values higher than 25% of the total variance, indicating a moderate spatial dependence (Cambardella et al. 1994). This percentage of nugget for the MSL was similar of that obtained by Baluja et al. (2012b), also was the range for this vegetative variable. Cerovic et al. (2009) also reported a similar value of percentage of nugget for the NBI_G_{AD} and for cluster number per vine. The exception were the two normalised indices that showed a low percentage of nugget, which indicates a strong spatial dependence.

All spectral indices (figure 12), vigour parameters (figure 13), fluorescence-based indices (figure 14) and yield variables (figure 15) showed marked spatial structure. Maps of vigour spectral indices exhibited maximum and minimum values at the East and West sides of the vineyard, respectively (figure 12).

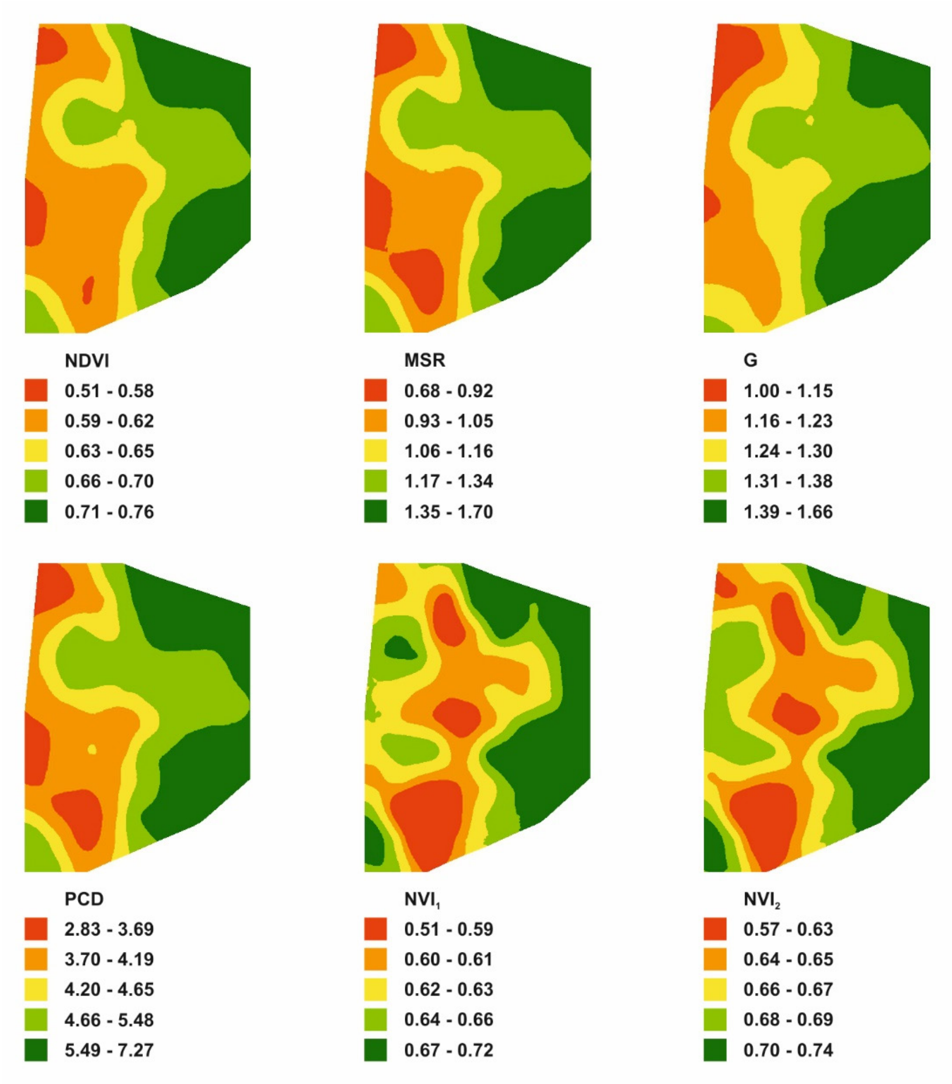

Figure 12. Maps obtained for spectral indices (NDVI, MSR, G, PCD, NVI_1 and NVI_2) in a Tempranillo (*Vitis vinifera* L.) vineyard. Maps were represented by quantiles.

This spatial pattern matched that of vigour parameters, especially those related to main and secondary shoot length and leaf area (figure 13).

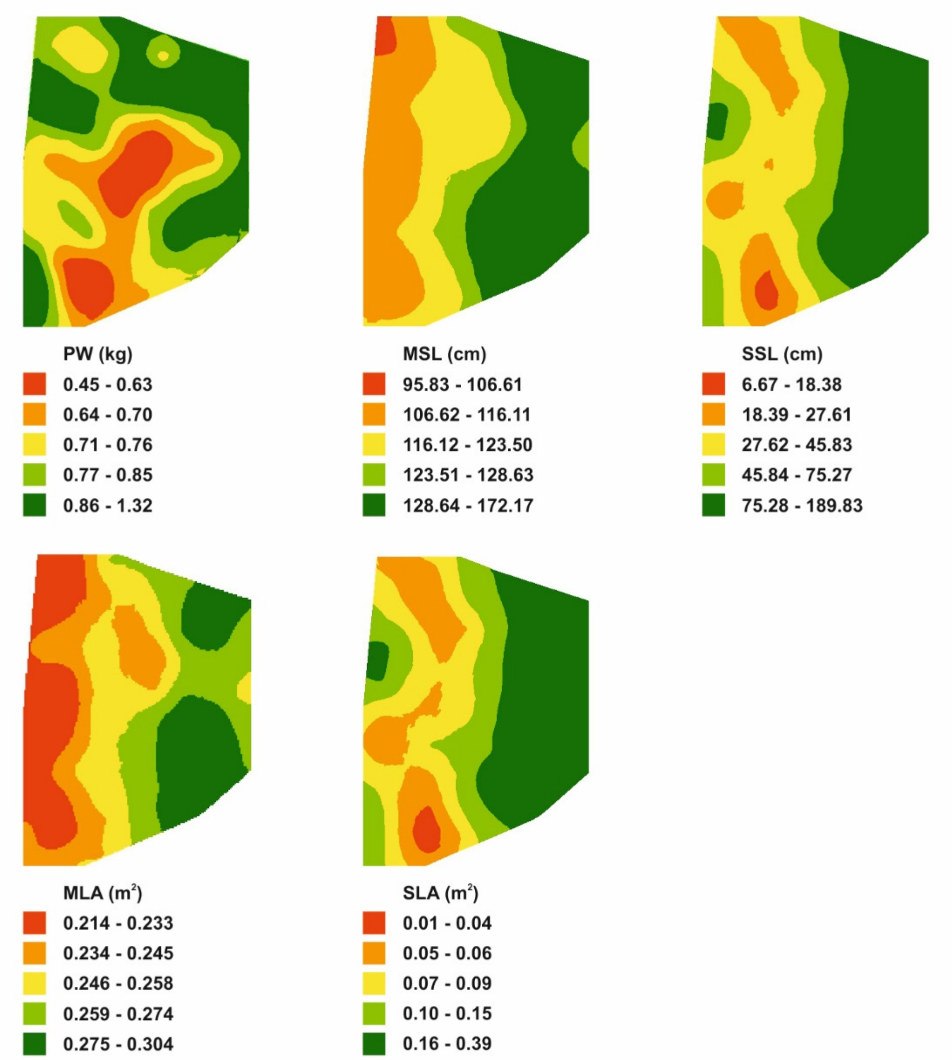

Figure 13. Maps obtained for vigour and vegetative parameters (pruning weight per vine –PW-, main shoot length –MSL-, secondary shoot length –SSL-, mail leaf area per shoot –MLA- and secondary leaf area per shoot –SLA-) in a Tempranillo (*Vitis vinifera* L.) vineyard. Maps were represented by quantiles.

Normalised indices NVI_1 and NVI_2 showed the highest values at the East of the plot and lowest at the centre and West zones, and this behaviour better agreed with the spatial variability shown by the pruning weight (figure 13). In general, fluorescence-based indices were also showing larger values on the East than on the West side of the vineyard (figure 14).

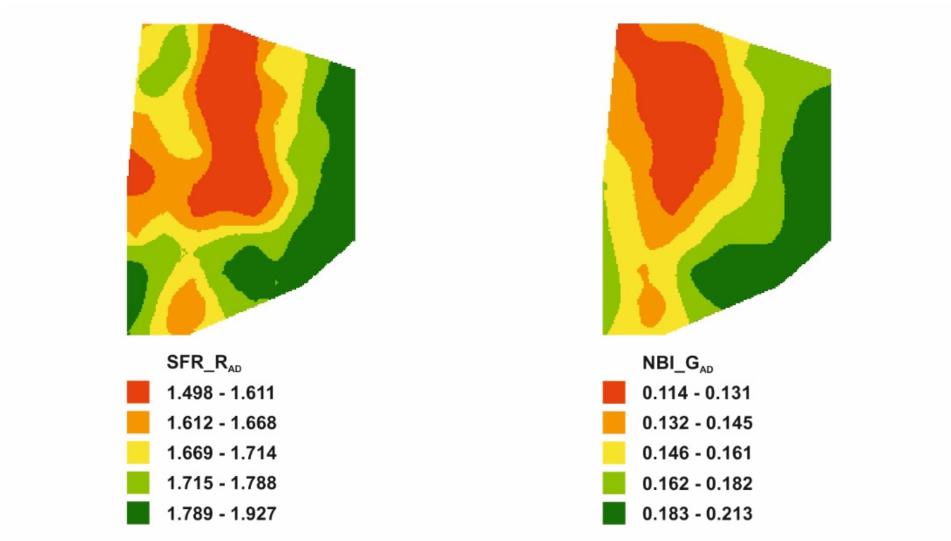

Figure 14. Maps obtained for vigour and fluorescence-based indices of nitrogen balance index (NBI_G_{AD}) and leaf chlorophyll content (SFR_R_{AD}) in a Tempranillo (*Vitis vinifera* L.) vineyard. Maps were represented by quantiles.

Among yield parameters, only cluster weight exhibited a similar spatial pattern to that of vigour variables and spectral indices (figure 15), while grapevines of the West part of the vineyard were found to be more productive in terms of yield per vine and number of clusters per vine, and less productive on the East side of the plot (figure 15). High values of leaf area do not always result in increased yield outputs as may occur in other crops such as cereals. In grapevines, yield is also related to the exposed leaf area (ELA), not only to total leaf area. As total leaf area increases, in Vertical Shoot Positioned systems, ELA reaches a point at which it cannot continue increasing, so the leaves that are not exposed may become a sink for the nutrients and energy of the plant competing with the clusters for these resources (Martínez de Toda 2011).

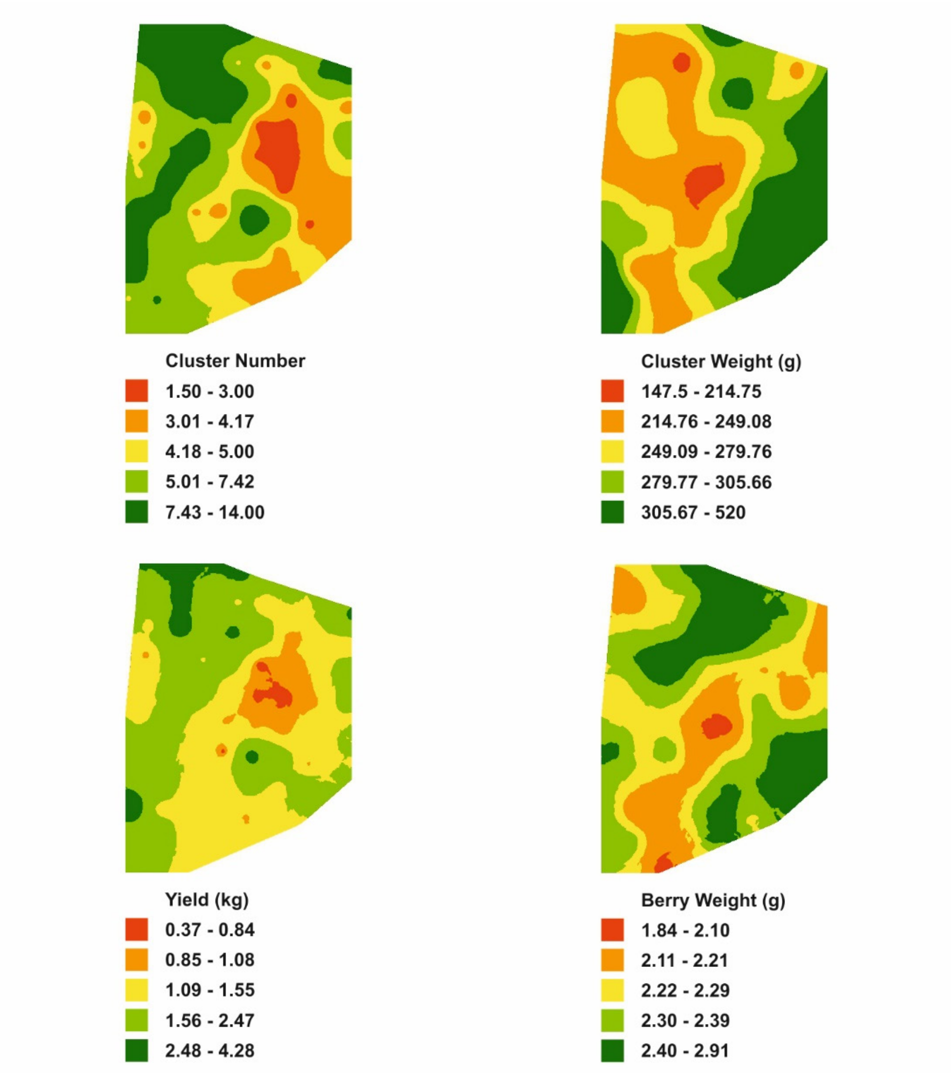

Figure 15. Maps obtained for yield parameters (cluster number per vine, cluster weight –CW, yield per vine and berry weight –BW-) in a Tempranillo (*Vitis vinifera* L.) vineyard. Maps were represented by quantiles.

Table 6 collects Pearson correlation coefficients values significant at *P<0.01* between the spectral indices and crop variables measured in the field. Correlations with vigour parameters were positive and moderate. The best performance was observed for the normalised spectral indices, NVI_1 and NVI_2, followed by the NDVI and MSR. From all the vigour variables studied, pruning weight exhibited the highest "r" values, although quite similar to those for shoot length and leaf area parameters. Other authors (Baluja et al. 2012b, Dobrowski et al. 2003, Proffitt and Malcolm 2005) have also shown significant correlations between NDVI and pruning weight.

No significant relationships were found between the spectral indices and yield components such as the number of clusters and yield per vine, while the spectral indices were positively correlated with cluster weight and berry weight, with "r" values ranging from 0.42 to 0.69. The best correlations were observed for NVI_1 and NVI_2. Moderate relationships between NDVI and PCD, and yield per vine have been reported in earlier studies (Hall et al. 2011, Martínez-Casasnovas and Bordes 2005). The strong positive correlation (r=0.90) between yield per vine and the number of clusters per plant (table 6), together with the lack of significance with cluster weight and very poor (r=0.35) with berry weight, suggest that the number of clusters per vine was the key component in determining the final grape production per plant, at the expense of average berry and cluster weight. This is an interesting outcome, as the number of clusters per vine is directly impacted by bud fertility, which is determined at the flowering period in the previous season (Keller et al. 2010) (May – June 2010). The weather conditions in Spring 2010 at the vineyard of study were characterised by intense rainfall (94.9% of average historical rainfall in these two months), low radiation and cooler temperatures than mean historical values for this area (-1.9 ºC in May and -0.6 ºC in June). These adverse conditions may have imparted stronger differences in bud fertility among the grapevines within the vineyard of study, leading to the bigger variability described for the number of clusters per vine, and the critical role of this yield component in final yield per vine. It may also explain the lack of significance of the correlations between yield and the vigour spectral indices derived from RPAS imagery, as the time period mainly influencing yield was Spring 2010, while Spring – Summer 2011 was determining grapevine vigour. This was also reflected in the lack of coincidence between areas of the vineyard, as the larger values of cluster number and yield per vine were in the East side of the plot, while the larger values for the vigour spectral indices were in the West side (figures 12 and 15).

Regarding the fluorescence-based indices, low to moderate positive correlations were observed between the spectral indices NVI_1 and NVI_2 with SFR_R_{AD} and NBI_G_{AD}. More classical vigour spectral indices like NDVI, MSR and PCD were only significantly correlated with NBI_G_{AD}, showing an inverse relationship. SFR_R_{AD} has been reported as a precise indicator of the chlorophyll content in kiwi leaves (Tremblay et al. 2012) and in grapevine leaves (see Chapter 2). The SFR_R_{AD} index is based on the partial reabsorption of red fluorescence by the chlorophyll itself (Buschmann 2007, Gitelson et al. 1999), which depends upon the pigment content, while the FRF (far red fluorescence) is not re-absorbed. As a consequence, SFR_R increases when the chlorophyll concentration increases.

The fluorescence-based indices were calculated from measurements taken at leaf level, while the spectral indices were calculated from data at canopy level, so the latter are affected by additional factors such as leaf angle distribution and the presence of stems, shadows, background, etc., which may result in a reduction of the correlation values.

Table 6. Pearson correlation coefficients (all with *p* value < 0.01) for vigour and vegetative parameters (pruning weight –PW- , main shoot length –MSL-, secondary shoot length –SSL-, main leaf area per shoot –MLA- and secondary leaf area per shoot –SLA-), fluorescence-based indices (nitrogen balance index, NBI_G_{AD}, and chlorophyll content in leaves, SFR_R_{AD}), yield parameters (cluster number per vine, cluster weight, yield per vine and berry weight) and for spectral indices (NDVI. $MTVI_1$. G. PCD. NVI_1 and NVI_2) in a Tempranillo (*Vitis vinifera* L.) vineyard.

	NDVI	MSR	G	PCD	NVI_1	NVI_2	PW	MSL	SSL	MLA	SLA	NBI_G_{AD}	SFR_R_{AD}	Cluster Nº/vine	Cluster weight	Yield/ vine
NDVI	---															
MSR	0.99	---														
G	0.84	0.84	---													
PCD	0.98	0.99	0.83	---												
NVI_1	0.81	0.82	0.54	0.82	---											
NVI_2	0.80	0.81	0.56	0.80	0.98	---										
PW	0.55	0.53	0.36	0.52	0.58	0.58	---									
MSL	0.49	0.49	0.54	0.48	0.34	0.32	0.19	---								
SSL	0.50	0.50	0.48	0.49	0.52	0.50	0.33	0.61	---							
MLA	0.49	0.50	0.54	0.49	0.35	0.33	NS	1.00	0.60	---						
SLA	0.49	0.50	0.47	0.49	0.52	0.50	0.33	0.61	1.00	0.60	---					
NBI_G_{AD}	0.40	0.46	NS	0.44	0.54	0.53	0.32	NS	0.48	NS	0.48	---				
SFR_R_{AD}	NS	NS	NS	NS	0.43	0.44	NS	NS	NS	NS	NS	0.46	---			
Cluster Nº/vine	NS	NS	NS	NS	NS	NS	NS	-0.37	-0.50	-0.35	-0.50	-0.34	NS	---		
Cluster weight	0.59	0.59	0.51	0.58	0.65	0.69	0.59	NS	0.45	NS	0.45	0.56	0.47	NS	---	
Yield/vine	NS	NS	NS	NS	NS	NS	0.35	NS	-0.31	NS	NS	NS	NS	0.90	NS	---
Berry weight	0.41	0.42	0.32	0.41	0.38	0.42	0.52	NS	NS	NS	NS	NS	NS	NS	0.45	0.35

* NS: not significant correlation with *p* value < 0.01.

Overall, moderate correlations were found between vegetation spectral indices and all field vigour indices, which were calculated on a "per-shoot" basis. This modest performance may also be influenced by the experimental conditions and adjustments of the image acquisition system from a RPAS platform. Despite greater independence and flexibility of RPAS systems, one of their disadvantages is that they are significantly affected by the wind, even by light wind, generating movements of the platform that were reflected in the images as geometric distortions. In addition, a lower flying height would result in a higher spatial resolution of the images, which should reduce the problem of the spectral mixture, providing pure vine pixels. These facts may lead to improved correlations between spectral indices and in-field variables. Furthermore, RPAS image quality is usually constrained by hardware limitations and requires very complex processing (geometric and radiometric) to be useful for precise quantitative measurements. In the specific case of the Tetracam Mini-MCA multi-spectral sensor used in this work, the six spectral bands were acquired by different lenses and complementary metal-oxide-semiconductor (CMOS) sensors; this leads to alignment problems between bands. Other problems related to image quality that were probably responsible for the moderate correlation between image properties and field variables are spatially heterogeneous response of the CMOS, heterogeneous CMOS response across bands, *vignetting* effects and lens distortions. Most of this inconveniences might be avoided by the used of ground sensors, either manual or on-the-go, especially for vineyards trained to a vertically shoot-positioned.

