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Comparison of plant proximal sensing approaches for nitrogen supply detection in crops

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Abstract

Nondestructive proximal sensors can be an efficient source of information of N status in crops for localized and rapid adjustment of fertilization applications. The aim of this study was to compare two transmittance/reflectance-based sensors (SPAD, ASD) and a florescence-based sensor (Multiplex) in their ability to measure N content in corn (*Zea mays* L.), spring and winter barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.), both at the leaf and canopy level. Measurements of leaves and canopies from six fertilization field trials in 2019 and 2020 were analyzed to establish relationships between sensor information and laboratory-determined N content in crops. Analyses included linear regression for single sensor variables and machine learning for multivariate approaches, to assess the relative accuracy of the proximal sensors to measure N. The ASD is time-intensive and requires post hoc analyses of the spectra. However, the spectral outputs of this device were clearly correlated with the N status of leaves and canopies. At the leaf level, SPAD showed higher accuracy than any of the single Multiplex variables to predict plant N. Multiplex performance could be improved by combining three of its variables. At the canopy level, interpolated SPAD values and the best-performing Multiplex variables showed similar accuracy. It could be concluded that the relationship sensor-N status is species specific. Despite the high standard deviation recorded in some raw Multiplex variable, the derived indices showed a comparable low standard deviation. At both, leaf and canopy levels an integrated sensor solution would combine the multidimensionality of Multiplex and ASD, and the accuracy and practicality of SPAD.

1 | INTRODUCTION

Fast and reliable ways of measuring N content in plants are necessary to monitor plant development and to efficiently manage crop production, especially in the case of precision

Abbreviations: FLAV, flavonoid index; GPR, Gaussian processes regression; NBI, nitrogen balance index; NRMSE, normalized root mean square error.

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agriculture. While in traditional agriculture a general knowledge of the field suffices to optimize crop production, precision agriculture needs efficient methods to determine the needs for resources at multiple sites on the field and at different times. To this end, non-destructive proximal sensors seem particularly suited for efficient assessment of crops' N status, for local, rapid adjustment of fertilization applications. Following this, the measuring device, Multiplex (Force-A), based on leaf fluorescence was developed and commercialized. Multiplex was designed to estimate plant chlorophyll (Chl) and other pigments by measuring fluorescence directly on leaves or fruits, or on parts of the canopy. SPAD (SPAD-502, Konica Minolta Sensing, Inc.) is a well-known, nondestructive Chl meter, widely used for N measurements in plants (Padilla, Gallardo, et al., 2018). Given the small size of SPAD's sampling area, however, the sensor is very sensitive to leaf irregularities, veins and spots, and therefore, some experience and multiple measurements are necessary to obtain a reliable reading that can be compared with other situations. In this context, the development of new analytical methods offers an opportunity to explore the performance of such devices.

The focus of this study was to determine the relative accuracy of Multiplex to estimate N in crops by applying the device in the so-called leaf and canopy mode. Based on the principle that the production of pigments in plants is intimately correlated to the N content, and therefore, that lower amounts of the former is a good indicator of N deficiencies, it is worth exploring if this dynamic is depicted when the measurement target differs.

Previous studies have tested Multiplex as a predictor of N content in turfgrass species (Agati et al., 2013), rice (*Oryza sativa* L.) (Li et al., 2013), corn (*Zea mays* L.) (Longchamps & Khosla, 2014), wheat (*Triticum aestivum* L.) (Martinon et al., 2011; Zecha et al., 2017), sweet pepper (*Capsicum* spp.) (Padilla, de Souza, et al., 2018) and cucumber (*Cucumis sativus* L.) (Padilla et al., 2016), in different settings, with different objectives and varied results. One of the challenges with previous studies is the lack of clear contrast between canopy and leaf level measurements, which makes it difficult to understand the exact conditions at which Multiplex can be most efficient. In this study, whenever possible, we analyzed both targets of measurements, leaf and canopy.

Most studies assume some Multiplex indices, such as the Nitrogen Balance Index, the Simple Chlorophyll Fluorescence Ratio, and the Flavonoid Index, to be the best correlated with N content and consequently measuring accuracy tests are based on one or more of these indices. These assumptions tend to disregard the potential use of any of the remaining 12 or more parameters provided in Multiplex readings. In this study, the possibility of combining variables and indices is considered to fully exploit Multiplex's potential. Secondly, the objective was to test the accuracy and applicability of SPAD and Multiplex to measure N. Besides measurement accuracy, the sensor applicability is closely linked to the target of measurement sensors are designed for. For example, Multiplex can measure both individual leaves and canopies, whereas SPAD is only designed for leaf measurements. A question that follows is whether SPAD measurements can be effectively interpolated to estimate canopy N values. In this study, relationships between sensors and N content were addressed for leaf and canopy levels separately. Exemplarily, the performance of a field spectrometer was assessed for comparison at selected trials.

Core Ideas

- Results confirm crop-specific relations between sensor signals and total N content.
- Results show clear differences in this relation between leaf and canopy level.
- To exploit the total output of Multiplex a multivariate approach is recommended.
- At leaf level SPAD reflects most accurately crop N status, but requires an active user involvement and knowledge.
- At canopy level SPAD and Multiplex seem similarly accurate, although Multiplex measures faster and more reliable.

2 | MATERIAL AND METHODS

2.1 | Sensors

The Multiplex sensor (Force-A) (Ben Ghozlen et al., 2010) was used to measure leaf and canopy fluorescence and determine its relationship with crop N content. Multiplex records fluorescence at three different wavelengths, blue-green, red, and infrared, which can be excited with ultraviolet, blue, green, and red wavelengths, producing an excitation-emission matrix of 12 basic or "raw" variables. Additionally, Multiplex provides several band combinations or indices: two chlorophyll indices, a flavonoid index, a "FER" index, two anthocyanin indices, and two variants of the Nitrogen Balance Index (Ben Ghozlen et al., 2010). Due to an acquisition time of more than 250 measurements per second, standard deviations of all parameters are also provided in the Multiplex outputs. Leaf measurements were done by placing leaves' adaxial side up on a black background (which was previously used as reference for calibration) and resting the device on each leaf. The Multiplex sensor also allows for canopy measurements by holding the sensor unit horizontally (90° from nadir) to the

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TABLE 1 Overview of trials and available data at the leaf and canopy level; trial names are explained in Section 2.2

