

Direct seeding under cover crops: a solution to optimize the potential for adaptation of transgenic field crops to water stress in a context of glyphosate exposure

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Abstract. This study conducted in open fields cultivated with glyphosate-resistant soybean and corn crops (GR cultivars) aimed at comparing the effects of two weed management practices (WMP) namely: conventional direct seeding (DS) and direct seeding under cover crop (DSCC). Stomatal development and stomatal activity were analyzed and used as indicators of the potential of a plant to assimilate CO₂ and manage H₂O when the vapor pressure deficit (Vpd) is increasing. These indicators were also used to compare the effects of glyphosate-based herbicides (GBH) on GR cultivars among the WMP. The stomatal development is based on measures of stomatal density (StoDensity) on both foliar surfaces (adaxial (Ad) and abaxial (Ab)), stomatal size (StoSize) and stomatal index (StoIndex). A generalized linear model (GLM) of the stomatal conductance (Gs) as a function of the Vpd was used to predict Gs following a GBH exposure in all plots. The results obtained in this study showed that plants growing in a DSCC system seem prompter to execute gas exchange. This can be observed through the development of bigger stomata and less sensitive stomatal activity to vapor pressure deficit increase on the abaxial foliar surface. DSCC corn plants also have bigger leaves which can give an advantage for the dry matter production during the fill grain period and positively influence the yield.

Keywords: Stomatal density, stomatal conductance, vapor pressure deficit, glyphosate-based herbicide, soybean, corn, maize, glyphosate-resistant, plant physiology.

INTRODUCTION

Direct seeding (DS) in field crops is a weed management system that is based on soil conservation principles such as reduced tillage, rotation in crop varieties and retention of residues from previous crops on the ground (Nichols *et al.*, 2015). Over the last two decades, this system expanded in Quebec and elsewhere in the world due to its efficiency (ease of implementation, less tillage and less workforce for weed management) as well as reduced machinery operating costs (workforce, gas and maintenance costs) (Derpsch *et al.*, 2010). Between 1999 and 2009, the area of field crops cultivated using DS

system have increased by 6 million hectares per year on a global scale. This expansion was made easier by the growing use of herbicides in the production systems (Derpsch *et al.*, 2010). In 2016, it was estimated that globally 180M ha were on direct seeding and represented 12.5% of the global cropland (Kassam *et al.*, 2019). The most common practice consists of using glyphosate-based herbicides (GBH) in association with transgenic glyphosate-resistant cultivars (GR) crops. Despite the benefits such as reducing erosion incidence, DS system presents shortcomings such as topsoil compaction and

reduced water infiltration, increased use of herbicide and higher number of weeds resistant to GBH (Triplett and Dick, 2008). Moreover, the use of GBH can impact protein contents, chlorophyll, photosynthesis, water use, gas exchange and agronomic performance of GR crops (Albrecht *et al.*, 2014; Krenchinski *et al.*, 2017; Smedbol *et al.*, 2019) and these impacts may be greater when combined with other plant stresses caused by abiotic factors (Petter *et al.*, 2016).

Compared with DS system, direct seeding under cover crops (DSCC) system is still unfrequently used in Quebec. It is also an operationally more complex weed management practice (WMP) (Lemessa and Wakjira, 2015). The DSCC system differs from the traditional DS system by the fact that it relies on the addition of cover / catch / companion crops during and / or between crop production periods. The addition of cover crops can bring agronomic benefits, which will contribute to the development of the crop plants (Amsili and Kaye, 2021; Ogilvie *et al.*, 2021; Wagg *et al.*, 2021). In addition, cover crops can limit the impacts of hazardous weather conditions such as extreme temperature variations and water depletion (Robertson *et al.*, 2014). The presence of cover plants limits soil compaction at the surface as well as increases its porosity and water infiltration. Water access and management are both essential processes for plants (Are *et al.*, 2021; Bowsher *et al.*, 2018; Liu *et al.*, 2005). Any soil disturbance can affect the soil-plant-atmosphere continuum, which can be tracked using the leaf stomatal conductance (Gs) (Williams *et al.*, 1996) since stomata are the main channel through which the plant and the atmosphere exchange gases (CO₂, O₂ and H₂O) (Zeiger *et al.*, 1987). CO₂ assimilation and transpiration are both intrinsically influenced by the stomatal development and activity (Zhao *et al.*, 2015).