CONCLUSIONS

The results presented in this study confirm the potential of multi-sensor RPAS systems to assess the vineyard vegetative status and its spatial variability in precision viticulture. Significant and moderate correlations were found between spectral indices and field variables. The two developed vegetation spectral indices, NVI_1 and NVI_2 were those best correlated with in-field vigour and fluorescence-based parameters. Moreover, the spatial pattern of the spectral indices was similar with that of the vegetative and vigour variables measured in the vineyard. The possibility of acquiring "on-demand" images with unprecedented high spatial resolution will certainly promote its operational application to vineyard management in order to improve crop productivity and sustainability.

The difficulties found in this study, such as mixed pixels due to the narrow vine architecture, misalignments between bands due to the use of individual sensors for each band, geometric distortions due to platform movements produced by wind, could be overcome, but still require a very complex processing and very skilled technicians.

3.2. Chapter 2

Calibration and evaluation of the performance of a hand-held fluorescence sensor for the assessment of grapevine leaf chlorophyll, flavonol and nitrogen status in the field

ABSTRACT

Background and Aims: Precision viticulture requires a large amount of reliable data to delineate different management zones within the vineyard. For that purpose, proximal sensing techniques allow to measure a high number of plants in a fast and non-destructive way. The goal of this work was to calibrate and evaluate the performance of a hand-held, non-destructive, fluorescence sensor for the assessment of chlorophyll, flavonol and nitrogen in grapevine leaves within a vineyard.

Methods and Results: The experiment was carried out in a commercial vineyard planted with nine different red *Vitis vinifera* L. grapevine varieties. The vineyard was monitored with the hand-held fluorescence sensor Multiplex™. The reference method selected to calibrate it was the optical leaf-clip sensor, Dualex4™. The results demonstrated that the fluorescence sensor is a reliable device to assess the grapevine leaf chlorophyll, epidermal flavonol and nitrogen content. The fluorescence indices studied yielded high R^2 values against the reference method: 0.92 for SFR_R_T, related to chlorophyll content; 0.78 for $FLAV_T$, linked to the leaf epidermal flavonols; and 0.93 for $NBI_C\_R_T$, indicator of the plant nitrogen status. Among the different possibilities to calculate the nitrogen balance index (NBI), the one calculated as the ratio of chlorophyll-to-flavonols was the one that reported the best correlations. The calibration equations for grapevine leaf chlorophyll, flavonol and nitrogen fluorescence indices between the fluorescence sensor object of this study and the reference are presented here.

Conclusions: The hand-held fluorescence sensor and its fluorescence indices proved to be appropriate to assess the leaf chlorophyll, epidermal flavonol and nitrogen content in grapevine leaves under field conditions. This sensor enables the implementation of precision viticulture, due to its capacity of non-destructively measuring large amount of plants in a short time.

Keywords: Grapevine, nitrogen, non-destructive sensor, vegetative status, precision viticulture

INTRODUCTION

Leaf chlorophyll, flavonol and nitrogen are key physiological compounds in grapevines. Chlorophyll is the pigment responsible for the photosynthesis. It increases until grapevine leaves are fully expanded and starts to decrease afterwards, as soon as it attains its maximal value (Kriedemann et al. 1970). Flavonols comprise a class within the flavonoids, a secondary metabolite group of compounds sharing a three-ring phenolic structure. Flavonols in plants are attributed to display a wide range of physiological functions, involving microbial interactions (Koes et al. 1994) and free radical scavenging (Markham et al. 1998), but their most prevalent role seems to be as UV screening agents (Flint et al. 1985, Smith and Markham 1998). Flavonol biosynthesis is upregulated not only because of UV-radiation, but also in response to other biotic and abiotic stresses, such as nitrogen/phosphorus depletion (Lillo et al. 2008), cold temperatures (Olsen et al. 2009) and salinity/drought stress (Agati et al. 2011a, Tattini et al. 2004). Leaf chlorophyll and flavonol contents on a surface basis depend on leaf age and the amount of light radiation received during their development. Both increase with age and light exposure, while afterwards, leaf chlorophyll usually decreases and flavonol remain unvaryingly high (Louis et al. 2009). Nitrogen is considered to be one of the most important factors for biomass production (Agati et al. 2013b, Lemaire et al. 2008) and grapevine metabolism, as it is crucial for vine development and fruit yield (Guilpart et al. 2014). In grapevines, excessive N can be even more damaging than N deficiency because vines would be more prone to disease and insect infestations due to an increase in the density of the canopy or the clusters (Dordas 2009). Over-fertilization usually produces lower quality grapes (Keller 2010), higher susceptibility to flowering abortion and reduced fruit set (Vasconcelos et al. 2009). Therefore, the assessment of the vineyard chlorophyll and flavonol content and nitrogen status is necessary and very helpful to delineate fertilization and canopy management strategies intended to improve the grapevines balance and fruit composition.

Cartelat et al. (2005) have shown that the flavonol and chlorophyll contents are both important for the assessment of the nitrogen status of the plant. This ratio is known as the Nitrogen Balance Index (NBI = Chlorophyll / Flavonols) and its relation with the N content has also been reported for different species (Tremblay et al. 2012) and recently for grapevine (Cerovic et al. 2015). Chlorophyll content increases whereas flavonol content decreases with increased N application so that NBI increases with N fertilization. Therefore, in the framework of precision farming, the epidermal content of flavonols and leaf chlorophyll content are of first importance for nitrogen management (Tremblay et al. 2012) as they allow to calculate the NBI.

Leaf chlorophyll, flavonols and nitrogen are usually assessed using destructive wet chemistry procedures. Compared to these, optical methods provide much faster assessment, becoming feasible the analysis of the whole plot. Optical methods are based on leaf transmittance, reflectance or fluorescence, and are susceptible of being used as proximal sensors.

Proximal sensing, which includes all detecting technologies that retrieve information from an object when the distance between the sensor and the object is less than, or comparable to, some of the dimensions of the sensor, have emerged as an alternative to remote sensing in viticulture. Proximal sensing provides a successful solution to most of the drawbacks (large percentage of background noise in the images; limited temporal flexibility; elevated cost of aerial monitoring) of remote sensing in vertically trellising vineyards worldwide. Specifically, it avoids some of the inconveniences found in chapter 1 for the use of multispectral images taken from a RPAS in vertically trellising vineyards, such as mixed pixels of vegetation and soil, and image geometric distortions due to platform movements produced by the wind. Among the wide variety of technologies used in proximal sensing, the chlorophyll fluorescence technique has been implemented in a commercial hand-held sensor, Multiplex™, that has been developed to be used either in the laboratory or in the field. It has been introduced in viticulture for the assessment of anthocyanin accumulation (Baluja et al. 2012c, Ben Ghozlen et al. 2010a), the assessment of vine nitrogen status (Garcia et al. 2012, Serrano et al. 2010), and the control of fungal diseases in plants (Agati et al. 2008, Bellow et al. 2012, Latouche et al. 2013). Various authors have reported the calibration equations of the fluorescence-based indices related to berry anthocyanin content against a reference method (Baluja et al. 2012b, Ben Ghozlen et al. 2010b, Bramley et al. 2011a). However, when it comes to the variables related to the vegetative and nutritional status of the vineyard, more research is needed. The fluorescence-based indices related to leaf epidermal flavonol and nitrogen content in grapevines have already proved to be reliable indicators of these variables when provided by the optical sensor Dualex4™ (Cerovic et al. 2015, Cerovic et al. 2012). Regarding the Multiplex™ sensor, it has been mainly used for the assessment of the nitrogen status of grapevines (Serrano et al. 2010), but its fluorescence indices have not been yet calibrated for grapevine leaves against a reference method.

The main goal of the present study was to calibrate (against an optical sensor, used as reference) and evaluate the performance of a portable, non-destructive, hand-held fluorescence sensor for the assessment of chlorophyll, flavonol and nitrogen contents in grapevine leaves within a vineyard.

MATERIALS AND METHODS

Site description

The study was carried out in 2012 during the last week of September and first week of October at a commercial vineyard of 1.43 ha located in Vergalijo (Lat. 42º 27' 45.96", Long. 1º 48' 13.42", Alt. 325 m), Navarra, Spain. The vineyard was planted with nine different red international *Vitis vinifera* L. cultivars: Cabernet Sauvignon, Carmenere, Caladoc, Grenache, Marselan, Maturana Tinta, Pinot Noir, Tempranillo and Syrah. Grapevines were trained to a vertically shoot-positioned trellis system, north-south row orientation at 2 m x 1 m inter and intra row distances. Grapevines were planted on rootstock Richter 110, with the exception of Tempranillo vines, which were planted on rootstock 3309. Irrigation was routinely and uniformly applied across the season for all cultivars.

Fluorescence sensors and indices

An optical ground sensor was used to monitor the experimental vineyard: the hand-held Multiplex™ (MX_H). The leaf clip optical sensor Dualex4™ (DX4) served as the reference method.

Leaf-clip fluorescence sensor Dualex4™ (FORCE-A, Orsay, France), DX4 hereafter, is a leaf-clip sensor with a measuring surface of 6 mm (figure 16), which operates on transmittance mode for the assessment of leaf chlorophyll content and measures leaf epidermal flavonols by the chlorophyll fluorescence screening method (Bilger et al. 2001, Goulas et al. 2004). It provides three indices: CHL index (eq. 6) for the leaf chlorophyll concentration, displayed in chlorophyll units (Cerovic et al. 2012); FLAV index (eq. 7) for the epidermal flavonol concentration in absorbance units; and NBI index (eq. 8), as the ratio of chlorophyll to flavonol (Cartelat et al. 2005), which refers to the leaf nitrogen content (Cerovic et al. 2015, Cerovic et al. 2012).

$$CHL = \frac{(T_{850} - T_{710})}{T_{710}} \quad (6)$$

$$FLAV = \log\left(\frac{FRF\_R}{FRF\_UV}\right) \quad (7)$$

$$NBI_T = \frac{Chl}{Flav} = \frac{(CHL_{AD} + CHL_{AB})/2}{FLAV_{AD} + FLAV_{AB}} \quad (8)$$

where T_{850} and T_{710} are the leaf transmittance at 850 nm and 710 nm, respectively; FRF is the far-red fluorescence emission (>710 nm) excited by red (_R, 650 nm) or UV (_UV, 375 nm) light; and the subscripts AD and AB refer to the adaxial and abaxial side of the leaf, respectively.

The DX4 CHL and FLAV indices have been validated by Cerovic et al. (2012) against chlorophyll extracts and Dualex3 FLAV index, respectively. The NBI index has been previously validated by Cartelat et al. (2005) to assess the nitrogen status of wheat and very recently it has been validated and calibrated by Cerovic et al. (2015) for the assessment of grapevine leaf N content against leaf N content.

Figure 16. Optical sensor Dualex4™ (left) and an example of the measurement of a grapevine leaf (right) (Force-A).

Hand-held fluorescence sensor Multiplex™ (FORCE-A, Orsay, France), MX_H hereafter, is a hand-held, multi-parametric fluorescence sensor based on light-emitting-diode (LED) excitation and filtered-photodiode detection that is designed to work in the field under daylight, on leaves, fruits and vegetables (Ben Ghozlen et al. 2010a). The sensor illuminates a surface of 8 cm of diameter at a 10-cm distance from the source (figure 17).

Figure 17. Fluorescence sensor Multiplex™ (left) and an example of the measurement of a grapevine leaf (right) (Force-A).

This device provides twelve signals, which combined produce different ratios, among them the indices which are the object of the present study: SFR (eqs. 9, 10), FLAV (eq. 11) and NBI (eqs. 12-14), which are defined as:

$$SFR\_R = \frac{FRF\_R}{RF\_R} \tag{9}$$

$$SFR\_G = \frac{FRF\_G}{RF\_G} \tag{10}$$

$$FLAV = \log\left(\frac{FRF\_R}{FRF\_UV}\right) \tag{11}$$

$$NBI\_R = \frac{FRF\_UV}{RF\_R} \tag{12}$$

$$NBI\_G = \frac{FRF\_UV}{RF\_G} \tag{13}$$

$$NBI_C = \frac{Chl}{Flav} = \frac{SFR_{AD}+SFR_{AB}}{FLAV_{AD}+FLAV_{AB}} \tag{14}$$

The emission ratio (SFR) is linked to the chlorophyll content of leaves. It is a simple chlorophyll fluorescence ratio (SFR) of far-red emission (FRF, 735 nm) divided by red emission (FR, 685 nm) under red excitation (FRF_R and FR_R, respectively) (eq. 9) or green excitation (FRF_G and RF_G, respectively) (eq. 10). Due to the overlap of the chlorophyll absorption and emission spectrum, re-absorption occurs at shorter wavelengths (RF) but not at longer wavelengths (FRF) (Gitelson et al. 1999, Pedrós et al. 2010). Therefore, SFR increases with increasing sample chlorophyll content.

The FLAV index (eq. 11) compares the chlorophyll fluorescence intensity emitted as far-red fluorescence under ultraviolet (FRF_UV) and red excitation (FRF_R), which represents a differential absorption measurement (in accordance with the Beer–Lambert's law) that is proportional to the flavonol content (Agati et al. 2011b, Ounis et al. 2001).

The Nitrogen Balance Index (NBI) (Cerovic et al. 2008) displayed in eq.(12) and eq.(13) is related to the nitrogen status of the plant and proportional to the chlorophyll-to-flavonol ratio proposed by Cartelat et al. (2005). It utilises only two signals as the ratio of far-red emission under UV excitation (FRF_UV) and red emission under red excitation (RF_R) in NBI_R, or green excitation (RF_G) for NBI_G.

The NBI_C index of Eq. (14) is the hand-held Multiplex index that calculates the N content of the leaves in the same way as it is calculated with the DX4 (eq. 8). It takes into account the total chlorophyll content of the leaf (in the numerator), as the sum of the SFR index of the adaxial and abaxial side of the leaves, and the total epidermal flavonol content of the leaves (in the denominator), as the sum of the epidermal flavonols of the abaxial and the adaxial side of the leaf.

Besides the NBI_R and NBI_G given by Eq. (12) and Eq. (13), respectively, we calculated also the NBI index separately for the adaxial and the abaxial leaf side based on the SFR and FLAV index of the MX_H, which corresponds with the NBI_{AD} and NBI_{AB} of DX4. Equations 15 and 16 show the formulae for the computation of the NBI_C index for the adaxial leaf side.

$$NBI_C\_R_{AD} = \frac{Chl}{Flav_{AD}} = \frac{SFR\_R_{AD}+SFR\_R_{AB}}{FLAV_{AD}} \tag{15}$$

$$NBI_C\_G_{AD} = \frac{Chl}{Flav_{AD}} = \frac{SFR\_G_{AD}+SFR\_G_{AB}}{FLAV_{AD}} \tag{16}$$

$$NBI_{C2}\_R_{AD} = \frac{Chl_{AD}}{Flav_{AD}} = \frac{SFR\_R_{AD}}{FLAV_{AD}} \tag{17}$$

$$NBI_{C2}\_G_{AD} = \frac{Chl_{AD}}{Flav_{AD}} = \frac{SFR\_G_{AD}}{FLAV_{AD}} \tag{18}$$

$$NBI_C\_R_T = \frac{Chl}{Flav} = \frac{SFR\_R_{AD}+ SFR\_R_{AB}}{FLAV_{AD}+ FLAV_{AB}} \tag{19}$$

$$NBI_C\_G_T = \frac{Chl}{Flav} = \frac{SFR\_G_{AD}+ SFR\_G_{AB}}{FLAV_{AD}+ FLAV_{AB}} \tag{20}$$

Another possibility to compute the NBI index was explored here, similar to that of eq. (15) and eq. (16). In this case, in the denominator, was not the sum of SFR_{AD} and SFR_{AB}, but the SFR for the adaxial or for the abaxial side, according to the FLAV employed in the denominator. Equations 17 and 18 show the formulae for this NBI computation for the adaxial leaf side. In eq. (19) and eq. (20) the total NBI_C index of eq. (14) is re-written explicitly for the red (R) and green (G) excitation in $MX_{H,}$ respectively.

An exhaustive description of all formulae and equations of the fluorescence indices provided and calculated from the two sensors, DX4 and MX_H can be found in Table S1 of the supplementary data.

The comparison among these NBI indices would enable knowing which one better estimates the N status by the MX_H with respect to the NBI given by the DX4, which is considered as the reference. Towards that aim, the indices corresponding to the adaxial (named using AD as subscript) and abaxial (named using AB as subscript) sides of the leaves, independently, and total indices (named using T, for total, as subscript) for the whole leaf were taken into account.

Fluorescence measurements

Twenty four rows of the vineyard plot under study were manually monitored with both the MX_H and the DX4 (figure 18). In each row, 13 sampling points, each one comprising 3 adjacent vines, were defined. Measurements with MX_H and DX4 were conducted on the east side of the rows, on 12 leaves per sampling point (4 leaves per vine). The same leaf was measured once with the MX_H and twice with the DX4 (figure 18). Both sensors measured both sides of the leaf, abaxial and adaxial.

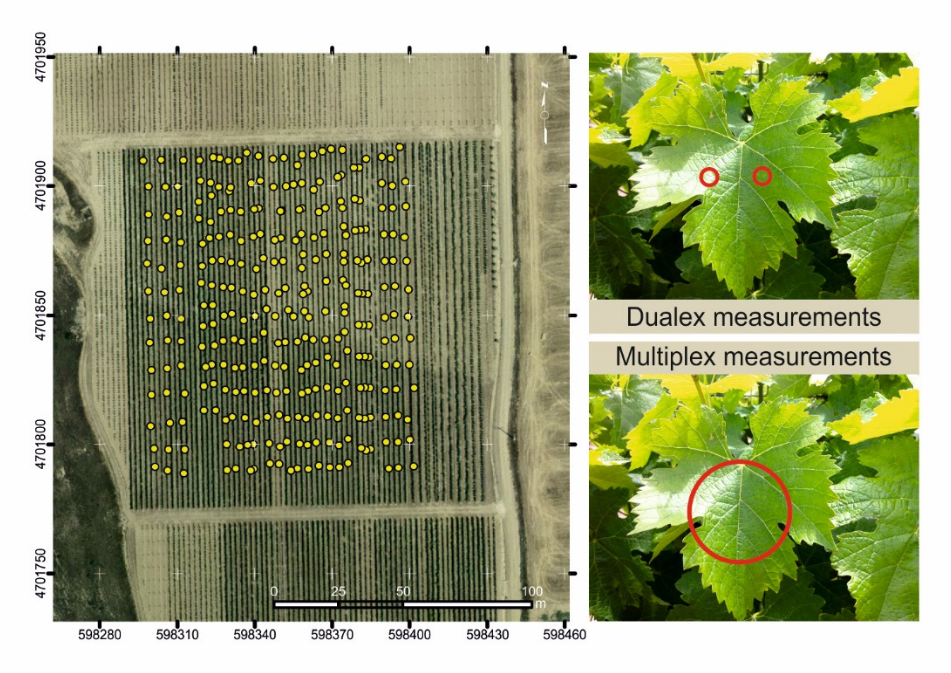

Figure 18. Distribution of the sampling points (yellow points) within the vineyard studied (left). Representation of the measurements done with the Dualex (top right) and with the Multiplex (bottom right) on the adaxial side of the leaf.

A total of 3744 manual measurements (24 rows x 13 sampling points x 12 leaves) on the abaxial, and 3744 measurements on the adaxial sides of leaves with the $MX_{H,}$ and 7488 measurement (24 rows x 13 sampling points x 12 leaves x 2 measurements) on each leaf side with the DX4 were taken.

Data treatment and statistical analysis

The data obtained with the MX_H were filtered by discarding readings higher than 4200 mV to avoid possible nonlinearity in the sensor response. The values lower than 10 mV, which correspond to the residual offsets, and the readings with a coefficient of variation of the FRF_R signal larger than 20 % were also removed, because this indicates that the sensor shifted during measurement acquisition, or that fluctuations in variable chlorophyll fluorescence were too large. After the filtering, the data were standardised against a blue plastic-foil standard (Force-A, Orsay, France) in order to compare the data obtained with other sensors and data collected under other measuring conditions. Prior to any statistical analysis, the data obtained with the two devices were corrected for the day of measurement by applying the Standard Normal Variate transformation (Legendre and Legendre 1998). Outliers were identified and excluded from the dataset by applying the Tukey's method (Tukey 1977).

