



Use of fluorescence indices as predictors of crop N status and yield for greenhouse sweet pepper crops

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Abstract

To increase nitrogen (N) use efficiency and reduce water pollution from vegetable production, it is necessary to optimize N management. Fluorescence-based optical sensors are devices that can improve N fertilization through non-destructive field monitoring of crop variables. The aim of this work was to compare the performance of five fluorescence indices (SFR-R, SFR-G, FLAV, NBI-R, and NBI-G) to predict crop variables, as dry matter production, crop N content, crop N uptake, Nitrogen Nutrition Index (NNI), absolute and relative yield, in sweet pepper (*Capsicum annuum*) crops grown in greenhouse. Fluorescence measurements were periodically made with the Multiplex® 3.6 sensor throughout three cropping cycles subjected to five N application treatments. The performance of fluorescence indices to predict crop variables considered calibration and validation analyses. In general, the five fluorescence indices were strongly related with NNI, crop N content and relative yield. The best performing indices to predict crop N content and NNI at the early stages of the crops (i.e., vegetative and flowering phenological stages) were the SFR indices, both under red (SFR-R) and green (SFR-G) excitation. However, in the final stage of the crop (i.e., harvest stage), the best performing indices were NBI, both under red (NBI-R) and green (NBI-G) excitation, and FLAV. The two SFR indices best predicted relative yield of sweet pepper at early growth stages. Overall, the fluorescence sensor and the fluorescence indices evaluated were able to predict crop variables related to N status in sweet pepper. They have the capacity to be incorporated into best N management practices.

Keywords *Capsicum annuum* · Flavonols meter · Fluorimeter · Nitrogen Nutrition Index · Vegetable crops · Yield

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Introduction

Nitrogen (N) is an essential nutrient and is one of the most important elements for plant development and growth, affecting yield and fruit quality (Greenwood et al., 1991; Lemaire et al., 2008). To guarantee high yields, N fertilizer is commonly applied in high amounts in vegetable production systems (Neeteson et al., 1999). Commonly, in vegetable production, the combined N supply, consisting of fertilizer N plus available soil N, appreciably exceed crop N requirements (Ju et al., 2006; Thompson et al., 2017b). The excess N is susceptible to nitrate (NO_3^-) leaching losses (Thompson et al., 2007; Zotarelli et al., 2009), and subsequent environmental contamination (Meisinger et al., 2008). Nitrate contamination of groundwater, from vegetable production, has been reported for diverse regions, such as south-eastern (SE) Spain (Pulido-Bosch et al., 2000), SE United States (Zotarelli et al., 2009), and China (Ju et al., 2006).

Matching N supply with crop N requirements quantitatively over time will optimize N use and decrease the associated environmental problems (Samborski et al., 2009; Thompson et al., 2017b; Zhang et al., 2012). Vegetable production systems increasingly have the technical potential to match N supply to crop requirements through the combined use of localized irrigation and fertigation systems (Thompson et al., 2017a, 2017b). Tools that can assess crop N status are required to ensure optimal N management in intensive production systems, by permitting correction of N fertilizer additions added frequently through combined fertigation and localized irrigation.

A convenient way to determine crop N nutrition status on farm is through the use of portable optical sensors. There are several types of proximal optical sensors with the capacity to evaluate crop N status; the most important are chlorophyll meters, canopy reflectance sensors, and flavonols meters (Padilla et al., 2018; Samborski et al., 2009; Tremblay et al., 2012). These sensors estimate the N content through the use of optical properties of the light which interact with compounds sensitive to N content (Fox & Walthall, 2008; Tremblay et al., 2012). The major advantages of these sensors are speed and simplicity of measurement, and that they can determine crop N status non-destructively (Samborski et al., 2009; Tremblay et al., 2012).

Two groups of compounds in plants are particularly sensitive to plant N content, chlorophyll and polyphenols (Agati et al., 2013; Fox & Walthall, 2008; Tremblay et al., 2012). Within polyphenols, flavonols are the compounds that are easier to estimate by using optical tools (Meyer et al., 2006). Fluorescence-based sensors can estimate chlorophyll and flavonols contents of leaf tissue *in-situ* (Padilla et al., 2018). These sensors estimate chlorophyll content from the chlorophyll fluorescence emission ratio of red (RF), and far-red radiation (FRF) emitted from chlorophyll after excitation with ultraviolet (UV), red, green or blue radiation (Tremblay et al., 2012). Flavonols content in leaves has the opposite behaviour to that of chlorophyll as function of N, and can also be directly affected by other environmental factors such as light. When N is deficient, flavonols content increases and chlorophyll content decreases (Bragazza & Freeman, 2007; Liu et al., 2010). The Nitrogen Balance Index (NBI) is calculated as the ratio between chlorophyll and flavonols contents; it has been shown to be a very sensitive indicator of crop N status (Cartelat et al., 2005; Padilla et al., 2016; Samborski et al., 2009).

Fluorimeters are a kind of optical sensors which provide indirect measurements of both chlorophyll and flavonols contents from the fluorescence properties of the leaves (Padilla et al., 2018; Thompson et al., 2017b). These sensors can also be used to estimate other crop properties not dependent on the chlorophyll content such as leaf health status and light use

efficiency (Schreiber & Bilger, 1987). Two fluorimeters developed for agriculture are the Dualex, a leaf-clip sensor, and the Multiplex, both produced by Force-A (Orsay, France). The Multiplex is a proximal sensor that take measurements at a distance of 10 cm from the leaf (Padilla et al., 2018; Thompson et al., 2017b).

The evaluation of fluorescence measurements as predictors of crop variables that are important for crop N management has been made by establishing relationships between fluorescence measurements and crop variables (Padilla et al., 2016). Most of the literature has focused on the prediction of crop N status but it remains largely unaddressed if measurements of fluorescence-based sensors can predict other crop variables such as crop N uptake and yield (Huang et al., 2019).

This manuscript evaluates the capacity of five fluorescence indices, measured with the Multiplex sensor, to predict the following crop variables: dry matter production, crop N content, crop N uptake, Nitrogen Nutrition Index (NNI), and yield (both absolute and relative yield respect to the maximum yield), in sweet pepper crops. Subsequently regressions were validated for phenological stages. The work was carried out in an intensive vegetable production system in Almería, SE Spain. This area is one of the most important for production of vegetables crops in greenhouse in Spain, occupying 30,000 ha. Within the vegetable crops, sweet pepper is one of the most important crops, occupying an area of 8,000 ha (Valera et al., 2017).

Material and methods

Experimental crops and N treatments

This study was carried with sweet pepper (*Capsicum annuum* cultivar ‘Melchor’) grown in three different years at the Experimental Station of the University of Almería (36°51’N, 2°16’W and 92 m elevation), in Retamar, Almería, Spain. The crops were grown in soil in a plastic greenhouse. The soil was an artificial layered “enarenado” typical of this area (Thompson et al., 2007). A complete description of the greenhouse and the enarenado soil are presented in Thompson et al. (2007). A complete list of acronyms used is presented in Table 1.