It is hypothesized that the presence of cover crops will benefit the main crop while limiting the combined effect of stress following the application of GBH during a critical period for crop's water management. Modelling the relation between the vapor pressure deficit (Vpd) and the stomatal conductance (Gs) should be useful to obtain the necessary information to illustrate and better understand how WMP and the environment affects the stomatal activity (Krober and Bruelheide, 2014; Domec *et al.*, 2009). The vapor pressure deficit is a main environmental factor influencing stomata and their loss of water at midday (Domec *et al.*, 2009; Driesen *et al.*, 2020). Since the last decades it has been observed that Vpd has risen globally and it is now considered as a primary factor of exacerbated trees mortality and lower crops production (Seager *et al.*, 2015; Zhao *et al.*, 2017). The Vpd is the difference between the water-vapor pressure in the immediate environment at a given temperature and the water-vapor pressure in a leaf (Sinclair *et al.*, 2017). As Vpd is a function of temperature and relative humidity, higher Vpd values are mostly associated to a drier environment which causes sensitivity to plants and influences the gas

exchange potential. Stomata play a protection role for plants from immediate or long-term damages caused by condition changes in the environment (Devireddy *et al.*, 2020). Plant can respond to those changes by a rapid systemic response, which can influence stomatal activity and development (Devireddy *et al.*, 2020; Driesen *et al.*, 2020). When the Vpd increases, the plant closes its stomata to avoid any excessive loss of water, which on the other hand also impact its CO₂ assimilation (Sinclair *et al.*, 2017). The use of GBH can also affect the stomatal activity in RR cultivars (Albrecht *et al.*, 2014; Krenchinski *et al.*, 2017; Smedbol *et al.*, 2019; Zobiolo *et al.*, 2010). The stomatal development analysis is also a useful tool to strengthen and improve the interpretation of the stomatal activity data collected across the various WMP in interaction with the Vpd. Moreover, these foliar traits can be used as indicators of historic stressors in crops (Zhao *et al.*, 2015). They can provide useful information on the implementation potential of an agricultural practice and how it benefits the stress tolerance of crop plants at crucial growth stages. Therefore, this study aimed at analyzing the aptitude of crop plants to manage the water-CO₂ trade-off in different WMP contexts while being exposed to GBH in open field plots.

MATERIALS AND METHODS

Study Site

This study took place over the summer of 2017 in field plots of corn and soybean located in Sainte-Marthe, Quebec (45°40'05.12"N; 74°34'82.92"W) in a Ste-Rosalie type clay soil. The size of each experimental plot was 190 m long by 12 m wide (2280 m²). Each plot was associated with a different cultivar-WMP pair. The WMP were comprised of a DSCC system and two DS systems (Table 1). In the two DS plots, corn plots were cultivated on wheat residues and soybean plots were cultivated on corn residues. In DSCC plots, alfalfa seeds were broadcasted (12 kg ha⁻¹) as cover crops at the beginning of the fall of 2016 in corn and soybean plots. A second alfalfa seeding was realized on June 6th 2017 at a lower seedling rate depending upon the alfalfa spring regrowth in those plots. Glyphosate-resistant RR crops were used in the corn (P9188AM®) and soybean (PIONEER, P90Y90®) plots (DS and DSCC systems) (Table 1). A starter fertilizer (Alpine G24®) was used during the sowing period (10.09 kg/ha of nitrate; 40.41 kg/ha of phosphorus and 10.09 kg/ha of potassium) (6-24-6 + Zn, 57 L/ha). A post sowing ammonium thiosulfate (ATS) fertilizer (174 kg/ha of nitrate) (28-0-0 + S, 620 kg/ha) was also added in the corn plots. Corn was seeded on May 21st at the density of 82 500 plants ha⁻¹ while soybean was seeded on June 4th in all plots at the density of 382 000 plants ha⁻¹.

All corn and soybean crops in DS and DSCC plots were treated with GBH (WeatherMax®). In the corn DS plots,

Table 1. Weed management practices applied to the Sainte-Marthe direct seeding (DS) and direct seeding on cover crops (DSCC) plots during the summer of 2017.

