POTENTIAL INDICATORS BASED ON LEAF FLAVONOIDS CONTENT FOR THE EVALUATION OF POTATO CROP NITROGEN STATUS

F. Ben Abdallah

Crop Science Department, Phytotechnie Unit
Gembloux Agro-Bio Tech (GxABT), University of Liège
Gembloux, Belgium

J.P. Goffart

Production and Sectors Department, Crops Production Systems Unit
Walloon Agricultural Research Centre (CRA-W)
Gembloux, Belgium

ABSTRACTS

The assessment of in season crop nitrogen status (CNS) implies the use of plant-based indicators. Leaf flavonoids content appears as a valuable indicator of CNS. Flavonoids compounds can be measured by rapid and non-destructive optical methods generating chlorophyll fluorescence. The objective of this research was to compare the use of leaf flavonoids content as potential indicators for the evaluation of CNS with other recognized indicators such as chlorophyll content. Trials were conducted in Belgium in 2010 on two potato [*Solanum tuberosum* (L.)] cultivars: Charlotte and Bintje. The experiment included five nitrogen (N) rates for each cultivar. Leaf flavonoids content was determined using two devices, the Dualex and Multiplex (Force-A, Paris, France). Leaf chlorophyll content was measured with a SPAD/HNT chlorophyll-meter (Yara, Oslo, Norway), a Cropscan radiometer (Cropscan, Rochster, USA), Dualex and Multiplex devices. The measurements were made periodically during the potato growth cycle. Plant tissue samples were collected and analyzed allowing the calculation of the biomass N concentration and the N nutrition index. The indexes, provided by optical devices and related to N-status indicators, were evaluated by ANOVA and orthogonal contrast (SAS software) on the basis of three criteria: 1) sensitivity to N; 2) earliness of the diagnosis and 3) stability of the diagnosis. Neither flavonoids nor chlorophyll content of the leaf, considered alone, was able to address successfully all requirements. The combination of leaf flavonoids content to leaf chlorophyll content as SPAD/FLV and NBI suggests that these indexes could be used as valuable tools to assess the potato CNS.

Keywords: Flavonoids, Nitrogen, Potato

INTRODUCTION

Nitrogen (N) is an essential nutrient in plant growth affecting yield and quality. Moreover, potato crop have low nitrogen use efficiency due to its poorly developed root system increasing the N losses by leaching or evaporation. In order to maintain yield and quality and to preserve environmental pollution, there is a need to improve the crop nitrogen use efficiency. This implies fine management of N fertilization by the determination of the optimum rates and dates of fertilizer applications. The determination of optimum N rate can be calculated by software like Azobil (INRA, Laon, France) on the basis of different factors as estimated yield, variety, soil properties and cultural practices (Goffart et al., 2008). However, the application of the total N recommended rate at planting seems inappropriate. In fact, it is impossible to predict the total crop N requirements during the growing season in a context where weather conditions have a major impact on N mineralisation and crop N uptake (Goffart et al., 2008).

Thus, N strategies combining splitting of the N recommended rate and the assessment, during the growth cycle, of potato crop N status (CNS) allow to better match needs and supply (Goffart et al., 2008). Therefore several in season plant based methods have been developed to assess this CNS. These methods can help to decide on the need for supplementary fertilizer N application.

The Nitrogen Nutrition Index (NNI) is a reference method for CNS defined as the ratio between the actual N concentration and the critical N concentration (N_c) for a given biomass production. N_c is the minimum N concentration necessary to achieve maximum growth rate of the crop (Ulrich, 1952). A NNI lower than 1 indicates that N limits crop growth and a value higher than 1 indicates that N is in excess. A value near 1 indicates a sufficient N concentration in the crop. However, the establishment of the NNI at the farm level requires destructive and chemical analysis and does not suit for a quick assessment of CNS. The NNI can be used as a reference to calibrate other non-invasive methods for quick and simple in season monitoring of CNS (Goffart et al., 2008)

Therefore indicators which are closely related to the N nutrition of the crop were introduced and estimated using hand held devices on the basis of leaf/canopy spectral characteristics.

The leaf chlorophyll content was shown to be highly correlated to nitrogen status as most of the N in leaves is associated with photosynthetic machinery (Evans, 1983). It is already established that chlorophyll content decreased for nitrogen deficiency conditions. Chlorophyll content can be assessed based on leaf transmittance by a chlorophyll-meter (Minotti et al., 1994) or a specific device named Dualex (Force-A, Orsay, France). Chlorophyll content can also be assessed on the basis of crop light reflectance by a radiometer (Goffart et al., 2010). Besides transmittance and reflectance, leaf chlorophyll content can also be measured from its fluorescence properties by Multiplex device (Force-A, Orsay, France).

The leaf polyphenolics (Phen) content was also investigated as potential indicators for CNS. Nitrogen deficiency induces an increase in leaf Phen content (Bongue-Bartelsman and Phillips, 1995, Stewart et al, 2001). Phen include several families of chemical compound such as flavonoids (one of the largest classes of Phen). Devices using chlorophyll fluorescence have been developed to assess leaf Phen content (mainly flavonoids content in relation with the used wavelength) such as Dualex (Goulas et al., 2004, Cerovic et al., 2005, Cartelat et al., 2005, Tremblay et al.,2007,) and Multiplex (Ben Ghozlen et al., 2010, Zhang et al., 2012).

The chlorophyll/flavonoids ratio is suggested as a good and promising indicator for CNS. This ratio would alleviate at least partially the problem of chlorophyll and flavonoids gradients along leaves and would increase the discriminancy among levels of crop N deficiencies thanks to the opposite effect of N nutrition on chlorophyll and Phen (Cartelat *et al.*, 2005, Tremblay, 2007).

The objective of the current study was to evaluate the potentialities of leaf flavonoids content considered alone or combined to leaf chlorophyll content for the evaluation of potato CNS. On the basis of a comparative study of indexes provided by optical devices, three criteria were evaluated: 1) The sensitivity of the studied index; 2) the earliness of the diagnosis and 3) the stability of the index.