The sweet pepper crop was repeated over three cropping seasons (2014, 2016, and 2017; Table 2, Fig. 1), with a summer–winter cycle. Each crop was subjected to five different N treatments, applied throughout the crop in the nutrient solution applied by a combined drip irrigation and fertigation system. The N treatments were very N deficient (N1), N deficient (N2), conventional N (N3), excessive N (N4) and very excessive N (N5) (Table 2). The majority of N was applied as nitrate (90%), and the rest as ammonium (NH_4^+). In addition to N, the other macronutrients applied remained constant in all treatments in the following concentrations: H_2PO_4^- , 1.75 mmol L⁻¹; K^+ , 4 mmol L⁻¹; Ca^{+2} , 4 mmol L⁻¹; Mg^{+2} , 1.5 mmol L⁻¹; SO_4^{-2} , 2.35 mmol L⁻¹; on average for the three cropping seasons. The different N treatments were applied in every irrigation, which were made every 1–4 days. The irrigation was scheduled using tensiometers (Irrometer, Co., Riverside, Ca, USA) that were installed at 15 cm depth. Crop management followed local practices.

The experimental design was a randomized block design, with four replications per treatment. Each replicate plot measured 6 m by 6 m. There was a total of 20 plots. In each

Table 1 List of acronyms used

List of acronyms		Units
SFR	Simple Fluorescence Ratio	Dimensionless
SFR-R	Simple Fluorescence Ratio under red excitation	Dimensionless
SFR-G	Simple Fluorescence Ratio under green excitation	Dimensionless
FLAV	Flavonols	Dimensionless
NBI	Nitrogen Balance Index	Dimensionless
NBI-R	Nitrogen Balance Index under red excitation	Dimensionless
NBI-G	Nitrogen Balance Index under green excitation	Dimensionless
NNI	Nitrogen Nutrition Index	Dimensionless
DMP	Dry Matter Production	t ha ⁻¹
Nup	Crop Nitrogen uptake	kg N ha ⁻¹
N	Crop Nitrogen Content	%
I	Integrated values	Depends on the variable
Y	Absolute Yield	t ha ⁻¹
RY	Relative Yield	%
RMSE	Root mean square error	Depends on the variable
RE	Relative error	%
SE	Standard error of the mean	Depends on the variable

replicate plot there were 72 plants with a density of two plants m⁻². One emitter with a flow of 3 L h⁻¹ was immediately adjacent to each plant.

Dry matter production, crop N uptake, crop N content, NNI and yield

Periodic above-ground biomass samplings were made in each of the three crops. In the 2014–2015 crop, the biomass samplings were made every 21 days, in the 2016–2017 every 23 days, and in the 2017–2018 crop every 24 days.

At each sampling date, dry matter production (DMP) and N content were determined from two complete plants for each replicate. The dry weights (oven-dried at 65°C) of the different plant organs (stems, leaves, and fruits) were determined. Additionally, harvested fruit and pruned shoot material were periodically removed from eight marked plants for replicate plot throughout each crop. The total amounts of harvest fruit and pruned material were calculated by summing the data per plot per treatment. Subsamples of oven-dry material were ground to a fine powder prior to analysis of N content (%) in a Dumas-type elemental analyzer (Rapid N, Elementar Analysensysteme GmbH, Hanau, Germany).

The total N in each organ was calculated multiplying the %N of the sub-sample by the corresponding dry matter mass. Total crop N uptake (kg N ha⁻¹) was the sum of N in all relevant components. Total crop N content (%N) was calculated as total crop N uptake divided by total DM.

The NNI was calculated from the critical N curve derived for greenhouse-grown sweet pepper crop (Critical N = $4.71 \cdot \text{DMP}^{-0.22}$; Rodríguez et al., (2020)). The NNI was calculated as:

Table 2 Characteristics of the three cropping cycles and the corresponding N treatments applied in the five N treatments

Crop	Cycle	Duration (days)	Beginning of N treatments	N treatment	Mineral N concentration of fertigation (mmol N L ⁻¹)	Amount of mineral N applied (kg N ha ⁻¹)	Absolute yield (t ha ⁻¹)	Relative yield (%)
2014–2015	12 Aug – 29 Jan	170	1 DAT	N1	2.4	64	38.7 ± 1.1	65.5 ± 1.9
				N2	6.2	189	52.2 ± 1.5	88.3 ± 2.6
				N3	12.6	516	52.9 ± 2.3	89.5 ± 3.8
				N4	16.1	804	51.1 ± 0.3	86.4 ± 0.4
				N5	20.0	990	46.4 ± 2.1	78.5 ± 3.4
2016–2017	19 July – 24 Mar	248	9 DAT	N1	2.0	88	67.2 ± 1.8	66.2 ± 1.7
				N2	5.3	302	86.4 ± 3.1	85.2 ± 3.0
				N3	9.7	561	91.5 ± 4.2	90.1 ± 4.1
				N4	13.5	1052	94.2 ± 2.7	92.8 ± 2.6
				N5	17.7	1320	89.7 ± 4.0	88.5 ± 3.9
2017–2018	21 July – 20 Feb	214	10 DAT	N1	2.0	86	33.3 ± 3.4	45.5 ± 4.5
				N2	5.7	304	54.4 ± 4.5	74.3 ± 6.1
				N3	9.7	519	61.0 ± 1.7	83.3 ± 2.3
				N4	13.1	870	65.1 ± 1.5	89.0 ± 1.9
				N5	16.7	1198	68.9 ± 1.5	94.2 ± 2.0

Values are means ± SE, when applicable.

DAT days after transplanting

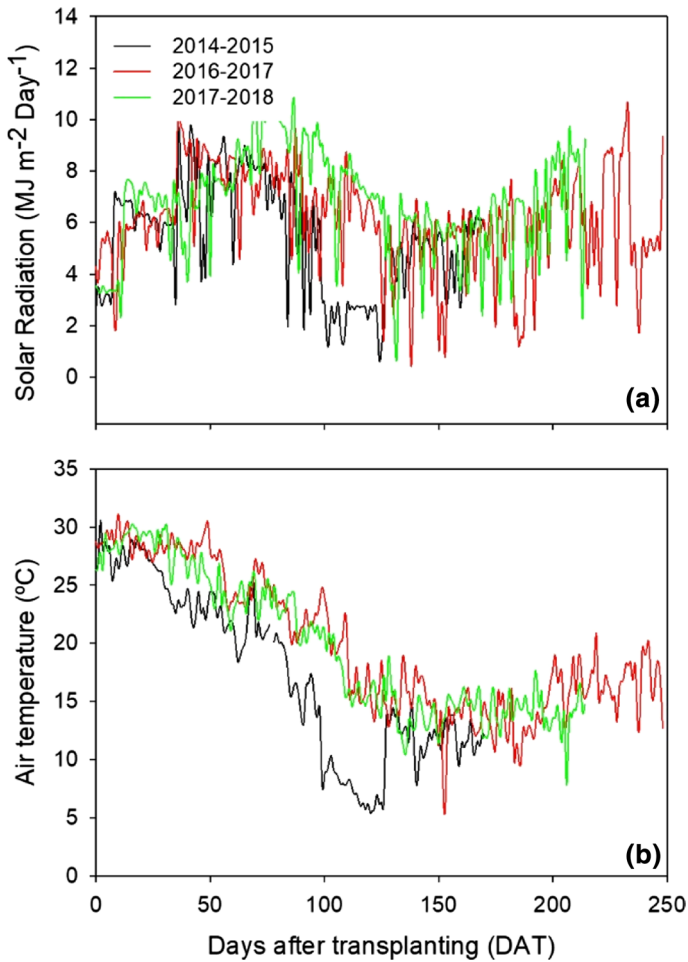


Fig. 1 **a** Daily integral of solar radiation and **b** daily average air temperature, in the greenhouse during the three cropping seasons (2014–2015, 2016–2017, and 2017–2018), from transplant to end of the crop