Both devices automatically provide the indices for the side from which the leaf is measured, adaxial or abaxial. To obtain calculated (C) and total (T) leaf indices, the indices of the adaxial and the abaxial side must be added (cf. eqs. 14, 19 and 20). Indeed, each side of the leaf, either the palisade (adaxial) or the spongy mesophyll (abaxial) is different (Vogelmann and Evans 2002) and will have a fluorescence SFR and FLAV index specific to that side of the leaf. An exception is the CHL_T index of the DX4, which was used as the average of the adaxial and the abaxial side (eq. 8) because the DX4 measures the chlorophyll in transmittance mode, so regardless the side from which the leaf is measured, the chlorophyll index reflects chlorophyll concentration of the whole leaf (Cerovic et al. 2012).

Once data were properly pre-processed, the correlations between the same indices obtained by DX4 (reference method) and by the MX_H were computed and analysed. Correlations were separately computed for the indices of the adaxial and abaxial sides of the leaf and for the total leaf indices.

Data pre-treatment and statistical analysis were carried out using the software Microsoft Office Excel 2013 (Microsoft Corporation, Washington, USA) and Statistica 9 (Stat Soft Inc., Tulsa, USA).

RESULTS

Chlorophyll indices. The indices related to the leaf chlorophyll content were CHL in DX4 (reference) and SFR in MX_H. Figure 19 shows the correlations between these two indices, determined for the whole leaf and for adaxial and abaxial sides individually. Figure 19(A) evidenced no differences between CHL_{AD} and CHL_{AB} measured by DX4 (R^2=0.99, P<0.001). This was an expected result as DX4 works on transmittance mode, hence regardless the side of the leaf measured, the whole leaf chlorophyll concentration is determined. Figure 19(B) shows that the indices SFR_R_T and SFR_G_T, acquired with the MX_H were similar with an R^2 of 0.99 (P<0.001) and a small offset in favour of G excitation. Figure 19(C) and 19(D) show the strong positive correlation between the SFR index with the CHL_T index, yielding a R^2 of 0.93 (P<0.001) for SFR_G_T and a R^2 of 0.92 (P<0.001) for SFR_R_T. Both plots show an offset of 10 and 15 respectively; which is explained by the fluorescence reabsorption method in the SFR index. The correlation between the SFR index under red or green excitation from the adaxial side with the CHL_T index (figure 19E, F) was equivalent to the correlation of CHL_T with SFR_R_T (figure 19D) or SFR_G_T (figure 19C), exhibiting a strong positive correlation. These results indicate that the SFR indices from the adaxial side, which are reflecting the chlorophyll content of the palisade mesophyll, are the component mostly determining its variability among leaves, so the SFR_{AD} can be used as a proxy of the leaf chlorophyll content. Furthermore, comparing the adaxial with the abaxial side of the SFR index (SFR_G or SFR_R), a strong correlation was obtained with a determination coefficient of 0.77 (P<0.001) (figure 19G) and 0.74 (P<0.001) (figure 19H), respectively. A correlation matrix showing all possible relations between the chlorophyll related indices of DX4 and MX_H is included in Figure S1 of the supplementary data.

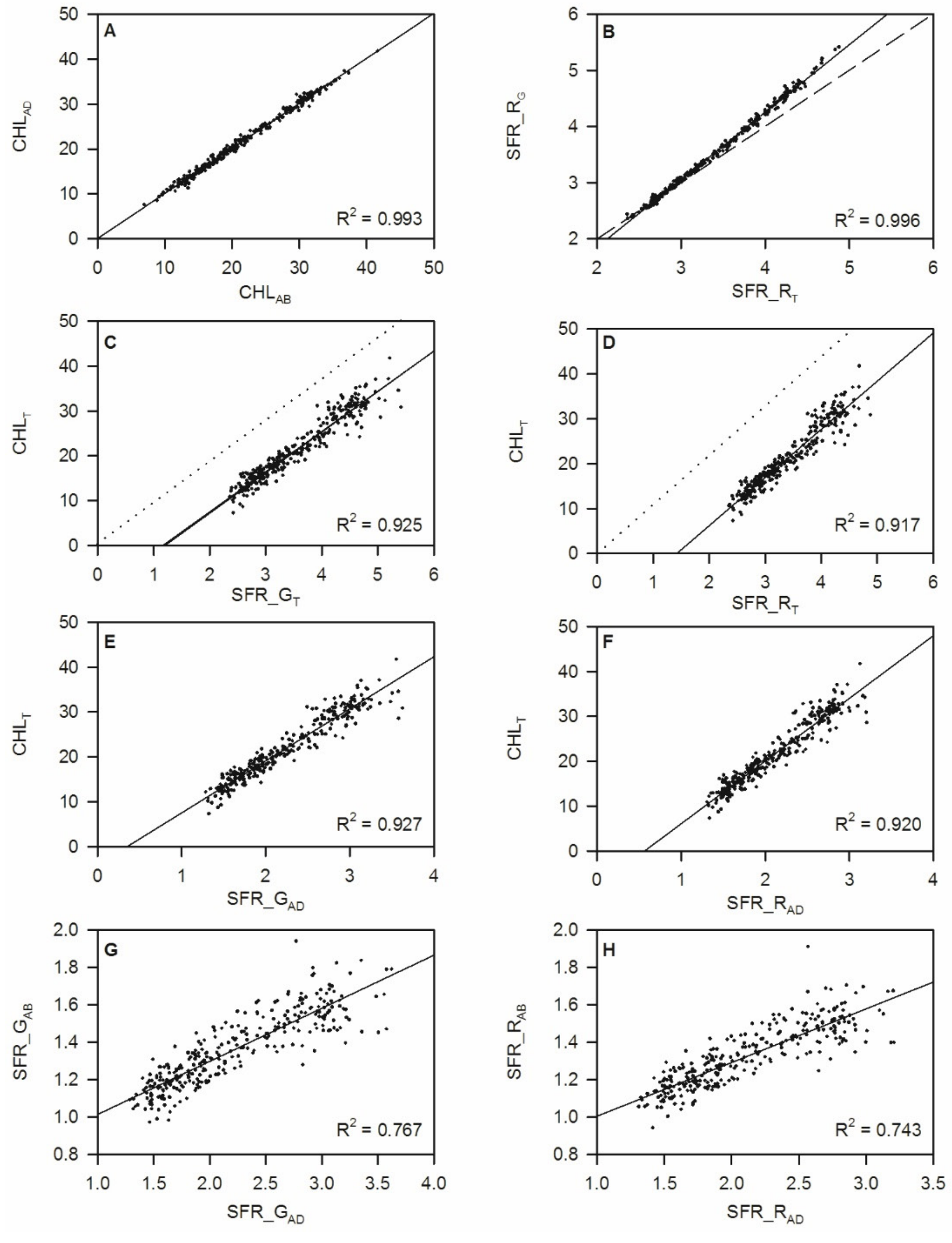

Figure 19. Correlations between the indices related to the leaf chlorophyll content obtained with the MX_H and DX4 devices. Coefficients of determination (R^2) were significant at $P<0.001$ (N=302). AD: Measurement taken on the adaxial side of the leaf; AB: measurement taken on the abaxial side of the leaf. T: Global index including measurements on adaxial and abaxial sides of the leaf. _R: Fluorescence measurements using a Red excitation source; _G: Fluorescence measurements using a Green excitation source. Dashed line in (B) represents the 1:1 line. Dotted lines in (C) and (D) represent the regression lines of the same slope without the offset of the observed correlations.

Flavonol indices. FLAV is the fluorescence index related to the leaf epidermal flavonols. Figure 20 shows the correlations between the FLAV indices measured with the two hand-held devices, DX4 and MX_H. Regarding the DX4 measurements, $FLAV_{AB}$ yielded a strong correlation (R^2=0.96 at P<0.001) with $FLAV_T$, which is the sum of $FLAV_{AD}$ and $FLAV_{AB}$ and represents the total amount of flavonols in the leaf (figure 20A), while $FLAV_{AD}$ poorly correlated with $FLAV_T$ (R^2=0.27 at P<0.001) (figure 20B) and $FLAV_{AB}$ (figure 20C), due to the reduced variation of flavonols on this side of the leaf, which is more exposed to sunlight and tend to have a maximum epidermal flavonol content. The same behaviour can be seen for the FLAV indices obtained with the MX_H (figure 20A, 20B and 20C), where the flavonols determined on the abaxial side of the leaf ($FLAV_{AB}$) seem to be those driving the variation of the total leaf flavonols ($FLAV_T$), with a R^2 = 0.93 (*P*<0.001). Accordingly, the $FLAV_{AB}$ measured with MX_H correlated better with $FLAV_T$ of the DX4 (R^2=0.82, P<0.001) (figure 20D) than the $FLAV_T$ measured with MX_H with the same $FLAV_T$ of the DX (R^2=0.78, P<0.001) (figure 20E), due to the reduction in the span when $FLAV_{AD}$ is added to $FLAV_{AB}$. The relationship between $FLAV_T$ measured with DX4 and MX_H shows a 1.4 offset, which can be traced to the difference in wavelengths and therefore extinction coefficients for flavonols used in DX4 and MX_H, which were 375 nm for DX4 and 385 for MX_H in the UV region, and 650 nm for DX4 and 630 nm for MX_H in the red part of the spectrum. A correlation matrix showing all possible relationships between the FLAV indices of DX4 and MX_H is included in Figure S2 of the supplementary data.

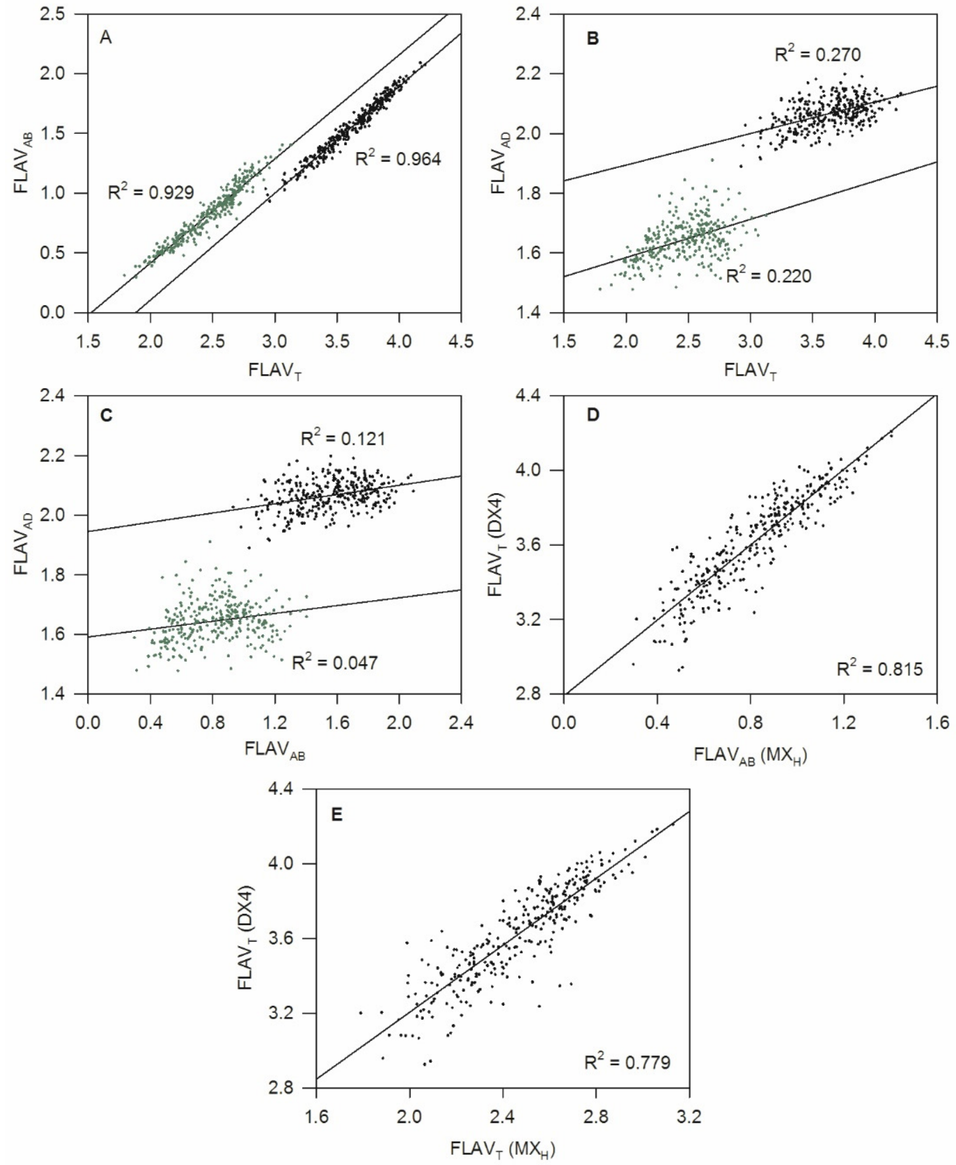

Figure 20. Correlations between the indices related to the leaf flavonols content obtained with the MX_H and DX4 devices. Coefficients of determination (R^2) were significant at P<0.001 (N=302). AD: Measurement taken on the adaxial side of the leaf; AB: Measurement taken on the abaxial side of the leaf. T: Global index including measurements on adaxial and abaxial sides of the leaf. In (A), (B) and (C) black dots refer to DX4 and green dots refer to MX_H.

Nitrogen indices. Regarding the NBI, two different NBI indices have been compared. The NBI index and the NBI_C index. The first one is the NBI index provided by the MX_H, which is computed using the equations 12 and 13. The second one (NBI_C) is a NBI index calculated for each side of the leaf, or the total NBI, using the equations 15 to 20, which are based on the equation given by Cartelat et al. (2005). The NBI_C index from the MX_H (eq. 19 and 20) is the same as the NBI_T in equation 8, provided by the DX4.

Figure 21 shows the correlations between the NBI_R and NBI_C_R indices, which are derived when red excitation was used, with the NBI_T from the DX4. It should be noted that the global NBI_T index from the DX4 strongly correlated with either NBI_{AD} (R^2=0.98, P<0.001) or NBI_{AB} (R^2=0.97, P<0.001) of the same device (figure 21A and B) and that the determination coefficient between NBI_{AD} and NBI_{AB} provided by the DX4, was also very high (R^2=0.90, P<0.001) (figure 21C). This means that the chlorophyll (represented by the CHL index in numerator) is the component that is mostly determining the NBI value, because the flavonol content, represented by the FLAV index in the denominator, is the only component of the ratio which is different. Regarding the MX_H, a similar result can be seen when analysing the correlations between the global NBI_C_R_T with NBI_C_R_{AB} (R^2=0.87, P<0.001, figure 21D) and NBI_C_R_{AD} (R^2=0.92, P<0.001, figure 21E). Indices NBI_C_R_{AB} and NBI_C_R_{AD} have been calculated as for DX4, the numerator being global SFR_T and the denominator being the epidermal flavonols of either the abaxial ($FLAV_{AB}$) or the adaxial side ($FLAV_{AD}$). The strong correlations of the total NBI_C_R_T with the indices of the two sides of the leaf, where again the only factor varying was the FLAV index, indicated that the chlorophyll concentration (SFR index) was the component driving the NBI index. Moreover, when looking at the NBI_{C2}_R_{AD}, it can be seen the high correlation with the NBI_T of the DX (R^2=0.90, P<0.001, see figure S3 of the supplementary data) which agrees with the fact established above that the SFR_{AD} can be used as a proxy of the leaf chlorophyll content. The study of the relationships between the abaxial and adaxial versions of the provided NBI index by the MX_H (NBI_R_{AB} and NBI_R_{AD}, respectively) with the abaxial and adaxial versions of the calculated NBI_C index from MX_H (NBI_C_R_{AB} and NBI_C_R_{AD}) revealed strong significant correlations with R^2 higher than 0.88 at P<0.001 (See figure S3 of the supplementary data). Regarding the comparison of the two NBI index of the MX_H (NBI and NBI_C) with the reference, a higher value of the determination coefficient corresponded to the NBI_C_R_T calculated as the chlorophyll index (SFR_R_T) divided by the flavonol index ($FLAV_T$) with a R^2 of 0.93 (P<0.001) (figure 21F), while the NBI_R_{AD} and the NBI_R_{AB} yielded determination coefficients of 0.75 (P<0.001) and 0.67 (P<0.001), respectively. Therefore, in the case of the MX_H, the most suitable NBI index for the assessment of the N status of the grapevine leaves would be the NBI_C_R_T. The same result was yielded when the green excitation was used to calculate all the possible NBI equations. In this case, the most suitable NBI index for the assessment of the N status of the grapevine leaves would also be the NBI calculated for the total leaf (adaxial and abaxial), NBI_C_G_T (figure S4 of supplementary data).

The NBI_G index was also studied, but as it yielded very similar results to the same index under red excitation (NBI_R), we decided better to not explain it, as it would be redundant. In any case, the correlation matrix showing all possible relationships between the NBI indices of DX4 and MX_H when green excitation (_G) was used instead of red excitation (_R) is included in the supplementary data (figure S4).

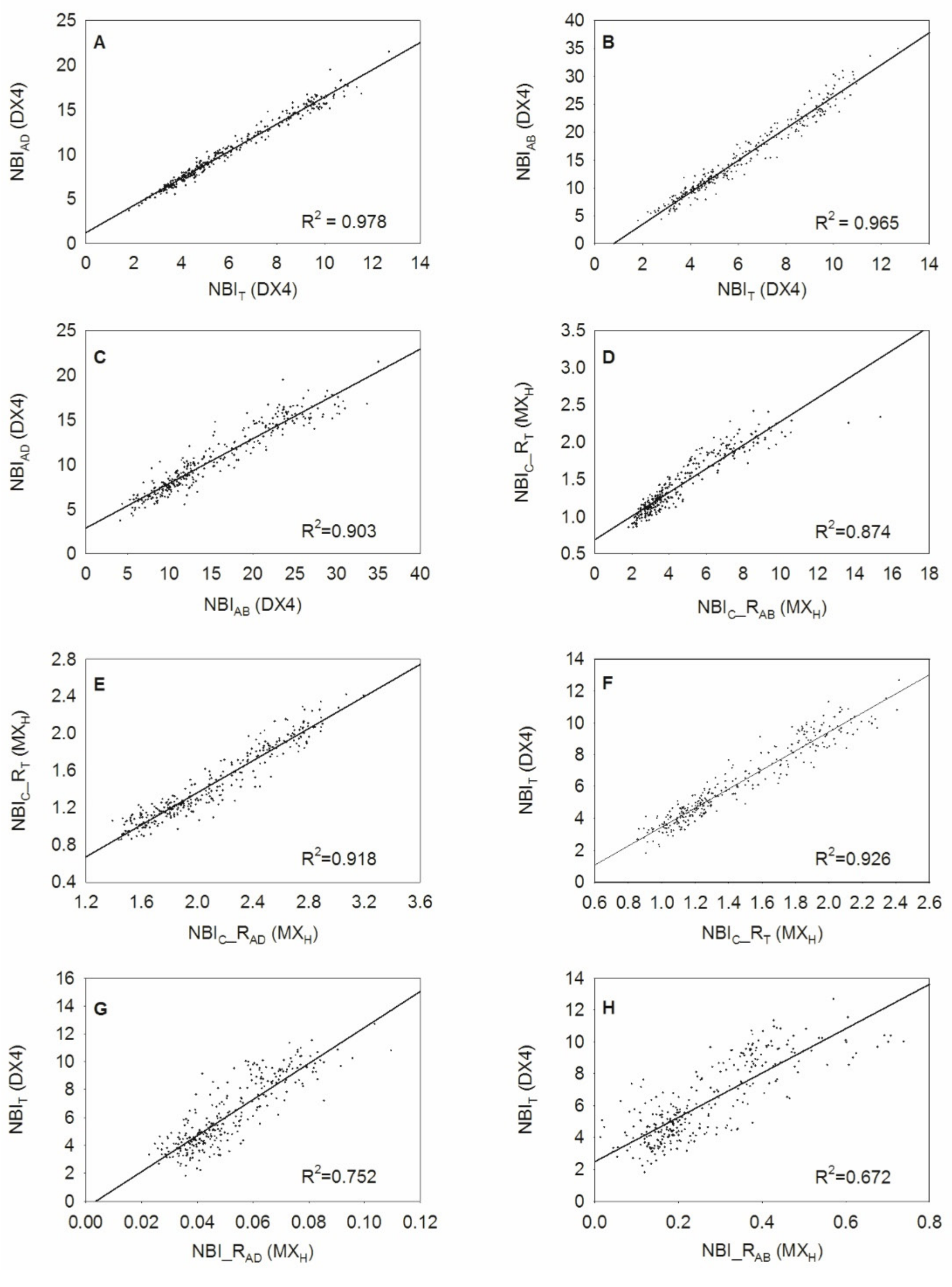

Figure 21. Correlations between the indices related to the leaf nitrogen balance obtained with the MX_H and DX4 devices. Coefficients of determination (R^2) were significant at $P<0.001$ (N=302). AD: Measurement taken on the adaxial side of the leaf; AB: Measurement taken on the abaxial side of the leaf. T: Global index including measurements on adaxial and abaxial sides of the leaf. _R: Fluorescence measurements using a Red excitation source; _G: Fluorescence measurements using a Green excitation source.