Feature	MUCO19	MACO20	MUCO20	MUSB19	MUWB19	MAWR19
Trial	Corn	Corn	Corn	Spring barley	Winter barley	Winter rye
	3 treatments (0, 120, and 160 kg N ha^{-1})	4 treatments (0, 70, 140, and 160 kg N ha^{-1})	1 treatment (120 kg ha^{-1})	2 treatments (0 and 90 kg N ha^{-1})	2 treatments (0 and 80 kg N ha^{-1})	4 treatments (0, 90, 120, and 160 kg N ha^{-1})
Measurements	Multiplex, SPAD, ASD, N	Multiplex, SPAD, N	Multiplex, SPAD, N	Multiplex, SPAD, ASD	Multiplex, SPAD, ASD	Multiplex, N
Leaf level	N = 75 (5 leaves x 5 plots x 3 treatments)	N = 16 (2 leaves, pooled x 4 plots x 4 treatments)	N = 6 (2 leaves, pooled x 6 plots)	N = 40 (20 leaves per treatment)	N = 20 (10 leaves per treatment)	—
Canopy level	N = 15 (5 plots x 3 treatments)	—	—	N = 8 (2 replicates x 4 treatments)	N = 4 (2 replicates x 2 treatments)	N = 12 (3 samples x 4 treatments)

standing crops with a measuring distance of approximately 10 cm. Canopy measurements were conducted stationary at different locations per plot. Sampling approaches varied with respect to the settings and plot sizes of considered field trials (see following sections).

The SPAD-502 device (Konica Minolta Sensing, Inc.) (Parry et al., 2014) measures leaf photosynthetically active radiation transmittance through a sensor that comes into contact with both sides of the leaf, providing a unit-less value correlated with leaf chlorophyll content (Frampton et al., 2013). Measurements on selected leaves were done midway between the base and the tip of each leaf, avoiding leaf veins. SPAD values were interpolated to a canopy by simply calculating the average out of single leaf measurements to allow for a comparison of SPAD and Multiplex values at the canopy level.

The FieldSpec 4 field spectrometer (ASD Inc.) was used to measure leaf and canopy reflectance in wavelengths from 350 to 2500 nm. For leaf measurements, a leaf clip was attached to the sensor to provide an enclosed environment with artificial illumination. The device was set to provide the average of 10 automatic readings per leaf sample (previously calibrated with a white reference enclosed in the leaf clip). Proximal canopy reflectance was measured by holding the FieldSpec's optic perpendicular to the ground surface at approximately 50 cm above the canopy. Field spectrometer measurements were conducted only at selected trials for exemplary comparison with SPAD and Multiplex (see below).

2.2 | Field trials and total N

Sensor measurements were conducted on six field trials, covering four crops (corn, spring and winter barley [*Hordeum vulgare* L.], and winter rye [*Secale cereale* L.]) at up to four different N fertilization levels. Data was gathered during the vegetation period of 2019 and 2020 and together with the corresponding sensor measurement leaf and biomass samples were collected for analyzing the reference total N content of the crop by applying a general elemental analysis method. Throughout the text it is referred to as crop N as well as canopy or leaf N content, depending on the respective target of sensor measurements. For comparison with leaf-based measurements a specified number of representative leaves has been collected, measured directly with sensors, and prepared for N analysis. For comparison with canopy-based measurements aboveground biomass samples were taken, weighted, and prepared for N analysis. Due to varying settings and plot sizes of field trials the sampling approach differed. Detailed information is provided in the following subsections and Table 1 provides an overview of the data finally available for evaluation.

2.2.1 | Long-term field experiment Muencheberg (MUCO19)

The long-term field experiment (LTFE) at experimental station Muencheberg, Germany, consists of 168 plots of 5 by 5 m size each, treated with randomly assigned levels of mineral and organic N fertilization (Ellerbrock et al., 2016; Ellerbrock et al., 1999; Rogasik et al., 1997). Treatments sampled in this study included: no N fertilization ("zero"), 120 kg ha^{-1} kg of mineral N fertilization ("conventional"), and 160 kg ha^{-1} of mineral N fertilization plus 12.8 t ha^{-1} manure ("high"). In 2019, when corn was grown, 15 plots at the three fertilization levels (five plots each) were randomly chosen and Multiplex measurements in canopy mode were carried out by taking five readings at the center of each plot. Five plants from the central rows of each plot were selected, and from them the most recent, fully expanded healthy leaf was harvested (15 × 5 = 75) and measured directly with Multiplex

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in leaf mode and SPAD. Out of the 15 plots, a subsample of nine plots, three from each of the treatment levels was taken to measure leaf spectra with ASD FieldSpec spectrometer (5 leaves × 9 plots = 45 spectra). All 75 leaves were then taken to the lab for total N analysis.

2.2.2 | Field trial project I4S (MAWR19 and MACO20)

The field trial at experimental station Marquardt, Germany, was established within the I4S project (BMBF BonaRes program 031B0513I) in 2017 to observe the effects of mineral N fertilization on different crops. The entire 0.3 ha large field is divided into 16 plots of 12 × 4.5 m size at four different fertilization levels ("zero", "low", "conventional", "high") with four repetitions. In 2019, winter rye was grown at four fertilization levels: no N input, 90, 120, and 160 kg ha^{-1} N. Multiplex measurements were taken five times along a central transect of each plot in the canopy mode. A sample of aboveground biomass at two central, regularly spaced quadrats of 0.50 m^2 was taken at each of the 16 plots, pooled per plot and taken to the lab for N analysis (MAWR19).

In 2020, silage corn was grown and each of the four repetitions was fertilized with 0, 70, 140 and 160 kg ha^{-1} N. At mid-season, when corn was about 1 m tall, one point at each plot was randomly placed to collect the most recent, fully expanded healthy leaf from two plants to be measured with Multiplex in leaf mode and SPAD. After measurements, each leaf pair was pooled and taken to the lab for N analysis (MACO20).

