EVALUATION OF THE MULTIPLEX® FLUORESCENCE SENSOR 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 about 20 parameters descriptive of plant physiological status. The Multiplex is of potential value for in-season assessment of crop nitrogen status, but no evaluation has been released for that matter as of yet. An experiment was therefore 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. However, FERARI (fluorescence excitation ratio anthocyanin relative index), SFR-G (simple fluorescence ratio under green excitation), ANTH (anthocyanins content) and FLAV (flavonol content) were found to be particularly sensitive and consistent among sampling dates. Multiplex readings made on single leaves were found to be more related to N treatments than those made on the top of the plant. The overall ability of the instrument to distinguish N treatments was equally good when the measurements were made in shadow or full sunlight, and at any time during the day.

Keywords: Multiplex, nitrogen status, fluorescence, fertilization, corn

INTRODUCTION

The need for quick and accurate non-destructive alternatives for nitrogen status detection has led to several research initiatives. The Minolta SPAD meter is

a hand-held spectrophotometer that measures the relative greenness of leaves in a rapid and non-destructive manner and that was found to be a good estimator of crop N status (Schepers et al., 1992; Blackmer and Schepers, 1995; Bausch and Duke, 1996; Bullock and Anderson, 1998). However, Martinez and Guiamet (2004) suggested that relative water content (RWC) and irradiance influence chlorophyll meter values in corn and wheat. Goulas et al. (2004) developed a portable leaf-clip device, the Dualex (Force-A, Orsay, France), which can assess crop N status through the detection of leaf polyphenolic content. Tremblay et al (2007) demonstrated that the Chl/Phen (SPAD/Dualex) ratio was a very good indicator of corn N status, with even a greater sensitivity range than either Dualex or SPAD readings. The use of hand-held sensors offers several advantages over conventional soil or tissue testing procedures for detecting N deficiencies. The most obvious advantages are portability and rapid assessment of N status in the field without destruction of plant tissue.

Multiplex® is a new hand-held multi-parameter optical sensor from Force-A (Orsay, France). The instrument generates fluorescence in the plant tissues using multiple excitation light sources (ultraviolet, blue, green and red) to estimate 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 (Table 1). Chlorophyll content in the leaves and canopy are known to be related to crop nitrogen availability (Schlemmer et al., 2005). Polyphenolics (including anthocyanins and flavonols) in the epidermal layers, which are issued from secondary plant metabolism, are also affected by plant N availability (Cerovic et al., 1999).

Both Chl and Phen were shown to be highly correlated with leaf N concentration. According to Cartelat et al (2005), nitrogen deficiency induces a decrease in leaf Chl and an increase in leaf Phen contents. Cerovic et al. (2008) showed that the Multiplex ANTH index provided a good estimation of the skin ANTH content of whole bunches of wine grapes throughout the maturation period.

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, there are no reports on how it should be used in the field or about its sensitivity to agronomic measures such as fertilization, irrigation, planting density, etc.

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; and 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.

Table 1. Description of the parameters provided by the Multiplex

Multiplex parameter	Description	Excitation	Description
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		

MATERIALS AND METHODS

Experimental Design

The experiment on corn was conducted at the Agriculture and Agri-Food Canada experimental farm in L'Acadie (73°20'14.45''W, 45°17'44.70''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).

This experiment established four nitrogen treatments 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 × 10 m and consisted of four rows with 0.75 m inter-row spacing. Corn cultivar 'Pioneer 38M58,' was sown on 14 Aug. 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 Sept. to 2 Oct. 2009 (V2 to V6 stage), Multiplex readings were obtained five times, at 21, 27, 34, 42, and 49 days after sowing (DAS) (6–8-d intervals), respectively. On each date, measurements were made on 20 representative plants in the center two rows of each plot. For each selected plant, a Multiplex measurement was first made at the longitudinal center 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 Sept. 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 Sept. 2009 (V4), in the same selection of treatments, Multiplex, Dualex and SPAD readings were obtained four times from 0900h to 1800h at 3-h intervals. For all instruments, measurements were made from the same area of the 20 uppermost fully developed leaves per plot.

Statistical Analysis

The readings of Multiplex parameter ANTH were slightly positive or negative due to the very low levels of anthocyanins in corn plants, and the fact that a correction of original data was made using a blue standard. Therefore, a constant "1" was added to all ANTH results in order to eliminate negative numbers. 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 (SAS Institute, 1990).

RESULTS AND DISCUSSION

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. N fertilization treatments were apparent from most Multiplex parameters, regardless of whether the measurements were made from the leaves (Table 2) or from the tops of the plants (data not shown) across sampling dates. At the first sampling date (21DAS) (V2), a few parameter measurements made from leaves, such as FLAV (Table 2), RF-UV, FRF-UV, BGF-G, RF-G and RF-R (data not shown), and BGF-G, RF-R, SFR-G, SFR-R, BRR-FRF, FLAV and NBI-G made from plants (data not shown), 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, data not shown), 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 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 the plants 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; 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.