$$NNI = \frac{N_{act}}{N_c} \quad (1)$$

where N_{act} is the real N measured and N_c is the critical N content calculated from the critical N curve. The NNI value for each day of the crop was calculated by interpolating values between two consecutive biomass samplings (Padilla et al., 2017).

Multiplex measurements

A Multiplex® 3.6 sensor (Force-A, Orsay, France) was used to measure chlorophyll fluorescence throughout the three cropping seasons. This sensor is a portable device with four emission light sources (UV, green, red and blue) that induce fluorescence in plant tissues (Huang et al., 2019). Detailed descriptions of the sensor and its operation are available in

Bürling et al. (2013) and Tremblay et al. (2012). The measurements were made weekly in the 2014–2015 crop and every two weeks for the other two crops. Measurements started at 15, 25 and 21 DAT in the first, second and third crops, respectively.

Measurements were made throughout the growing cycle in 16 marked plants per replicate plot. In each of these 16 plants, measurements were made on the most recently fully expanded leaf, following the protocols of Padilla et al., (2018). The measurement area was an 8 cm diameter circle of leaf surface.

Of the various indices measured by the Multiplex sensor (Huang et al., 2019), the present work focused on five indices that are most sensitive to plant N status (Tremblay et al., 2012). These indices were: i) the Simple Fluorescence Ratio (SFR), both under red (SFR-R) and green (SFR-G) excitation, indicative of leaf chlorophyll content (Tremblay et al., 2012); ii) the FLAV index, indicative of leaf flavonols content (Ceric et al., 2002); and iii) the Nitrogen Balance Index (NBI) (Cartelat et al., 2005), either under red (NBI-R) or green (NBI-G) excitation.

Data analysis

Sweet pepper is an indeterminate crop with multiples harvests during the cycle. The major phenological stages considered were: vegetative, flowering, early fruit growth, and harvest (de Souza et al., 2019). Integrated values (I_v) of fluorescence indices, DMP, crop N content, crop N uptake and NNI for each phenological stage, were calculated as:

$$I_v = 1/D \cdot \Sigma(V \cdot ds) \quad (2)$$

where D was the duration of the phenological stage, V was the value of fluorescence indices or crop variable for each day of measurement, and ds was the duration between two successive measurements (de Souza et al., 2019).

Yield data were considered both in absolute terms (kg m^{-2}) and in relative terms (%). In each crop, relative yield was calculated for each plot by dividing yield of each of the 20 plots by the maximum yield recorded in a plot of that particular crop, expressed as a percentage.

Data of integrated fluorescence indices and integrated crop variables were pooled across the three crops, for each phenological stage. Within each phenological stage, pooled data were randomly divided into two groups, one group was used for calibration and the other group was used for validation. The calibration group had approximately 2/3 of the data; the remaining data was in the validation group. For the calibration group, simple linear regression analyses were performed between each integrated fluorescence index, as the independent variable, and each integrated crop variable, as the dependent variable. These relationships assessed and compared the capacity of each fluorescence index to predict each crop variable. The CurveExpert Professional@2.2.0 software (Daniel G. Hyams, MS, USA) was used to retrieve statistical data of these relationships.

The relationships between each integrated fluorescence index and each integrated crop variable, at each phenological stage, were validated using the validation group. Validation consisted of calculating the predicted value of each integrated crop variable using the calibration equation, and then comparing with the observed values of each integrated crop variable of the validation dataset. Linear regression analysis was established between observed (independent variable) and predicted (dependent variable) values of each crop variable, and the root mean square error (RMSE) of the estimated crop variable was calculated as:

$$RMSE = \sqrt{\sum_{i=1}^n \frac{(P_i - O_i)^2}{n}} \quad (3)$$

where n is the number of samples, P_i is the predicted value of the relationship, and O_i is the observed value (Zhao et al., 2018). Additionally, the relative error (RE) of the validation regression was calculated between values of each observed and predicted crop variables as:

$$RE : \frac{RMSE}{\overline{O_i}} \quad (4)$$

where $\overline{O_i}$ is the average of observed values.

The performance of fluorescence indices to predict crop variables was evaluated taking into account both calibration and validation results (Xin-feng et al., 2013). Coefficient of determination (R^2) and RMSE values of linear regression of the calibration dataset, and R^2 , RMSE, absolute values of slope-1, and absolute values of intercept, of linear regression of the validation dataset, were used (Xin-feng et al., 2013). Slope-1 is the absolute value of the slope after subtracting one from the slope of the linear regression. The use of this parameter effectively normalizes slope values and enabled ranking of all integrated vegetation indices from lowest to highest values (de Souza et al., 2020). The performance of each fluorescence index was calculated by: (i) sorting R^2 in decreasing order and RMSE in ascending order, for the calibration and validation datasets separately, and (ii) sorting absolute slope-1 values and absolute values of intercept in ascending order, for the validation dataset. After sorting these parameters, each one was assigned an ascending number (i.e., 1, 2, 3, etc.) starting from the beginning. The best performing fluorescence indices were those that had the lowest sum of numbers assigned in the six factors (Xin-feng et al., 2013).

Results

Calibration relationships between fluorescence indices and crop variables for each phenological stage

Results of linear regression between the five integrated fluorescence indices (SFR-R, SFR-G, FLAV, NBI-R, NBI-G) and the six crop variables (DMP, crop N content, crop N uptake, NNI, absolute yield and relative yield) are shown in Table 3 and Table 1S. SFRi and NBIi indices were positively related with all crop variables, whereas the FLAVi index was inversely related with these variables. Most of the relationships between integrated fluorescence indices and crop variables were statistically significant, in all phenological stages (Table 3 and Table 1S).