2.2.3 | Field trials project BarleyIT (MUSB19 and MUWB19)

Two barley field trials established within the BarleyIT project at ZALF experimental station Muencheberg, Germany, were used for measurements. Eight spring barley plots of 15 × 15 m each were selected for measurements, two without fertilizer, two with 30 kg ha^{-1}, two with 60 kg ha^{-1}, and two with 90 kg ha^{-1} mineral N. The corresponding treatments are named "zero", "conventional" (30 and 60 kg N ha^{-1} plots merged), and "high", respectively. Additionally, four winter barley plots, 30 × 20 m each, two with no fertilization ("zero" treatment) and two with 80 kg ha^{-1} ("conventional" treatment) of mineral N were selected. At each of the 12 plots, 10 readings of Multiplex in canopy mode at the central part of the plot were taken. Also, three ASD FieldSpec canopy measurements were taken at the center of the same plots. Then at each plot, all plants from four regularly spaced quadrats of 0.25 m^2 were harvested, pooled per plot and taken to the lab for N analysis. For the Multiplex leaf mode measurements, the most recent, fully expanded healthy leaf from 10 plants from each described spring and winter barley plot was randomly collected. Leaves were read additionally with SPAD and with the ASD FieldSpec spectrometer.

2.2.4 | Field trial ZALF corn (MUCO20)

A field (160 × 46 m) sown with silage corn at ZALF experimental station Muencheberg, Germany, in 2020 was chosen to assess the intrinsic variability in uniform corn fields (no treatments). Six points regularly spaced, each representing an area of 53 × 23 m were placed on the field, and on each point the most recent, fully expanded leaf from two plants was collected for Multiplex measurements in leaf mode and SPAD, and then taken to the lab for N analysis.

2.3 | Data analysis

Leaf and canopy N content was considered as the reference variable to be compared with sensor measurements. The ASD field spectrometer measurements were used to generally understand the reflectance properties of leaves and canopies with different N content. The SPAD values and individual Multiplex variables (both, raw sensor outputs and indices) measured in crop leaves and canopies were contrasted with N content at the leaf level. Multiplex canopy level measurements were compared with plot averages of SPAD and N leaf measurements to interpolate to the canopy level. Variables were tested for normality using the Shapiro–Wilk test (Shapiro & Wilk, 1965). Spearman's correlation coefficient was chosen to determine the tightness of relationships. Additionally, Tukey's HSD test was applied to determine the significance of differences between values observed at varying N fertilization levels. Overall, the significance level was defined by $p = .05$. In case of high correlations, regression analyses were conducted and the adequacy of the model was assessed by the coefficient of determination (R^2) and the residuals' distribution. Analyses were done using the R software (R Core Team, 2014).

For the multivariate analyses to relate Multiplex outputs with N at the leaf level, first, a ranking of relevant fluorescence variables was established by means of the Gaussian processes regression (GPR) algorithm, a nonparametric machine learning procedure appropriate for tests with smaller sample sizes (Sinha et al., 2020; Upreti et al., 2019; Verrelst et al., 2015). Relevance was determined by building N ∼ fluorescence models first with all Multiplex variables and gradually reducing the number of variables until reaching a univariate model. To assess the relative performance of models with different dimensionality, alternative models were compared by the normalized root mean square error (NRMSE) (Equation 1),

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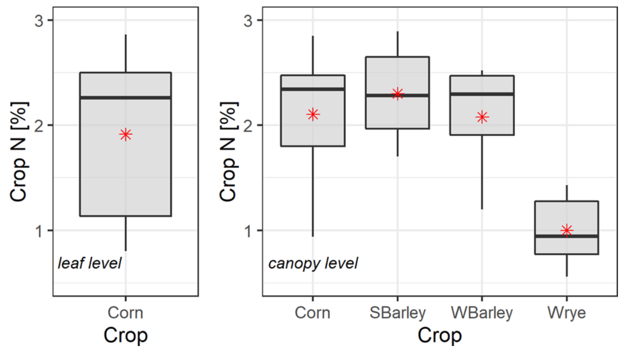

FIGURE 1 Overall variability of measured crop N content based on the leaf level (left side: MUCO19, MUCO20, MACO20) and canopy level (right side: MUCO19, MUSB19, MUWB19, MAWR19); boxplots display the median, two hinges (25th and 75th percentile), and two whiskers (minimum, maximum); red asterisk = mean value

$$\mathrm{NRMSE} = \frac{\sqrt{\frac{\left(X_{\mathrm{pred}} - X_{\mathrm{obs}}\right)^2}{N}}}{\left(X_{\max} - X_{\min}\right) 100} \tag{1}$$

where X_{pred}, X_{obs}, $X_{\max}$, and $X_{\min}$ are predicted, observed, maximum, and minimum values of N, respectively, and N is the number of observations. Once a set of best performing models was chosen, nine machine learning algorithms were applied on these selected models to evaluate the possibility of improving the relationship fluorescence-N by choosing the appropriate algorithm. Algorithms used included: Bagging Trees, Boosting Trees, GPR, Gradient Boosting/Boosted Trees, Partial Least Square Regression, Principal Component Regression, Random Forest Tree Bagger, Regression Trees, and Relevance Vector Machine (Upreti et al., 2019; Verrelst et al., 2015). Model and algorithm performances were assessed by their adjusted coefficient of determination (R^2) and the NRMSE obtained from training and validation data, each split of 70 and 30%, respectively. A K-fold cross validation approach (with K = 10) was applied on selected models to further assess their accuracy in a comparative way, independently of the algorithm used to produce them. The implementation of all the MLRAs was performed with the Matlab ARTMO Toolbox version 3.28. With respect to the canopy-based observations, crop-related sample sizes were not sufficient for conducting the described multivariate analysis.

3 | RESULTS

3.1 | Plant N content

The range of observed N content was highest for corn (both for leaf and canopy; Figure 1), followed by winter barley (1.20–2.52 % N), spring barley (1.70–2.89 % N), and winter rye (0.56–1.43 % N). The lower level of N supply for winter rye compared with the other crops might be due to sampling at the time of ripening. In terms of the distribution of N for each crop, the difference between median and mean is the highest for corn. In order to set up valid regression functions for the prediction, the distribution of N measurements per crop and level (leaf, canopy) was tested for normality. Results of Shapiro–Wilk's normality tests indicated normally distributed N values for winter rye, winter barley, and spring barley (Table 2), whereas leaf and canopy samples of corn showed non-normally distributed N values.