The comparison of the indices retrieved with the MX_H and DX4 enabled the definition of the calibration equations (table 7) to transform the SFR index (provided by MX_H) into absolute units of chlorophyll, the FLAV index into absorbance units, and to link the NBI_C (computed from the MX_H measurements) with the NBI index provided by DX4.

Table 7. Calibration linear models for the fluorescence indices derived from the hand-held Multiplex™ sensor (MX_H) ($P < 0.001$ for all models) using the indices obtained with the reference, Dualex4™ (DX4). The 95 % confidence intervals for the fit coefficients are indicated in brackets. Residual sum of squares (RSS), root mean square error (RMSE), bias (BIAS), standard error of prediction corrected for bias (SEPC) are shown.

Equations	N	Model parameters		Model statistics				
		Intercept	Slope	R^2	RSS	RMSE	BIAS	SEPC
CHL_T (DX4) = a + b*SFR_R_T (MX_H)*	302	-15.32 (-16.60, -14.03)	10.76 (10.38, 11.12)	0.917	1286.298	2.064	0.038	2.064
CHL_T (DX4) = a + b*SFR_G_T (MX_H)*	302	-10.41 (-11.47, -9.35)	8.96 (8.67, 9.25)	0.924	1165.282	1.964	0.007	1.964
$FLAV_T$ (DX4) = a + b*$FLAV_T$ (MX_H)	302	1.41 (1.28, 1.55)	0.90 (0.84, 0.95)	0.778	4.452	0.121	0.008	0.121
NBI_R_T (DX4) = a + b*$NBI_C\_R_T$ (MX_H)	302	-2.50 (-2.78, -2.20)	5.97 (5.78, 6.16)	0.926	126.432	0.647	0	0.647
NBI_G_T (DX4) = a + b*$NBI_C\_G_T$ (MX_H)	302	-1.63 (-1.89, -1.38)	5.15 (4.99, 5.31)	0.929	121.687	0.635	0	0.635

* The calibration equations for the SFR_R_T and SFR_G_T indices transform the values of these indices into chlorophyll units (g/cm^2), as the DX4 is already calibrated to yield the CHL index in chlorophyll units (g/cm^2).

DISCUSSION

This study presents the first calibration of the fluorescence-based Multiplex™ sensor, used manually (MX_H) on grapevine leaves, against the Dualex4™ (DX4) as the reference. In this work, the MX_H has been studied to determine its capability to properly measure the grapevine leaf chlorophyll, epidermal flavonol and nitrogen concentrations.

The DX4 was chosen as the reference for several reasons. First, its performance to yield a reliable and accurate measurement of the chlorophyll content (Cerovic et al. 2012), epidermal flavonols (Cerovic et al. 2012) and nitrogen concentration (Cerovic et al. 2015) has been shown even in grapevine leaves. Secondly, the efficiency of leaf extraction by organic solvents may be a potential problem for the calibration of sensors (Lashbrooke et al. 2010). In the same way, according to Casa et al. (2014), the calibration between chlorophyll meter values and actual chlorophyll concentration is affected by different issues, including chloroplast movement in response to light and temperature (Nauš et al. 2010), method of extraction, type of solvent used and even the specifications of the spectrophotometer (Coste et al. 2010, Wellburn 1994). Casa et al. (2014) reported higher average coefficient of variation values for chlorophyll assessment using wet chemistry methods than using DX4 in four different crops.

The MX_H has been used for the study of leaf flavonols (Agati et al. 2011b, Müller et al. 2013) and nitrogen status of different species as potato (Ben Abdallah and Goffart 2012), turfgrasses (Agati et al. 2013c), rice (Li et al. 2013), maize (Longchamps and Khosla 2014) or corn (Zhang and Tremblay 2010, Zhang et al. 2012). While its application on vineyards has been widely focused on grape clusters to assess their anthocyanin content (Agati et al. 2013b, Baluja et al. 2012c, Ben Ghozlen et al. 2010a) few studies have been carried out in grapevine leaves (Garcia et al. 2012, Serrano et al. 2010). In other crops, the only work reporting the assessment of chlorophyll by MX_H was conducted by Tremblay et al. (2012), who validated the SFR_G_{AD} index against chlorophyll extractions for kiwi leaves. In the present study, the two SFR indices (_R or _G) have been calibrated against the CHL_T index provided by the DX4 and the calibration equations for the global SFR_R_T and SFR_G_T were obtained. This means that the SFR index provided by the MX_H can now be translated into chlorophyll units for grapevine leaves. Moreover, $FLAV_T$ was also calibrated against the $FLAV_T$ index from the DX4 and the calibration equation provided. This equation will allow to transform the $FLAV_T$ index provided by the MX_H into absorbance units (AU), so the flavonol content assessed by MX_H could be compared to that carried out by the DX4 or any other different sensor that measures it in AU. In the same way, the calibration equations for the $NBI_C\_R_T$ and the $NBI_C\_G_T$ against the reference, NBI_T from the DX4, are also provided so these indices could be translated into the same units as the NBI provided by the DX4.

To be able to calculate the chlorophyll and flavonol indices at the whole leaf level, the two sides of the leaf have been measured. This is important as the light does not penetrate into the entire leaf depth and the chlorophyll fluorescence recorded corresponds to either the palisade or spongy mesophyll (Karabourniotis et al. 2000, Koizumi et al. 1998, Vogelmann and Evans 2002).

The indices related to the epidermal content of flavonols and chlorophyll are of first importance for nitrogen management (Tremblay et al. 2012) as they allow to calculate the NBI index. The latter, as an indicator of the nitrogen status of the plant, has been here analysed by comparing different sensors and ways to calculate it. The NBI_C is the one that yielded the better correlation with the reference, hence it would be a more accurate index of the nitrogen status of the plant. It is based on the inverse dependence of N on chlorophyll and flavonols, which increases the dynamic range, lessens the effect of leaf position and differential exposure, and finally, avoids the effect of the leaf mass per area.

Diagnosis of plant N status is important for rational management of nitrogen in a sustainable fertilization context. Overfertilization results in lower nitrogen use efficiency, high levels of residual N after harvest, and losses in the environment (leading to groundwater pollution due to NO_3-N leaching) (Hashimoto et al. 2007), while N deficiency may lead to photosynthesis diminishment and may jeopardize final grape yield in terms of quantity and quality. Precise quantification of the N content can be conducted with MX_H. In addition, MX_H may be used as phenotyping tool, enabling a rapid, reliable and non-destructive assessment of the vegetative and nutritional status within a vineyard at several timings within the season.

CONCLUSIONS

This study has proved that the hand-held fluorescence sensor Multiplex™ is a reliable tool for the non-destructive assessment of the grapevine leaf chlorophyll and epidermal content and nitrogen status of the vine under field conditions.

The defined calibration equations allow reporting the SFR index in chlorophyll content units, the FLAV index in absorbance units and to link the nitrogen-related index with the NBI of a contrasted optical device, such as the Dualex. This is an important outcome, as it will allow to know, in an absolute way, the leaf chlorophyll content of the vines. It will enable its comparison with other vineyards and analytical methods or the analysis and subsequent establishment of absolute thresholds, which in turn would lead to the appropriate management strategy for each case.

The nitrogen balance index (NBI) measured with the hand-held Multiplex™ has been proved to be a good estimator of the nitrogen status of the plant. Specifically, among the different possibilities to calculate this index, the NBI_C index calculated as chlorophyll-to-flavonol ratio, was the one that better correlated with the reference. Therefore, it is advisable to use the NBI_C for the assessment of nitrogen status of the vine.

Overall, the fluorescence indices SFR, FLAV and NBI from the hand-held Multiplex™ demonstrated to be suitable indicators for the assessment of leaf chlorophyll content, epidermal flavonol content and nitrogen status of the vine. Therefore, this hand-held fluorescence sensor is appropriate to be used by grapegrowers in the field, enabling them to assess the vegetative status of the vineyards in a fast and non-destructive way, and making feasible the implementation of precision viticulture.

3.3. Chapter 3

Analysis of the spatio-temporal variation of chlorophyll and nitrogen status in vineyards using a hand-held fluorescence sensor

ABSTRACT

Background and aims: Vineyards are spatially variable and the characterization of their vegetative and nutritional parameters requires an extensive sampling and analyses, therefore the use of non-invasive and rapid sensors can be valuable. The goal of this work was to study the spatial and temporal variability of the leaf chlorophyll and nitrogen contents in grapevines using a hand-held fluorescence sensor, and their relation to an indicator of the plant vegetative growth in the vineyard (*Vitis vinifera* L.).

Methods and results: Fluorescence measurements were taken along five dates, from veraison to harvest, on 72 sampling points delineated on a regular grid across the vineyard. Shoot pruning weight was also measured at the end of the season for each sampling point as indicator of the grapevine vegetative growth. Geostatistical analysis was applied to interpret and model the spatial variability of the fluorescence indices and the shoot pruning weight. The coefficient of variation (around 10%) and the spread of the fluorescence-based indices showed that their variability increased during the ripening period, reaching a maximum prior to harvest. The detailed variographic analysis of the variables has shown that the leaf chlorophyll content had a similar spatial behaviour at all timings, while the nitrogen balance index showed a variable spatial behaviour along time. The k-means clustering analysis indicated that the nitrogen balance index could be used to delineate two different vegetative growth management areas. The fluorescence sensor was a powerful phenotyping tool, which enabled the assessment of the spatio-temporal variability of the nitrogen and chlorophyll contents within a vineyard in a fast and non-invasive way, and yielded valuable information to support decision making regarding management strategies in subsequent seasons.

Conclusions: The hand-held fluorescence sensor can be used in precision viticulture for assessing the spatio-temporal variability of the nitrogen and chlorophyll contents during the growing season.

Keywords: proximal sensing, plant phenotyping, vegetative growth, geostatistical analysis, grapevine.

INTRODUCTION

Nitrogen has been proved to be one of the most important factors for biomass production (Agati et al. 2013c, Lemaire et al. 2008) and grapevine metabolism, as it is crucial for vine development and fruit yield (Guilpart et al. 2014). Furthermore, chlorophyll is the pigment responsible for the photosynthesis. Therefore, both components are key physiological compounds in grapevines and the assessment of the spatial variability of chlorophyll and nitrogen status is necessary and very helpful to delineate fertilization and canopy management strategies intended to improve the grapevines balance and fruit composition.

Vineyards are demonstrated to be spatially variable. Hence, large amounts of data collected from their vegetative status, yield and grape composition are required to optimise their management (Proffitt et al. 2006). Such information may lead to the demarcation of management zones within the vineyard for adapting cultural practices to the different necessities in each area (Bramley 2010a). Moreover, vineyards are non-continuous crops, where grapevines are usually arranged in narrow rows of 30-40 cm width of vegetation. In this context, proximal sensing has proved as a successful alternative to retrieve productive and vegetative growth-related data in precision viticulture (Baluja et al. 2012c, Bramley 2010a, Proffitt et al. 2006). To analyse the spatial and temporal variability of experimental data, geostatistics have been applied in precision agriculture since the late 1980s, allowing the production of maps and the delineation of the managements zones within the plots (Oliver 2010).

Chlorophyll and nitrogen are associated to grapevine vegetative growth-related variables such as the vine pruning weight (Lemaire et al. 2008), which is a measure of the plant vegetative growth during the season (Smart and Robinson 1991) and may provide useful ancillary information for the delineation of different vineyard management zones with oenological significance (Urretavizcaya et al. 2014). With this regard, it is important to study, not only on a temporal basis, but also spatially-speaking, how these components vary along the plot during the season. The classical methods for assessing chlorophyll and nitrogen contents in grapevine leaves are based on wet chemistry analysis, which are time and labour demanding, making the analysis of a high number of samples during the growing season not feasible (Muñoz-Huerta et al. 2013). Therefore, the use of rapid and non-invasive sensors can be valuable to assess these key physiological factors. In the previous chapter, the hand-held fluorescence sensor based on the chlorophyll fluorescence emission (Ben Ghozlen et al. 2010a) has proved to be a very powerful, rapid and efficient phenotyping tool to determine the chlorophyll content and the nitrogen status in grapevine leaves. This hand-held sensor has also been used to assess the chlorophyll and flavonol content and nitrogen status of leaves of different species (Agati et al. 2013c, Tremblay et al. 2012, Zhang et al. 2012). Regarding its application to assess the spatial variability within vineyards, it has been successfully used for the appraisal of the spatial and temporal variability of grape composition (Baluja et al. 2012c, Cerovic et al. 2009), but there are no examples regarding the heterogeneity of the vegetative status within vineyards.

The goal of the present work was to study the spatial and temporal variability of grapevine leaf chlorophyll content and nitrogen status using a hand-held fluorescence sensor and their relationship with the vegetative growth in a (*Vitis vinifera* L.) vineyard.

MATERIALS AND METHODS

Experimental site and layout

The study was conducted in a commercial Tempranillo (*Vitis vinifera* L.) vineyard located in Navarra (42º38' N, 2º2' E, 518 m a.s.l.), Spain, during season 2011 (figure 22). Grapevines were planted in a sandy-clay soil in 2004, at 2.4 x 1.6 m (inter- and intra-row) with north-south orientation. Vines were trained to a vertically shoot-positioned, spur-pruned cordon retaining 16 nodes per vine and uniformly irrigated twice across the season. Veraison occurred on the 17 of August and harvest was carried out on the 17 October.

Figure 22. Commercial vineyard where the experiment was conducted.

A regular sampling grid was defined, consisting on 72 sampling points at 20 m intervals, following the sampling grid strategy described in Baluja et al. (2012b), Baluja et al. (2012c). Each sampling point was constituted by three adjacent vines, where the central one was georeferenced using a Leica Zeno 10 Global Positioning System (GPS) (Heerbrug, St. Gallen, Switzerland), with real time kinematic correction and working at <30 cm precision. The values obtained for each of the three vines that define a sampling point were averaged to have one averaged value per experimental point.

Shoot pruning weight measurements

The pruning weight of each vine was manually measured using a hanging scale (20 November). The shoot pruning weight per vine was calculated by dividing the values measured by the number of shoots per vine.

Proximal sensing measurements

Measurements of the chlorophyll content and nitrogen status in grapevine leaves were taken using the hand-held fluorescence-based proximal sensor Multiplex3™ (Force-A, Orsay, France). This non-invasive active device generates fluorescence in plant tissues by the excitation at four different wavelengths: UV_A (375 nm), blue (450 nm), green (530 nm) and red (630 nm). The sensor includes three different detectors with filters to record the fluorescence emission at three different bands: blue-green (447 nm) or yellow (590 nm) depending whether blue excitation is used or not respectively, red (665 nm) and far-red (735 nm) (Ben Ghozlen et al. 2010b).

The fluorescence-based indices studied were the nitrogen balance index (NBI) that follow the eq. 17 described in Chapter 2 ($NBI_{C2}\_R_{AD}$) and the simple fluorescence ratio (SFR), defined as follows:

$$SFR\_R_{AD} = \frac{FRF\_R_{AD}}{RF\_R_{AD}} \tag{21}$$

where FRF_R refer to the far red fluorescence evolved from red excitation and RF_R refers to red fluorescence excited by red light (Ben Ghozlen et al. 2010a).

Measurements were carried out on three main leaves per vine, adaxial side, between the 8^{th} and the 10^{th} node, along five dates during the season (17 August, 2 September, 14 September, 5 October and 11 October), from veraison until six days prior to harvest.

Statistical and geostatistical analysis

In a first step, potential outliers were identified and removed using box and whisker plots. Samples with a value higher than two standard deviations were removed. Once the database was refined, the measurements were averaged to have one value per sampling point, which involves a total of three vines. After that, descriptive statistics were calculated to have a first view of the individual variable behaviour at each date. In addition, the spread of the distributions was calculated as the ratio between the range and the median value (Bramley 2005).

A detailed geoestatistical analysis (Chilès and Delfiner 2008a, Chilès and Delfiner 2008b) of the spatio-temporal variability of the experimental variables was performed. Experimental variograms were computed for all the variables at each date. For the indices measured, relative variograms and relative variograms of the mean for all dates were plotted to compare each index among its different measurement dates. The relative variogram is calculated by dividing the data of the experimental variogram by the variance of the same variable. The best model for the experimental variograms was selected based on a best visual fit for an omnidirectional variogram and taking into account the cross-validation results. All fitted variogram models (stationary models) are described by three parameters: nugget effect, sill and range. The nugget represents the variability at distances smaller than the sample spacing; the sill is the semivariance value at which the variogram reaches stationarity; and the range is the distance at which the semivariogram reaches the sill value and corresponds to the maximum autocorrelated distance. Based on these variograms and their descriptive parameters (nugget, sill and range), the Cambardella index (CmbI) was computed (eq. 22). The Cambardella index (Cambardella et al. 1994) is a ratio between the nugget variance C_0 and total variance (C_0+C_1) expressed as a percentage, which provides information on the spatial dependence of the variable defining it as strong (less than 25%), moderate (between 25% and 75%) or weak (more than 75%).

$$\text{CmbI} = \left(\frac{C_0}{(C_0+C_1)}\right) \quad (22)$$

where C_1 is the sill and C_0 is the nugget effect of the variogram.

These variograms were also used for applying the spatial interpolation method of ordinary kriging. This method was used to estimate a continuous surface of the indices and the vegetative variable (Z). For every variable, the estimation model was validated applying the cross-validation technique and studying its mean error (ME), the mean square error (MSE) and the mean square standardised error (MSSE), defined as follows:

$$ME = \frac{1}{n}\sum_{i=1}^{n}[Z_k\,(x_i) - Z(x_i)] \quad (23)$$

$$MSE = \frac{1}{n}\sum_{i=1}^{n}[Z_k\,(x_i) - Z(x_i)]^2 \quad (24)$$

$$MSSE = \frac{1}{n}\sum_{i=1}^{N}[((Z_k)(x_i) - Z(x_i))/\sigma_k]^2 \quad (25)$$

where n is the size of the population, Z_k (x_i) corresponds to the ordinary krigged datum of the experimental value (Z (x_i)) and σ_k is the standard deviation of the kriging estimation. Variogram analysis and ordinary kriging interpolation were carried out using ArcGis 9.3 (ESRI, Redlands, USA).

The fluorescence-based indices temporal trends were also analysed by the non-parametric Mann-Kendall test at two different scales, for the whole range and for each sampling point (Kendall 1975, Mann 1945).

Finally, different subareas within the plot that showed heterogeneous and uniform-like pattern were labelled by clustering. The k-means (Lloyd 1982), used in precision viticulture by Bramley and Hamilton (2004), was the algorithm applied for this purpose. This non-hierarchical algorithm for data aggregation maximises the Euclidean distance between the cluster means and minimises the distances within the clusters. The k-means was applied to the point-to-point data by using the software Statistica 9 (Stat Soft Inc., Tulsa, USA), and t test was applied to determine statistical significance.

RESULTS

The descriptive statistics of the vegetative growth and the fluorescence indices over time are shown in table 8. The largest values of the coefficient of variation (CV) and the spread were observed for shoot pruning weight (SPW), whereas SFR_R_{AD} and $NBI_{C2}\_R_{AD}$ had a CV that oscillated around 10%. It is worth highlighting that for the two indices studied the coefficient of variation and the spread increased as the ripening season progressed from veraison to harvest. In particular, their variability increased until the fourth date of measurement (5 October), and stabilised thereafter. Regarding the spatial variability, all the variables were fitted to a spherical model (table 8). The range of the SFR_R_{AD}, the index that reflects the leaf chlorophyll content, increased as the season progressed. While for the $NBI_{C2}\_R_{AD}$, the range showed in general the opposite behaviour, decreasing as harvest was getting closer, with the exception of the high range of the third date in comparison with the other dates of measurement. The CmbI showed an alternation from strong ($CmbI<25$) to moderate spatial correlation ($25<CmbI<75$) for SFR_R_{AD} index and for $NBI_{C2}\_R_{AD}$, while CmbI was found to be moderate for SPW.

Table 8. Descriptive and spatial statistics for shoot pruning weight (SPW) and fluorescence-based indices of leaf chlorophyll content (SFR_R_{AD}) and nitrogen balance index ($NBI_{C2}\_R_{AD}$) in Tempranillo leaves at five dates from veraison to harvest.