MATERIELS AND METHODS

Experimental design

The experiment was conducted at the experimental site of the Walloon Agriculture Research Center in Gembloux (Belgium) in 2010. The experiment included two potato [*Solanum tuberosum* (L.)] cultivars: Charlotte and Bintje. Five increasing N rates for each cultivar were applied at planting. A completely randomized block design with three replications was used in the field. The soil was loam. The cropping characteristics of the trial are given in Table 1. Each replication was divided into two parts. The first part (5m*3m) was used for the optical measurements and the assessment of final yield and the second part (7m*3m) was used for plant sampling during the season.

Optical data collection and analysis

Optical measurements were carried periodically during the potato growth from mid of June to end of July. All the optical measurements were made on uniform appearance plants in the four rows of each plot avoiding the borders lines.

Table 1. Cropping characteristics of 2010 potato trial

Variety	Bintje	Charlotte
Planting date	April 26	April 26
Planting density (plant.ha^{-1})	35088	41667
Full emergence date	May 26	June 3
N Recommended rate (kg .ha^{-1})=T_3†	165	140
N rate (kg .ha^{-1})	T_1 : 0	T_1 : 0
	T_2 : 115	T_2 : 100
	T_3 : 165	T_3 : 140
	T_4 : 251	T_4 : 180
	T_5 : 248	T_5 : 210

†Nitrogen recommended rate established by the software AZOBIL (INRA, Laon France)

The measurements were collected on the same stage with the different sensors but not on the same leaves/plants. This also was the case for the different dates, where different plants were considered. The obtained data summarised in Table 2 are expressed as index.

The chlorophyll-meter Hydro N Tester (SPAD/HNT, Yara, Oslo, Norway) and the Dualex (Force-A, Orsay, France) are leaf clip sensors (corresponding respectively to 2-3 mm^2 and 19-20 mm^2 of leaf area). The measurements were made on distal leaflet of the first fully developed leaf from the top of the canopy (corresponding to the 4th or 5th leaf from the apex of a main stem) avoiding midribs. The measurements concern the upper face of the leaf. On each date, 60 individual readings were collected for each plot and averaged across the replications of a same N treatment. These sensors provide an estimation of leaf chlorophyll content by the SPAD index (Hydro N Tester) and by the CHL index (Dualex). The measurement is based on transmittance of two wavelengths, one in the red and another one in the near infrared. The red light is absorbed by chlorophyll while at the infrared light no absorption occurs and the light is then transmitted. The used wavelengths provided by the Hydro N Tester differ from the used wavelengths provided by the Dualex.

Besides the CHL index, the Dualex sensor provides two other indexes: FLV and NBI. The leaf flavonoids content, estimated by the FLV index, is deduced from flavonoids UV absorbing properties. This approach is based on fluorescence technique using two excitation wavelengths: one in the UV wavelength (375 nm) that is absorbed by flavonoids, mainly located in epidermis, and one reference wavelength that go through the epidermis without being absorbed before reaching chlorophyll in the mesophyll. The ratio between CHL/FLV enables the estimation of the NBI index.

The Multiplex (Force-A, Orsay, France) is a handheld multi-parametric fluorescence sensor which provides measurements on leaves. The sensor generates four excitations on the UV (375 nm), the blue (B), the green (G) and the red (R) wavelength and detect the yellow (YF), the red (RF) and the far-red (FRF) fluorescence (Ben Ghozlen et al., 2010). Measurement distance is 10 cm from light sources and the measurement surfaces is 50 cm^2. The sensor was downward in contact to the leaf surface. The Multiplex readings were obtained on the fully developed leaf from the top of the canopy as Dualex and SPAD readings. On each date, 60 individual measurements were made and averaged across the replications of a same N treatment. Fluorescence ratios are computed directly by the Multiplex and give information on chlorophyll content and flavonoids content. The chlorophyll content is estimated by the SFR-R index defined as the simple fluorescence emission ratio FRF-R/RF-R. This parameter is based on increasing chlorophyll fluorescence re-absorption at its shorter wavelength peak when chlorophyll content increases (Buschmann et al., 2007). The Multiplex flavonoids content is estimated by the FLAV index based on the same principle than the Dualex FLV index. The chlorophyll to flavonoids ratio is estimated by NBI-R index computed as the ratio FRF-UV /RF-R.

The Cropscan is a passive near remote sensing measuring crop light reflectance. The radiometer is extended on a boom to a height of 2 m above the ground (±1.5 m above canopy) providing a circular field area of 1 m^2.

Table 2. Description of indexes provided through the optical devices

Optical devices	Index	Indicators	Formula†
Hydro N Tester	SPAD	chlorophyll	T_{650}/T_{940}
Cropscan	R	chlorophyll	$\{[(RE_{610}/I_{610})*100]+[(RE_{650}/I_{650})*100]\}/2$
	NIR	chlorophyll	$\{[(RE_{760}/I_{760})*100]+[(RE_{810}/I_{810})*100]\}/2$
Dualex	CHL	chlorophyll	$(1/T_R)-(1/T_{IR})$
	FLV	flavonoids	Log(FRF-R/FRF-UV)
	NBI	chlorophyll and flavonoids	CHL /FLV
Multiplex	SFR-R	chlorophyll	FRF-R/RF-R
	FLAV	flavonoids	Log(FRF-R/FRF-UV)
	NBI-R	chlorophyll and flavonoids	FRF-UV/RF-R
Hydro N Tester and Dualex	SPAD/FLV	chlorophyll and flavonoids	SPAD/FLV
Hydro N Tester and Multiplex	SPAD/FLAV	chlorophyll and flavonoids	SPAD/FLAV

†T_{650} and T_{940}: Transmittance respectively at 650 and 940 nm, RE_{610}, RE_{650}, RE_{760}, and RE_{810}: Reflected Light at respectively 610, 650, 760, and 810 nm, I_{610}, I_{650}, I_{760}, and I_{810}: Incident Light at respectively 610, 650, 760 and 810 nm, T_R and T_{IR}: Transmittance respectively at Red and Infra Red wavelength, FRF-R : Far Red Fluorescence under Red excitation, FRF-UV : Far Red Fluorescence under UV excitation, RF- R : Red Fluorescence under Red excitation .