In general, most of the integrated fluorescence indices had low R^2 values for their relationship with DMP, regardless of phenological stage (Table 3). The average R^2 value of relationships of all five fluorescence indices with DMP, across the four phenological stages, was 0.37 ± 0.05 (\pm SE). Results were similar for the relationships of each of the five integrated fluorescence indices with absolute yield (Table 3 and Table 1S), with an average R^2 value of relationships of all integrated indices and phenological stages of 0.33 ± 0.04 . Regarding relative yield, the average R^2 value of relationships between each integrated fluorescence index and relative yield, across all four phenological stages, was 0.54 ± 0.03 ,

Table 3 Equations, coefficient of determination (R^2) and root mean square error (RMSE) of linear regressions between SFR-R, FLAV, and NBI-R integrated indices (x variable) and each integrated crop variable (y variable) at different phenological stages, for calibration data (n = 40)

Phenological stage	Dry matter production (DMPi)				Crop N content (Ni)				Crop N uptake (Nupi)			
	Index	Equation	R ²	RMSE	Equation	R ²	RMSE		Equation	R ²	RMSE	
Vegetative	SFR-Ri	DMPi = 0.53x - 0.63	0.26**	0.11	Ni = 4.87x - 4.57	0.68***	0.40		Nupi = 33.84x - 46.52	0.41***	4.84	
	FLAVi	DMPi = -1.14x + 0.32	0.44***	0.09	Ni = -3.81x + 4.08	0.16 ns	0.65		Nupi = -51.41x + 13.59	0.35***	5.07	
	NBI-Ri	DMPi = 0.25x - 0.13	0.45***	0.09	Ni = 1.15x + 1.98	0.31***	0.59		Nupi = 12.31x - 8.851	0.44***	4.74	
Flowering	SFR-Ri	DMPi = 2.22x - 2.72	0.64***	0.27	Ni = 3.36x - 2.91	0.54***	0.51		Nupi = 128.68x - 191.95	0.78***	11.10	
	FLAVi	DMPi = -0.06x + 1.69	0.00 ns	0.45	Ni = -3.91x + 4.40	0.67***	0.43		Nupi = -64.40x + 73.92	0.18**	21.46	
	NBI-Ri	DMPi = 0.14x + 1.48	0.02 ns	0.45	Ni = 1.35x + 1.78	0.76***	0.36		Nupi = 25.85x + 25.55	0.27**	20.17	
Early fruit growth	SFR-Ri	DMPi = 4.30x - 4.74	0.42***	0.81	Ni = 3.39x - 4.37	0.74***	0.33		Nupi = 253.03x - 408.31	0.61***	32.94	
	FLAVi	DMPi = -5.44x + 6.56	0.53***	0.73	Ni = -3.70x + 4.33	0.68***	0.36		Nupi = -301.11x + 250.27	0.67***	30.24	
	NBI-Ri	DMPi = 2.22x + 2.29	0.51***	0.75	Ni = 1.56x + 1.38	0.70***	0.35		Nupi = 126.25x + 10.48	0.68***	29.79	
Harvest	SFR-Ri	DMPi = 1.71x + 5.20	0.06 ns	2.17	Ni = 0.90x + 0.78	0.41***	0.33		Nupi = 103.69x + 20.72	0.15 ns	76.11	
	FLAVi	DMPi = -17.81x + 17.10	0.69***	1.25	Ni = -3.36x + 4.20	0.66***	0.25		Nupi = -694.61x + 560.65	0.77***	39.73	
	NBI-Ri	DMPi = 7.56x + 2.91	0.51***	1.56	Ni = 1.76x + 1.27	0.74***	0.22		Nupi = 320.71x - 12.751	0.68***	46.87	
NNli												
Equation	Absolute yield (Y)				Relative yield (RY)							
	R ²	Equation	R ²	RMSE	Equation	R ²	RMSE		Equation	R ²	RMSE	
NNli = 1.03x - 0.97	0.68***	Y = 5.32x - 3.17	0.13 ns	1.65	RY = 100.66x - 98.04	0.70***	7.89					
NNli = -0.81x + 0.87	0.16 ns	Y = -19.06x + 6.28	0.62***	1.09	RY = -131.25x + 8.77	0.44***	10.70					
NNli = 0.24x + 0.42	0.31***	Y = 3.91x - 0.84	0.56***	1.17	RY = 32.31x + 21.89	0.58***	9.33					
NNli = 0.98x - 1.06	0.67***	Y = 6.23x - 6.07	0.33***	1.44	RY = 71.41x - 60.65	0.65***	8.43					
NNli = -0.92x + 1.04	0.54***	Y = -3.31x + 6.85	0.09 ns	1.69	RY = -42.85x + 88.10	0.22**	12.71					
NNli = 0.33x + 0.41	0.65***	Y = 1.33x + 4.35	0.13 ns	1.64	RY = 17.90x + 54.90	0.36***	11.49					
NNli = 1.16x - 1.64	0.73***	Y = 6.28x - 7.33	0.33***	1.44	RY = 74.81x - 81.45	0.72***	7.65					
NNli = -1.30x + 1.36	0.70***	Y = -9.24x + 9.65	0.56***	1.17	RY = -72.03x + 107.04	0.52***	9.98					
NNli = 0.55x + 0.32	0.72***	Y = 3.83x + 2.34	0.56***	1.17	RY = 30.78x + 49.09	0.54***	9.69					

Table 3 (continued)

NNfi Equation	Absolute yield (Y)		Relative yield (RY)	
	R ²	RMSE	Equation	RMSE
NNfi = 0.33x + 0.21	0.31***	0.15	RY = 30.70x + 17.91	10.83
NNfi = -1.51x + 1.60	0.74***	0.09	RY = -92.86x + 124.57	10.61
NNfi = 0.75x + 0.32	0.76***	0.09	RY = 51.70x + 41.14	9.29

Significance of regressions are indicated with asterisks close to R² values

*** p < 0.001; ** p < 0.01, ns: not significant at p > 0.05

with stronger relationships for SFR-Ri in the vegetative (R^2 of 0.70), flowering (R^2 of 0.65) and early fruit growth (R^2 of 0.72) stages (Table 3). In the relationships between integrated fluorescence indices and crop N uptake, the average R^2 values of relationships for all integrated indices throughout the four phenological stages was 0.50 ± 0.05 (Table 3 and Table 1S). The indices NBI-Ri (R^2 of 0.44), SFR-Ri and SFR-Gi (both with R^2 of 0.78), NBI-Gi (R^2 of 0.69), and FLAVi (R^2 of 0.77), were most strongly related to crop N uptake in the vegetative, flowering, early fruit growth and harvest stages, respectively (Table 3 and Table 1S).

In general, the strongest relationship of integrated fluorescence indices with crop variables occurred with crop N content and NNi (Table 3 and Table 1S). The average R^2 value of relationships of all five fluorescence indices with crop N content and NNi, across the four phenological stages, was 0.60 ± 0.04 for crop N content and for NNi. The SFR and NBI indices had one of the highest R^2 values in relationships with crop N content and NNi in all four phenological stages (Table 3). For relationships with crop N content, the SFR-Gi had stronger relationships in the vegetative (R^2 of 0.70) and early fruit growth (R^2 of 0.77) stages, and the NBI-Gi in the flowering (R^2 of 0.77) and the harvest (R^2 of 0.75) stages (Table 3). For relationships with NNi, the SFR-Gi had stronger relationships in the vegetative (R^2 of 0.70), flowering (R^2 of 0.68) and early fruit growth (R^2 of 0.76) stages, and the NBI-Ri in the harvest stage (R^2 of 0.76) (Table 3 and Table 1S).