Variable	Date	Mean	CV (%)*	Spread (%)	Range	CmbI*
SPW (g)	20 November	51.44	26.08	142.17	115	46.76
SFR_R_{AD}	17 August	1.84	6.35	24.15	85	25.64
	2 September	1.90	6.35	32.64	90	11.76
	14 September	1.70	8.06	38.65	95	20.00
	5 October	1.54	11.79	57.71	95	30.00
	11 October	1.49	11.47	62.64	115	22.73
$NBI_{C2}\_R_{AD}$	17 August	1.613	11.07	52.23	65	27.03
	2 September	1.552	10.08	53.11	45	25.81
	14 September	1.484	14.85	62.06	140	23.08
	5 October	1.461	13.04	71.79	35	39.29
	11 October	1.415	12.23	67.97	30	24.32

*CV: coefficient of variation and CmbI: Cambardella Index.

The temporal variation of SFR_R_{AD} and $NBI_{C2}\_R_{AD}$ was illustrated by box plots (figure 23). The leaf chlorophyll content increased until September 2, when it started to decrease until harvest. On the other hand, the leaf nitrogen content steadily diminished, although moderately, from veraison to harvest.

The spatio-temporal behaviour of the chlorophyll and nitrogen indices was studied by variographic analysis and their data were fitted to spherical variograms (figure 24). The SFR_R_{AD} index showed similar values of nugget and increasing range as season advanced. It showed a similar structure during the five dates of measurement (figure 24A-E). The experimental variogram of the mean of the five dates exhibited a nugget effect smaller than that of the individual dates (figure 25A), as the average reduces the variability in origin.

Regarding the $NBI_{C2}\_R_{AD}$ index, the experimental (figure 24F-J) and relative (figure 25B) variograms of the five dates of measurements showed a different behaviour between dates, but nearly all of them had a short range of less than 65 m, approximately.

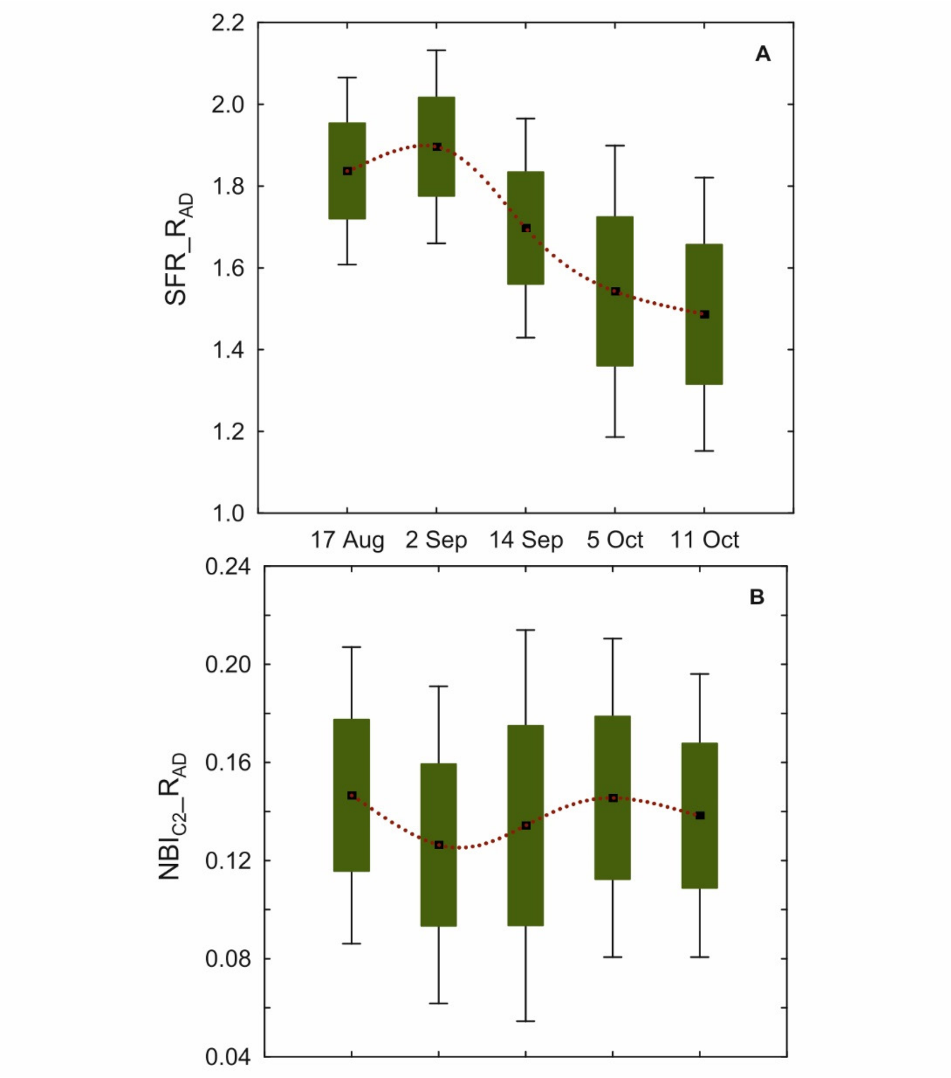

Figure 23. Temporal variation of leaf chlorophyll content index –SFR_R_{AD}- (A) and nitrogen balance index –$NBI_{C2}\_R_{AD}$- (B) measured along five dates from veraison to harvest, for all the 72 sampling points in the vineyard. Black dots represent the mean values, the boxes represent the standard deviation for each date, and whiskers represent twice the standard deviation.

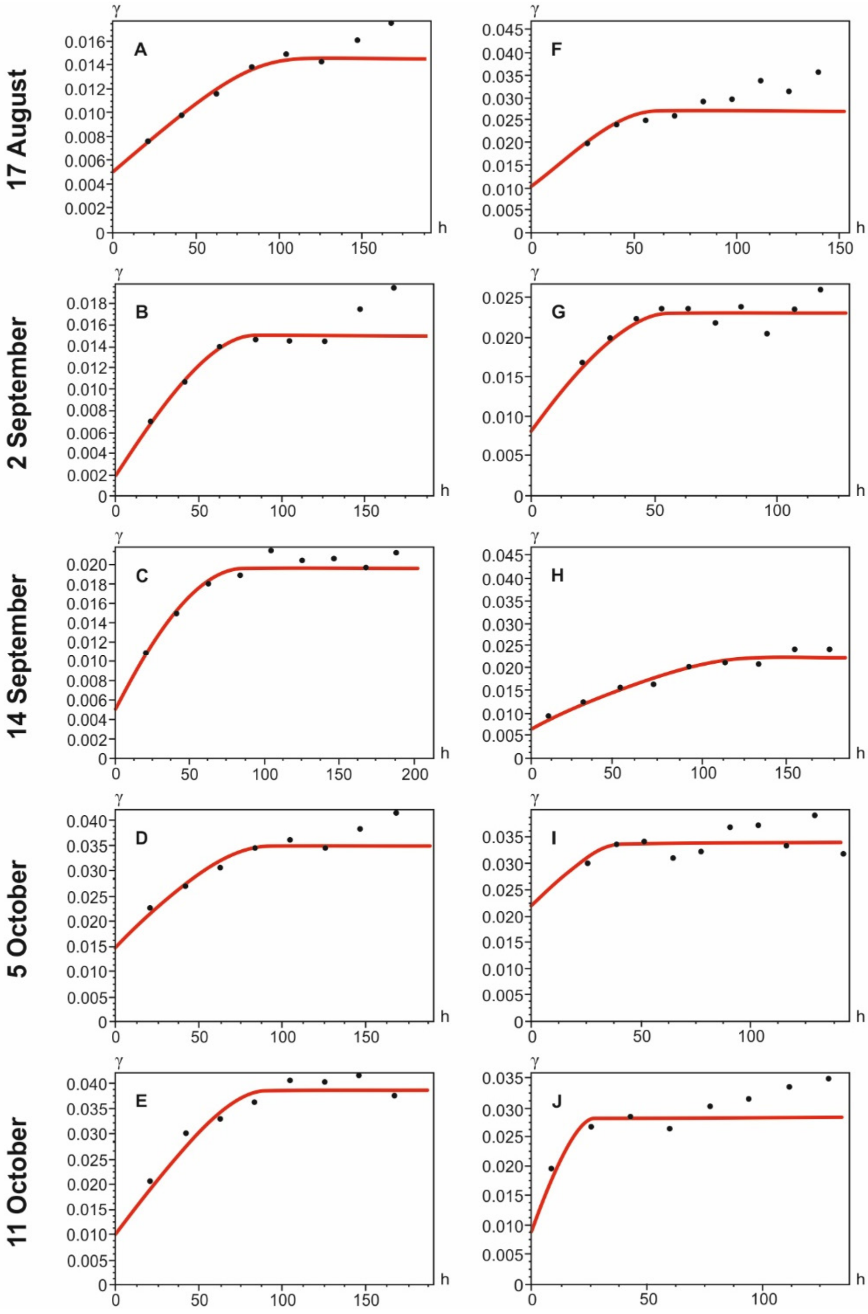

Figure 24. Experimental variograms (black dots) of the five dates for both fluorescence indices, SFR_R$_{AD}$ (A-E) and nitrogen balance index (NBI$_{C2}$_R$_{AD}$) (F-J) fitted to spherical models (solid red line).

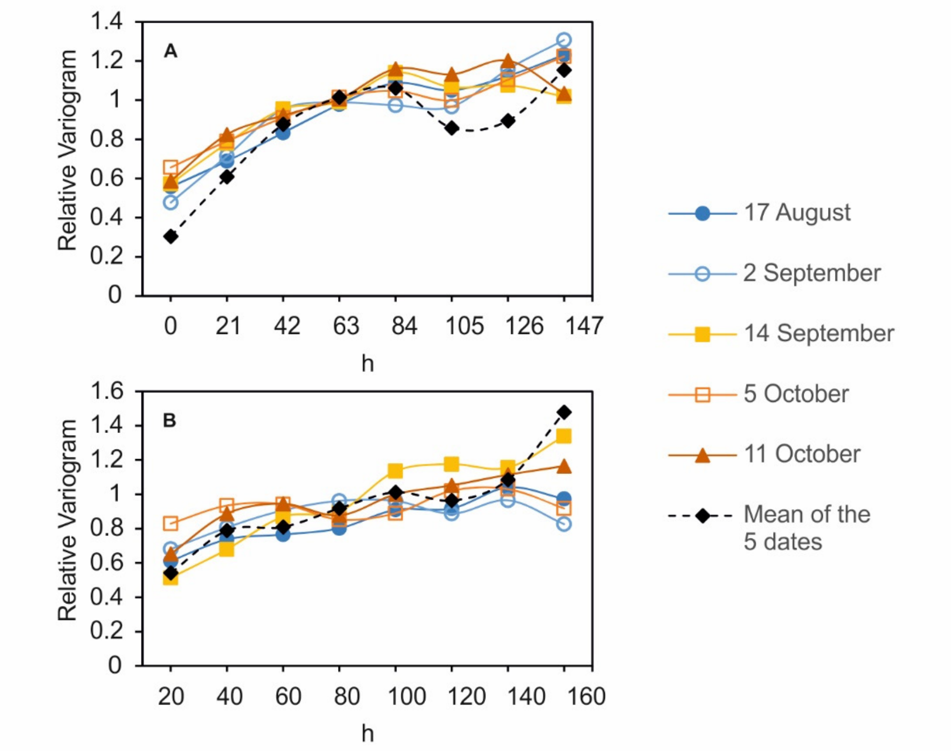

Figure 25. Comparison of the relative variograms of the five dates of measurement with the relative variogram of the average value of the five dates of measurements for the leaf chlorophyll index, SFR_R_{AD} (A); and for the nitrogen balance index, $NBI_{C2}\_R_{AD}$ (B).

In order to improve the thorough understanding of spatial and temporal dynamics along the vineyard, interpolated surfaces were built for each index and date. As a preliminary step, all the variogram models were validated. A representative case of the validation is shown in table 9. In this case the validation of the ordinary kriging method applied corresponds to the SFR_R_{AD} index measured at veraison. The first model was selected as the best one, according to the mean error (ME) value closer to 0 and the mean square standardised error (MSSE) value closer to 1.

Table 9. Example of the validation of the best model by applying ordinary kriging to the SFR_R_{AD} index measured at veraison. For every model, the mean error (ME), the mean square error (MSE) and the mean square standardised error (MSSE) were calculated by the cross-validation method to select the model with the lowest error. The different models are variations of the minimum and maximum number of sample points taken into account for the kriging prediction. In this example, the first case was the one selected to map the variable.

Index	Model	ME	MSE	MSSE
SFR_R_{AD} 17 August	5/13	-0.00039	0.0086	1.063
	9/13	-0.00052	0.0086	1.073
	9/21	-0.00052	0.0086	1.073

Figure 26 shows the interpolated surfaces of the SFR_R_{AD} index and the $NBI_{C2}\_R_{AD}$ index at the five measurement dates. The higher values for the two indices were obtained at veraison and decreased until harvest. These maps evidenced the asynchronous development between vines, since the maximum values were located in the west part of the plot at veraison and the least values started to appear in the east part of the plot as harvest was getting closer.

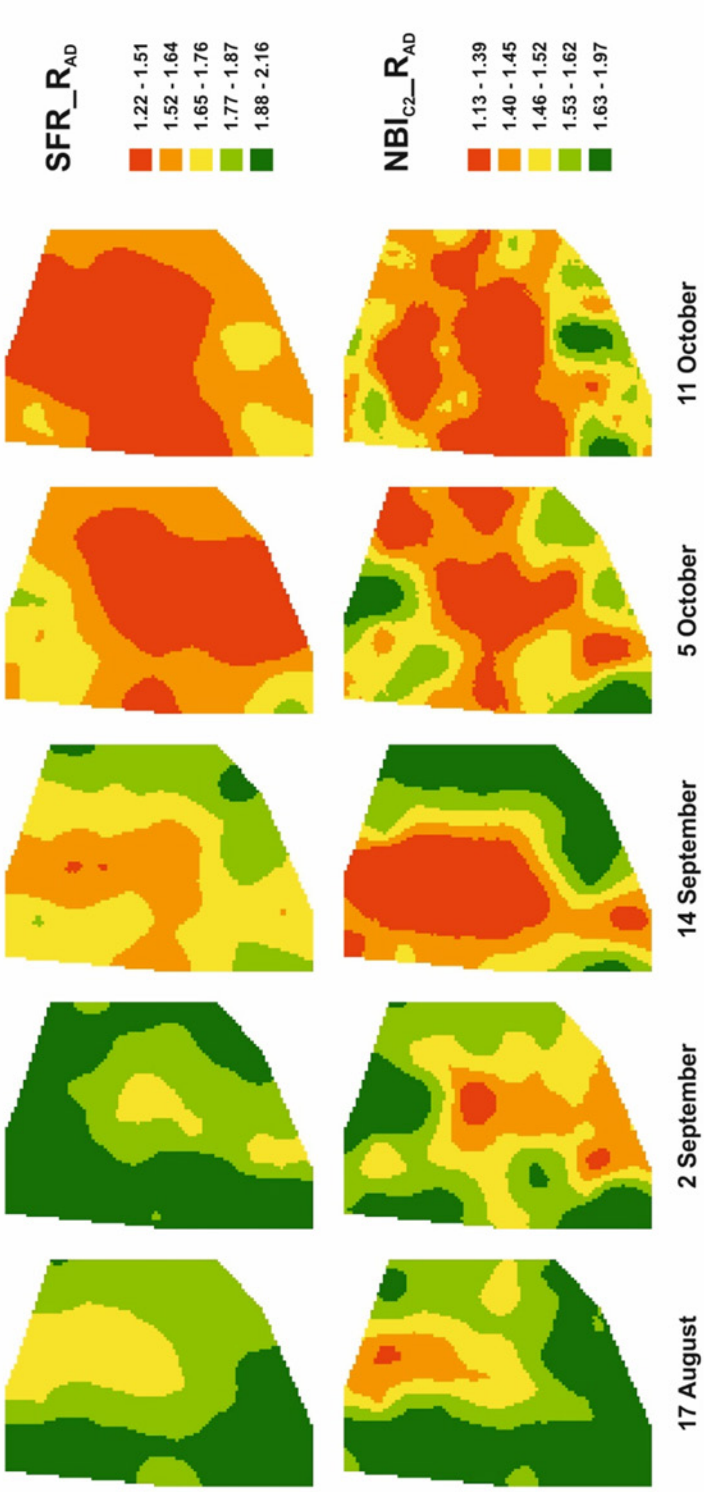

Figure 26. Interpolated surfaces of the fluorescence indices from veraison to harvest. Leaf chlorophyll content index (SFR_R$_{AD}$) kriged surfaces and nitrogen balance index (NBI$_{C2}$_R$_{AD}$) kriged surfaces obtained from the 72 sampling points in a Tempranillo vineyard from veraison to harvest.

The $NBI_{C2}\_R_{AD}$ index spatio-temporal behaviour (figure 26) in the plot showed a large dispersion in accordance with the spreading illustrated in the box-plots (figure 23B). Figure 27 depicts the spatial variability of the shoot pruning weight (SPW), which showed its lowest values (37.28 -45.14) at the centre and south parts of the vineyard, while the highest values appeared mainly on the north area (57.23 – 69.07).

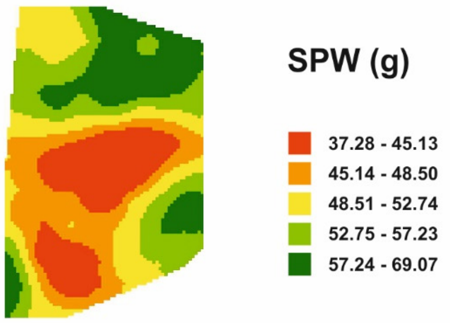

Figure 27. Interpolated surface of the vegetative growth variable. Shoot pruning weight (SPW) kriged surface map of the 72 sampling points in a Tempranillo vineyard. The map is represented by quantiles.

The temporal trend pattern displayed in the interpolated maps was statistically studied with the Mann-Kendall test. This test was calculated at two levels for each index: at each individual sampling point; and for the average value of the whole vineyard. At the entire plot level, the observed decreasing trend pattern for the two fluorescence-based indices along the season was confirmed, as the Mann-Kendall scores (S) were -8 (P = 0.09) for SFR_R_{AD} and – 10 (P = 0.03) for $NBI_{C2}\_R_{AD}$. The temporal trend results obtained for each sampling point are summarized in figure 28. There was not statistical significant result, but nearly every point showed a decreasing trend pattern. SFR_R_{AD} reported a homogenous decreasing trend within the entire vineyard while $NBI_{C2}\_R_{AD}$ exhibited an important spatio-temporal variability within the plot.

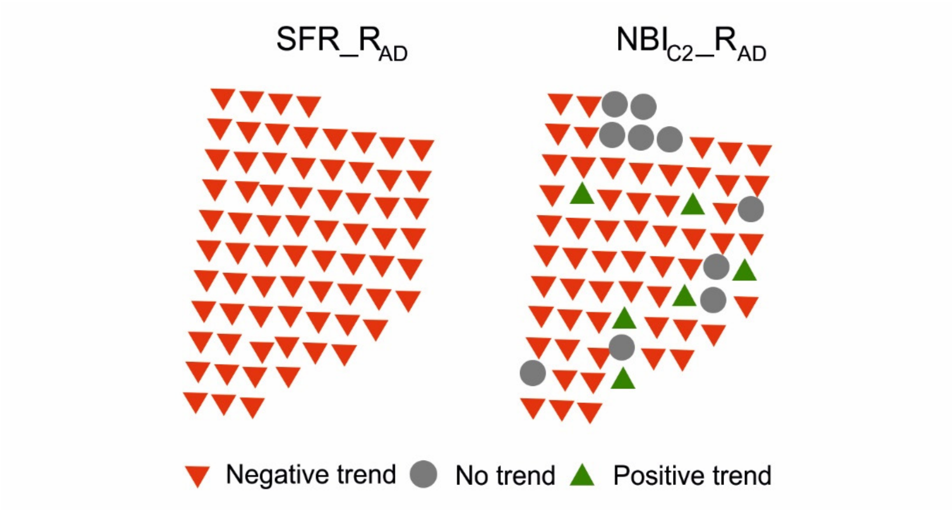

Figure 28. Temporal trend of the fluorescence indices. Mann-Kendall test results for the analysis of the temporal variation trend of the (a) leaf chlorophyll content –SFR_R_{AD}, and (b) nitrogen balance index –NBI_{C2}_R_{AD} from veraison to harvest.

The SFR_R_{AD} and NBI_{C2}_R_{AD} indices were used to define different management zones within the vineyard that better identified the diverse zones of shoot pruning weight. As shown in figure 29, the clustering analysis yielded statistically different areas for SPW. Two different sectors were established according to the NBI_{C2}_R_{AD} index measured on the 2 September and 5 October, at P <0.05.