TABLE 2 Results of the Shapiro–Wilk test for normality of measured N content in crop; overall *p*-value canopy = .002

Crop	Level	W	*p* value
Corn	leaf	.87	<.05
Corn	canopy	.84	<.05
Spring barley	canopy	.95	.71
Winter barley	canopy	.83	.17
Winter rye	canopy	.91	.19

Significant differences in the mean of observed N contents per treatment can be seen in selected trials (Supplemental Figure S1). Corn leaf N contents at MUCO19 ranged from 0.81 to 2.86 N percentage dry weight, with a clear effect of fertilization, where the zero, conventional, and high treatment showed means of 1.06, 2.18 and 2.49 N percentage dry weight, respectively. Here, Tukey's HSD test explicitly identified differences between all the three treatment groups. At MACO20, Tukey's HSD revealed significant differences in N contents between the zero treatment and all others. The respective N means are 1.83 N percentage dry weight for the zero treatment, 2.39 N percentage dry weight for the low, 2.56 N percentage dry weight for the conventional, and 2.65 N percentage dry weight for the high treatment. At the same field, winter rye canopy N contents significantly differed between the zero, the low, and the two highest N fertilization treatments (conventional and high) resulting in 0.59 N percentage, 0.85 N percentage, 1.17 N percentage, and 1.38 N percentage dry weight, respectively.

3.2 | Plant N content and spectral properties

The ASD reflectance spectra indicated that leaf and canopy reflectance varied concomitantly with N content in tissues. There was a clear leaf optical properties response to fertilization treatments, which in turn were reflected in differences in N content. Leaf spectra showed a trend towards higher absorbance at all wavelengths with increasing N in leaves (Supplemental Figure S2). Also, higher canopy N content seemed to correspond to decreases in red reflectance, increase

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in near-Infrared reflectance and a corresponding increase in the near-infrared/red ratio (Supplemental Figure S3). Both leaf and canopy spectral variability at the wavelengths at which Multiplex (435, 685, and 735 nm for emission bands B, R, and FR respectively) and SPAD (650 and 942 nm) operate, led to the expectation of these sensors to be sensitive enough to changes in N content in crops. A trend towards increasing green and NIR reflectance with increasing N was evident. This however, rather than corresponding to the typical spectral response to changes in chlorophyll is more likely the effect of spectral mixture with soil background, as lower chlorophyll contents also correspond with lower plant density.

3.3 | Leaf N content and sensors' outcome

3.3.1 | Univariate analysis

In contrast to the SPAD device, Multiplex produces a total of 21 variables at each measurement. Considering each of the 21 output variables separately, the two nitrogen balance indices (NBI-R, NBI-G) and the flavonoid index (FLAV) showed tighter correlations with leaf N according to Spearman's rho (Supplemental Table S1), especially when taking all corn experiments together or focusing only on the MUCO19 trial. Thereby, FLAV decreases with increasing N content while NBI-R and -G increase with increasing N content. The same variables, however, showed rather low Spearman's rho at MACO20 and MUCO20. For the uniformly fertilized sample of MUCO20, the BGF-G, the SFR-G, the SFR-R, and ANTH-RG showed the highest values for Spearman's rho (−.73 to −.88). For the MACO20 sample Spearman's rho did not exceed −.54. The overall correlation results are mainly influenced by the relatively high sample size observed at MUCO19.

The SPAD measurements resulted generally in relatively higher and more consistent values for Spearman's rho, with coefficients of −.83(at MUCO19), −.73(at MACO20), and −.83(at MUCO20). Based on these results, Multiplex' leaf-based FLAV, NBI-R, NBI-G and SPAD values were analyzed for significant differences across fertilization treatment (Supplemental Figure S4). Because no N measurements were directly involved in this analysis, all leaf-based Multiplex measurements presented on Table 1 were used. Regarding the FLAV index observed on corn leaves, a significant difference of means could be seen between the conventional and high fertilization treatment ($p < .005$, Tukey HSD) as well as between the zero and high N supply ($p < .005$, Tukey HSD). For leaf-based NBI-R and NBI-G values, a significant difference could only be verified between the low and the conventional treatment ($p < .005$, Tukey HSD). Regarding the SPAD values measured on corn leaves, a significant difference of means was found between the two lower and the two higher fertilization treatments ($p < .005$, Tukey HSD). In the case of spring barley leaves, Tukey's HSD resulted in significant differences between all considered treatments for Multiplex' FLAV ($p < .005$) with lowest sensor values for the "conv" treatment. Similar results were observed for NBI-R, NBI-G, and SPAD: The zero-fertilization treatment differed significantly from the other treatments ($p < .005$). In the case of winter barley leaves, no significant difference was found for FLAV ($p = .07$), whereas the NBIs and SPAD values varied significantly between the two considered fertilization treatment ($p < .05$).

Focusing on the corn field trials (Supplemental Figure S5) at MACO20 neither FLAV, NBI-R nor NBI-G showed a significant difference between the zero-N application and the other treatments, contrasting with the N distribution per treatment observed previously (Supplemental Figure S1). In the case of field trial MUCO19, SPAD values more closely reflect the pattern of N than the NBI values. The variation between treatments was more pronounced for FLAV, albeit in the opposite direction. When considering the total corn sample, Shapiro–Wilk's test for normality confirmed a normal distribution for measured FLAV ($p = .09$) and rejected it for NBI-R, NBI-G, and SPAD ($p < .005$). Despite of these potential violation to the test's assumptions, a regression analysis for the prediction of N from each of these four variables was carried out for exploratory purposes. The regression model to determine N content from FLAV showed an exponential decline of this variable with increasing corn N content ($R^2 = -.65$) and a residual standard error of 0.43 N % dry weight (Figure 2). Both N balance indices (NBI-R and -G) showed a logarithmic relationship with a coefficient of determination of $R^2 = -.71$ and −.67, respectively, and a standard error of 0.35 and 0.38 N percentage dry weight. The N-NBI relationship was stronger at low N values, but above 2.00 percentage N content the prediction ability of NBI dropped. It has to be mentioned, that due to the selected fertilization treatments, there was a gap of N values between 1.50 and 2.00 N percentage dry weight. Hence, the effect of two point clouds corresponding to the zero and higher fertilization levels might contribute considerably to the strength of the relation. The SPAD measurements showed a tight relationship with N at the leaf level ($R^2 = -.87$) (Figure 2), best explained by an exponential function, with a residual standard error of 0.28 percentage dry weight.

