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A first comparison of Multiplex® for the assessment of corn nitrogen status

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Abstract

The Multiplex® is a new hand-held optical fluorescence sensor for non-destructive measurement of various parameters representative of plant physiological status such as anthocyanin, flavonol and chlorophyll content as well as chlorophyll fluorescence emission ratio and UV (ultraviolet)-excited blue-green fluorescence (BGF). The Multiplex® is of potential value for in-season assessment of crop nitrogen status, but no evaluation has been released for that matter. The objectives of this research were to establish the presence of significant relationships between Multiplex parameters, applied nitrogen rate and measurement dates in corn (*Zea mays* L.) in order to select the parameters of greatest interest, and to compare those selected Multiplex parameters with other recognized plant-based indicators: SPAD (chlorophyll meter), DUAD (Dualex reading from adaxial side of leaf) and NNI (nitrogen nutrition index). An experiment was conducted, which consisted of four nitrogen fertilization treatments with 0, 20, 50 (grower's practice) and 255 kg N ha^{-1} applied at sowing, and another treatment where straw was incorporated into the soil before sowing in order to stimulate N immobilization to maximize N deficiency. Statistical analysis of repeated measurement data showed that all Multiplex parameters measured from the leaf or from above the plant, from growth stages V2 to V6, were strongly influenced by applied N dose. The parameters FERARI (fluorescence excitation ratio anthocyanin relative index), SFR-G (simple fluorescence ratio under green excitation), SPAD/DUAD and SPAD were found to be reliable indicators for monitoring corn N status at early stages.

Key words: Multiplex®**,** nitrogen status**,** chlorophyll meter, diagnosis, Dualex, nitrogen nutrition index.

Introduction

There is a need to develop sustainable nitrogen (N) management systems that minimize environmental losses by maximizing the use efficiency of applied fertilizers, particularly in wide-row annual crops [1]. A key approach to achieving this is to match nitrogen supply with crop nitrogen demand using improved methods of fertilizer application, timing and rating. For crops with high N requirements such as corn, N application is often split in one or several applications, which can provide an opportunity to adjust the N requirements in response to unpredictable seasonal conditions [2]. According to Olfs *et al.* [3], the best N management practices need to consider both the soil and the crop status at each relevant point in time during the vegetation period to readjust the N application strategy. Ma *et al.* [4] hypothesized that the precision of N application can be improved by fine-tuning application rates according to crop-based indicators of N status.

Corn is the third most important cereal crop in the world, after rice and wheat. Although corn has a high requirement for nitrogen, many studies show that its utilization of fertilizer nitrogen is often lower than 55% [5]. This unused substantial mineral N that remains in the soil after harvest is at risk of being lost to the environment either as gas or through runoff and leaching, resulting in contaminated air and water resources and represents a substantial economic loss for producers.

Assessments of many soil and plant test methods for corn have been reported in the literature. The methods can be summarized under four major categories: 1) soil N tests [6]; 2) tissue N test such as the nitrogen nutrition index (NNI) [7-10] and the late season stalk nitrate (LSSN) [11]; 3) remote sensing indicators such as the normalized difference vegetation index (NDVI) [11-13] and spectral reflectance [13]; and 4) hand-held sensors such as the chlorophyll meter, SPAD [14-16], the Dualex [2, 17, 18] and the Multiplex®.

Of the above, NNI is probably considered the best available "reference" diagnostic tool for many crops such as wheat [19] and corn [8, 20]. NNI is strongly related ($R^2 = 0.89$) to relative yield in corn[9]. An NNI value of 1.0 or larger indicates a non-N-limiting situation, whereas NNI values lower than 1.0 correspond to a N-deficiency situation. However, soil and tissue N tests have their shortcomings, including short time between soil testing and fertilizer application, higher labour requirements, analytical expenses, time-consuming nature and generally high prediction errors, which limit the adoption of these methods by farmers [21, 22]. In recent years, hand-held sensors have been developing speedily due to their instantaneous, accurate, convenient and non-destructive features for crop nitrogen-status detection.

Chlorophyll content in the leaves and canopy are known to be related to crop nitrogen availability [23]. The Minolta SPAD meter is a hand-held spectrophotometer that measures the chlorophyll status of leaves in a rapid and non-destructive manner. SPAD meter readings have been found to correlate positively with leaf N and leaf chlorophyll concentration [14, 15]. Martínez and Guiamet [16] demonstrated that relative water content (RWC) and irradiance influence chlorophyll meter values in corn and wheat. SPAD measurements should therefore be made early in the morning, when irradiance is low and RWC is at its peak. Taking chlorophyll meter readings at a similar time of day and shielding the tool from the direct rays of the sun may diminish measurement errors.

Polyphenolics (including anthocyanins and flavonols) in the epidermal layers, which are issued from secondary plant metabolism, are also affected by plant N availability [24]. Nitrogen deficiency induces a decrease in leaf Chl and an increase in leaf Phen contents [25]. Therefore, crop N status can also be assessed through the detection of leaf Phen content. Goulas *et al.* [17] developed a portable leaf-clip device, the Dualex (Force-A, Orsay, France), which can assess crop N status through the detection of leaf Phen content. Tremblay *et al.* [18] compared Dualex to SPAD for N status determination in corn and demonstrated that the Chl/Phen ratio was a very good indicator of corn N status, with an even greater sensitivity range than either Dualex or SPAD readings (Chl/Phen is the ratio of SPAD to the sum of DUAD and DUAB readings. DUAD is the Dualex reading obtained from the adaxial side of a leaf and DUAB is the Dualex reading obtained from the abaxial side of a leaf). Cartelat *et al.* [25] showed that Phen measurements were stable when they were measured from 07:30 h to 11:00 h. Polyphenolic measurements in the field were not influenced by light environment in this time frame.