Validation of relationships between integrated fluorescence indices and crop variables for each phenological stage

Results of the validation analysis varied with phenological stages, fluorescence indices and crop variables. Considering all crop variables, validation had high R^2 values for crop N uptake, crop N content and NNi, with average R^2 values of 0.61 ± 0.05 , 0.64 ± 0.05 and 0.64 ± 0.05 , respectively, across all indices and phenological stages. Validation had low R^2 values for crop yield (average R^2 value across all indices and phenological stages of 0.38 ± 0.05), followed by dry matter production and relative yield (average R^2 values across all indices and phenological stages of 0.48 ± 0.06 and 0.51 ± 0.04 , respectively) (Table 2S).

According to higher R^2 and lower RMSE (Eq. 3) values of the validation analysis, results showed better validation for crop dry matter production in the vegetative stage than in others phenological stages, with best results for the NBI indices (Table 2S). For crop N content, the phenological stage with best validation results, considering all indices, was the early fruit growth; the index with best validation in this stage was SFR-Gi. The early fruit growth stage and the SFR-Gi index showed better validation results for crop N uptake. The best validation for NNi was early fruit growth, with best results for SFR-Gi. For absolute crop yield, the phenological stage with best validation results was early fruit growth, and the best performing index in this stage was NBI-Ri. For relative yield, best results were obtained in fruit growth stage with the SFRi indices.

Slope of regression between observed and predicted values of crop variables from fluorescence indices for each phenological stage

For all fluorescence indices and phenological stages, the slopes of linear regression between observed and expected values differed appreciably from 1 for absolute yield (Figs. 2–4, and Figs. 1 S and 2S).

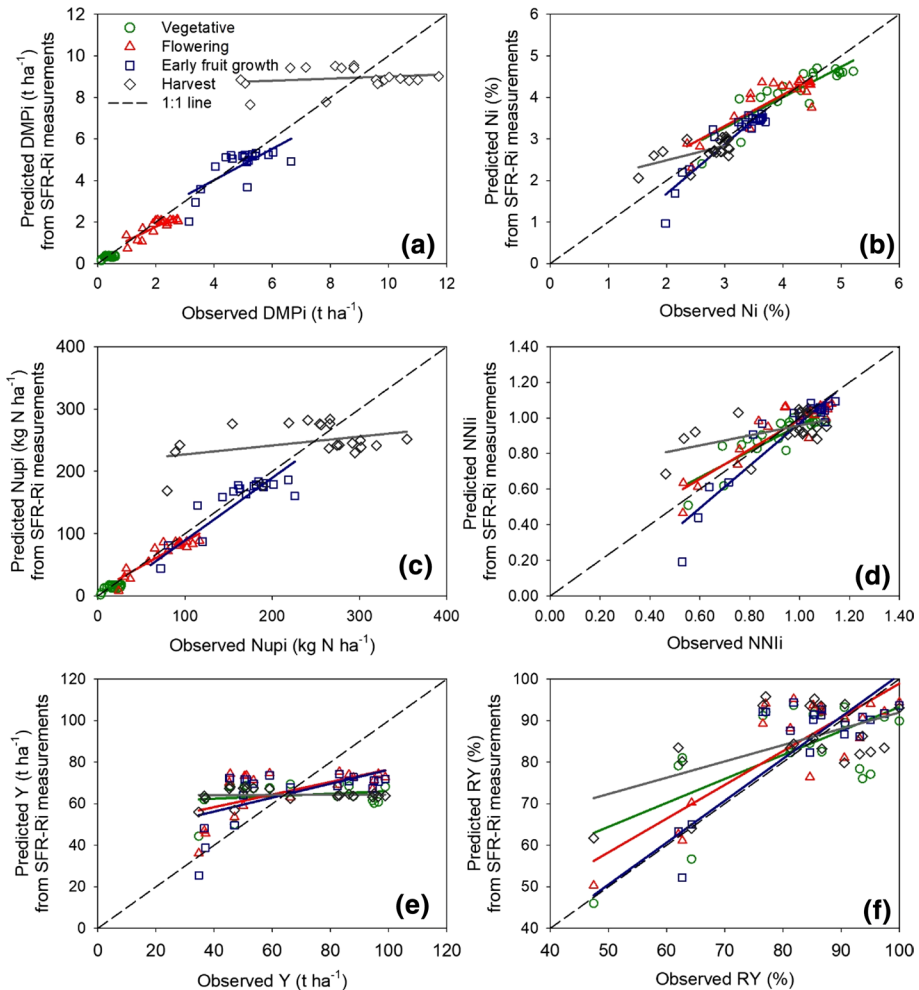


Fig. 2 Relationships between observed values and predicted values, from SFR-Ri measurements, of **a** dry matter production (DMPi), **b** crop N content (Ni), **c** crop N uptake (Nupi), **d** nitrogen nutrition index (NNli), **e** absolute yield (Y), and **f** relative yield (RY); for vegetative, flowering, early fruit growth and harvest stages, for validation data ($n=20$). Dashed line represents the 1:1 line. Regression results are in Table S1

In general, for the SFR-Ri and SFR-Gi indices, the slopes of the linear regressions between observed and predicted values of crop variables were closer to 1 particularly in the early fruit growth and flowering stages, followed by the vegetative stage, for most crop variables (Fig. 2 and Fig. 1S). For these indices, the slopes of the linear regressions between observed and predicted values of crop variables were clearly different to 1 in the harvest stage. It is notable that, in the flowering stage, the slopes for crop N uptake and relative yield were approximately 1 (Fig. 2 and Fig. 1S), indicating very good prediction of these crop variables.