For further assessment of the univariate regression models, a Shapiro–Wilk test of the residuals and residual plots were used. Test results suggested a normal distribution of residuals with p values of .16 (FLAV), .36 (NBI-R), .19 (NBI-G), and .50 (SPAD). In contrast, plots of residual vs. predicted values (Figure 3) seem to show some patterns, casting doubts on the appropriateness of the models (e.g., irregular scattering of residual values around the zero line).

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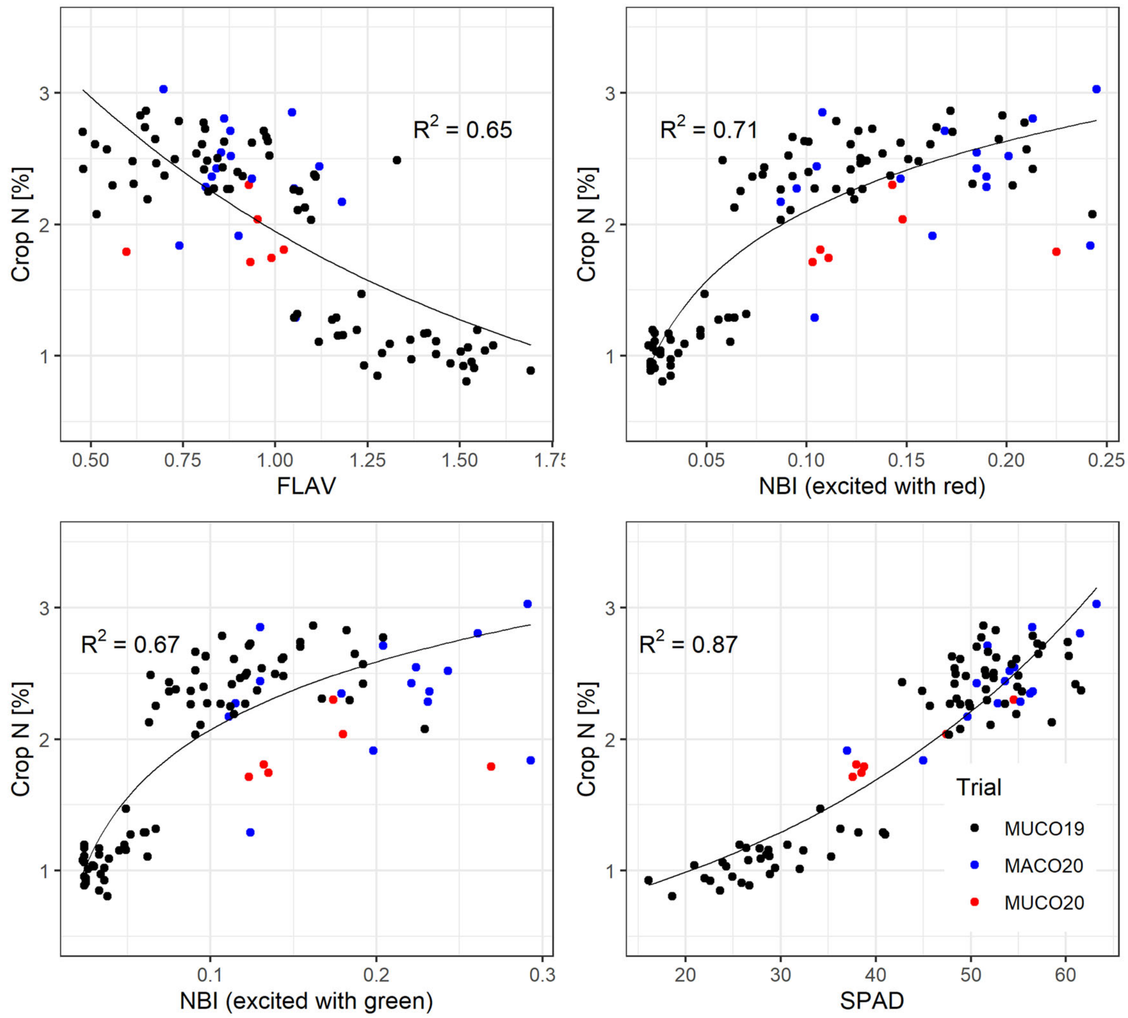

FIGURE 2 Regression models for N content vs. Multiplex flavonoid index (FLAV), Multiplex nitrogen index (NBI-R), NBI-G, and SPAD at leaf level in corn

3.3.2 | Multivariate analysis

The ranking of relevant Multiplex fluorescence variables carried out with GPR, showed that the lowest error (NRMSE = 17.27) corresponded to a model with three variables, FRF-R, SFR-G, and FLAV. The first of which is a "raw" variable; the second, a chlorophyll index; and the third, the flavonoid index that was among the variables with the highest N correlation values. Surprisingly, the NBI-G index, a widely used parameter to estimate N content was last included in the model with six variables. A model with only one variable, which was chosen to be FLAV, yielded a relatively very high error of 20.87, indicating that any univariate model to predict N may not be the most adequate option. Based on the GPR ranking, first, we selected the model with six variables in attention to the improvement in error observed between the use of seventh and sixth variables (Figure 4), and the fact that NBI was among the variables chosen. Nine ML algorithms were applied to this model to see whether algorithms other than GPR could produce better modeling results. The algorithm RVM represented an improvement respect to GPR (Table 3) whereby the NRMSE went from 18.42 to 17.66. When applied to the best, three-variable GPR model, RVM slightly outrun GPR, but a significant improvement in performance was obtained when the same model was tested with cross-validation (Figure 4). To test whether any of the nine ML algorithms could produce better models than GPR and, given the already known close relationship between FLAV and NBI to leaf N, both parameters were tested again in a univariate frame, this time with the nine algorithms and cross-validation. As a result, NBI performed slightly better than FLAV (NMRSE values of 16.03 and 16.22, respectively), but both notably worse than SPAD (NRMSE = 9.82 and R^2 = .89), whose model was used as a reference. The lower accuracy of the NBI model respect to the three-variable model and SPAD measurements can be