Multiplex® is a new hand-held multi-parameter optical sensor that was launched on the market by the Force-A company, Orsay, France. The instrument generates fluorescence in the plant tissues using multiple excitation light sources (ultraviolet, blue, green and red) to measure parameters in any plant material, leaves, coniferous needles, crops, turf, fruits, vegetables, grains, *etc.* Multiplex can measure simultaneously and non-destructively various compounds such as anthocyanin content (epidermal visible absorbance by FER method), flavonol content (epidermal UV absorbance by FER method), chlorophyll content, chlorophyll fluorescence emission ratio and UV-excited blue-green fluorescence (BGF), as well as several other fluorescence parameters (such as SFR-G, SFR-R, FERARI) that have been identified as indicative of plant physiological status. Twenty Multiplex parameters can be acquired simultaneously through each measurement (Table 1).

An appropriate tool for crop N diagnosis should be sensitive to applied nitrogen rate and robust through the whole season. It should be able to detect N deficiencies as early as possible. Since the Multiplex is a new instrument, to our knowledge, there are no reports on how it should be used in the field or about its sensitivity to N fertility conditions. No comparative studies on Multiplex parameters, SPAD and Dualex also have been undertaken for the assessment of corn N status. Therefore, the objectives of this study were: 1) to establish the presence of significant relationships between Multiplex parameters, applied nitrogen rate and measurement dates in corn in order to select the parameters of greatest interest; 2) to compare the sensitivity of the selected parameters to applied N dose; 3) to clarify the influence of irradiance and/or time of day on Multiplex readings in order to optimize the Multiplex measurement procedure for corn; 4) to characterize the nature of the relationships between Multiplex parameters, SPAD and DUAD, on one hand, and applied nitrogen rate as well as measurement dates in corn, on the other hand; 5) to compare the sensitivity of Multiplex® parameters, SPAD and DUAD to applied N dose; and 6) to identify correlations between Multiplex, SPAD, DUAD and NNI.

Materials and Methods

Experimental design: The experiment was conducted at the Agriculture and Agri-Food Canada experimental farm in L'Acadie (73º20´14.45" W, 45º17´44.70" N), Quebec, Canada, in 2009. The soil was a clay loam. Sweet corn was the previous crop. A soil test (0-30 cm layer) indicated the following mean values: Soil pH ($CaCl_2$) of 6.0, organic matter of 3.7%, nitrate (NO_3-N) of 6 mg kg^{-1}, available P of 81 mg kg^{-1} (Mehlich 3) and available K of 150 mg kg^{-1} (Mehlich 3).

Four nitrogen treatments were established, with total N doses of 0, 20, 50 and 255 (40+215) kg N ha^{-1} as NH_4NO_3 (27.0-0-0, 2.5 Mg), respectively, at sowing; another special treatment involved straw incorporated without nitrogen fertilizer before sowing. A completely randomized block design was used in the field and there were 20 experimental units in this field experiment. The plots were 3 m × 10 m and consisted of four rows with 0.75 m inter-row

Table 1. Description of the parameters provided by the Multiplex.

Multiplex parameter	Description	Excitation	Formula
BGF-UV	Yellow Fluorescence	UV	/
RF-UV	Red Fluorescence	UV	/
FRF-UV	Far-Red Fluorescence	UV	/
BGF-B	Yellow Fluorescence	Blue	/
RF-B	Red Fluorescence	Blue	/
FRF-B	Far-Red Fluorescence	Blue	/
BGF-G	Reflected Yellow-Green light	Green	/
RF-G	Red Fluorescence	Green	/
FRF-G	Far-Red Fluorescence	Green	/
BGF-R	Reflected Yellow-Red light	Red	/
RF-R	Red Fluorescence	Red	/
FRF-R	Far-Red Fluorescence	Red	/
SFR-G	Simple Fluorescence Ratio	Green	FRF-G/RF-G
SFR-R	Simple Fluorescence Ratio	Red	FRF-R/RF-R
BRR-FRF	Blue to Red Fluorescence Ratio	UV	BGF-UV/FRF-UV
FLAV	Flavonols	Red and UV	Log(FRF-R/FRF-UV)
ANTH	Anthocyanins	Red and Green	Log(FRF-R/FRF-G)
NBI-G	Nitrogen Balance Index	UV and Green	FRF-UV/RF-G
NBI-R	Nitrogen Balance Index	UV and Red	FRF-UV/RF-R
FERARI	Fluorescence Excitation Ratio Anthocyanin Relative Index		

spacing. Corn cultivar Pioneer 38M58 was sown on 14 August 2009 at a sowing density of 75,000 plants ha^{-1}. This sowing date was not typical of commercial corn production, but an earlier sowing was neither possible (late arrival of the Multiplex device) nor necessary, since the experiment was to end early in the corn growing cycle.

Multiplex testing: A Multiplex (Force-A, Orsay, France) sensor was used in this field experiment. From 4 September to 2 October 2009 (V2 to V6 stage), Multiplex readings were obtained five times; at 21, 27, 34, 42 and 49 days after sowing (DAS) (6-8 days intervals), respectively. On each date, measurements were made on 20 representative plants in the centre two rows of each plot. For each selected plant, a Multiplex measurement was first made at the longitudinal centre of the uppermost fully developed leaves, avoiding midribs. Then, a measurement was made at a vertical angle from above the top of the plant. The purpose was to determine the best procedure in terms of sensitivity to plant N status.

In order to determine whether Multiplex readings were influenced by irradiance, measurements were taken alternatively under full sunlight and in shaded conditions (shade created by the body of the operator). This was done at noontime on 17 September 2009 (growing stage V3) in the "straw" and "255 kg N ha^{-1}" treatment plots. A minimum of 20 leaves with uniform appearance in each plot was selected for Multiplex measurements. On 25 September 2009 (V4), in the same selection of treatments, Multiplex, Dualex and SPAD readings were obtained four times from 09:00 h to 18:00 h at 3 hours intervals. For all instruments, measurements were made from the same area of the 20 uppermost fully developed leaves per plot.