The Cropscan readings were obtained between noon and 2.00 pm (GMT). The radiometer was faced downward perpendicular to the crop surface (nadir view). On each date, 5 measurements were made and averaged across the replications. The sensor allows the use of eight wavebands from 460 nm to 810 nm at 50 nm intervals. Irradiance and radiance were stored for each waveband allowing the calculation of canopy reflectance as the ratio between radiance and irradiance. R and NIR indexes were computed respectively as canopy reflectance in the red (average of reflectance at 610 and 650 nm) and near infrared spectrum (average of reflectance at 760 and 810 nm). A leaf with high chlorophyll content exhibits higher reflectance in the near infrared spectrum than in the red spectrum.

SPAD/FLV and SPAD/FLAV indexes were calculated as the ratio between chlorophyll index provided by the Hydro N Tester and flavonoids index provided by Dualex or Multiplex.

Samples collection and analysis

After optical measurements were performed, plant tissue samples were collected for five dates. For each replication, eight whole plants were collected and carried to the laboratory. The plants were weighed for the determination of fresh weight and then dried at 80° C until constant dry weight. The dry matter (DM) content is then calculated. For the different plant tissue, crushed samples were subjected to laboratory analyses on the basis of near infrared reflectance spectroscopy allowing the determination of the total N for different parts of the plant (expressed as % DM). The NNI of the crop was determined by dividing the actual N concentration of total biomass (shoot and tuber biomass) by the N_c. As calculated by Lemaire and Gastal (1997), N_c was determined by equation1:

$N_c\% = a * (W)^{-b}$ Equation 1

Where: W: is the total shoot and tuber biomass expressed in t DM.ha^{-1}, *a*: represents the N concentration in the total biomass with 1 t DM ha^{-1} and *b*: represents the coefficient of dilution (decreasing N concentration with increasing biomass).

For a W below 1.3 t DM ha^{-1}, N_c takes a constant value of 4.5%. The coefficient *a* and *b* were estimated for Bintje as 5.21 and 0.56 respectively. In the absence of reference for Charlotte, these parameters take the same value than Bintje.

Statistical analysis

The statistical analysis was performed with the SAS software package (SAS 9.2). The studied indexes (including all the sampling dates) were first subjected to analysis of variance (ANOVA) using PROC MIXED (Tukey). The effect of N, DAS and N*DAE interaction were studied. Thereafter, data for each sampling dates were subjected to ANOVA and the N responses curves were analysed through polynomial contrasts. Finally, the studied indexes were compared according to the outlined criteria.

RESULTS AND DISCUSSION

Effect of nitrogen rate and whole sampling dates on diagnosis indexes

A statistical analysis of repeated measurements (including the eight measurement dates) showed that all the studied indexes were significantly influenced by applied N dose (Table 3). Differences for FLV, NBI, SFR-R and SPAD/FLV indexes were highly significant (P<0.001) for both varieties. Significant effects of DAE were observed for all variables. The interaction effects of N fertilizer and sampling dates was not significant for CHL, NIR and SPAD/FLV indexes for both varieties. The interaction was also not significant for both NBI and SFR-R indexes for Charlotte and for both SPAD and R indexes for Bintje.

Effect of nitrogen rate on diagnosis indexes for each date of sampling

Chlorophyll indicators

SPAD index showed generally low values for T_1 compared to the N fertilized levels (Table 4). For Charlotte, the significance appears at 36, 43 and 57 DAE. The relationship with N fertilization can be described as curvilinear.

Table 3. Responses of the studied indexes to N rates, DAE and to N*DAE interaction

	Fixed effect					
Variety	**Bintje**			**Charlotte**		
Index	**N**	**DAE**	**N*DAE**	**N**	**DAE**	**N*DAE**
CHL	***	***	NS	*	***	NS
FLV	***	***	***	***	***	**
NBI	***	***	**	***	***	NS
SPAD	*	***	NS	***	***	*
SFR-R	***	***	*	***	***	NS
FLAV	***	***	***	**	***	*
NBI-R	***	***	***	**	***	**
R	*	***	NS	***	***	*
NIR	**	***	NS	**	***	NS
SPAD/FLV	***	***	NS	***	***	NS
SPAD/FLAV	***	***	**	**	***	*

N: Nitrogen effect. DAE: Days After Emergence effect, N*DAE: interaction between N and DAE. *, ** and ***: statistical significance respectively at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$. NS indicates no significance at $P>0.05$.

For Bintje, the significant relationship was almost curvilinear at 34, 41, 48 and 58 DAE except at 17 and 20 DAE where linear relationships were found. The curvilinear shape of SPAD measurements was also reported for potato (Minotti et al., 1994) and corn (Tremblay et al., 2007, Zhang et al., 2012). Tremblay et al. (2007) explained the curvilinear response of the relationship by a relatively strong increase in SPAD values between non-fertilized treatment and medium rate of fertilizer and a low or no effect for the higher N rates. According to Shröder et al. (2000), SPAD index is a poor indicator of excess N because not all the N uptaken is converted to chlorophyll.

Starting from the second date of sampling, the T_1 showed low values of CHL index compared to moderate and high N treatments (Table 4). CHL index was generally not significantly affected by N treatments except at only two dates for Bintje (34 and 48 DAE). CHL index is therefore not a reliable indicator for the evaluation of N status among all the growth stages.

The non-fertilized plants (T_1) had generally lower values of SFR-R index than the N fertilized plants (Table 4). SFR-R index expressed a significant curvilinear response to N dose for Bintje at 28, 58, and 62 DAE. For Charlotte, this chlorophyll indicator showed a significant curvilinear response to N dose at 29, 47, and 57 DAE and a significant linear response at 36 DAE.

Higher average values of R index were obtained corresponding to T_1 while NIR index showed lower values (generally from the third sampling date) (Table 4). This opposite dependence to N levels of R and NIR indexes was also reported by Goffart et al. (2010). For Bintje, R index expressed a significant N response at 28, 34, 41, 48, and 62 DAE and NIR index at 34, 41, and 48 DAE. For Charlotte, a significant N response was found at 29, 36, and 43 DAE for R and NIR indexes and also at 57 DAE for NIR index. The response of R and NIR indexes were linear or curvilinear.