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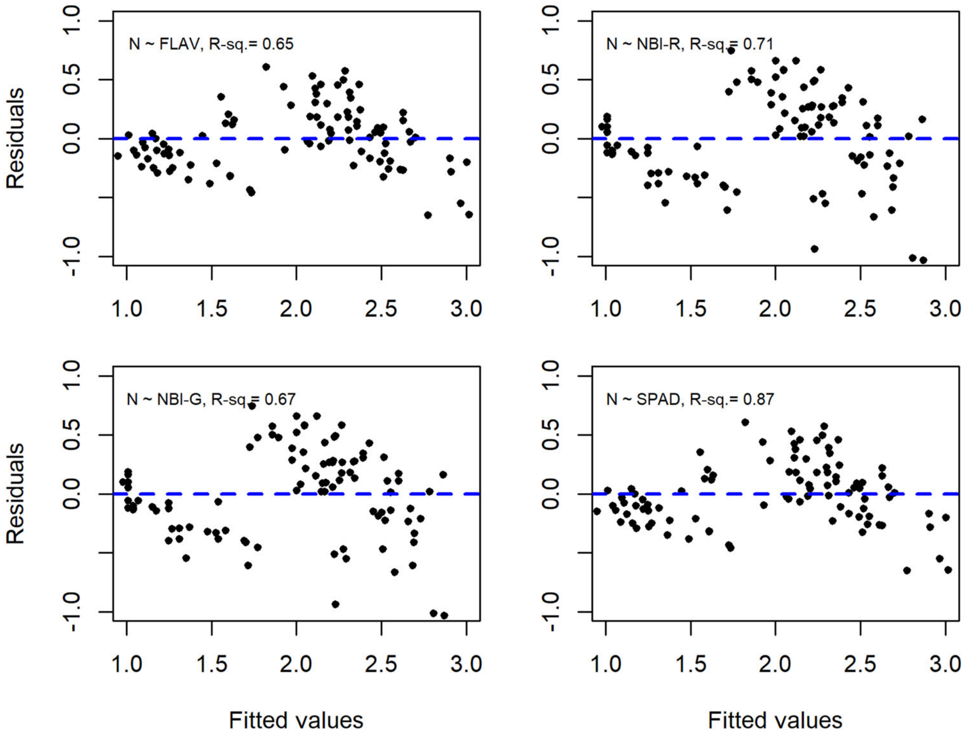

FIGURE 3 Residual plots of the regression models to retrieve N from proximal sensors

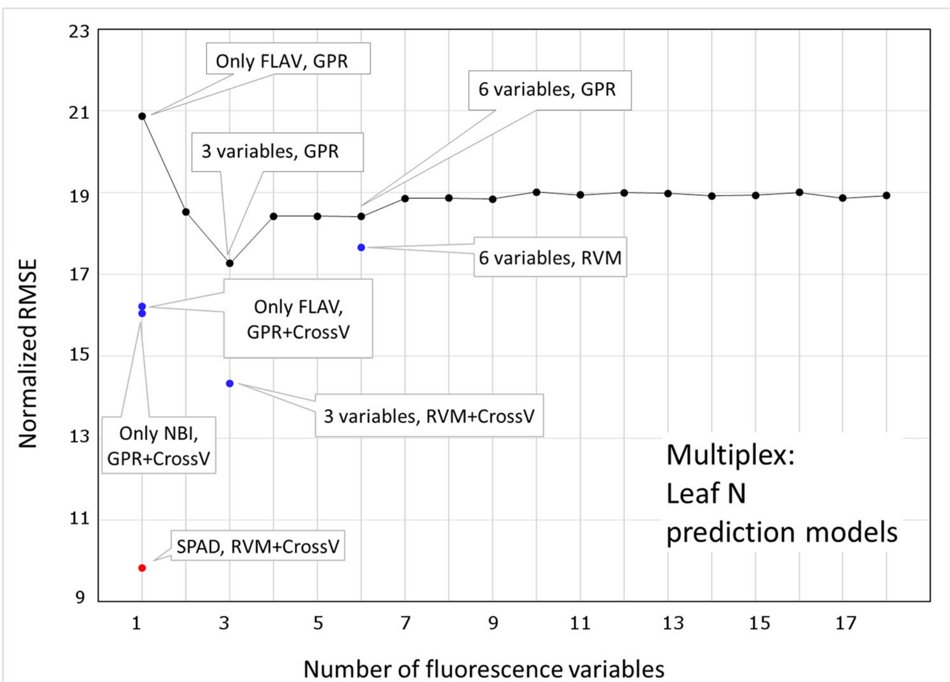

FIGURE 4 Result of the Multiplex variable relevance test, black line and dots indicate the error (normalized root mean square error [NRMSE]) from the Gaussian processes regression (GPR) method. Blue and red dots indicate additional tests with selected models and algorithms other than GPR. CrossV, cross-validation; FLAV, Multiplex flavonoid index; NBI, Multiplex nitrogen index

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TABLE 3 Machine learning model performances

Model	ML algorithm	NRMSE	Adj. R^2	Method
Six variables (includes NBI)	RVM	17.66	−.78	Training/validation data split
Three variables (FRF_R, SFR_G and FLAV)	RVM	14.34	−.76	Cross-validation
One variable, FLAV	GPR	16.22	−.70	Cross-validation
One variable, NBI	GPR	16.04	−.71	Cross-validation
SPAD (for comparison)	RVM	9.82	−.89	Cross-validation

Note. FLAV, Multiplex flavonoid index; GPR, Gaussian processes regression; NBI, Multiplex nitrogen balance index; NRMSE, normalized root mean square error; RVM, relevance vector machine (for other Multiplex acronyms see Supplemental Table S1).

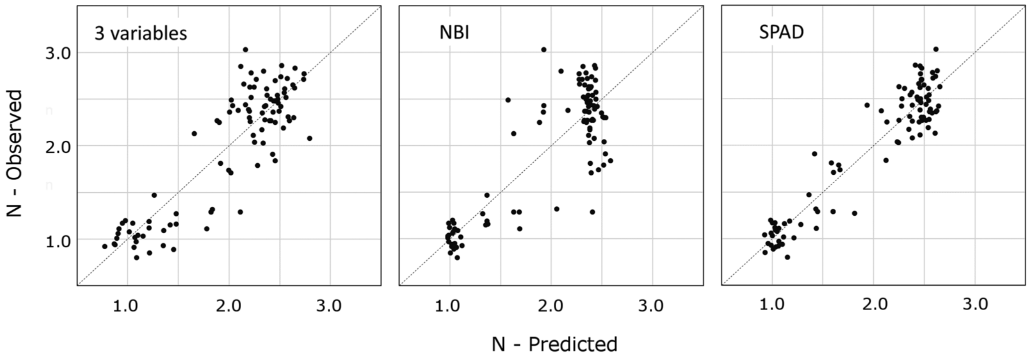

FIGURE 5 Accuracy of the three best performing (lower normalized root mean square error [NRMSE]; Table 3) models from the machine learning analysis

seen in predicted-vs.-observed plots (Figure 5). The robustness of the relationship SPAD to N content is particularly meaningful after the cross-validation test.