SPAD and Dualex testing: Minolta SPAD-502 meter (Soil Plant Analysis Development, Minolta Camera Co. Ltd., Japan) and Dualex (Force-A, Orsay, France) were used on the same stage and leaves as the Multiplex. A minimum of 20 leaves with uniform appearance in each plot was selected for SPAD and Dualex measurements. All measurements were made on the lamina, avoiding midribs.

Shoot biomass and tissue N assessments: After Multiplex, SPAD and Dualex measurements were completed in the field. Plant tissue samples of corn (whole above-ground parts) were collected from 1.5-m-long plant sections in the centre two rows of each plot, 21, 27, 34, 42 and 49 DAS. Samples were taken back to the laboratory to be weighed to determine fresh weight; they were then dried at 70ºC in a forced-draft oven for 7 days, weighed to determine dry weight (DW), ground to pass through a 2-mm screen in a Wiley mill and stored at room temperature prior to laboratory analyses. Samples of 0.5 g of dried and ground corn were digested in a solution of sulphuric and selenous acid at 370°C using a block digestor on the basis of the method of Isaac and Johnson [26]. Total N in plant tissue was determined by colorimetry using a Traacs 800 (Bran+Lubbe Analyzing Technologies Inc.).

Nitrogen nutrition index (NNI): The NNI of the crop on each sampling date was determined by dividing the N concentration of the shoot biomass by Nc (the minimum N concentration required to achieve maximum shoot growth) according to Ziadi *et al.* [9]. NNI is defined as a function of shoot biomass, as proposed for corn by Plenet and Lemaire [8] and validated for eastern Canada [20]. Since the biomass levels were below 1 Mg DM ha^{-1} on the sampling dates, NNI was calculated with a constant Nc of 3.4%, according to Lemaire and Gastal [7].

Statistical analysis: The readings of Multiplex parameter ANTH were positive or negative because of the very low levels of anthocyanins in the corn leaves and the fact that a correction of the raw data was made with a blue standard. An arbitrary constant (1) was added to all ANTH results. This allowed for the elimination of negative values in the statistical calculation without influencing the nature or significance of the relationships.

Data were subjected to ANOVA using PROC MIXED (Tukey) and orthogonal contrast analyses of linear, quadratic and residual effects for quantitative treatments. The statistical analysis was performed with the SAS software package [27].

Results and Discussion

It took about 28 days for the crop to evolve from V2 to V6 at this period in early autumn. This is almost twice as much as under normal conditions (sowing within the first half of May) where this was achieved in 12 and 18 days, in 2005 and 2006 respectively, from observations made at the same location for the establishment of a corn phenological model (Bourgeois, G. 2011, personal communication). According to this model; however, the 28 days duration was perfectly in line with the climatic conditions experienced between September 4th and October 2nd at this site. As well, no unusual leaf striking or other symptoms not related to N shortage were observed during the course of the experiment so that there is no reason to think that the behaviour of the crop to the imposed N treatments was atypical.

Comparison of Multiplex readings obtained from individual leaves and tops of plants: Multiplex parameters obtained from the surface of leaves were significantly different ($P \leq 0.05$) than those obtained from above the corn plants (data not shown). Exceptions were observed only for RF-UV at 21DAS, 27DAS and FRF-UV at 27DAS and 42DAS sampling dates. N fertilization treatments were apparent from most Multiplex parameters, regardless of whether the measurements were made from the leaves or from the tops of the plants (Table 2) across sampling dates. At the first sampling date (21DAS) (V2), a few parameter measurements, such as RF-UV, FRF-UV, BGF-G, RF-G, RF-R and FLAV made from leaves, and BGF-G, RF-R, SFR-G, SFR-R, BRR-FRF, FLAV and NBI-G made from plants, were not significantly influenced by N rate treatments. The effects of N fertilization treatments were particularly obvious at later stages (V3 to V6). The sensitivity of each Multiplex parameter was assessed on the basis of the overall *P* levels of the five N rates (straw, 0, 20, 50 and 255 kg N ha^{-1}). Out of a total of 100 possibilities (twenty parameters, five sampling dates), the *P* level was higher 46 times for the leaf-based measurement as compared with 40 times for the above-plant measurement. For the remaining cases, it was not possible to sort out the differences (similar *P* levels).

As a complement, the ability of the different Multiplex parameters to assess highly contrasting N status was evaluated. A criterion was therefore developed by determining the ratio between the results obtained from plots with high N deficiency (straw applications) and those obtained from plots under N saturation

Table 2. Significance level (ANOVA) of Multiplex parameters measured from the leaves and the plants, and the ratio between the "straw" and the "255 kg N ha^{-1}" treatment under different nitrogen treatments for five sampling dates.