Flavonoids indicators

FLV index showed higher values for T_1 comparatively to the N fertilized treatments, in agreement with the theory relating increases in Phen to higher N stress levels (Table 4). Previous studies have revealed this inverse relationship between leaf Phen and the level of N fertilization (Cartelat et al., 2005, Tremblay et al., 2007, Zhang et al., 2012). For Bintje, no significant relationship with N dose was found for 20 and 41 DAE. For the others sampling dates, the significance was curvilinear at 28, 34, 48, and 62 DAE and linear at 17 and 58 DAE. For Charlotte, the significance with N rate was curvilinear from 20 until 43 DAE and linear at 47 DAE.

FLAV index as FLV index are related to flavonoids content and then usually increased from the N fertilized treatments to T_1 (Table 4). However, Zhang et al. (2012) reported an increase in FLAV readings with N dose for corn. A strong curvilinear relationship with N doses was found for Bintje at all sampling dates except at 17 DAE where a significant linear response was found. For Charlotte, the response of FLAV index was less consistent than for Bintje expressing a significant linear or curvilinear relationship at 20, 43, 47, and 57 DAE.

Chlorophyll and flavonoids indicators

NBI index usually increased with N rates comparing the T_1 to the other increasing N treatments (Table 4). As N rates increased, chlorophyll content increased and flavonoids content decreased explaining then the increase of NBI index. For Bintje, NBI index was affected by linear and curvilinear components in relationship to N rates but not at 20 or 41 DAE. This index was also statistically significant for Charlotte at six dates of sampling, but not at 12 or 47 DAE. The relationship with N dose for Charlotte was linear except at 20 DAE where a curvilinear response was found.

Similarly to NBI index, NBI-R index increased with N rates from T_1 to N fertilized treatments (Table 4). For Bintje NBI-R expressed a strong curvilinear response to N rates from 20 until 62 DAE. For Charlotte, this index was less sensitive comparatively to Bintje expressing a linear or curvilinear response to N rates at 20, 29, 43, 47, and 57 DAE.

SPAD/FLV index expressed lower values for T_1 compared to moderate or high N levels (Table 4). More consistent increase for SPAD/FLV with N dose across all treatments have been reported by Zhang et al. (2012) in corn. With the exception of 20 and 62 DAE, SPAD/FLV index for Bintje expressed a linear or curvilinear significant response to N treatments. For Charlotte, a curvilinear relationship with N dose was found at 20, 29, 36, 43, and 57 DAE.

SPAD/FLAV index showed a similar response of SPAD/FLV index to N dose (Table 4). Strong linear or curvilinear relationship of SPAD/FLAV was found for Bintje from the first date of sampling until 58 DAE. SPAD/FLAV index expressed a significant relationship for Charlotte at 20, 36, 43, 47, and 57 DAE.

NNI varied from 0.88 to 1.56 across all treatments and sampling dates (Table 4). In our study, the increase of NNI was generally consistent between the T1 compared to the different level of N treatments. The NNI for all N treatments was optimum (above 1) at the first date of plant sampling. Thereafter, the NNI corresponding to T_1 was slightly below 1 indicating a small deficiency of the crop N nutrition.

Table 4. Responses of studied indexes to applied N dose at different Days After potato Emergence (DAE).

Variety		Bintje								Charlotte									
DAE	Index	Applied N dose(kg.ha^{-1})					ANOVA	Contrast analysis		DAE	Index	Applied N dose(kg.ha^{-1})					ANOVA	Contrast analysis	
		0	115	165	215	248	N	Linear	Quadratic			0	100	140	180	210	N	Linear	Quadratic
17	CHL	34,9	34,9	35,8	35,4	35,2	NS	NS	NS	5	CHL	33,9	33,7	34,2	34,6	33,6	NS	NS	NS
	FLV	0,63	0,60	0,60	0,59	0,59	*	**	NS		FLV	0,76	0,72	0,75	0,73	0,72	NS	*	NS
	NBI	56,0	58,9	60,7	60,4	60,1	*	**	NS		NBI	45,2	47,4	46,5	48,1	47,2	*	**	NS
	SPAD	43,2	43,3	44,0	44,2	44,1	*	**	NS		SPAD	45,5	45,9	46,6	45,9	46,1	NS	NS	NS
	SFR-R	2,65	2,64	2,64	2,68	2,66	NS	NS	NS		SFR-R	2,46	2,51	2,53	2,56	2,48	NS	NS	NS
	FLAV	0,34	0,33	0,33	0,34	0,32	*	**	NS		FLAV	0,49	0,49	0,48	0,51	0,48	NS	NS	NS
	NBI-R	1,22	1,25	1,25	1,25	1,28	NS	*	NS		NBI-R	0,75	0,78	0,79	0,75	0,78	NS	NS	NS
	R	10,0	9,8	9,8	9,7	10,3	NS	NS	NS		R	10,8	11,2	11,6	10,6	10,7	NS	NS	NS
	NIR	27,5	27,8	26,1	27,8	28,0	NS	NS	NS		NIR	25,3	25,2	24,6	24,3	26,0	NS	NS	NS
	SPAD/FLV	68,4	71,9	73,8	74,7	74,3	**	***	NS		SPAD/FLV	59,9	63,8	62,5	62,8	63,9	NS	NS	NS
	SPAD/FLAV	125,4	131,1	134,2	131,6	136,1	**	**	NS		SPAD/FLAV	92,3	94,6	97,2	90,2	95,8	NS	NS	NS
20	CHL	35,0	35,9	35,6	35,7	35,8	NS	NS	NS	12	CHL	35,1	36,3	36,5	36,3	36,9	NS	NS	NS
	FLV	0,92	0,91	0,90	0,91	0,90	NS	NS	NS		FLV	0,97	0,91	0,92	0,90	0,90	NS	**	NS
	NBI	39,0	40,7	40,5	40,5	40,7	NS	NS	NS		NBI	36,9	40,8	40,5	41,3	42,1	NS	NS	NS
	SPAD	41,0	42,3	42,4	42,7	42,9	*	**	NS		SPAD	43,1	43,6	43,6	44,2	43,8	NS	NS	NS
	SFR-R	2,57	2,58	2,61	2,60	2,61	NS	NS	NS		SFR-R	2,5	2,5	2,5	2,5	2,5	NS	NS	NS