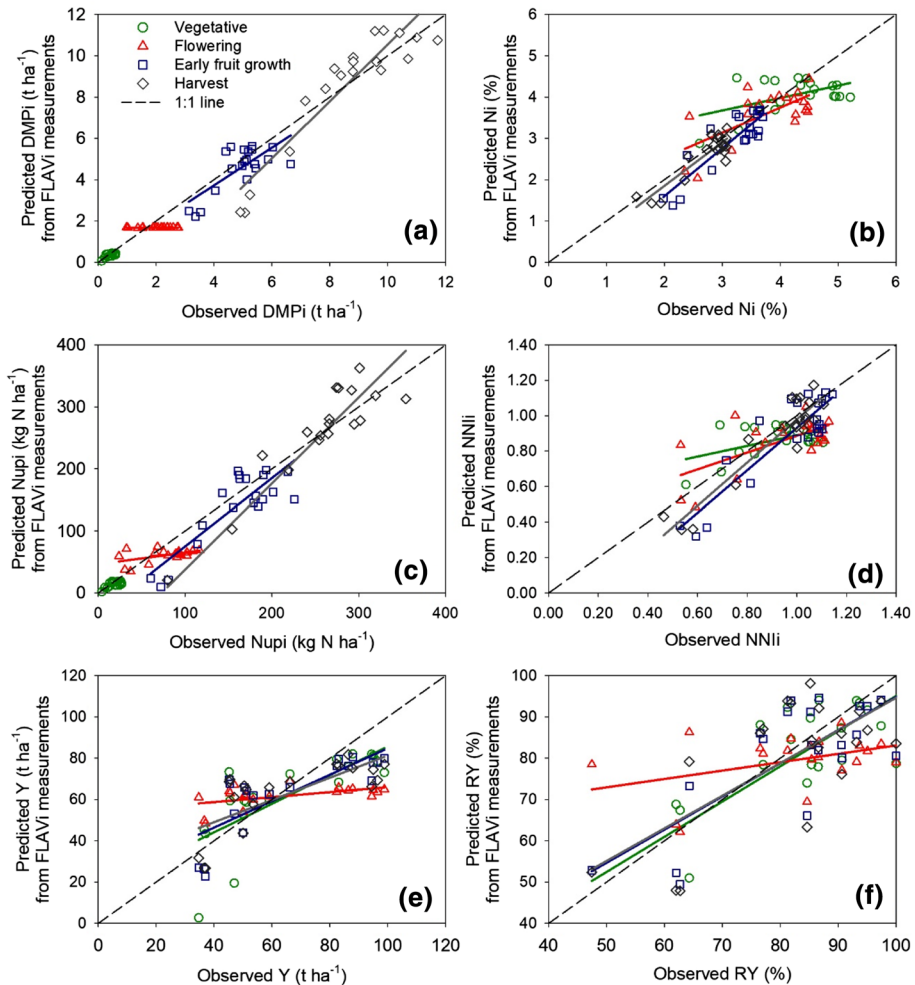


Fig. 3 Relationships between observed values and predicted values, from FLAVi measurements, of **a** dry matter production (DMPi), **b** crop N content (Ni), **c** crop N uptake (Nupi), **d** nitrogen nutrition index (NNli), **e** absolute yield (Y), and **f** relative yield (RY); for vegetative, flowering, early fruit growth and harvest stages, for validation data ($n=20$). Dashed line represents the 1:1 line. Regression results are in Table S1

For the FLAVi index, the slopes of the linear regression between observed and predicted values of crop variables were different to 1 particularly in the flowering stage, followed by the vegetative stage, for most crop variables (Fig. 3). Slopes of linear relationships were closer to 1 in the early fruit growth and harvest stages for most crop variables, particularly for crop DMP, crop N content, crop N uptake and NNli (Fig. 3).

Similar to FLAVi, slopes of linear regression between observed and predicted values of crop variables, from NBI-Ri and NBI-Gi measurements, differed from 1 in the flowering stage, followed by the vegetative stage (Fig. 4 and Figure 2S). Slopes

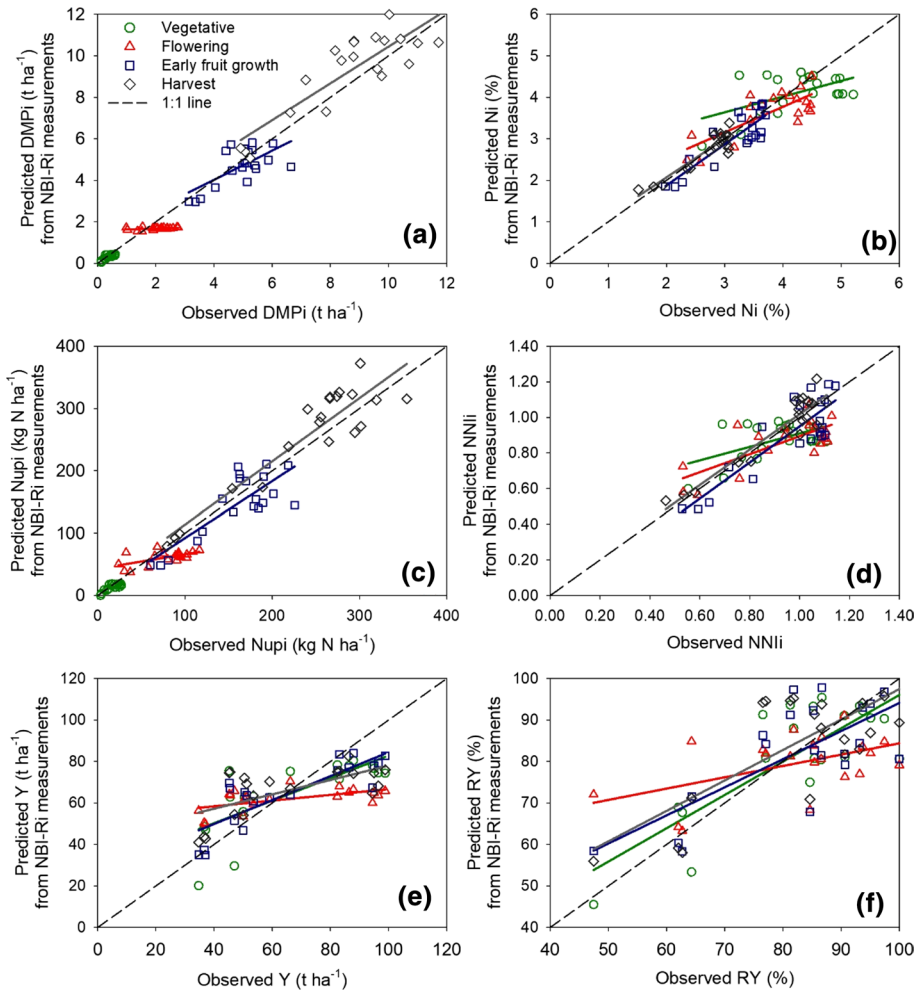


Fig. 4 Relationships between observed values and predicted values, from NBI-Ri measurements, of **a** dry matter production (DMPi), **b** crop N content (Ni), **c** crop N uptake (Nupi), **d** nitrogen nutrition index (NNli), **e** absolute yield (Y), and **f** relative yield (RY); for vegetative, flowering, early fruit growth and harvest stages, for validation data ($n=20$). Dashed line represents the 1:1 line. Regression results are in Table S1

of regression were closer to 1, for most crop variables, in the early fruit growth and harvest stages, particularly for crop N content, crop N uptake and NNli (Fig. 4 and Fig. 2S).