3.4 | Canopy N and sensors' outcomes

Multiplex variables showing consistently high correlation (whether negative or positive) across crops are: FLAV, NBI-G and -R, SFR-G and -R, and FRF-UV (Supplemental Table S2). These results, except for SFR, are similar to the results observed at the leaf level (Supplemental Table S1). Compared with the leaf-based observations, corn canopy measurements showed equally strong correlations for the same Multiplex variables. Two variables, FER-RG and ANTH-RG, showing the highest correlation coefficients had very low values at the leaf level. Winter barley showed higher correlations with almost all indices and even with some single variables such as far red excited with blue (RF-B), green (RF-G), and red (RF-R). One reason for that could be the very small sample size of $N = 4$. Spring barley, on the other hand, had in general less tight relationships than the other three crops with no correlation coefficient reaching −.80. Winter rye, with a larger sample size ($N = 12$), showed exceptionally high correlation coefficients for FLAV, ANTH-RG, and both N balance indices. Given the diversity of crops that this analysis comprises, the standard deviation (SD) of Spearman's rho provides a measure of the consistency of correlation across crops. A comparable low SD in connection with a relatively high Spearman's rho was found for the Multiplex parameters FLAV, SFR-G, NBI-R, and NBI-G (Supplemental Table S2). As described in the methodology, SPAD values for the canopy level are represented by the averages of the leaf-based measurements. The correlation coefficients obtained were −.89 for corn, −.86 for spring barley, and −.80 for winter barley. The SPAD sensor was not applied to the winter rye vegetation at Marquardt experimental station.

Regression analyses were carried out for the best-performing Multiplex variables: FLAV, NBI-G and SFR-G; and interpolated SPAD. NBI-R, also a well-performing variable, was not included given its similarity with NBI-G. Both, interpolated SPAD and selected Multiplex parameters, appeared to be linearly related with biomass N content (Figure 6). The high coefficient of determination for the corn models ($R^2 = −.91$ for FLAV, $R^2 = −.84$ for NBI-G, and $R^2 = −.94$ for SPAD) are obviously strongly influenced by the bimodal distribution of the value pairs. In the case of spring barley, NBI-G seems to be the best predictor compared with FLAV, SFR-G, and interpolated SPAD (R^2 value of −.70 vs. −.45, −.36, and −.54, respectively). Canopy values observed for the relatively small winter barley sample also showed a high bipolarity contributing to high R^2 values of SFR-G (−.89), NBI-G (−.91), and SPAD (−.80). The FLAV parameter performed less well resulting in $R^2 = −.45$. Despite the small sample size of winter rye, the linear relationships are characterized by a generally high coefficient of determination for all selected Multiplex parameters (R^2 of −.88 for FLAV, −.71 for SFR-G, and −.96 for NBI-G). In addition, N

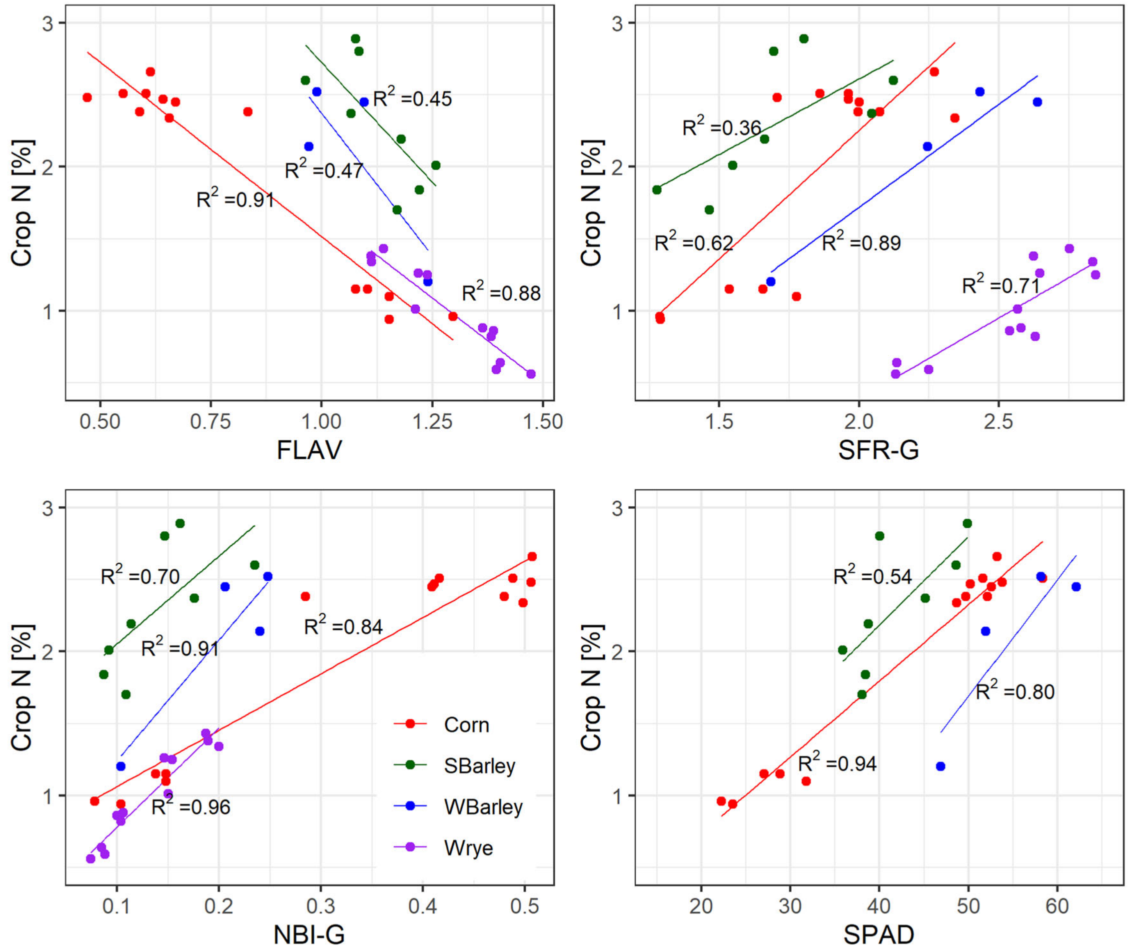

FIGURE 6 Crop-specific linear relationships between canopy N content and Multiplex flavonoid index (FLAV), Multiplex chlorophyll index (SFR-G), Multiplex nitrogen index (NBI-G), and interpolated SPAD values for different crops

contents of winter rye biomass are well distributed with no obvious bimodality.