	FLAV	0,50	0,47	0,47	0,47	0,47	**	***	*
	NBI-R	0,83	0,89	0,90	0,91	0,91	***	***	*
	R	7,5	6,7	7,1	6,7	6,9	NS	NS	NS
	NIR	37,7	38,2	36,8	38,3	41,0	NS	NS	NS
	SPAD/FLV	44,6	46,7	46,9	47,2	47,6	NS	*	NS
	SPAD/FLAV	82,1	90,2	90,3	91,8	92,2	***	***	*
	INN	1,34	1,41	1,41	1,45	1,43	NS	NS	NS
28	CHL	36,5	38,8	39,3	38,9	39,0	NS	NS	NS
	FLV	1,28	1,15	1,12	1,11	1,11	***	***	**
	NBI	29,1	34,2	35,7	35,9	35,8	*	**	NS
	SPAD	45,6	47,8	47,5	47,8	47,9	NS	*	NS
	SFR-R	2,73	2,85	2,83	2,85	2,81	*	*	*
	FLAV	0,75	0,67	0,64	0,64	0,64	***	***	**
	NBI-R	0,51	0,64	0,68	0,68	0,67	***	***	**
	R	5,5	4,7	4,8	4,4	4,6	**	**	NS
	NIR	45,1	47,4	48,9	50,5	50,0	NS	NS	NS
	SPAD/FLV	35,7	41,5	42,5	43,2	43,2	**	***	NS
	SPAD/FLAV	60,8	71,7	74,6	74,8	74,6	**	***	*
34	CHL	58,9	67,4	70,4	67,7	68,5	**	***	**

		8	3	4	7	6			
	FLAV	0,64	0,60	0,63	0,63	0,60	NS	NS	NS
	NBI-R	0,57	0,60	0,57	0,57	0,61	NS	NS	NS
	R	8,8	8,6	9,3	7,9	8,2	NS	*	NS
	NIR	37,4	34,9	37,8	37,1	35,0	NS	NS	NS
	SPAD/FLV	44,4	48,1	47,4	48,9	48,7	NS	*	NS
	SPAD/FLAV	67,9	72,7	69,1	70,2	72,5	NS	NS	NS
	INN	1,15	1,37	1,38	1,38	1,40	**	***	*
20	CHL	39,2	40,4	41,1	41,6	40,9	NS	*	NS
	FLV	1,26	1,06	1,07	1,06	1,06	**	**	**
	NBI	31,4	38,5	38,8	39,9	39,3	**	***	*
	SPAD	46,4	48,5	49,2	49,3	48,7	NS	*	NS
	SFR-R	2,69	2,73	2,72	2,75	2,69	NS	NS	NS
	FLAV	0,88	0,75	0,80	0,78	0,76	**	**	*
	NBI-R	0,34	0,48	0,42	0,46	0,46	**	**	*
	R	6,8	5,8	6,1	5,7	5,7	NS	NS	NS
	NIR	42,0	44,4	45,2	45,1	45,0	NS	NS	NS
	SPAD/FLV	36,7	45,6	45,8	46,5	46,0	**	***	*
	SPAD/FLAV	52,6	64,8	61,2	63,6	64,0	**	**	*
29	CHL	66,3	71,0	70,6	71,9	70,6	NS	*	NS

	FLV	1,36	1,22	1,20	1,19	1,17	***	***	*		FLV	1,36	1,18	1,20	1,21	1,16	**	***	*
	NBI	44,3	57,2	60,3	58,0	60,1	***	***	*		NBI	49,6	61,6	59,8	60,8	62,3	**	**	NS
	SPAD	48,1	53,2	53,4	53,2	53,7	***	***	**		SPAD	55,4	57,9	57,4	58,1	58,2	NS	*	NS
	SFR-R	2,77	2,93	2,92	2,89	2,91	NS	*	NS		SFR-R	2,67	2,81	2,80	2,80	2,78	**	**	**
	FLAV	0,85	0,70	0,66	0,64	0,63	***	***	*		FLAV	0,92	0,83	0,84	0,81	0,80	NS	**	NS
	NBI-R	0,41	0,60	0,65	0,68	0,70	***	***	*		NBI-R	0,31	0,41	0,39	0,42	0,43	*	**	NS
	R	7,2	5,4	5,5	5,1	5,1	***	***	*		R	7,6	5,5	6,0	5,5	5,3	**	***	NS
	NIR	38,5	41,2	42,0	42,5	43,8	**	***	NS		NIR	39,9	44,9	44,4	43,9	44,6	*	**	NS
	SPAD/FLV	35,3	43,8	44,7	44,6	46,0	***	***	*		SPAD/FLV	40,6	49,1	47,7	48,1	50,2	**	***	NS
	SPAD/FLAV	56,7	76,0	80,5	82,9	85,6	***	***	*		SPAD/FLAV	60,5	70,2	68,2	72,1	72,8	NS	**	NS
	INN	0,92	1,21	1,26	1,29	1,42	**	***	NS		INN	0,88	1,29	1,31	1,11	1,24	***	***	*
41	CHL	71,8	75,2	79,0	77,1	77,2	NS	*	NS	36	CHL	65,2	68,7	69,5	70,3	68,8	NS	NS	NS
	FLV	1,24	1,18	1,17	1,15	1,13	NS	*	NS		FLV	1,41	1,23	1,28	1,22	1,22	**	***	*
	NBI	59,5	65,5	69,0	68,1	69,5	NS	*	NS		NBI	47,0	57,0	55,3	58,6	57,5	*	**	NS
	SPAD	45,9	51,0	51,5	51,1	51,9	***	***	***		SPAD	51,8	56,8	56,7	56,2	56,3	**	***	**
	SFR-R	2,80	2,88	2,90	2,88	2,87	NS	*	NS		SFR-R	2,49	2,59	2,57	2,63	2,59	*	**	NS
	FLAV	0,80	0,70	0,68	0,66	0,66	***	***	*		FLAV	0,86	0,80	0,79	0,78	0,78	NS	*	NS
	NBI-R	0,46	0,59	0,62	0,64	0,64	***	***	*		NBI-R	0,34	0,40	0,40	0,42	0,42	NS	*	NS
	R	4,7	3,8	3,8	3,6	3,7	***	***	**		R	7,0	4,6	5,0	4,8	4,8	***	***	**
	NIR	47,3	52,3	50,8	53,4	52,4	***	***	*		NIR	40,	46,	45,	46,	46,	*	**	NS