Relative error (RE) of prediction of crop variables from fluorescence indices for each phenological stage

The RE (Eq. 4) values of the prediction of crop variables from fluorescence indices were lower for crop N content, relative yield and NNli, regardless of the fluorescence index and

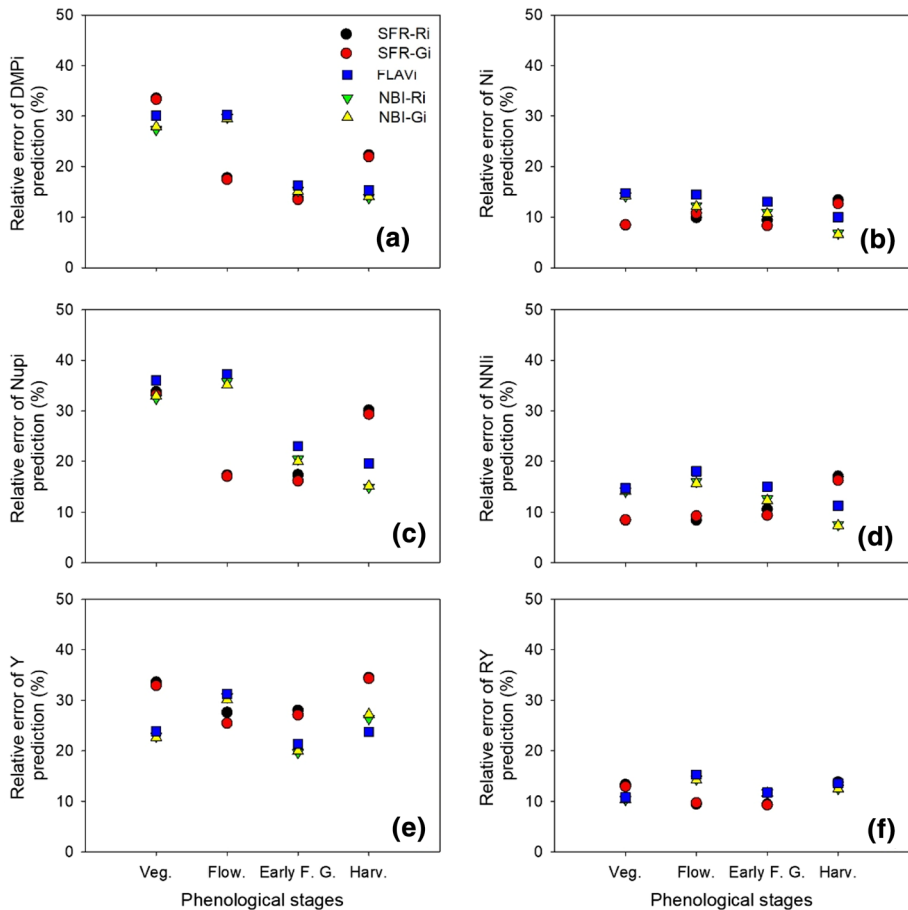


Fig. 5 Relative error, expressed in percentage, of prediction of crop variables from measurements with each fluorescence index at different phenological stages for validation data ($n=20$). **a** Dry matter production (DMPi), **b** crop N content (Ni), **c** crop N uptake (Nupi), **d** nitrogen nutrition index (NNIi), **e** absolute yield (Y), and **f** relative yield (RY). Veg: vegetative stage, Flow: flowering stage, Early F. G.: early fruit growth stage, Harv: harvest stage

phenological stage (Fig. 5). Average RE values across fluorescence indices and phenological stages were 11.06 ± 0.19 , 12.02 ± 0.20 and $12.36 \pm 0.26\%$, for crop N content, relative yield and NNIi, respectively. The RE of prediction of DMP, crop N uptake and absolute yield ranged 13.49–33.55, 14.94–37.30 and 19.65–34.49%, respectively. In general, for most of the crop variables, the fluorescence index that presented lower RE was SFR-Ri, mainly in the flowering and early fruit growth stages (Fig. 5), and NBI-Ri, mainly in the harvest stage (Fig. 2S). In the vegetative stage, there was not an individual fluorescence index that stood out for most crop variables (Fig. 5).

Performance of fluorescence indices

An overall assessment of the performance of fluorescence indices for each phenological stage and crop variable was made considering R^2 and RMSE values of linear regression analysis of the calibration and validation datasets, and the slope and intercept values of linear regressions of the validation dataset (Table 3S). One of the best performing indices for most of the variables and phenological stages was SFRi (both under red and green excitation), followed by NBIi (both under red and green excitation) (Table 3S). FLAVi was the best performing index for absolute yield in most of the phenological stages (i.e., vegetative, early fruit growth and harvest stage). The same procedure explained before was calculated to know An assessment of the phenological stage in which each fluorescence index performed best was also made considering R^2 and RMSE values of linear regression analysis of the calibration and validation datasets, and the slope and intercept values of linear regressions of the validation dataset (Table 4S). In general, both SFR-Ri and SFR-Gi had their best performance in the flowering and early fruit growth stages (Table 4S). FLAVi had its best performance in the vegetative and harvest stages. The best performance of both NBI-Ri and NBI-Gi was in the vegetative, early fruit growth and harvest stages (Table 4S).

Discussion

Calibration relationships between integrated fluorescence indices, measured with the Multiplex® 3.6 sensor, and crop variables were variable in terms of R^2 values across phenological stages and the indices. In general, SFRi, NBIi and FLAVi were related to all six crop variables from the vegetative stage through to the harvest stage. With rice (Huang et al., 2019), these three indices were also correlated with above-ground biomass, leaf and plant N content, plant N uptake, and NNI. In the present study, SFRi and NBIi were positively related with crop variables, whereas FLAVi was inversely related with crop variables. These results concur with mainstream literature showing that chlorophyll indices, such as SFR, increases with N availability, whereas flavonols indices, such as FLAV, decreases with N availability (Cerovic et al., 2002; Gabriel et al., 2017; Tremblay et al., 2012).

Of all crop variables evaluated, the best calibration relationships were, in general, those between the indices and crop N content and NNIi. These results are in agreement with the study of Huang et al., (2019), which showed high correlation between fluorescence indices and plant N concentration in rice. They also agree with the results of Agati et al., (2015) and Cerovic et al., (2015) regarding the correlation of fluorescence indices to leaf N content. Calibration relationships between integrated indices and NNIi were generally equal to or slightly better than those with crop N content, in the four phenological stages. Similar results were obtained with muskmelon (Padilla et al., 2014). Calibration relationships with absolute yield indicated a relatively low capacity for fluorescence indices to predict yield in sweet pepper, which could be due to the differences in the length of the crop cycles and of yield between the crops, since sweet pepper is an indeterminate crop with multiple fruit harvests throughout the crop cycle. All fluorescence indices were better able to predict relative yield compared to absolute yield. However, only SFRi had a strong (i.e., R^2 value > 0.60) prediction capacity in most phenological stages, including the vegetative stage. This finding has implications for crop management because it suggests that SFRi could be useful to predict yield of sweet pepper, in relative terms, at early growth stages.

In general, the best performance of SFRi to predict crop N content, crop N uptake, NNli, and relative yield occurred in the first half of the crop cycle (i.e., vegetative, flowering, and early fruit growth stages). These results are consistent with studies in maize where the best relationships of SFR with crop N status were during the earlier stages of the crop (Gabriel et al., 2017; Quemada et al., 2014; Zhang et al., 2012). However, working with cucumber, contradictory results were reported by Padilla et al., (2016), where SFR was not a good predictor of crop N status in the vegetative stage. The discrepant results of Padilla et al., (2016) may be because of the time required to fully establish the different N treatments at the beginning of the transplanted cucumber crops.