Table 2. Significance level of Multiplex parameters and the ratio between the "straw" and the "255 kg N ha^{-1}" treatment under different nitrogen treatments for five sampling dates. ANTH, Anthocyanins; FERARI, Fluorescence Excitation Ratio Anthocyanin Relative Index; FLAV, Flavonols; SFR-G, Simple Fluorescence Ratio (Green)

			Multiplex parameters			
	Date	DAS† (Growth stage)	SFR-G	FLAV	ANTH	FERARI
P ‡	4/9	21 (V2)	*	N.S.	*	***
	10/9	27 (V3)	***	**	**	***
	17/9	34 (V4)	**	***	**	***
	25/9	42 (V5)	**	***	*	***
	2/10	49 (V6)	***	***	***	***
Ratio §	4/9	21 (V2)	0.62	0.82	0.96	0.42
	10/9	27 (V3)	0.60	0.85	0.94	0.28
	17/9	34 (V4)	0.70	0.91	0.94	0.19
	25/9	42 (V5)	0.68	0.84	0.96	0.17
	2/10	49 (V6)	0.60	0.92	0.96	0.20
	Avg.		0.64	0.87	0.95	0.25

† DAS indicates days after sowing.

‡ *, **, *** 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).

§ 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.

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, Table 2) at all sampling dates. It was followed in this respect by SFR-G and FLAV, then by ANTH.

Table 3. Repeated measurement analysis results of Multiplex parameters across all five sampling dates after sowing (DAS). Straw treatment included. ANTH, Anthocyanins; FERARI, Fluorescence Excitation Ratio Anthocyanin Relative Index; FLAV, Flavonols; SFR-G, Simple Fluorescence Ratio (Green)

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

* Significant at $P \leq 0.05$; ** Significant at $P \leq 0.01$; *** Significant at $P \leq 0.001$; † 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). Multiplex measurements in the field therefore appear to be stable under different light environments. Cartelat et al. (2005) also showed that polyphenol measurements on leaves were stable from 0730h to 1100h.

Daytime Effects: Multiplex, Dualex and SPAD Readings

Water status and irradiance may influence chlorophyll meter values (Samborski et al., 2009). Martinez and Guiamet (2004) reported that 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 1500h in the straw treatment, and was significantly lower than that observed at 1200h or 1800h. 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 (Martinez and Guiamet, 2004). 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.

Table 4. Dynamics of Multiplex selected parameters, SPAD and Dualex readings for the various sampling times of day (42DAS †, V5). ANTH, Anthocyanins; FERARI, Fluorescence Excitation Ratio Anthocyanin Relative Index; FLAV, Flavonols; SFR-G, Simple Fluorescence Ratio (Green); SPAD, chlorophyll meter

Treatment	Time	SFR-G	ANTH	FLAV	FERARI	SPAD	Dualex
Straw	0900h	1.634‡	0.984	1.018	0.752	18.0	1.561
	1200h	1.643	0.983	1.012	0.796	20.3	1.537
	1500h	1.981	0.998	1.099	0.685	16.3	1.593
	1800h	2.164	0.974	1.047	0.760	20.4	1.746
	MSD (0.05) §	0.625	0.062	0.282	0.385	3.7	0.230
255 kg N ha^{-1}	0900h	2.505	0.958	1.158	0.128	35.9	1.652
	1200h	2.633	0.953	1.220	0.233	35.1	1.397
	1500h	2.938	0.956	1.259	0.110	37.9	1.349
	1800h	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

† DAS indicates days after sowing.
‡ Data is the mean for 20 leaves at each sampling time of day in each plot.
§ MSD (0.05) indicates Minimum Significant Difference at 5% level by Tukey test.

CONCLUSION

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.

Comparison of Multiplex parameters with other known indicators of N status determination would be warranted.

REFERENCES

Bausch W.C., Duke H.R. 1996. Remote sensing of plant nitrogen status in corn. Transactions of the American Society of Agricultural Engineers 39:1869-1875.

Blackmer T.M., Schepers J.S. 1995. Use of a chlorophyll meter to monitor nitrogen status and schedule fertigation for corn. Journal of Production Agriculture 8:56-60.

Bullock D.G., Anderson D.S. 1998. Evaluation of the Minolta SPAD-502 chlorophyll meter for nitrogen management in corn. Journal of Plant Nutrition 21:741-755.

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

Cerovic Z.G., Moise N., Agati G., Latouche G., Ben Ghozlen N., Meyer S. 2008. New portable optical sensors for the assessment of winegrape phenolic maturity based on berry fluorescence. Journal of Food Composition and Analysis 21:650-654.

Cerovic Z.G., Samson G., Morales F., Tremblay N., Moya I. 1999. Ultraviolet-induced fluorescence for plant monitoring: Present state and prospects. Agronomie 19:543-578.

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

Martinez D.E., Guiamet J.J. 2004. Distortion of the SPAD 502 chlorophyll meter readings by changes in irradiance and leaf water status. Agronomie 24:41-46.

Samborski S.M., Tremblay N., Fallon E. 2009. Strategies to make use of plant sensors-based diagnostic information for nitrogen recommendations. Agronomy Journal 101:800-816.

Schepers J.S., Francis D.D., Vigil M., Below F.E. 1992. Comparison of corn leaf nitrogen concentration and chlorophyll meter readings. Communications in Soil Science and Plant Analysis. 23:2173-2187.

Schlemmer M.R., Francis D.D., Shanahan J.F., Schepers J.S. 2005. Remotely measuring chlorophyll content in corn leaves with differing nitrogen levels and relative water content. Agronomy Journal 97:106-112.

Tremblay N., Wang Z., Belec C. 2007. Evaluation of the Dualex for the assessment of corn nitrogen status. Journal of Plant Nutrition 30:1355-1369.