Item	Date	DAS [a] (growth stage)	BGF-UV	RF-UV	FRF-UV	BGF-B	RF-B	FRF-B	BGF-G	RF-G	FRF-G	BGF-R	RF-R	FRF-R	SFR-G	SFR-R	BRR-FRF	FLAV	ANTH	NBI-G	NBI-R	FERARI
			Multiplex parameters from leaves																			
P [b]	4/9	21 (V2)	***	N.S.	N.S.	***	*	**	N.S.	N.S.	**	**	N.S.	*	*	*	***	N.S.	*	**	**	***
	10/9	27 (V3)	**	N.S.	**	***	***	***	**	***	***	***	***	***	***	***	***	**	**	**	*	***
	17/9	34 (V4)	***	**	***	***	***	***	***	***	***	**	**	***	**	*	***	***	**	***	**	***
	25/9	42 (V5)	N.S.	N.S.	**	***	**	***	**	**	***	N.S.	**	***	**	**	***	***	*	**	***	***
	2/10	49 (V6)	**	*	***	*	***	***	**	***	***	*	***	***	***	***	***	***	***	**	***	***
Ratio [c]	4/9	21 (V2)	-0.10	1.00	0.60	0.37	0.45	0.37	0.79	0.49	0.34	0.67	0.56	0.38	0.62	0.61	-0.24	0.82	0.96	0.76	0.80	0.42
	10/9	27 (V3)	0.62	0.70	0.39	0.46	0.38	0.29	0.82	0.41	0.26	0.68	0.51	0.32	0.60	0.60	0.25	0.85	0.94	0.80	0.80	0.28
	17/9	34 (V4)	0.74	0.55	0.35	0.49	0.35	0.31	0.78	0.38	0.28	0.72	0.46	0.33	0.70	0.70	0.25	0.91	0.94	0.96	0.95	0.19
	25/9	42 (V5)	0.92	0.76	0.41	0.68	0.40	0.34	0.88	0.42	0.30	0.86	0.48	0.34	0.68	0.69	0.47	0.84	0.96	0.88	0.89	0.17
	2/10	49 (V6)	0.73	0.80	0.42	0.91	0.51	0.37	0.92	0.53	0.32	0.92	0.62	0.38	0.60	0.60	0.61	0.92	0.96	0.85	0.77	0.20
	Avg.		0.58	0.76	0.44	0.58	0.42	0.34	0.84	0.44	0.30	0.77	0.53	0.35	0.64	0.64	0.27	0.87	0.95	0.85	0.84	0.25
			Multiplex parameters from plants																			
	Date	DAS (growth stage)	BGF-UV	RF-UV	FRF-UV	BGF-B	RF-B	FRF-B	BGF-G	RF-G	FRF-G	BGF-R	RF-R	FRF-R	SFR-G	SFR-R	BRR-FRF	FLAV	ANTH	NBI-G	NBI-R	FERARI
P	4/9	21 (V2)	*	*	**	***	*	**	N.S.	*	**	***	N.S.	**	N.S.	N.S.	N.S.	N.S.	*	N.S.	*	**
	10/9	27 (V3)	***	*	**	***	*	**	***	*	**	***	N.S.	**	***	***	***	**	***	**	**	**
	17/9	34 (V4)	**	**	***	***	**	***	***	**	***	***	**	***	***	***	***	***	***	***	***	***
	25/9	42 (V5)	***	***	***	***	**	***	***	**	***	***	N.S.	***	***	***	***	***	***	***	***	***
	2/10	49 (V6)	N.S.	*	***	**	**	***	***	*	***	***	N.S.	***	***	***	***	***	**	***	***	***
Ratio	4/9	21 (V2)	0.00	0.58	0.42	0.31	0.55	0.43	0.82	0.58	0.40	0.62	0.70	0.46	0.66	0.65	0.41	0.94	0.94	0.73	0.65	0.62
	10/9	27 (V3)	0.56	0.27	0.22	0.38	0.57	0.49	0.76	0.59	0.44	0.51	0.73	0.52	0.70	0.67	0.11	0.69	0.93	0.37	0.31	0.67
	17/9	34 (V4)	0.73	0.28	0.23	0.47	0.45	0.42	0.79	0.47	0.38	0.61	0.54	0.42	0.77	0.75	0.17	0.77	0.95	0.49	0.44	0.59
	25/9	42 (V5)	0.72	0.71	0.55	0.42	0.81	0.76	0.74	0.82	0.68	0.53	0.94	0.75	0.82	0.81	0.38	0.85	0.96	0.66	0.59	0.82
	2/10	49 (V6)	0.86	0.78	0.47	0.61	0.78	0.70	0.81	0.79	0.64	0.62	0.88	0.70	0.81	0.80	0.42	0.86	0.94	0.64	0.58	0.81
	Avg.		0.57	0.52	0.38	0.44	0.63	0.56	0.78	0.65	0.51	0.58	0.76	0.57	0.75	0.73	0.30	0.82	0.96	0.58	0.51	0.70

[a]DAS indicates days after sowing. [b]*, **, *** and N.S. indicates significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$and not significant ($P > 0.05$) respectively (F test).
[c]Ratio is the value of the "straw" treatment divided by the "255 kg N ha^{-1}" treatment or conversely for Multiplex parameters. Thus, the ratio value was always less than 1.

levels (255 kg N ha^{-1} at sowing) (Table 2). The smaller this ratio between low N and high N, the higher the sensitivity of the parameter under consideration. The value of the ratio was lower 55 times for the leaf-based measurement as compared with 45 times for the above-plant measurement. This confirms the fact that Multiplex measurements made from corn leaves are more able to distinguish plant N status than those made from above the plants. Measurements from leaves are made in close contact with the plant tissues, while those made from above are made from a small distance, which may explain the lower level of accuracy. However, such measurements from a distance pave the way to "on-the-go" uses of the instrument in a precision farming context. For the sake of further discussion, from this point on, only leaf-borne measurements will be used, unless otherwise specified.

Selection of Multiplex parameters for corn N status assessment: Among the parameters provided by the Multiplex instrument, a selection was made on the basis of the following criteria: 1) sensitivity to N treatment; 2) consistency of significant effects to N treatments among growth stages; 3) earliness of diagnosis; and 4) absence of interaction between N treatment and growth stages. The application of these criteria should result in a selection of parameters sensitive to N status as early as possible and consistently among growth stages.