In the present work, the relationships between SFRi and all crop variables, including absolute yield and relative yield, were weak in the harvest stage. However, there were stronger relationships with FLAVi and NBli in the early fruit growth and harvest stages, mostly in the relationships with crop N content, crop N uptake, and NNli. In the vegetative stage, the relationships between FLAVi and NBli and all crop variables, including absolute yield and relative yield were relatively weak. These results are consistent with those of Gabriel et al., (2017) and Quemada et al., (2014) in maize, where the performance of FLAV index to estimate leaf N content increased as the cropping season advanced. The better performance found in the first half of the crop cycle for the chlorophyll index SFRi could be due to leaf chlorophyll content increasing steadily in the first half of the cycle (de Souza et al., 2019), and a decrease in chlorophyll content thereafter (de Souza et al., 2019), likely because of translocation of N from leaves to fruits (da Cunha et al., 2015). This could have caused the weaker performance of SFRi indices in the harvest stage. In contrast, there was better performance of FLAVi and NBli in the harvest stage, compared to SFRi. A likely explanation for this contrasting behaviour may be in the dynamics of flavonols content throughout the crop. The FLAV index, measured with the Multiplex sensor as an indicator of flavonols content, was not different between N treatments in the vegetative stage (data not shown), but differences between treatments appeared at the middle of the crop and were maintained during the harvest stage and until the end of the crop (data not shown). This could explain the poor performance of FLAVi and NBli in the vegetative stage and the better performance in later phenological stages.

The validation analysis showed that lower relative errors (mostly < 20%) were found in the prediction of crop N content, NNI and relative yield. According to the classification of Yang et al., (2016), these relative error values indicate good prediction capacity. However, relative errors were, in general, > 20% for the prediction of absolute yield, indicating a relatively poor capacity to predict absolute crop yield in sweet pepper. These results contradict Tremblay et al. (2010) who reported that fluorescence indices were able to predict yield in wheat. The likely explanation for this discrepancy may be the kind of crop; wheat is a determinate crop with a single harvest at the end of the crop, and greenhouse sweet pepper is an indeterminate crop with multiple fruit harvests that can span a period of 3–4 months.

Generally, the behaviour of fluorescence indices in the validation and calibration analysis were similar. The prediction of crop N content, crop N uptake, NNli and relative yield, from SFRi values generally had slopes close to one and lower relative error values in the vegetative, flowering and early fruit growth stages. For FLAVi and NBli, poorer prediction occurred in the vegetative and flowering stages, and the prediction notably improved (i.e., slopes took values closer to one and relative error values were lower) in the early fruit growth and harvest stages. Slopes close to one and lower RE values represent excellent validation of regression equations (Gallardo et al., 2014; Pifeiro et al., 2008). The analysis of the performance of fluorescence indices to predict crop variables considering both calibration and validation results was mostly consistent with the analysis conducted

for calibration and validation separately (de Souza et al., 2020; Xin-feng et al., 2013). The best predictions of crop N content and NNli in the first half of the crop cycle (i.e., vegetative, flowering and early fruit growth stages) were with SFRi followed by NBI and FLAVi. However, in the second half of the crop cycle (i.e., harvest stage), the best predictions of crop N content and NNli were with NBI and FLAVi, followed by SFRi.

These results show that it is possible to predict crop N status in sweet pepper, throughout the crop, using fluorescence indices. As NNI was accurately estimated by fluorescence indices, and NNI is a good indicator for diagnosis of crop N nutrition (Lemaire et al., 2008), values of fluorescence indices can be used to determine if the amounts of N being applied throughout the crop cycle are optimal or not. Since optimal crop N status occurs when NNI values are equal to 1, and deficient crop N status when NNI is < 1 , on-going monitoring with fluorescence indices enables regular assessment of the adequacy of N fertilization. Knowing sweet pepper N status will contribute to appreciably improved crop N management, since vegetable growers commonly apply excessive amounts of N fertilizer as a risk avoidance strategy because they have no quantitative information of actual crop N status (Thompson et al., 2007, 2017b). This issue is more relevant in zones with a high density of sweet pepper crops, such as south-east Spain, where this crop is one of the most important with more than 8000 ha cultivated every year (Valera et al., 2017).

With all the fluorescence indices considered in this work, the SFR under both excitations was able to predict relative yield in the earlier phenological stages. This is important for growers because this early information on potential yield can guide N management at early phenological stages.

In general, in this work, the performance of SFR and NBI under red or green excitation were very similar in terms of R^2 values for the relationships of these indices with crop variables, as was reported for maize (Quemada et al., 2014) and cucumber (Padilla et al., 2016). The SFR under both excitations are related to the leaf chlorophyll concentration (Tremblay et al., 2012). The difference between red and green light is that green light penetrates more in the leaf tissues (Terashima et al., 2009) giving slightly different values of SFR because of different reabsorption of fluorescence by chlorophyll. Since the SFR under red or green excitation was very similar in the current work, it shows that the structure and chlorophyll concentration of sweet pepper does not affect much the SFR indices. The similitude between NBI under red or green excitation is explained because they are derived from SFR-R and SFR-G divided by the same parameter (FLAV) so that their difference in the estimate performance is expected to be similar to SFR indices. Therefore, the use of either red or green excitation with these indices is equally effective for monitoring and predicting sweet pepper N status.

In terms of crop N management, the high cost of flavonols meters (3,000–14,000€ in Europe, depending on the model) may make them unattractive to farmers. It is necessary to determine if the use of these sensors could make it attractive given the economic benefit of the reduction in the use of nitrogen fertilizers when using these tools. An alternative for the use of flavonols meters in farmers' best N management practices is through the service of technical advisors or consulting companies that provide flavonols meters measurements on farms.

Overall, the results of this work show the potential of fluorescence indices to predict crop N content, crop N uptake, NNli, and relative yield. It has been shown that selecting an optimized index for each phenological stage could be a potentially useful approach to derive different crop variables related to crop N content of sweet pepper. The use of these indices would be useful for the estimation of N status in the field, for guiding farmers in an

accurate application of N fertilisation throughout the crop cycle, and for increasing N use efficiency. The SFRi was most effective for prediction of crop N content and NNli at earlier stages of the crop (i.e., vegetative and flowering phenological stages), with either red (SFR-Ri) or green (SFR-Gi) excitation. In the harvest stage, the best capacity for prediction was with NBIi, both under red (NBI-Ri) or green (NBI-Gi) excitation, and with FLAVi. However, the high economical cost of fluorescence sensors can be an issue that hampers adoption by farmers.

Conclusions

- Selecting the best performing index in each phenological stage could improve the N use efficiency in sweet pepper crops.
- The SFRi indices showed the best performance at earlier phenological stage to predict crop N status.
- The NBIi indices and the FLAVi index showed the best performance at harvest stage to predict crop N status.
- The SFRi indices could be useful to predict relative yield of sweet pepper at early growth stages.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11119-021-09837-4>.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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