3.5 | Multiplex readings uncertainty

Because each Multiplex value is the result of the average of 500 readings, an analysis of the deviation or dispersion of these readings provided by the device, may indicate the reliability of the measurements. The standard deviation, SD (or coefficient of variation, CV, in the case of indices), provided by Multiplex at each measurement were used for the analysis. Measurements were grouped and averaged by leaf ($N = 179$) or canopy ($N = 199$) levels (Supplemental Table S3). Average SD of three digits (126.6–679.9) were obtained on some basic ("raw") variables measured in corn at the leaf level (Supplemental Table S3). The other three crops never exceeded an average of 19.56 both, at the leaf or canopy levels, and an average of 31.66 for corn at the canopy level. All the Multiplex variables belonging to the index type of parameter had SD or CV values ranging from .00 to .58.

4 | DISCUSSION

4.1 | Multiplex

Out of the three variables (FRF-R, SFR-G, and FLAV) included in the best-performing multivariate model, only FLAV appeared as a single variable with relatively high correlation with leaf N content (Supplemental Table S1). This variable, together with NBI also showed the best regression models to estimate leaf N (Figure 2). At the canopy level, FLAV and NBI-G were the best correlating variables with N, if we consider the SD column (Supplemental Table S2) a good indicator of consistent correlation across crops. Agati et al. (2013) also found that NBI and FLAV were consistently among the most accurate variables to estimate N in leaves of turfgrass species. Similar results were reported by Li et al. (2013), although the testing of potential Multiplex predictors was restricted to SFR-R, FLAV, and NBI-R. Song et al. (2017) found both -G and -R NBIs to be a slightly better predictor of canopy N density in corn than SFR, however all indices showed a relatively loose correlation with the N

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parameter. In that paper, FLAV was not reported to have been tested. Martinon et al. (2011) found canopy Multiplex regression coefficients of −.45 to −.74 for NBI, FLAV, and SFR with percentage N, being NBI the best and SFR the weakest. Zecha et al. (2017) presented one of the few research works combining Multiplex variables. The authors claimed that by building a model with NBI and FLAV, the prediction of wheat yield improved significantly. In conclusion, the three Multiplex variables most frequently tested in previous works seem to produce the best results, although a thorough screening of other variables is not common in literature, and even less frequent, a combination of variables. In our case, results indicate that a multiple variable approach seems to be the most appropriate to predict N when measuring with Multiplex. Both the univariate regression analyses and the ML approach yielded similar coefficient of determination values when only one variable (NBI or FLAV) was considered. Therefore, if one variable instead of many Multiplex variables is to be used to estimate N, either FLAV or NBI seem to be the most appropriate. At the canopy level, even though more data would be necessary to arrive to more definite conclusions, our results seem to indicate that also NBI and FLAV have the best N prediction capabilities. From our results it is also clear that crops have species-specific fluorescence-N relationships. The level of uncertainty (variability) of some Multiplex readings in corn leaves were strikingly high when compared to most other variables in canopy and the other three crops. This could be due to some intrinsic properties of corn leaves in terms of their production of red and infrared fluorescence. The reading variability was much lower for the indices, even when highly variable parameters are used to build them. The relatively low variability of canopy readings was also unexpected, considering the potential sources of instability during measurements, such as hand movement, wind, variable background, etc.

4.2 | Multiplex vs. SPAD

The SPAD-N relationship curve resembled the type of relationship between SPAD and Chlorophyll as reported in previous studies (Cartelat et al., 2005; Hunt et al., 2013; Parry et al., 2014; Uddling et al., 2007). At the leaf level, SPAD reproduced N content more accurately than Multiplex regardless of how many of its variables were included, and independently of the method used to build the relationships. According to Li et al. (2013), also SPAD performed slightly better than Multiplex variables in rice, although their measurements were made at the canopy level. Due to the smaller sample size at the canopy level compared with the leaf level and the fact that SPAD canopy readings represent interpolated values, a direct comparison of sensory measurement accuracy was not appropriate. However, based on our results it seems likely that with an appropriate leaf sampling protocol, SPAD can be used to characterize the N status at the canopy level. The problem of the canopy representativity is much more sensitive in the case of interpolated SPAD than Multiplex, because Multiplex seems to be better suited for direct canopy readings. The sensor reading mechanics has also a big relevance for the leaf level. In comparison with Multiplex, the SPAD device has a very small point of contact with the leaf. As a consequence, SPAD readings are more subject to local variations in leaf surface such as small nerves, chlorotic spots, or imperfections, which can drastically affect the results. For this reason, to make sure that the value obtained is representative of the leaf nutritional status, the user needs to be careful and sometimes repeat measurements when spurious readings are suspected. The level of uncertainty (variability) of some Multiplex readings in corn leaves were strikingly high when compared to most other variables in canopy and the other three crops. This could be due to some intrinsic properties of corn leaves in terms of their production of red and infra-red fluorescence. The reading variability was much lower for the indices, even when highly variable parameters are used to build them. The relatively low variability of canopy readings was also unexpected, considering the potential sources of instability during measurements, such as hand movement, wind, and variable background. The results of this study suggest that, at least at the leaf level, fluorescence-based measurements are not able yet to compete in accuracy with a transmittance/reflectance-based device like SPAD. To this respect, a hyperspectral sensor like ASD mounted on a device apt for fast and easy measurements on the field, would be able to combine the multidimensionality of Multiplex and ASD, and the accuracy and practicality of SPAD.

AUTHOR CONTRIBUTIONS

Pablo Rosso: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Visualization; Writing – original draft. Evelyn Wallor: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Visualization; Writing – original draft. Lars Richter: Data curation; Formal analysis. Marc Wehrhan: Data curation; Formal analysis.

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CONFLICT OF INTEREST

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