												4	6	6	6	4			
	SPAD/FLV	37,2	43,4	44,0	44,3	45,8	**	***	NS		SPAD/FLV	36,7	46,3	44,4	46,3	46,1	**	***	*
	SPAD/FLAV	57,2	73,2	75,7	77,6	78,6	***	***	**		SPAD/FLAV	60,6	71,5	71,8	71,9	72,0	*	**	NS
	INN	0,90	1,18	1,27	1,28	1,37	**	***	NS		INN	0,94	1,31	1,18	1,28	1,36	**	***	NS
48	CHL	61,7	66,2	69,8	70,0	67,9	*	**	NS	43	CHL	66,2	68,1	69,5	69,7	69,5	NS	NS	NS
	FLV	1,33	1,14	1,12	1,12	1,10	***	***	**		FLV	1,27	1,13	1,15	1,11	1,11	**	**	*
	NBI	47,1	60,2	64,3	64,2	63,6	**	***	*		NBI	53,2	61,3	62,1	64,0	64,0	*	**	NS
	SPAD	46,9	50,4	51,3	50,6	51,2	***	***	***		SPAD	48,0	52,0	51,4	51,5	51,2	*	**	*
	SFR-R	2,44	2,60	2,65	2,66	2,66	NS	NS	NS		SFR-R	2,56	2,63	2,69	2,70	2,66	NS	NS	NS
	FLAV	0,77	0,65	0,68	0,67	0,68	**	**	*		FLAV	0,74	0,63	0,66	0,65	0,66	*	*	*
	NBI-R	0,42	0,60	0,56	0,59	0,57	**	***	**		NBI-R	0,45	0,58	0,55	0,57	0,55	*	*	*
	R	6,3	5,9	5,6	6,1	5,9	*	*	*		R	5,6	5,3	4,8	4,8	5,0	*	**	NS
	NIR	35,1	38,2	39,4	39,0	38,6	*	**	*		NIR	34,7	40,9	42,0	41,2	41,6	*	**	NS
	SPAD/FLV	35,2	44,4	45,8	45,2	46,5	***	***	**		SPAD/FLV	37,7	46,1	44,8	46,3	46,0	*	**	*
	SPAD/FLAV	60,8	77,3	75,3	76,1	75,9	**	***	**		SPAD/FLAV	65,2	82,6	78,2	79,7	77,6	**	***	*
	INN	0,90	1,26	1,23	1,36	1,35	***	***	*		INN	0,99	1,21	1,28	1,20	1,27	**	***	*
58	CHL	62,1	66,4	70,0	69,6	69,6	NS	**	NS	47	CHL	60,7	61,1	62,1	64,1	61,5	NS	NS	NS
	FLV	1,36	1,20	1,14	1,13	1,10	**	***	NS		FLV	1,29	1,16	1,18	1,16	1,16	*	**	NS
	NBI	47,1	57,1	63,2	63,8	65,6	*	**	NS		NBI	48,2	54,2	54,2	56,8	54,8	NS	*	NS
	SPAD	47,5	51,2	51,1	51,7	51,9	***	***	*		SPAD	47,	49,	49,	49,	50,	NS	*	NS

												5	8	9	7	1			
	SFR-R	2,51	2,78	2,79	2,77	2,83	***	***	**		SFR-R	2,22	2,34	2,31	2,32	2,33	**	**	*
	FLAV	0,94	0,74	0,72	0,75	0,73	***	***	***		FLAV	0,84	0,76	0,77	0,77	0,76	**	**	*
	NBI-R	0,30	0,52	0,55	0,51	0,54	***	***	***		NBI-R	0,33	0,40	0,39	0,40	0,40	**	***	**
	R	6,5	5,7	5,1	5,8	5,6	NS	*	NS		R	7,0	6,2	6,9	6,8	6,7	NS	NS	NS
	NIR	48,0	49,7	52,4	50,2	51,5	NS	NS	NS		NIR	41,4	44,3	42,3	42,1	42,5	NS	NS	NS
	SPAD/FLV	34,9	42,7	44,8	45,9	47,2	***	***	NS		SPAD/FLV	36,8	43,0	42,4	42,8	43,1	NS	*	NS
	SPAD/FLAV	50,6	69,2	71,2	69,5	71,2	***	***	***		SPAD/FLAV	56,6	65,4	64,6	64,6	65,5	*	**	NS
	INN	-	-	-	-	-	-	-	-		INN	0,95	1,23	1,20	1,26	1,29	***	***	NS
62	CHL	61,9	68,4	71,3	72,2	70,7	NS	**	NS	57	CHL	67,7	73,9	73,4	72,0	72,8	NS	*	NS
	FLV	1,41	1,20	1,13	1,10	1,09	***	***	*		FLV	1,33	1,18	1,21	1,20	1,18	NS	*	NS
	NBI	45,1	59,6	65,2	68,8	66,8	**	***	NS		NBI	52,5	65,5	63,3	63,0	64,0	*	*	NS
	SPAD	56,2	58,6	54,5	49,0	50,7	NS	NS	NS		SPAD	43,3	47,9	48,7	47,5	47,8	***	***	***
	SFR-R	2,53	2,78	2,77	2,81	2,81	**	**	*		SFR-R	2,41	2,65	2,59	2,62	2,58	***	***	***
	FLAV	0,93	0,70	0,67	0,69	0,68	***	***	**		FLAV	0,92	0,82	0,84	0,77	0,81	*	**	NS
	NBI-R	0,31	0,59	0,62	0,60	0,61	***	***	**		NBI-R	0,29	0,39	0,37	0,45	0,39	*	**	NS
	R	5,9	4,5	4,6	4,2	4,3	***	***	**		R	5,1	4,4	4,3	4,1	4,4	NS	*	NS
	NIR	46,2	51,0	52,3	54,0	52,6	NS	*	NS		NIR	37,4	46,7	47,2	46,1	44,6	*	*	*
	SPAD/FLV	39,7	49,0	48,1	44,6	46,5	NS	NS	NS		SPAD/FLV	32,5	40,8	40,3	39,5	40,5	**	**	*
	SPAD/FLAV	60,7	83,6	81,1	70,9	75,1	NS	NS	NS		SPAD/FLAV	47,1	58,3	58,3	61,7	58,9	**	**	NS