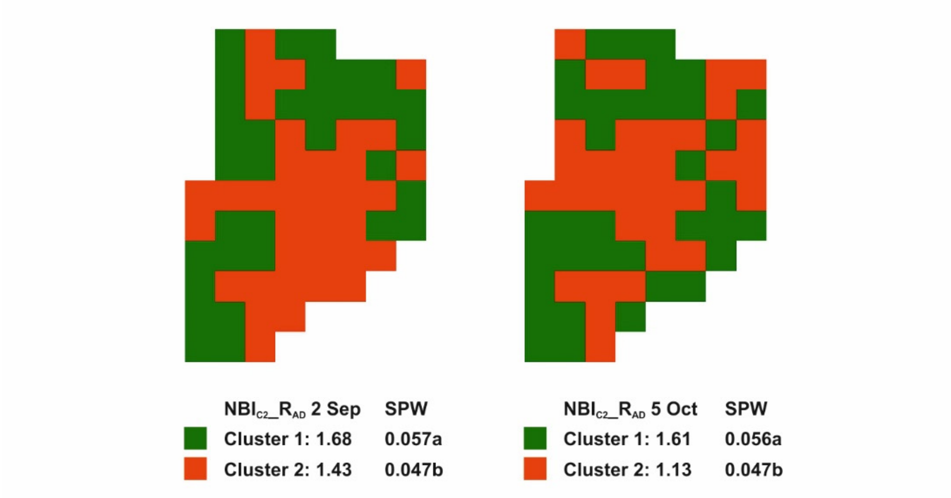

Figure 29. Management zones maps applying k-means clustering algorithm, describing variation in shoot pruning weight (SPW) by means of the nitrogen balance index (NBI$_{C2}$_R$_{AD}$). The k-means algorithm was apply to the individual sampling points. (Different letters indicate statistically different mean values at $P < 0.05$).

DISCUSSION

Leaf chlorophyll content and nitrogen status of the vineyard were measured by a proximal fluorescence sensor to assess their spatio-temporal variability from veraison to harvest.

In general, the leaf chlorophyll content displayed less gross variability than the shoot pruning weight and the nitrogen status of the vines. The high unevenness for shoot pruning weight was in agreement with the high variability found by Tisseyre et al. (2008) for this variable within the vineyard. The fluorescence-based indices variability changed with time, getting higher from veraison onwards and stabilising prior to harvest.

The leaf chlorophyll content assessed by the fluorescence index SFR_R$_{AD}$ was the variable that showed the most coherent spatial behaviour across dates. The relative variograms identified very similar structures of spatial variability along time, that is, the spatial behaviour of the SFR_R_{AD} index was similar from veraison to harvest. It will be interesting, for future studies, to analyse the possibility of the generation of a prediction model for any date between these two phenological stages.

The leaf nitrogen content steadily diminished from veraison to harvest, in agreement with the results obtained by Prieto et al. (2012) who reported a decrease in the N content in grapevine leaves during ripening and also observed a high N variability existing within the canopy. In the same way, Gastal and Lemaire (2002) demonstrated that the N content of well illuminated young leaves at the top of the canopy remains more or less constant despite the overall decline in N concentration at the plant level. In addition to the effects of leaf senescence in the N content, its high spatial variability and its different spatial behaviour along time could also be related to the leaf flavonol content along the vineyard, as it is inversely related with the nitrogen content of the plant (Cartelat et al. 2005). Also, it could be explained by some events of weak mineralization of soil nitrogen in some areas of the vineyard, which would prevent it from being available for plant nutrition, as postulated by Garcia et al. (2012), especially in dry and hot seasons. In this regard, the weather conditions during season 2011 at the experimental vineyard can be described as very dry in comparison to historical data, as it has already been explained in chapter 1.

The interpolated maps showed a negative trend along the vineyard for chlorophyll content and nitrogen status as ripening progressed, as expected in a senescence process. For the two indices, and in spite of the variability differences, their decrease started at the centre of the plot before it spread throughout the rest of the vineyard. Nitrogen content was the variable that exhibited the largest variability, which was also evidenced by its spatial behaviour, as it was the variable that presented the most heterogeneous pattern for almost all the dates.

The fluorescence-based indices were useful to delineate vegetative management areas. The clustering analysis suggests that the variation in shoot pruning weight could be driven, at least partially, by the nitrogen status on two different moments of the ripening period: 16 days after veraison and 12 days prior to harvest. From a practical point of view, the establishment of vine vigour management areas by the use of a fluorescence based sensor could be very interesting, as it enables a non-destructive and fast monitoring of the vineyard vegetative growth at earlier dates. Characterizing the variability of the vineyard vegetative growth provides useful information to support decision taking regarding fertilization and canopy management practices, such as defoliation, shoot thinning, hedging and cluster thinning, oriented to improve vine balance and fruit quality for subsequent seasons. The inclusion of vegetative growth data is very valuable as ancillary information when a stratified sampling procedure strategy for subzone delineation in precision viticulture is used (Urretavizcaya et al. 2014). As studied in this work, spatial vegetative growth knowledge may be fast and efficiently gathered at different timings across the season using non-invasive proximal sensing, and this technology may potentially be used as a phenotyping tool to characterize the vineyards in a flexible and easy way. As a further step, the use of this fluorescence-based sensor on board of a vehicle equipped with a GPS would allow a fast, on-the-go characterization of the canopy chlorophyll and nitrogen content distribution within the vineyard during the growing season.

CONCLUSIONS

This study confirms the effectiveness of a hand-held fluorescence sensor in assessing the spatio-temporal variability of chlorophyll content and nitrogen status of grapevine leaves under field conditions.

The existence of spatial and temporal variation of leaf chlorophyll content and nitrogen status within a vineyard was demonstrated for the ripening period. It is important to note that leaf chlorophyll content and nitrogen status spatial variability within the vineyard increased as the season advanced and stabilised prior to harvest. The nitrogen status showed the largest variability across the vineyard at all dates. On the contrary, the leaf chlorophyll content assessed by chlorophyll fluorescence had similar spatial behaviour along the ripening period. The possibility of developing a spatio-temporal model for the leaf chlorophyll content from veraison to harvest should be studied in future works.

The clustering analysis suggested that the nitrogen balance index (NBI) could be a suitable indicator to describe the shoot pruning weight variability within the plot and could be useful to delineate vigour and vegetative growth management zones within the vineyard before the data could be assessed by direct and destructive measurements. Our research suggests that the use of proximal sensors in precision viticulture should be encouraged for monitoring the spatio-temporal variability in vineyards.

3.4. Chapter 4

On-the-go assessment of leaf chlorophyll, flavonol content and nitrogen status in the vineyard using a fluorescence sensor

ABSTRACT

Background and Aims: The study of the spatial variability of a vineyard requires a large amount of georeferenced measurements. To cover this necessity, proximal sensors could be mounted on a vehicle to gather georeferenced data of the vineyard with high spatial resolution. The main goal of the present study was to evaluate the performance of a non-destructive fluorescence sensor (on a motorized platform) for the on-the-go assessment of the spatial variability and mapping of chlorophyll, flavonol and nitrogen in grapevine leaves.

Methods and Results: The experiment was carried out in a commercial vineyard planted with grapevines of nine different red varieties of *Vitis vinifera* L. The vineyard was monitored with a proximal fluorescence sensor mounted on a quad. The results demonstrated the capability of the fluorescence sensor to estimate on-the-go the grapevine leaf chlorophyll, epidermal flavonol and nitrogen content. The fluorescence indices studied yielded significant R^2 values against the reference: 0.75 for SFR_R, indicative of the leaf chlorophyll content; 0.52 for FLAV, related to the leaf epidermal flavonol content; and 0.77 for NBI_R, linked to the nitrogen status. In addition, the maps have revealed that the indices measured using the mounted fluorescence sensor evidenced the same spatial variability as the reference method.

Conclusions: This work has demonstrated that the mounted fluorescence sensor is a suitable device to assess the leaf chlorophyll, epidermal flavonol and nitrogen status of a vineyard on-the-go. Moreover, it allows a reliable appraisal of the spatial variability of the vegetative and nutritional status of the vineyard. Therefore, the mounted fluorescence sensor enables a fast, non-destructive, reliable, on-the-go assessment of the spatial and temporal variability of crucial indicators of the grapevine vegetative and nutritional status, being of interest for the application of precision viticulture.

Keywords: Grapevine, vegetative status, proximal sensing, spatial variability, precision viticulture

INTRODUCTION

The knowledge and the study of the spatial variability of the different features of a vineyard allow a differentiated, optimized management, which is known as precision viticulture. For this purpose, the collection and use of large amounts of data related to the plant physiological status, yield and grape composition is needed (Proffitt et al. 2006).

Proximal sensors are capable to provide numerous and spatially widespread monitoring of plant nutrient status, in comparison to destructive time-consuming wet chemistry analyses (Muñoz-Huerta et al. 2013). These sensors can be either hand-held or mounted onto a machine, allowing a non-destructive acquisition of data (Tisseyre 2013). In the case of mounted sensors, they might be able to provide a larger amount of data related to the vineyard with higher spatial and temporal resolution than the hand-held devices; data that can be georeferenced and comprehensive maps of the vineyard condition could be generated when this sensors are integrated with a GPS (Tremblay 2013). In fact, a significant portion of the current efforts in precision viticulture research is focused on the development of sensors able to be mounted or embedded on vehicles and monitor the vineyard parameters, such as vegetative, nutritional and water status, yield and grape composition, on-the-go.

It has already been proved in Chapter 2, the suitability of the hand-held fluorescence sensor MX_H to assess the leaf chlorophyll, epidermal flavonol and nitrogen status of a vineyard. In the present chapter, it is explored the use of this fluorescence sensor mounted on a vehicle to assess these nutritional leaf components and their spatial variability in a fast and continuous way. Nevertheless, the step from the manual use of the fluorescence sensor to an on-the-go operation may cope with some possible inconveniences. This sensor, when mounted on a vehicle, will be recording the fluorescence emitted by different kind of leaves, primary and secondary leaves; shaded leaves and sun-exposed leaves; leaves with different orientation; not being able to fully targeting the adaxial or the abaxial side of the leaf and, therefore, not being possible to measure both sides of the leaf. All these characteristics could lead to less accuracy of the fluorescence indices related to the leaf attributes that are the object of this work.

Few studies have been published about the use of this fluorescence sensor mounted on a vehicle in precision viticulture. Bramley et al. (2011a) mounted the Multiplex™ sensor on a harvester to assess the berry anthocyanin content on-the-go. Regarding the vegetative status of the vineyard, only two congress communications have been published using this sensor along with another optical sensor, both mounted on a caterpillar, to assess the nitrogen status (Garcia et al. 2012) and the vine vigour spatial variability (Debuisson et al. 2010) in a Champagne vineyard. However, the capability of this sensor to reliable assess the vegetative and nutritional status of the vineyard on-the-go has not been addressed yet.

The main goal of the present study was to evaluate the performance (against an optical sensor, used as a reference) of a fluorescence sensor (on a motorized platform) for the assessment of chlorophyll, epidermal flavonol and nitrogen content in grapevine leaves on-the-go and mapping their spatial variability within a vineyard.

MATERIALS AND METHODS

Site description

The study was carried out in 2012 during the last week of September and first week of October at a commercial vineyard of 1.43 ha located in Vergalijo (Lat. 42º 27’ 45.96”, Long. 1º 48’ 13.42”, Alt. 325 m), Navarra, Spain. The vineyard was planted with nine different red international cultivars: Cabernet Sauvignon, Carmenere, Caladoc, Grenache, Marselan, Maturana Tinta, Pinot Noir, Tempranillo and Syrah. Grapevines were trained to a vertically shoot-positioned trellis system, with north-south row orientation at 2 m x 1 m inter and intra row distances. Grapevines were planted on Richter 110, with the exception of Tempranillo vines, which were planted on rootstock 3309. Irrigation was routinely and uniformly applied across the season for all cultivars.

Fluorescence sensor and indices

The experimental vineyard was monitored using the fluorescence sensor Multiplex on-the-go™ (MX_M) to assess the spatial variability of chlorophyll, epidermal flavonol and nitrogen content. This vineyard was also monitored with the leaf clip sensor, Dualex4™ (DX4), which served as the reference method and was already described in chapter 2.

The version of the Multiplex™ sensor adapted to be used mounted on a quad or a tractor, named Multiplex On-The-Go™ (Multiplex 321 LD, FORCE-A, Orsay, France) or mounted Multiplex, MX_M hereafter, is synchronised with a GPS that allows georeferencing the fluorescence measurements (figure 30). Along with the fluorescence sensor and the GPS, the FA-BOX is incorporated, which is the component that records the data and synchronize the Multiplex sensor continuous measurements with the GPS. This device measures a surface of 10 cm of diameter from a distance of approximately 20 cm.

Figure 30. The three components of the fluorescence sensor mounted on a quad: Multiplex™ sensor, FA-BOX and GPS (Force-A).

The fluorescence signals and indices provided by the MX_M are the same as those yielded by the hand-held Multiplex™ (MX_H). In this study: SFR, FLAV and NBI, among others. The measurements recorded with the mounted fluorescence sensor are a mix of the adaxial (AD) and the abaxial (AB) sides of the leaves. In this case, unlike the indices from the MX_H that could correspond to the adaxial side, the abaxial side or the whole leaf, there is only one version of each index that will correspond to a mix between the signals of the adaxial and abaxial sides of the leaves.

$$SFR\_R = \frac{FRF\_R}{RF\_R} \tag{9}$$

$$FLAV = \log\left(\frac{FRF\_R}{FRF\_UV}\right) \tag{11}$$

$$NBI\_R = \frac{FRF\_UV}{RF\_R} \tag{12}$$

$$NBI_C\_R = \frac{Chl}{Flav} = \frac{SFR\_R}{FLAV} \tag{26}$$

The explanations about these fluorescence indices and the different variables of their equations have already been descripted in Chapter 2. An exhaustive description of all formulae and equations of the fluorescence indices provided and calculated from the three sensors, DX4, MX_H and MX_M can be found in table S1, in the supplementary data.

Fluorescence measurements

Twenty four rows of the vineyard plot under study were manually monitored with the DX4 as described in Chapter 2. The leaves measured with this device were located at the mid-upper height of the canopy, to satisfy the condition of being at the same height targeted and measured by the MX_M. All rows were monitored on both sides of the canopy with the MX_M.

Data processing and statistical analysis

The data obtained with the MX_M were filtered by discarding readings higher than 4200 mV to avoid possible nonlinearity in the sensor response. A histogram was computed to identify the data corresponding to leaves and to canopy gaps, the latter were removed (figure 31). After the filtering, the data were standardised against a blue plastic-foil standard (Force-A, Orsay, France) in order to compare the data obtained with other fluorescence sensors and data collected under other measuring conditions. Prior to any statistical analysis, outliers were identified and excluded from the dataset by removing those outside two standard-deviation interval.

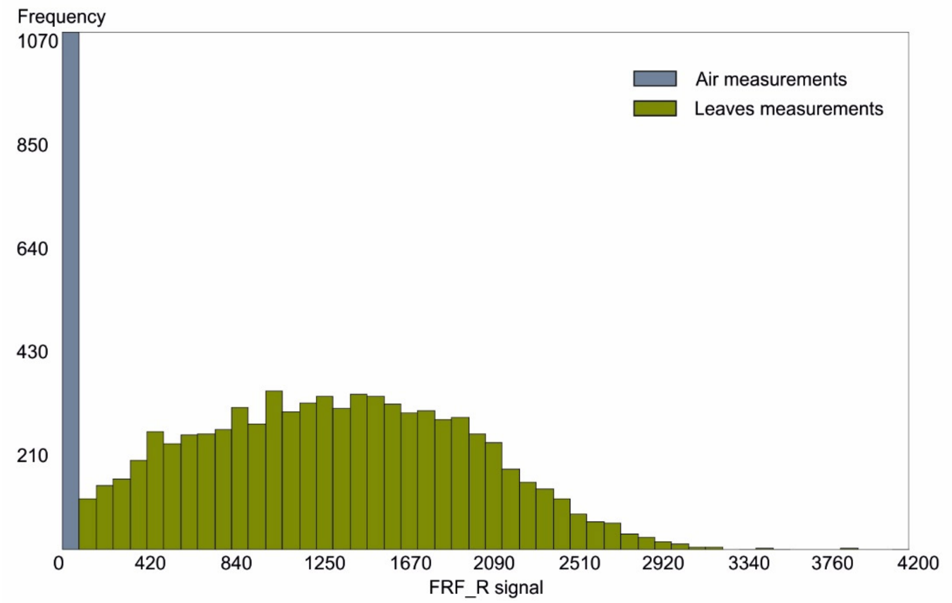

Figure 31. Histogram of the FRF_R signal (far red fluorescence exited by red light) computed for the MX_M data to identify and removed the gaps data from the leaves data.

The next step involved the calculation of the correlations between the MX_M indices and those retrieved with the DX4. For that purpose, as there were more data from the MX_M than from the DX4, and with different geographical coordinates, the data from each device were combined into a grid, generated by aggregation of the nearest points (figure 32). This grid allowed having a common framework to analyse the correlations between MX_M with DX4.

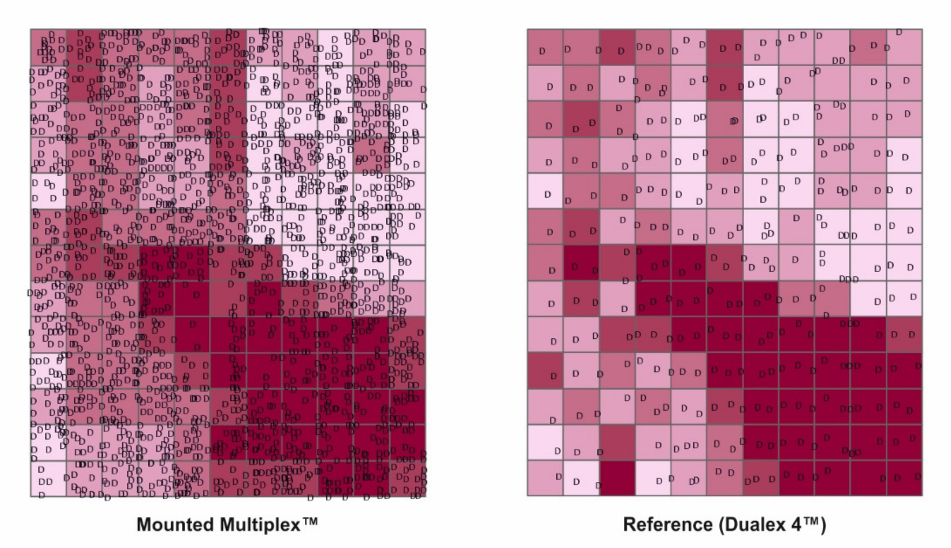

Figure 32. Example of the grid generated to create a common framework for the MX_M (left) and the reference, DX4 (right) data.

Classical global linear correlation models adjusted by ordinary least squares (OLS) were computed to analyse the relationships between the same indices measured with the MX_M and with the reference, DX4. The strength and direction of the association was indicated by the determination coefficients (R^2). Data pre-processing and statistical analysis were carried out using the software Microsoft Office Excel 2013 (Microsoft Corporation, Washington, USA) and Statistica 9 (Stat Soft Inc., Tulsa, USA).

Spatial variability analysis

The spatial behaviour of the indices obtained by the two devices was first analysed by computing the experimental variograms and fitting the best model to it. The parameters of the fitted variograms, range, sill and nugget, were then used to apply the interpolation method of ordinary kriging. These analyses were carried out using the software ArcGis 9.3 (ESRI, Redlands, USA).

RESULTS

Calibration of the mounted fluorescence sensor (MX_M)

In order to ensure the reliability of the MX_M to assess the chlorophyll, nitrogen and flavonol content in grapevine canopies in a dynamic and continuous way, calibration between the indices retrieved with the MX_M and the reference DX4 was needed. The relationships between the MX_M indices and the DX4 indices were studied applying the global regression model, OLS. All the correlations were significant at $P<0.001$ (table 10).

The chlorophyll-related SFR index obtained on-the-go with the MX_M proved to be well correlated with CHL_T from the reference DX4, with R^2 of 0.75. FLAV showed a moderate correlation with the reference. It correlated better with $FLAV_{AB}$ (R^2 = 0.47) than with $FLAV_{AD}$ (R^2 = 0.32). On the other hand, good correlations were also found for the NBI indices (with R^2 from 0.74 to 0.77) (table 10).

Concerning the two different possibilities explored here for the NBI, while for the MX_H the NBI_C was the one reporting the better correlations with the reference (Chapter 2, figure 21), for the MX_M both NBI, the provided by the sensor (eq. 12) and the calculated one (eq. 26), yielded similar results.

Table 10. Global linear models adjusted by ordinary least squares (OLS) for the three indices studied (SFR, FLAV and NBI) derived from measurements with the mounted Multiplex™ (MX_M) versus the reference Dualex4 (DX4). The root mean square error (RMSE) is shown in brackets for each relationship. All the coefficients of the model are significant at *P*-value < 0.001.