A repeated data statistical analysis, including the "straw" treatment, was conducted (Table 3) showing that all Multiplex parameters measured from the leaf were strongly influenced by applied N dose across sampling dates (V2 to V6). Significant effects of DAS were obtained for all Multiplex parameters except FLAV. Only SFR-G, FLAV, ANTH and FERARI expressed no significant interaction effects of nitrogen fertilizer and growth stage. This is an indication that the results obtained for these parameters are consistent across sampling dates. Among the four parameters selected, only SFR-G, ANTH, and FERARI were significantly related to N treatments at the first sampling date (21DAS, V2) (Table 2). FLAV was significantly related to N treatments from the second sampling date (27 DAS, V3). FERARI was particularly able to reveal differences between contrasting N fertility conditions (small value of the ratio "straw"/"N saturated treatment") at all sampling dates (Table 2). It was followed in this respect by SFR-G, FLAV, and then by ANTH.

Table 3. Repeated measurement analysis results of Multiplex parameters obtained from leaves across all five sampling dates after sowing (DAS). Straw treatment included.

Multiplex parameters	Fixed effect		
	Nitrogen	DAS	N x DAS
BGF-UV	**	***	***
RF-UV	***	***	**
FRF-UV	***	***	***
BGF-B	***	***	***
RF-B	***	***	*
FRF-B	***	***	**
BGF-G	***	*	*
RF-G	***	***	*
FRF-G	***	***	**
BGF-R	***	***	*
RF-R	***	***	**
FRF-R	***	***	**
SFR-G	***	***	NS
SFR-R	***	***	*
BRR-FRF	***	***	***
FLAV	***	NS	NS
ANTH	***	***	NS
NBI-G	***	***	*
NBI-R	***	***	*
FERARI	***	***	NS
SPAD	***	*	***
DUAD	***	***	NS
SPAD/DUAD	***	*	*
DW	**	***	**
NNI	***	***	***

* Significant at $P\leq0.05$. *** Significant at $P\leq0.001$.
** Significant at $P\leq0.01$. NS: Not significant ($P>0.05$).

Effects of shadowing on Multiplex readings: No significant difference was observed in the four selected Multiplex parameters (SFR-G, FLAV, ANTH and FERARI) under either sunlight or shadow (data not shown). Therefore, Multiplex measurements in the field appear to be stable under different light environments. Cartelat *et al.* [25] also showed that polyphenol measurements on leaves were stable from 07:30 h to 11:00 h.

Daytime effects - Multiplex, Dualex and SPAD readings: Water status and irradiance may influence chlorophyll meter values [22]. Martínez and Guiamet [16] reported relative leaf water content (RWC) and irradiance influence chlorophyll meter measurements in corn. In our study, SPAD value varied with the time of day in the straw treatment, but not in the 255 kg N ha^{-1} treatment (Table 4). Minimum SPAD value was obtained at 15:00 h in the straw treatment, and was significantly lower than that observed at 12:00 h or 18:00 h. This occurred probably as a result of irradiance, air temperature and/or water status effects. SPAD measurements should therefore be made before noon, when irradiance is low and RWC is high. Making chlorophyll meter measurements at a similar time of the day and shielding the tool from the direct rays of the sun may diminish measurement errors [16]. No significant differences ($P>0.05$) were obtained for SFR-G, FLAV, ANTH, FERARI or Dualex at any time during the day in either treatment. It follows that the Multiplex and Dualex instruments can be used without restrictions as to the time of day chosen for measurement.

Effects of applied nitrogen dose and sampling dates on Multiplex parameters, SPAD, DUAD, SPAD/DUAD and NNI: Statistical analysis of repeated measurement data for all five treatments showed that all variables were strongly influenced by applied N dose across sampling dates (V2 to V6) (Table 3). Such relationships for SPAD, DUAD, SPAD/DUAD, DW, NNI and N fertilization have been reported in corn [9, 10, 14, 18]. Significant effects of DAS were observed for all variables but FLAV. Both SFR-G and DW increased gradually with DAS, while FERARI, DUAD and NNI decreased. ANTH decreased gradually from 21 DAS to 42 DAS, but reached a peak value at 49 DAS. SPAD, SPAD/DUAD, DW and NNI were subjected to a significant N×DAS interaction, but the Multiplex parameters (SFR-G, FERARI, ANTH and FLAV) and DUAD were not. The significant interaction for SPAD, SPAD/DUAD and DW illustrated the fact that the parameters generally increased over DAS as N dose increased and the opposite occurred for low N doses. NNI tended to decrease for all treatments from the first to the second date. From the second date on, NNI increased for the two lowest N treatments (straw and 0 kg N ha^{-1}) but remained stable for the other treatments.

Table 4. Dynamics of Multiplex selected parameters, SPAD and Dualex readings for the various sampling times of day (on the 42nd day after sowing, V5).

Treatment	Time	SFR-G	ANTH	FLAV	FERARI	SPAD	Dualex
Straw	09:00 h	1.634 [a]	0.984	1.018	0.752	18.0	1.561
	12:00 h	1.643	0.983	1.012	0.796	20.3	1.537
	15:00 h	1.981	0.998	1.099	0.685	16.3	1.593
	18:00 h	2.164	0.974	1.047	0.760	20.4	1.746
	MSD (0.05) [b]	0.625	0.062	0.282	0.385	3.7	0.230
255 kg N ha^{-1}	09:00 h	2.505	0.958	1.158	0.128	35.9	1.652
	12:00 h	2.633	0.953	1.220	0.233	35.1	1.397
	15:00 h	2.938	0.956	1.259	0.110	37.9	1.349
	18:00 h	2.521	0.954	1.222	0.108	36.7	1.434
	MSD (0.05)	0.451	0.023	0.134	0.149	6.1	0.309

[a]Data is the mean for 20 leaves at each sampling time of day in each plot.
[b]MSD (0.05) indicates minimum significant difference at 5% level by Tukey's test.

Response of measured parameters to levels of N availability: ANOVA, including the straw treatment, showed highly significant effects of treatments on DW, NNI, SPAD, DUAD, SPAD/DUAD, SFR-G, FLAV, ANTH and FERARI on the five sampling dates, except for DUAD at 21 and 42 DAS and FLAV at 21 DAS (Table 5). These differences were primarily attributable to the important growth-reducing effect of the straw treatment, which was likely due to soil N immobilization.