	INN	0,98	1,35	1,53	1,49	1,56	**	***	NS	INN	-	-	-	-	-	-	-	-

N: Nitrogen effect. DAE: Days After Emergence. *, **, and *** indicate statistical significance respectively at P ≤0.05, P ≤ 0.01, and P ≤ 0.001. NS indicates no significance at P>0.

The NNI corresponding to N fertilized treatments showed data greater than 1 indicating that N was not limiting growth. Significant N dose were found with linear or curvilinear components for both varieties (except at 20 DAE for Bintje). The values slightly below 1 indicate that the effect of N deficiency was less notable and could be related to an important N contribution from the soil or could reflect an inconsistency of NNI since visual symptoms of chlorosis have appeared for T_1 during the growth cycle. It would be necessary then to establish the critical N curve for our pedoclimatic conditions and for the studied cultivars.

Ideally the response of indexes would be linear with N doses relating to the sensitivity of indicators among all N treatments. In this study, the shape of indexes response was mainly curvilinear indicating a low difference between the medium and high N rates. This could reflect a lack of sensitivity of indexes to increasing N rates or could be related to the drying conditions in 2010, in June and early July, limiting the crop N uptake.

Comparison of studied indexes among the criteria

This work is a preliminary step to evaluate the quality of the studied indicators on the basis on sensitivity, earliness of diagnosis and stability of the indicator. The sensitivity of an indicator reflects how quickly it reacts to change in nutrition (Cartelat et al., 2005). The earliness of the response implies that the indicator should allow early detection of nitrogen deficiency long before symptoms of chlorosis appear in the foliage. The stability of an indicator reflects its reliability during the growth cycle.

Sensitivity

Comparing the studied indexes for Bintje, FLAV index was able to reveal significant N response for all the sampling dates followed by NBI-R and SPAD/FLAV indexes (significant N response at seven of eight measurements dates) and then NBI, SPAD/FLV, FLV, and SPAD indexes (significant N response at six dates). Taken into account the studied indexes for Charlotte, NBI showed a significant N response for six dates of measurements. Then, NBI-R, SPAD/FLAV, SPAD/FLV, and FLV indexes were able to reveal significant N response at five of eight measurements dates.

In order to study further the sensitivity of indexes over a wide range of N availability, a Sensitivity Index (SI) was therefore calculated (Table 5). The SI corresponds to the ratio between changes in the response of the measuring index divided by the corresponding change of N concentration in shoots (Equation 2).

$$SI = \frac{(Index_{T_5} - Index_{T_1})/(Index_{T_1})}{(N_{T_5} - N_{T_1})/(N_{T_1})} \qquad \text{Equation 2}$$

Where:

$Index_{T5}$ and $Index_{T1}$: Studied index corresponding respectively to T_5 and T_1 treatments.

N_{T5} and N_{T0}: Nitrogen concentration in leaves corresponding respectively to T_5 and T_1 treatments (% of DM).

Table 5. Sensitivity index of the studied indexes across the five date of sampling for each variety.

SI†	Variety	Bintje						Charlotte					
	DAE	20	34	41	48	62	AV‡	12	29	36	43	47	AV‡
CHL		0,39	0,43	0,25	0,34	0,26	0,33	0,25	0,23	0,19	0,22	0,05	0,19
FLV		-0,31	-0,38	-0,29	-0,58	-0,42	0,39	-0,35	-0,52	-0,45	-0,56	-0,38	0,45
NBI		0,69	0,94	0,56	1,17	0,89	0,85	0,69	0,9	0,76	0,91	0,53	0,76
SPAD		0,73	0,31	0,43	0,31	-0,18	0,32	0,07	0,18	0,29	0,3	0,22	0,21
SFR-R		0,23	0,13	0,08	0,31	0,2	0,19	-0,04	0,14	0,13	0,18	0,2	0,12
FLAV		-1,06	-0,69	-0,59	-0,42	-0,5	0,65	-0,26	-0,44	-0,29	-0,46	-0,34	0,36
NBI-R		1,53	1,8	1,28	1,07	1,54	1,45	0,35	1,33	0,72	1,04	0,95	0,88
R		-1,25	-0,78	-0,73	-0,22	-0,49	0,69	-0,34	-1,07	-1,07	-0,46	-0,18	0,62
NIR		1,35	0,36	0,35	0,34	0,25	0,53	-0,31	0,42	0,5	0,9	0,1	0,32
SPAD/FLV		1,04	0,8	0,77	1,08	0,31	0,8	0,46	0,83	0,86	0,99	0,67	0,76
SPAD/FLAV		1,92	1,34	1,24	0,83	0,44	1,16	0,33	0,72	0,63	0,86	0,61	0,63

†Sensitivity index ‡AV: Average absolute Values

Negative values of SI have appeared in Table 5 for FLV, FLAV, and R indexes confirming the negative relationship with N dose at all the sampling dates. A negative SI was also obtained for SPAD, SFR-R, and NIR indexes but only at one date of sampling showing inconsistency of the optical measurements. The SI allows comparing the sensitivity of the parameters according to the degree of their response to low and high N fertility conditions. The higher is the absolute values of SI, the higher is the impact of N concentration on the studied index. The absolute values of SI above 1 were removed from the comparison. The data corresponding to a higher variation of index response than variation of leaves N concentration are not consistent or could be related to a lack of specificity of corresponding indicators. The average absolute values of SI across the five sampling dates allowed to classify the studied indexes according to a decreasing degree of response. The indexes were listed as follow for Bintje: NBI> SPAD/FLV> R> FLAV> NIR> FLV> CHL> SPAD>SFR-R. And for Charlotte the indexes were listed as: NBI-R> NBI and SPAD/FLAV> SPAD/FLV> R, FLV> FLAV> NIR>SPAD> CHL> SFR-R. The SI classified, therefore, the combined chlorophyll to flavonoids indexes as the higher sensitive indexes**.**