Mounted Multiplex™		**Reference (Dualex4™)**		
		CHL_T		
SFR_R	R^2	0.75		
	RMSE	(3.25)		
		$FLAV_{AD}$	$FLAV_{AB}$	$FLAV_T$
FLAV	R^2	0.32	0.47	0.52
	RMSE	(0.035)	(0.15)	(0.15)
		NBI_{AD}	NBI_{AB}	NBI_T
NBI_R	R^2	0.75	0.74	0.76
	RMSE	(1.68)	(3.21)	(1.08)
NBI_C_R	R^2	0.77	0.74	0.77
	RMSE	(1.61)	(3.21)	(1.04)

*The indices with the subscript AD were only measured on the adaxial side of the leaf; the AB subscript indicate that these indices were measure only on the abaxial side of the leaves; and the subscript T indicate that the indices have been calculated for the whole leaf, abaxial and adaxial. The subscript C in the NBI index indicate that this index has been calculated as a division of SFR to FLAV, to differentiate it from the NBI provided by the Multiplex™ device (see Table S1 in the supplementary data).

Comparison of the Multiplex™ sensor used manually and on-the-go.

The comparison of the relationships obtained here between the mounted Multiplex™ (MX_M) against the DX4 with the ones obtained in Chapter 2 between MX_H with the reference, DX4 (figures 19, 20 and 21), evidenced the loss of explained variability when the fluorescence sensor is mounted on a vehicle. The relationships between the SFR_T indices from MX_H and MX_M with the CHL_T from the reference DX4 showed higher R^2 for the MX_H device (R^2=0.92, figure 19D) than for MX_M (R^2=0.75, table 10). These results indicate that a loss of 17 % of information occurred when the MX_M operated on-the-go. Regarding the FLAV index, higher correlations were obtained for the MX_H device (R^2=0.78, figure 20E), in comparison to the MX_M (R^2=0.52, table 10). In this case, the loss of information was 26 % when the MX_M operated on-the-go. Finally, for the NBI, when the NBI_C was calculated, the relationship also reported better results for the MX_H (R^2=0.93, figure 21F) than for the MX_M (R^2=0.77, table 10), losing about 16 % of information when measuring on-the-go. But the NBI_R_{AD} of the MX_H (R^2=0.75, figure 21G) performed the same as the NBI_R retrieved from the MX_M measurements (R^2=0.76, table 10).

Spatial variability

The variograms of the indices studied (Figure 33) showed a similar range, between 55 and 70 m, for nearly all of them. The exception was $FLAV_{AD}$ from DX4, which showed a shorter range (40 m) and the highest nugget effect of all indices (74 % of nugget against less than 50 % for the other indices (data not shown)), which might be related with the short span of the epidermal flavonols of the adaxial side of the leaf, already mentioned in chapter 2. The nugget effect was found to be lower for the indices measured with the DX4, than for the same indices measured with the MX_M. This is probably related with the fact that the MX_M recorded measurements of either adaxial or abaxial sides of the leaves and primary and secondary leaves, shaded and sun-exposed leaves; while the DX4 measured selected primary leaves on both sides, adaxial and abaxial.

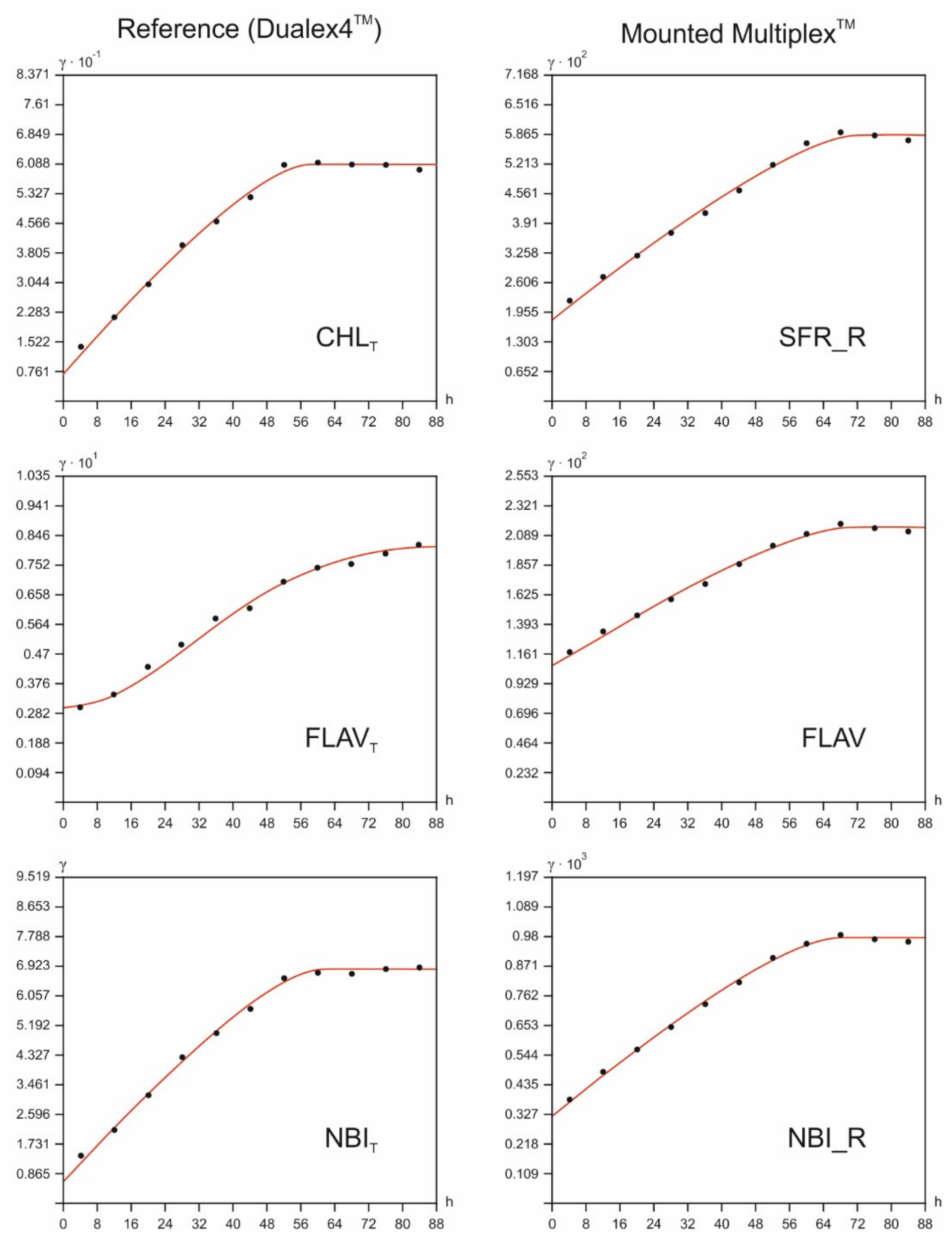

Figure 33. Experimental variograms (black dots) and fitted models (solid red line) for the chlorophyll index (CHL and SFR_R), the epidermal flavonol index (FLAV) and the nitrogen balance index (NBI) of the mounted Multiplex**™** (MX_M) and the reference, DX4.

Figure 34 depicts the krigged maps for the global indices of the two sensors. The maps showed a similar spatial distribution for the three indices studied, independently of the device used. Three differential subareas could be identified. Two of them are located in the upper half of the plot and follow three grapevine rows that were identified as Tempranillo and Grenache rows, going from West to East, respectively. In these two zones, the chlorophyll and nitrogen balance indices showed the highest values and the lowest for flavonols. The third area can be drawn in the lower half of the plot, with an irregular shape perpendicular to the grapevine rows direction, following a sharp change in soil characteristics, regardless the grapevine cultivar or clone planted.

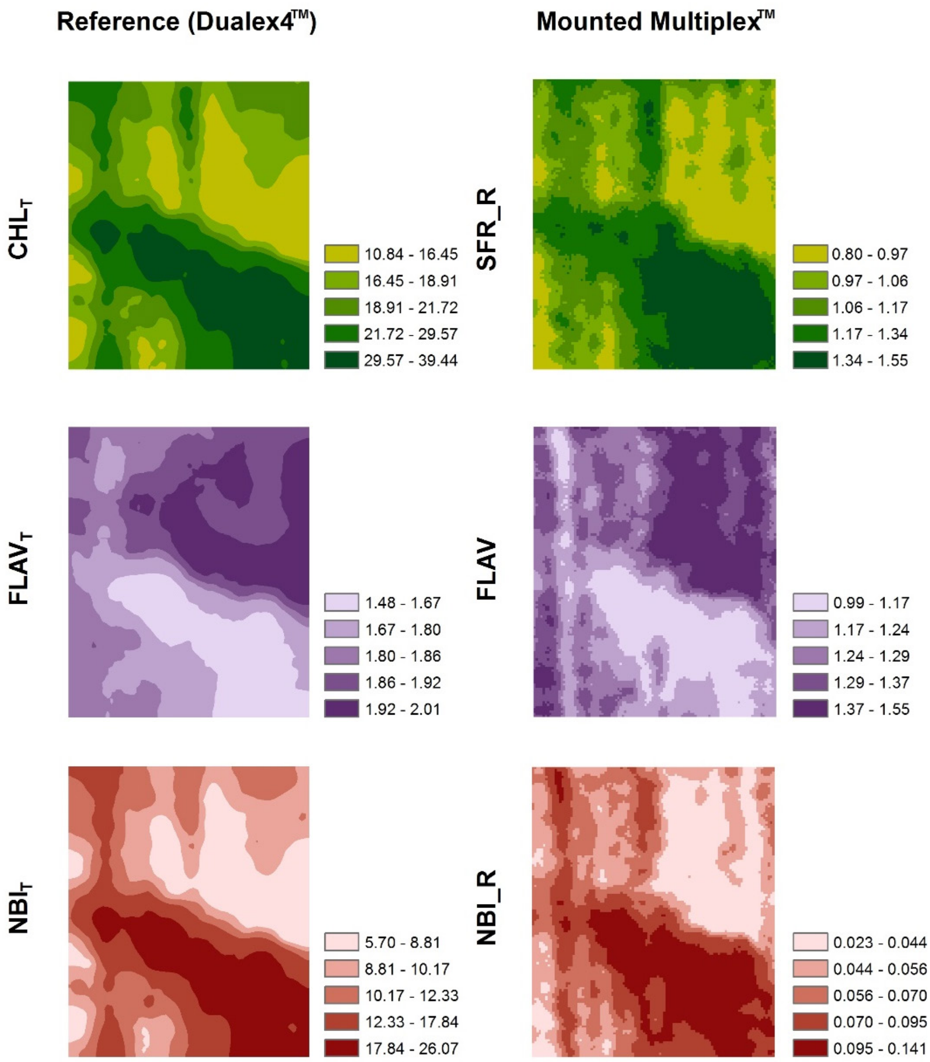

Figure 34. Interpolated surfaces by ordinary kriging of chlorophyll indices (CHL and SFR), epidermal flavonol index (FLAV) and nitrogen balance index (NBI) acquired by DX4 and MX_M. Maps were represented by quantiles.

DISCUSSION

On-the-go monitoring vineyard key parameters related to plant vigour and nutritional status, in a non-destructive, fast and reliable way, would enable the mapping and characterization of the spatio-temporal variability of these variables. This information can be of invaluable help to: i) optimize vineyard management (reduction of inputs and other management costs and application of variable fertilization rates) making it more sustainable; ii) to identify homogeneous-management zones within a vineyard, and iii) to improve the quality of grapes and wine. For these reasons, the evaluation of performance of the mounted Multiplex™ device against a widely verified reference, such as the DX4, to characterize the chlorophyll, epidermal flavonols and nitrogen content in grapevine leaves, was attempted.

Calibration of the mounted fluorescence sensor (MX_M)

This work presents the first evaluation of the on-the-go fluorescence-based Multiplex™ sensor on grapevine leaves, against the Dualex4™ as the reference. The DX4 was chosen as the reference for several reasons already exposed in Chapter 2. In this work, the MX_M has been studied to determine its capability to properly estimate the grapevine leaf chlorophyll, epidermal flavonol and nitrogen concentrations and their spatial variability.

The results obtained in the present study showed that the fluorescence indices measured with the MX_M successfully explained a high percentage of the variance of the same indices measured by the DX4, therefore confirming the capability of the MX_M to assess the chlorophyll, epidermal flavonol and nitrogen contents in grapevine leaves on-the-go. The moderate correlation showed by the FLAV index measured with MX_M with the reference was expected. It is due to the incidence of the light on the adaxial side of the leaves that leads to an accumulation of epidermal flavonols, even saturating. These high values all over the vineyard result in a short span of this index. This fact also explains that the FLAV index measured with MX_M correlated better with the $FLAV_{AB}$ than with $FLAV_{AD}$ of the DX4, as the abaxial side of the leaf is not directly exposed to light and reflects better the variability of the epidermal flavonols, as mentioned for the hand-held devices correlations (Chapter 2).

Several factors can affect the fluorescence measurements performed on-the-go with the MX_M. All of them involve leaf features, such as the side of the leaf exposed to the sensor, leaf exposure to sunlight during growth, and leaf age (i.e. leaf position on the shoot). These three factors are less controlled in on-the-go operations in comparison to manual measurements, as in the latter the leaves to be measured are susceptible to be chosen. Regarding the side of leaf that is measured, even though the MX_M was mainly targeting the adaxial side, their indices were highly correlated to those from the reference DX4. Light exposure of the measured leaves affects the fluorescence indices indirectly, by impacting the leaf sclerophylly. Sun-exposed leaves tend to be thicker than shaded leaves because light exposure induces an increase in the leaf mass per area (LMA), but also an increase in the leaf chlorophyll content (Posada et al. 2009). As regards the NBI index, since it is defined as the chlorophyll to flavonol ratio, the latter being a surrogate of the LMA (Meyer et al. 2006), the NBI index will not be affected by the increase in LMA. The third effect that influences the MX_M measurements would be the leaf age, as the sensor is measuring primary and secondary leaves, without distinction. Primary leaves are older, thicker and less photosynthetically active than secondary leaves. Similarly to the light exposure effect, the NBI, which is a ratio of chlorophyll and flavonol, will be less affected by the leaf age than simple surface-based indices like SFR and FLAV.

All these factors might have been responsible of the loss of explained variance (in terms or R^2) when the MX_M was used instead of the hand-held Multiplex™ (MX_H), reducing the accuracy of the MX_M measurements. With the two hand-held devices (DX4 and MX_H) both the abaxial and the adaxial sides of the leaf are accessible for measurements, therefore indices for the whole leaf can be obtained. By contrast, the MX_M targets the vines from a quad or a tractor in movement, at approximately 20 cm from the canopy leaves. The results showed that in spite of all the factors that are affecting the measurements with the $MX_{M,}$ causing a loss in the explained variability, this fluorescence sensor operating on-the-go is able to reliable estimate the leaf chlorophyll, epidermal flavonol and nitrogen content in grapevines. Therefore, it would be appropriate to apply the mounted fluorescence sensor in precision viticulture, as it will provide with reliable estimations of the vegetative and nutritional status of the vineyard in a continuous way.

Spatial variability

The variogram parameters and the krigged sufaces have revealed that the indices measured using the MX_M showed the same spatial variability as the indices measured by the reference, DX4. Even though the FLAV index measured with the MX_M yielded moderate correlations with the reference, the MX_M can be successfully used to estimate the spatial variability of the leaf epidermal flavonol content within a vineyard.

MX_M was also able to bring out the soil variability within the vineyard plot, and its effect in the plant vegetative growth, just like the reference DX4 did. The sharp soil change detected in the south middle part of the plot was obvious during the field measurements. This change in the soil characteristics was certainly affecting the leaf chlorophyll, flavonol and nitrogen content (van

Leeuwen 2010), therefore it was expected to be prompted by the fluorescence indices. The cultivar effect was also shown by Ben Abdallah and Goffart (2012) for two different varieties of potatoes. Different grapevine cultivars have different genotype that responds differently to the same environment, in the same way plants with the same genotype behave differently in different environments leading, both cases, to different phenotypes (Pearce and Coombe 2004). In the present study, two different varieties showed higher values of chlorophyll and nitrogen contents, and lower values of flavonoids than the rest of cultivars, but an exhaustive trial, including randomised block replication, would be needed to analyse this cultivar effect.

Efficient mapping of key leaf components was demonstrated in this work. Therefore, in the framework of precision farming, the MX_M enables a fast, non-destructive, and reliable on-the-go assessment of the spatial and temporal variability (as several measurements may be conducted along the season) of crucial indicators of the grapevine vegetative and nutritional status. In this way, detection of chlorotic vines, susceptible of additional iron or other mineral amendments may be carried out early in the season, and objective appraisal of the recovering of the plants after mineral spraying can be done with the $MX_{M.}$ It also allows assessing the total flavonols, which provides information about the leaves exposure to light and their potential susceptibility to diseases or pathogens (Agati et al. 2013a, Agati et al. 2008). Furthermore, MX_M allows the assessment of plant N status, which is important for rational management of nitrogen in a sustainable fertilization context. Overfertilization results in lower nitrogen use efficiency, high levels of residual N after harvest, and losses in the environment (leading to groundwater pollution due to NO_3-N leaching (Hashimoto et al. 2007), while N deficiency may lead to photosynthesis diminishment and may jeopardize final grape yield in terms of quantity and quality.

MX_M allows a rapid estimation of the distribution of chlorophyll content, epidermal flavonol content and N status within the vineyard and the delineation of homogeneous subzones for the application of variable rate fertilization strategies. Once the delineation of homogenous management zones is done, a precise quantification of these components may be needed to be able to know the exact concentrations and planed adequate strategies. It should be conducted with either MX_H or DX4 which, by measuring both sides of the leaf, provide the data needed to calculate the leaf chlorophyll, flavonol and N content. So the Multiplex sensor, by using it on-the-go or manually, will provide the grapegrower with two different kind of information, both very important and complementary, to manage the vegetative status of the vineyard: (i) when using it on-the-go, a rapid and reliable delineation of homogenous zones within the vineyard is achieved; (ii) and when using it manually, it allows the grapegrower to know the exact concentrations of leaf chlorophyll, flavonol and nitrogen, so precise interventions, if needed, can be carried out. In addition, either MX_H or MX_M may be used as phenotyping tools, the later in a faster and continuous way, enabling a rapid, reliable and non-destructive assessment of the spatial variability of the vegetative and nutritional status within a vineyard at several timings within the season.

CONCLUSIONS

An exhaustive verification was conducted for the first time to assess the chlorophyll and flavonol content as well as the nitrogen status of grapevine leaves in motion by the fluorescence Multiplex™ sensor.

The mounted fluorescence sensor has proved to be a trustworthy device to assess the chlorophyll, epidermal flavonol and nitrogen content on-the-go in grapevine leaves under field conditions. It was confirmed by the successful performance of the florescence indices provided by this device, despite of the several, not-controlled factors in on-the-go operations, potentially affecting the measurements.

The use of this fluorescence sensor mounted on a motorized vehicle allowed to map vineyard key parameters related to plant vigour and nutritional status in a non-destructive, fast and reliable way in precision viticulture. It will facilitate the study of the spatial variability over time, intra and inter-seasons, enabling the identification and delineation of homogeneous management zones within the vineyard. It has been proved as a suitable and appealing instrument to implement precision viticulture.

4 Conclussions

This PhD Thesis has established the usefulness of remote and proximal sensors to assess the spatial variability of the vegetative status in a vineyard in the scope of precision viticulture. Specifically, the conclusions obtained from this research work are detailed below:

Characterisation of vegetative growth by RPAS multispectral imagery

1. The spectral indices derived from the multispectral imagery obtained by RPAS showed moderate correlations with the vegetative parameters measured in the vineyard. The best correlations were addressed by two newly defined normalised spectral vegetation indices, named NVI_1 and NVI_2, with pruning weight, secondary shoot length, secondary leaf area, leaf chlorophyll content and nitrogen status.
2. Our results revealed the potential of multispectral imagery from RPAS in precision viticulture to assess the vineyard vegetative status but also brought to light some inconveniences related to technological and operational factors of this type of remote imagery.

Vegetative and nutritional status assessed by a hand-held fluorescence sensor

3. The hand-held fluorescence sensor has proved to be a reliable device for the assessment of the grapevine leaf chlorophyll, epidermal flavonol and nitrogen content under field conditions.

4. The computed calibration equations allowed to obtain the leaf chlorophyll concentration. This is an important outcome, as it will enable to determine, in an absolute way, the leaf chlorophyll content of the grapevines, making possible its comparison with the chlorophyll concentration obtained by analytical methods, or the analysis and subsequent establishment of absolute thresholds, which in turn, would support decision making regarding appropriate management strategy for each case.