The straw treatment was excluded for the purpose of determining the nature of relationships to N rates (from 0 to 255 kg N ha^{-1} at sowing). In this context, there were no significant effects of treatments on DW, except at 49 DAS, when a linear increase was seen with N rates (Table 5).

NNI varied from 0.52 to 1.05 across all treatments and sampling dates (Table 5), which is within the range reported for corn [9, 20]. NNI generally decreased gradually as corn developed and the time from N fertilization increased. As expected, maximum NNI values were obtained with 255 kg N ha^{-1} at 21 DAS. Highly significant increases in NNI with N doses were found with strong linear and quadratic components at all sampling dates, in agreement with Justes *et al.* [19] and Ziadi *et al.* [20]. Our results are consistent with a role for NNI as a "reference" diagnostic method for corn N status.

With or without the presence of the straw treatment in the statistical analysis, SPAD readings were also significantly influenced by applied N dose across sampling dates (Table 5). Increases in SPAD readings with N fertilization were often reported in corn [14, 27]. The shape of the SPAD relationship with N fertilization has been reported as linear [4, 23] but Bullock and Anderson [15] as well as Cerrato and Blackmer [29] suggested that a quadratic-plus-plateau function was more appropriate. Seasonal changes with quadratic components during the longer growing season for SPAD readings have been reported previously in corn [30, 31]. In our study, the relationship can be described as curvilinear. Similar results have been obtained for corn by Tremblay *et al.* [18], but only after 21 DAS. Waskom *et al.* [31] and Ziadi *et al.* [9] reported that SPAD readings were generally not significantly affected by N treatments at early sampling dates (shoot biomass <1 Mg DM ha^{-1}) because an N restriction was unlikely for very small plants. Our results show that N limitations can indeed be traced by SPAD and NNI at very early stages.

DUAD generally decreased with N dose at all sampling dates (Table 5), likely because it is N deficiency that stimulates the production and accumulation of Phen [33, 34]. Similar results have been reported by Tremblay *et al.* [18]. However, irregular changes were observed in DUAD depending on sampling dates. Significant linear or curvilinear relationships of DUAD with N dose were found at 27, 34 and 49 DAS, but not at 21 or 42 DAS. DUAD measurements were therefore not as consistent as SPAD or NNI for the growth stages considered in this study. SPAD/DUAD increased curvilinearly with N dose at all sampling dates (Table 5). Comparable to results reported by Tremblay *et al.* [18], values ranged from 10.7 to 27.5 across N dose treatments and sampling dates.

At 21 DAS, no Multiplex parameter was related linearly or quadratically to N doses (straw treatment excluded). After 21 DAS, significant linear and/or quadratic relationships were found (except for FERARI at 27 DAS and SFR-G at 34 DAS). The FLAV at 34 DAS as well as the FERARI, SFR-G and ANTH at 49 DAS expressed strong responses to N doses. SFR-G ranged from 1.41 to 3.21 depending on N application doses and sampling dates (Table 5). It generally increased with N dose, although this effect was not always statistically significant. The FLAV ranged from 1.11 to 1.45 and it too increased with N dose. Both FERARI and ANTH decreased with N dose at all sampling dates. There were no differences in SFR-G, FLAV, ANTH and FERARI above 50 kg ha^{-1} (no significant differences between the 50 and 255 kg ha^{-1} treatments, data not shown). This suggested that these parameters were not sensitive to luxury N uptake.

In order to compare the sensitivity of the diagnostic parameters over a wide range of N availability, the results obtained for the straw treatment were divided by those obtained for the N saturated treatment (255 kg N ha^{-1} at sowing). For the sake of facilitating comparison among parameters, the FERARI and DUAD results were calculated in a reciprocal manner since these parameters expressed a negative relationship with N dose (Table 5).

The average values of the ratio across the five sampling dates are indicative of the degree of contrast between low and high N fertility conditions and therefore of a parameter able to describe N status across a wide range of N status. In decreasing degree of contrast, we have: DW (0.11), FERARI (0.25), SPAD/DUAD (0.44), SPAD (0.50), NNI (0.60), SFR-G (0.64), FLAV (0.87), DUAD (0.88) and ANTH (0.95).

Correlations between Multiplex, SPAD, DUAD, SPAD/DUAD and NNI: Significant correlations were found between SFR-G, ANTH, FERARI, SPAD, DUAD; the ratio of SPAD/DUAD, on one hand, and NNI, on the other hand, across DAS, except for ANTH at 21 DAS, 42 DAS and DUAD at 42 DAS (Fig. 1). The FLAV correlated with NNI at 21 DAS only. The correlations with NNI were positive for SFR-G, SPAD and the SPAD/DUAD ratio and negative for ANTH, FERARI and DUAD. All parameters but ANTH and DUAD showed maximal correlation with NNI on the first sampling date (21 DAS). The |R| for SFR-G, FERARI, SPAD and SPAD/DUAD degraded with DAS, with the exception of the last date (49 DAS) for FERARI, SPAD and SPAD/DUAD. The relationship of |R| with NNI for ANTH and DUAD was erratic. FERARI, SFR-G, SPAD and the SPAD/DUAD ratio appeared to be reliable indicators of corn NNI before 30 DAS. Ziadi *et al.* [9] showed that SPAD values in corn were significantly related to NNI, but the intercepts and/or slope of the response curves varied with site-year and sampling dates.

Table 5. Responses of Multiplex parameters (SFR-G, ANTH, FLAV and FERARI), SPAD, DUAD, SPAD/DUAD and NNI to applied N dose at different days after corn sowing.