Earliness of Diagnosis

At the first measurement date (5 DAE), only the NBI index for Charlotte showed an effect of N fertilization. Then, at 20 DAE the significance concerned FLV, NBI, FLAV, NBI-R, SPAD/FLV, and SPAD/FLAV indexes. The chlorophyll indicators showed significance at later stages for 29 DAE and/or 36 DAE. For Bintje, FLV, NBI, SPAD, NBI-R, SPAD/FLV, and SPAD/FLAV indexes expressed significant effect of N fertilization at the first measurement date (17 DAE). R and SFR-G indexes showed significance at later stages (at 28 DAE). The combined chlorophyll and flavonoids indicators showed an early response to N doses even at 5 DAE (for Charlotte). However, this observed effect should be considered with caution since, at early emergence, the plant is dependent on the reserves of seed tubers. And thus there is no abrupt deficiency of N deficiency before 2-3 weeks after emergence.

Stability

The stability was assessed by the interaction between N rate and date of measurements. Only few combined chlorophyll to flavonoids indicators were stable in terms of their relationships with N level across sampling dates. SPAD/FLV index for Bintje and both NBI and SPAD/FLV indexes for Charlotte expressed no significant interaction effect of nitrogen fertilizer and growth stage.

Combining the three criteria, SPAD/FLV index for Bintje and NBI index for Charlotte were selected in this study.

CONCLUSION

Neither flavonoid nor chlorophyll content of the leaf, considered alone, was able to meet successfully all requirements. The combination of leaf flavonoids content to leaf chlorophyll content in the form of ratios (SPAD/FLV and NBI) suggests that these indicators could be used as valuable tools to assess potato CNS. This preliminary finding agrees with results of Tremblay et al.(2007), Cartelat et al. (2005) and Cerovic et al. (2005) and shows that the combined ratios improve the discrimination between N treatments. However the selection of index in this study on the basis of the analyzed criteria should not eliminate considerations of others in different context and have to be studied for further experimental years. The specificity is also a criteria required from an indicator for N diagnosis (Goffart et al., 2008). The specificity of the selected index was not investigated here, but, many factors could potentially influence the leaf Phen content (Stewart et al., 2001) or leaf chlorophyll content.

REFERENCES

Ben Ghozlen, N., Z.G. Cerovic, C. Germain, S. Toutain and G. Latouche. 2010. Non-destructive optical monitoring of grape maturation by proximal sensing. Sensors, 10:10040-10068.

Bongue-Bartelsman, M. and D. A. Phillips. 1995. Nitrogen stress regulates gene expression of enzymes in the flavonoid biosynthetic pathway of tomato. Plant. Physiol. Biochem., 33: 539–546.

Buschmann, C. 2007. Variability and application of the chlorophyll fluorescence emission ratio red/far-red of leaves. Photosynth. Res., 92:261–271.

Cartelat, A., Z.G. Cerovic, Y. Goulas, S. Meyer, C. Lelarge, J.-L Prioul, A. Barbottin, M.-H. Jeuffroy, P. Gate, G. Agati and I. Moya. 2005. Optically assessed contents of leaf polyphenolics and chlorophyll as indicators of nitrogen deficiency in wheat (Triticum aestivum L.). Field Crops Res., 91:35-49.

Cerovic, Z.G., A. Cartelat, Y. Goulas and S. Meyer. 2005. In-the-field assessment of wheat-leaf polyphenolics using the new optical leaf-clip Dualex, *In* J.V., Stafford (ed.) Precision Agriculture Wageningen Academic Publishers, Wageningen.,05:243-250.

Evans J.R. 1983. Nitrogen and photosynthesis in the flag leaf of wheat (Triticum aestivum L.). Plant Physiol., 72:297–302.

Goffart, J.P., L.Van Den Wyngaert, D. Buffet, A. Leonard and P. Defourny. 2010. SPOT 5 multispectral data potentialities to monitor potato crop nitrogen status at specified field scale. Paper presented at: 10 th International Conference on Precision Agriculture. Denver, Colorado, USA. July 18-21, 2010.

Goffart, J.P., M. Olivier and M. Frankinet. 2008. Potato Crop Nitrogen Status Assessment to Improve N Fertilization Management and Efficiency: Past–Present–Future. Potato Res., 51:355–383.

Goulas, Y., Z.G. Cerovic, A. Cartelat and I. Moya. 2004. Dualex: A new instrument for field measurements of epidermal UV-absorbance by chlorophyll fluorescence. App. Optics, 43 4488-4496.

Lemaire, G. and F. Gastal. 1997. N uptake and distribution in plant canopies. *In* G. Lemaire (ed.). Diagnosis of the Nitrogen Status in Crops. Springer-Verlag Berlin Heidelberg.pp3-43.

Minotti, P.L., D.E. Halseth and J.B. Sieczka. 1994. Field chlorophyll measurements to assess the nitrogen status of potato varieties. Hortsci., 29:1497–1500.

Schröder, J. J., J. J. Neeteson, O. Oenema, and P. C. Struik. 2000. Does the crop or the soil indicate how to save nitrogen in maize production? Reviewing the state of the art. Field Crops Res., 66, 277-278.

Stewart, A. J., W. Chapman, G.I. Jenkins, I. Graham and T. Martin. 2001. The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. Plant Cell Env, 24(11): 1189–1197.

Tremblay, N., Z. Wang. and C. Belec. 2007. Evaluation of the Dualex for the assessment of corn nitrogen status. J.Plant Nutri., 30:1355–1369.

Ulrich A.1952. Physiological Bases for Assessing the Nutritional Requirements of Plants. Annual Rev of Plant Physiol, 3: 207-228.

Zhang, Y., N. Tremblay and J. Zhu. 2012. A First Comparison of Multiplex® for the Assessment of Corn Nitrogen Status. J.F.A.E., 10(1):1008-1016.