5. The fluorescence indices, obtained by the hand-held fluorescence sensor, calculated for the whole leaf (adaxial and abaxial) were the best indicators of leaf chlorophyll, epidermal flavonol and nitrogen content. Among all the possibilities to calculate the nitrogen balance index (NBI), the one calculated as the chlorophyll-to-flavonol ratio for the whole leaf was revealed as the best indicator of the grapevine nitrogen status.

6. The hand-held fluorescence sensor allowed characterizing the spatial and temporal variability of leaf chlorophyll content and nitrogen status within a vineyard across the ripening period.

7. Leaf chlorophyll content and nitrogen status variability within the vineyard increased as the season advanced, until stabilising prior to harvest. The nitrogen status showed the largest variability across the vineyard at all dates. On the contrary, the leaf chlorophyll content had similar spatial behaviour along the ripening period.

8. The nitrogen balance index (NBI) could be a suitable indicator to describe the shoot pruning weight variability within the plot and, therefore useful to delineate homogeneous vigour and vegetative growth management zones within the vineyard.

On-the-go assessment of the vineyard vegetative and nutritional status

9. The reliable on-the-go estimation of the chlorophyll, epidermal flavonol and nitrogen content in grapevine leaves by the fluorescence sensor was demonstrated under field conditions.
10. The use of the fluorescence sensor on-the-go allowed to map vineyard key parameters related to plant vigour and nutritional status. Hence, it would enable the identification and delineation of homogeneous management zones within the vineyard.

Global conclusion

Remote and proximal sensing have been proved as a promising alternative to the traditional methods for appraising the vegetative status of the vineyard. Unlike the manual and analytical classical methods, these new technologies allow the measurement of large amounts of data at several timings, enabling the assessment of the spatial and temporal variability of the vineyard required in precision viticulture. The use of the fluorescence sensor would enable the grapegrower a reliable assessment of the vegetative and nutritional status of the vineyard. Furthermore, the successful performance of the fluorescence sensor mounted on a vehicle to assess the vegetative and nutritional status of the vineyard represents a significant advance in the implementation of non-destructive sensors on mobile platforms. This technological progress represents a step forward towards the application of precision viticulture techniques in commercial vineyards.

5 Supplementary Data

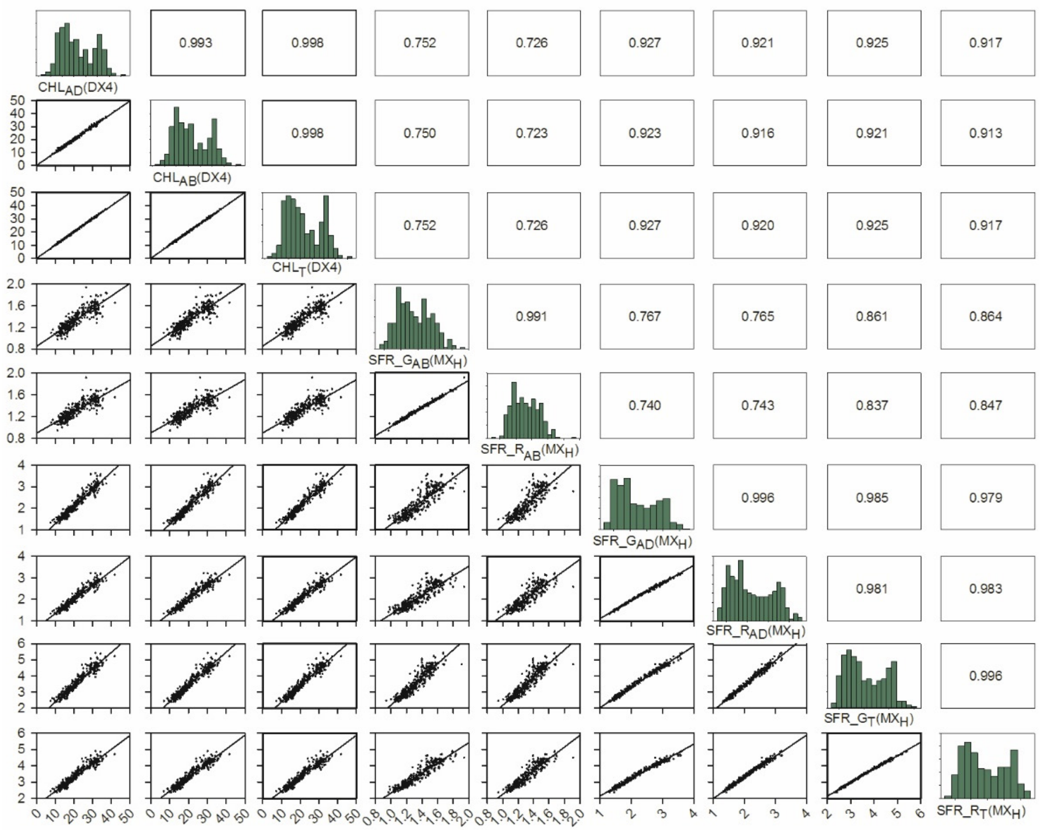

Figure S1. Correlation matrix between the chlorophyll indices of the MX_H and the reference (DX4). CHL_{AD} and CHL_{AB} are the chlorophyll indices for the adaxial and abaxial side of the leaf, respectively, given by the DX4 device. CHL_T is the sum of the adaxial and abaxial side. The simple fluorescence ratio (SFR_G and SFR_R) is the chlorophyll index yielded by the MX_H. Adaxial (SFR_R_{AD} and SFR_G_{AD}) and abaxial (SFR_R_{AB} and SFR_G_{AB}) sides are also taking into account as well as the index of the whole leaf as the sum of each side of the leaf (SFR_G_T and SFR_R_T). The determination coefficient (R^2) of each combination is indicated in the right side of the matrix diagonal, all of them are statistically significant at $P<0.001$ (N=302).

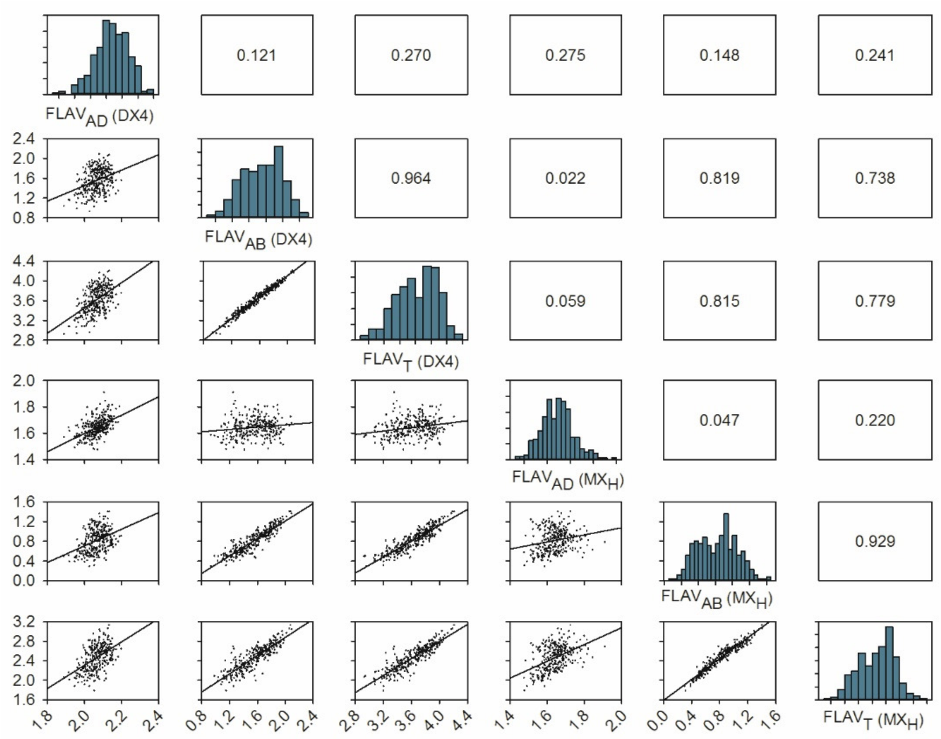

Figure S2. Correlation matrix between the epidermal flavonol indices of the MX_H and the reference (DX4). $FLAV_{AD}$ and $FLAV_{AB}$ are the epidermal flavonol indices for the adaxial and abaxial side of the leaf, respectively. $FLAV_T$ is the sum of the adaxial and abaxial side. The determination coefficient (R^2) of each combination is indicated in the right side of the matrix diagonal, all of them are statistically significant at *P*<0.001 (N=302).

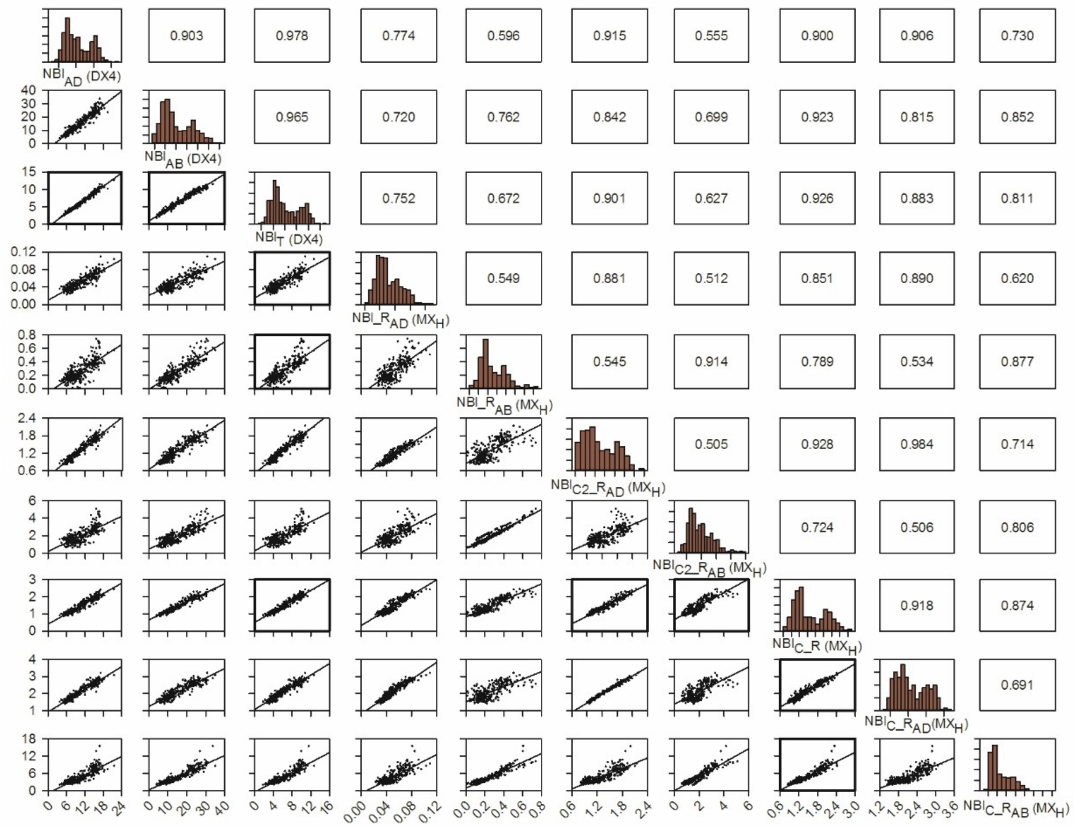

Figure S3. Correlation matrix between the nitrogen balance indices of the MX_H and the reference (DX4), using red as the excitation light. NBI_R_{AD} and NBI_R_{AB} are the nitrogen balance indices for the adaxial and abaxial side of the leaf, respectively. $NBI_C\_R_{AD}$ and $NBI_C\_R_{AB}$ are the nitrogen balance indices calculated as SFR_R_T divided to $FLAV_{AD}$ or $FLAV_{AB}$, respectively. $NBI_{C2}\_R_{AD}$ and $NBI_{C2}\_R_{AB}$ are the nitrogen balance indices calculated as SFR_R_{AD} or SFR_R_{AB} divided to $FLAV_{AD}$ or $FLAV_{AB}$, respectively. The determination coefficient (R^2) of each combination is indicated in the right side of the matrix diagonal, all of them are statistically significant at $P<0.001$ (N=302).

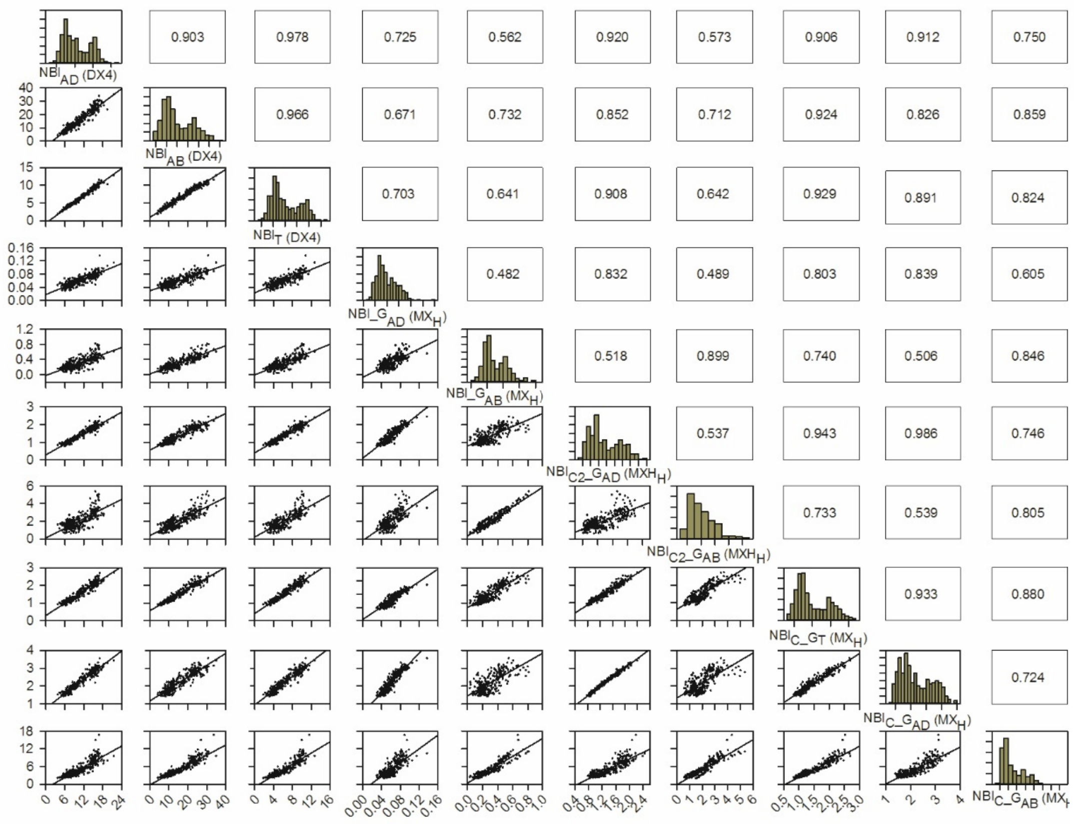

Figure S4. Correlation matrix between the nitrogen balance indices of the MX_H and the reference (DX4), using green as the excitation light. NBI_G_{AD} and NBI_G_{AB} are the nitrogen balance indices for the adaxial and abaxial side of the leaf, respectively. $NBI_C\_G_{AD}$ and $NBI_C\_G_{AB}$ are the nitrogen balance indices calculated as SFR_G_T divided to $FLAV_{AD}$ or $FLAV_{AB}$, respectively. $NBI_{C2}\_G_{AD}$ and $NBI_{C2}\_G_{AB}$ are the nitrogen balance indices calculated as SFR_G_{AD} or SFR_G_{AB} divided to $FLAV_{AD}$ or $FLAV_{AB}$, respectively. The determination coefficient (R^2) of each combination is indicated in the right side of the matrix diagonal, all of them are statistically significant at $P<0.001$ (N=302)

Table S1. Nomenclature and equations of the different chlorophyll, flavonol and nitrogen balance indices provided by the three sensors: Dualex4™ (DX4), hand-held Multiplex™ (MX_H) and mounted Multiplex™ (MX_M); and calculated from the provided indices. The indices with the subscript AD were only measured on the adaxial side of the leaf; the AB subscript indicate that these indices were measure only on the abaxial side of the leaves; and the subscript T indicate that the indices have been calculated for the whole leaf, abaxial and adaxial. The subscript C in the NBI index indicate that this index has been calculated as a division of SFR to FLAV, to differentiate it from the NBI provided by both Multiplex™ devices.

Chlorophyll Indices						
DX4	Adaxial (AD)	Abaxial (AB)	TOTAL			
Provided	CHL_{AD}	CHL_{AB}				
Calculated			$CHL_T = \frac{CHL_{AD} + CHL_{AB}}{2}$			
MX_{HH}	Red excitation (R)			Green excitation (G)		
	Adaxial (AD)	Abaxial (AB)	TOTAL	Adaxial (AD)	Abaxial (AB)	TOTAL
Provided	$SFR\_R_{AD} = \frac{FRF\_R_{AD}}{RF\_R_{AD}}$	$SFR\_R_{AB} = \frac{FRF\_R_{AB}}{RF\_R_{AB}}$		$SFR\_G_{AD} = \frac{FRF\_G_{AD}}{RF\_G_{AD}}$	$SFR\_G_{AB} = \frac{FRF\_G_{AB}}{RF\_G_{AB}}$	
Calculated			$SFR_{R_T} = SFR\_R_{AD} + SFR\_R_{AB}$			$SFR\_G_T = SFR\_G_{AD} + SFR\_G_{AB}$
MX_M	Red excitation (R)			Green excitation (G)		
Provided	$SFR\_R = \frac{FRF\_R}{RF\_R}$			$SFR\_G = \frac{FRF\_G}{RF\_G}$		

Flavonol Indices			
DX4	Adaxial (AD)	Abaxial (AB)	TOTAL
Provided	$FLAV_{AD}$	$FLAV_{AB}$	
Calculated			$FLAV_T = FLAV_{AD} + FLAV_{AB}$
MX$_{HH}$	Adaxial (AD)	Abaxial (AB)	TOTAL
Provided	$FLAV_{AD} = \log\left(\frac{FRF\_R_{AD}}{FRF\_UV_{AD}}\right)$	$FLAV_{AB} = \log\left(\frac{FRF\_R_{AB}}{FRF\_UV_{AB}}\right)$	
Calculated			$FLAV_T = FLAV_{AD} + FLAV_{AB}$
MX$_M$ (provided)	$FLAV = \log\left(\frac{FRF\_R}{FRF\_UV}\right)$		

NBI						
DX4	Adaxial (AD)	Abaxial (AB)	TOTAL			
Provided	$NBI_{AD} = \frac{CHL_T}{FLAV_{AD}}$	$NBI_{AB} = \frac{CHL_T}{FLAV_{AB}}$				
Calculated			$NBI_T = \frac{CHL_T}{FLAV_{AD} + FLAV_{AB}}$			
MX_H	Red excitation (R)			Green excitation (G)		
	Adaxial (AD)	Abaxial (AB)	TOTAL	Adaxial (AD)	Abaxial (AB)	TOTAL
Provided	$NBI\_R_{AD} = \frac{FRF\_UV_{AD}}{RF\_R_{AD}}$	$NBI\_R_{AB} = \frac{FRF\_UV_{AB}}{RF\_R_{AB}}$		$NBI\_G_{AD} = \frac{FRF\_UV_{AD}}{RF\_G_{AD}}$	$NBI\_G_{AB} = \frac{FRF\_UV_{AB}}{RF\_G_{AB}}$	
Calculated	$NBI_{C2}\_R_{AD} = \frac{SFR\_R_{AD}}{FLAV_{AD}}$	$NBI_{C2}\_R_{AB} = \frac{SFR\_R_{AB}}{FLAV_{AB}}$	$NBI_C\_R_T = \frac{SFR\_R_T}{FLAV_T}$	$NBI_{C2}\_G_{AD} = \frac{SFR\_G_{AD}}{FLAV_{AD}}$	$NBI_{C2}\_G_{AB} = \frac{SFR\_G_{AB}}{FLAV_{AB}}$	$NBI_C\_G_T = \frac{SFR\_G_T}{FLAV_T}$
	$NBI_C\_R_{AD} = \frac{SFR\_R_T}{FLAV_{AD}}$	$NBI_C\_R_{AB} = \frac{SFR\_R_T}{FLAV_{AB}}$		$NBI_C\_G_{AD} = \frac{SFR\_G_T}{FLAV_{AD}}$	$NBI_C\_G_{AB} = \frac{SFR\_G_T}{FLAV_{AB}}$	
MX_M	Red excitation (R)			Green excitation (G)		
Provided	$NBI\_R = \frac{FRF\_UV}{RF\_R}$			$NBI\_G = \frac{FRF\_UV}{RF\_G}$		
Calculated	$NBI_C\_R = \frac{SFR\_R}{FLAV}$			$NBI_C\_G = \frac{SFR\_G}{FLAV}$		

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