Date	Parameters	Applied N dose (kg N ha^{-1})					Contrast analysis by nitrogen [a]		P value [b]	Ratio [c]
		Straw	0	20	50	255	Linear	Quadratic		
21 DAS	DW [d]	1.17	5.36	6.39	5.76	7.42	NS	NS	0.0004	0.16
	NNI	0.54	0.75	0.94	1.01	1.05	***	***	<0.0001	0.52
	SPAD	21.3	29.9	34.5	35.9	35.8	**	***	<0.0001	0.60
	DUAD	1.89	1.70	1.62	1.68	1.60	NS	NS	0.0785	0.85
	SPAD/DUAD	11.56	17.63	21.27	21.46	22.39	*	*	<0.0001	0.52
	SFR_G	1.41	1.95	2.27	1.83	2.27	NS	NS	0.0420	0.62
	FLAV	1.14	1.44	1.43	1.15	1.39	NS	NS	0.4076	0.82
	ANTH	1.03	1.01	0.99	1.01	0.99	NS	NS	0.0355	0.96
	FERARI	0.78	0.37	0.38	0.32	0.33	NS	NS	<0.0001	0.42
27 DAS	DW	2.65	9.73	13.70	16.76	16.90	NS	NS	0.0172	0.16
	NNI	0.52	0.56	0.70	0.84	0.95	***	**	<0.0001	0.55
	SPAD	17.9	27.3	34.5	36.0	37.2	*	*	<0.0001	0.48
	DUAD	1.67	1.59	1.54	1.44	1.44	**	**	<0.0001	0.87
	SPAD/DUAD	10.80	17.36	22.47	25.11	25.79	*	**	<0.0001	0.42
	SFR_G	1.45	1.96	2.15	2.29	2.40	*	NS	0.0001	0.60
	FLAV	1.11	1.48	1.42	1.37	1.31	**	NS	0.0028	0.85
	ANTH	1.01	1.01	0.97	0.97	0.95	**	*	0.0040	0.94
	FERARI	0.80	0.28	0.24	0.18	0.22	NS	NS	<0.0001	0.28
34 DAS	DW	2.37	19.75	23.79	31.46	32.27	NS	NS	0.0004	0.07
	NNI	0.58	0.52	0.66	0.88	0.98	***	***	<0.0001	0.59
	SPAD	17.4	25.4	32.3	36.9	37.9	**	**	<0.0001	0.46
	DUAD	1.64	1.54	1.48	1.42	1.39	*	*	0.0005	0.85
	SPAD/DUAD	10.65	16.55	21.96	26.13	27.47	**	**	<0.0001	0.39
	SFR_G	1.69	1.99	2.17	2.49	2.43	NS	NS	0.0080	0.70
	FLAV	1.15	1.42	1.39	1.31	1.26	***	*	0.0005	0.91
	ANTH	1.03	1.01	0.99	0.97	0.96	*	*	0.0018	0.94
	FERARI	0.69	0.28	0.18	0.11	0.14	*	**	<0.0001	0.19
42 DAS	DW	3.99	26.93	22.51	40.58	43.90	NS	NS	0.0085	0.09
	NNI	0.63	0.59	0.68	0.81	0.95	***	**	<0.0001	0.67
	SPAD	19.7	25.3	27.8	36.1	39.7	***	**	<0.0001	0.50
	DUAD	1.57	1.58	1.57	1.51	1.52	NS	NS	0.6608	0.97
	SPAD/DUAD	12.57	16.02	17.80	23.92	26.15	***	***	<0.0001	0.48
	SFR_G	1.88	2.29	2.22	2.48	2.76	**	NS	0.0023	0.68
	FLAV	1.11	1.40	1.41	1.37	1.32	*	NS	<0.0001	0.84
	ANTH	0.99	0.99	0.98	0.97	0.96	*	NS	0.0150	0.96
	FERARI	0.67	0.25	0.17	0.08	0.11	NS	*	<0.0001	0.17
49 DAS	DW	3.66	18.46	31.42	31.78	50.19	*	NS	0.0032	0.07
	NNI	0.66	0.68	0.71	0.84	0.95	***	**	<0.0001	0.69
	SPAD	18.4	22.2	29.7	34.3	40.3	***	***	<0.0001	0.46
	DUAD	1.72	1.59	1.64	1.50	1.47	*	NS	0.0152	0.85
	SPAD/DUAD	10.80	13.89	18.35	22.89	27.50	***	***	<0.0001	0.39
	SFR_G	1.93	2.67	2.96	3.15	3.21	**	**	<0.0001	0.60
	FLAV	1.25	1.40	1.44	1.40	1.35	*	NS	<0.0001	0.92
	ANTH	1.06	1.05	1.04	1.02	1.00	***	**	<0.0001	0.96
	FERARI	0.57	0.34	0.21	0.17	0.12	**	**	<0.0001	0.20

* Significant at $P≤0.05$; ** Significant at $P≤0.01$; *** Significant at $P≤0.001$; NS: Not significant (P>0.05).
[a]Contrast analysis was made by nitrogen from 0 to 255 kg N ha^{-1} excluding the straw treatment. Both cubic and residual components for all parameters were not significant (P > 0.05).
[b]P value indicates the significant level of all parameters across treatments, including the straw treatment, at each sampling date (F-test).
[c]Ratio is the value of the straw treatment divided by 255 kg N ha^{-1} treatment for DW, NNI, SPAD, SPAD/DUAD, SFR-G and FLAV; the reciprocal calculation was made for the FERARI, DUAD and ANTH, in order to compare ratio values always smaller than 1.
[d]DW is the dry weight of shoot biomass (g DM per 1.5-m plant section in rows).

Table 6. Recommendations for use of the Multiplex instrument according to the findings of this study.

Element	Key finding	Recommendation
Valuable Multiplex indicators	Among the available Multiplex parameters, only two were able to meet all of the following criteria: 1) Sensitivity to N treatment, 2) consistency of significant effects to N treatments among growth stages, 3) earliness of diagnosis and 4) Absence of interaction between N treatment and growth stages. Those parameters were also not sensitive to luxury N uptake and appeared to be reliable indicators of corn NNI before 30 DAS.	Suggested Multiplex parameters: FERARI, SFR-G.
Time of day	No significant differences were obtained for the selected Multiplex parameters measurements at any time during the day.	Multiplex may be used at any time of the day.
Effect of shadow	No significant difference was observed in the four selected Multiplex parameters (SFR-G, FLAV, ANTH and FERARI) under either sunlight or shadow.	Multiplex may be used under different light environments.
Sampling	Multiplex readings made on single leaves were better related to N treatments than those made from tops of plants.	Measurements should be made directly on the uppermost fully developed leaves at the longitudinal center, avoiding midribs. Repeat for a total of 20 representative plants.

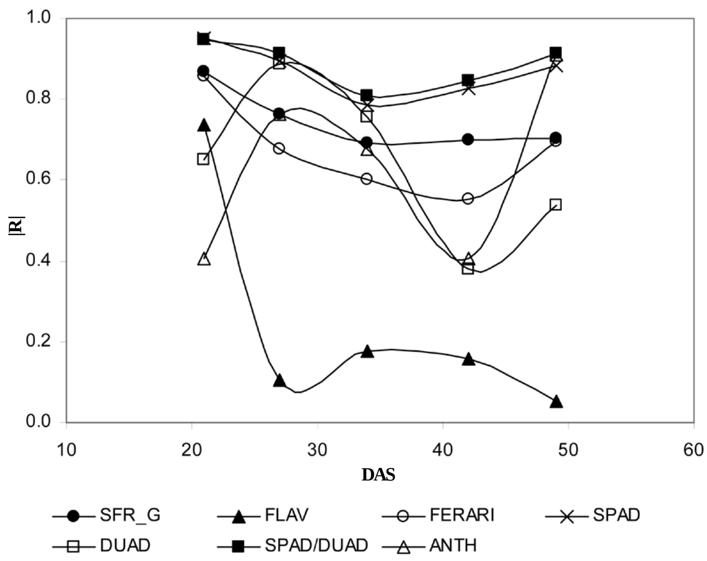

Figure 1. Evolution of Pearson's |R| with days after sowing (DAS) for SFR-G, ANTH, FLAV, FERARI, SPAD, DUAD and SPAD/DUAD. (|R| is the absolute value of the Pearson's correlation coefficient between each diagnostic parameter and NNI). The FERARI, ANTH and DUAD relationships with NNI are characterized by a negative R. All |R| are significant ($P \leq 0.05$), except FLAV>21 DAS, ANTH at 21 DAS, 42 DAS, and DUAD at 42 DAS.

Conclusions

All Multiplex parameters measured from corn leaves were strongly influenced by applied N dose across sampling dates (V2 to V6) in this study. But only FERARI, SFR-G, ANTH and FLAV were also stable in terms of their relationship with N treatments across sampling dates. However, most Multiplex parameters showed high sensitivity to experimental treatments, and the first selection of parameters achieved in this study should not eliminate consideration of others in a different context. Multiplex readings made on single leaves were found to be better related to N treatments than those made from tops of plants. The ability of Multiplex parameters to distinguish N treatments was equally good whether the measurements were made in shadow or full sunlight, and regardless of time of day. Guidelines from this study related to the proper use of the Multiplex are summarized in Table 6. The parameters SFR-F, FLAV, FERARI, SPAD, DUAD, SPAD/DUAD, DW and NNI were strongly influenced by nitrogen. All parameters except FLAV changed significantly with growth stage, but the Multiplex parameters and DUAD expressed no significant N×DAS interaction. Highly significant linear and quadratic relationships with N dose were observed for NNI, SPAD, DUAD, SPAD/DUAD, FERARI, SFR-G, ANTH, depending on sampling dates, but not for DW. SFR-G, SPAD and SPAD/DUAD increased with N dose, while the opposite was seen for FLAV, ANTH, DUAD and FERARI. The degree of contrast between low- and high-N fertility conditions was expressed by the parameter in this order: FERARI > SPAD/DUAD > SPAD > NNI > SFR-G > FLAV > DUAD > ANTH. Therefore, FERARI and SPAD/DUAD were better able to react to a range of N fertility conditions for corn than either SPAD or DUAD alone. FERARI, SFR-G, SPAD/DUAD and SPAD were reliable indicators of NNI across DAS.

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Abbreviations

ANTH, Anthocyanins; BGF-B, Yellow Fluorescence under blue excitation; BGF-G, Reflected Yellow-Green light under green excitation; BGF-R under red excitation, Reflected Yellow-Red light; BGF-UV under UV excitation, Yellow Fluorescence; BRR-FRF, Blue to Red Fluorescence Ratio; Chl, chlorophyll; DM, dry matter; DUAD, Dualex reading on adaxial side of leaf; DW, shoot dry weight; FERARI, Fluorescence Excitation Ratio Anthocyanin Relative Index; FLAV, Flavonols; FRF-B, Far-Red Fluorescence under blue excitation; FRF-G, Far-Red Fluorescence under green excitation; FRF-R, Far-Red Fluorescence under red excitation; FRF-UV, Far-Red Fluorescence under UV excitation; N, nitrogen; NBI-G, Nitrogen Balance Index under UV and green excitation; NBI-R, Nitrogen Balance Index under UV and red excitation; NNI, nitrogen nutrition index; Phen, polyphenolics; RF-B, Red Fluorescence under blue excitation; RF-G, Red Fluorescence under green excitation; RF-R, Red Fluorescence under red excitation; RF-UV, Red Fluorescence under UV excitation; SFR-G, Simple Fluorescence Ratio under green excitation; SFR-R, Simple Fluorescence Ratio under red excitation; SPAD, chlorophyll meter.