Parameter	Corn			Soybean		
	DS 1	DS 2	DSCC	DS 1	DS 2	DSCC
Cultivar	P9188 AM			Pioneer 90Y90		
Seedling Date	May 21 st			June 12 th		
Type of herbicid and quantity applied	Weather max [®] (1.35 L*ha ⁻¹)			Weather max [®] (1.35 L*ha ⁻¹) + Classic [®] (0.032 kg*ha ⁻¹)		
First application of GBH	May 24 th - Weather max (1.35 L*ha ⁻¹)			July 6 th		
Second application of GBH	June 26 th - Weather max (1.35 L*ha ⁻¹)	n/a		n/a		
First sampling Date	June 13 th			June 29 th		
Second sampling Date	July 13 th			July 13 th		

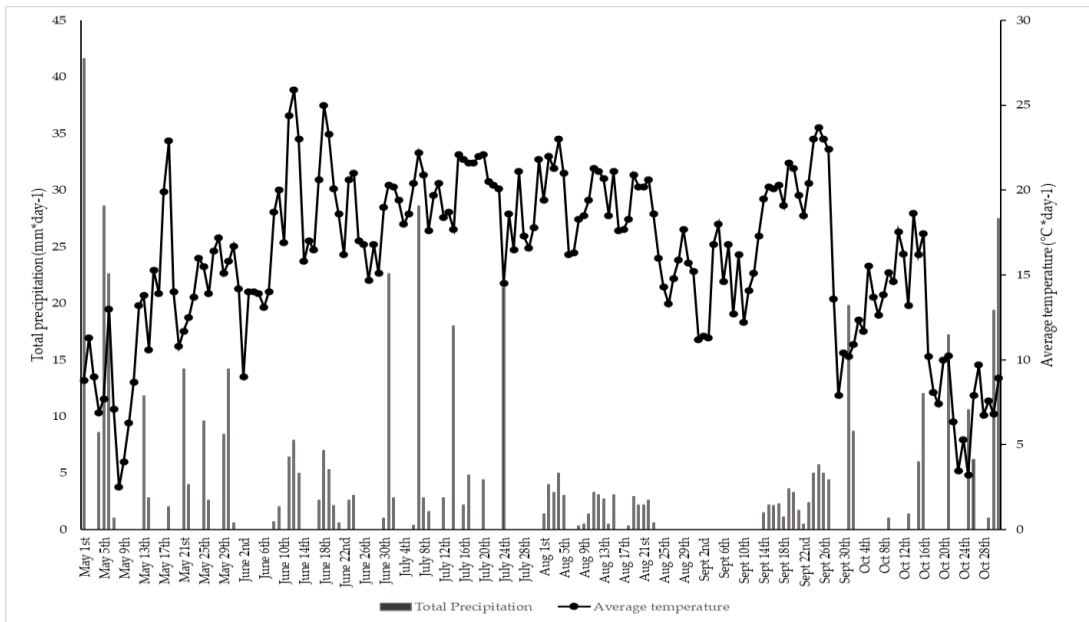


Figure 1 Meteorological data for the period of the essay in 2017 from the Environment Canada Rigaud 2 weather station, about 20 km from Sainte-Marthe, Qc. Daily total precipitations in mm (histogram) and mean temperatures in °C (curves with markers). Data retrieved from <http://climat.meteo.gc.ca> on April 23th 2019.

two post-sowing GBH applications (1.35 L ha⁻¹; 729 g a.i. ha⁻¹) (May 24th and June 26th) and in the DSCC plots one GBH application (1.35 L ha⁻¹; 729 g a.i. ha⁻¹) (May 24th) were realized. In soybeans plots, we applied one post-sowing spray (July 6th) of GBH (WeatherMax[®] at 1.35 L ha⁻¹; 729 g a.i. ha⁻¹) combined to Classic[®] herbicide with

sulfonylureas as active ingredients (0.032 kg ha⁻¹) in DS and DSCC plots. Daily meteorological conditions (mean temperatures and total precipitations) were recorded at the Environment Canada Rigaud 2 weather station (45°28'57.000"N, 74°17'33.000"W) throughout the summer of 2017 (Figure 1).

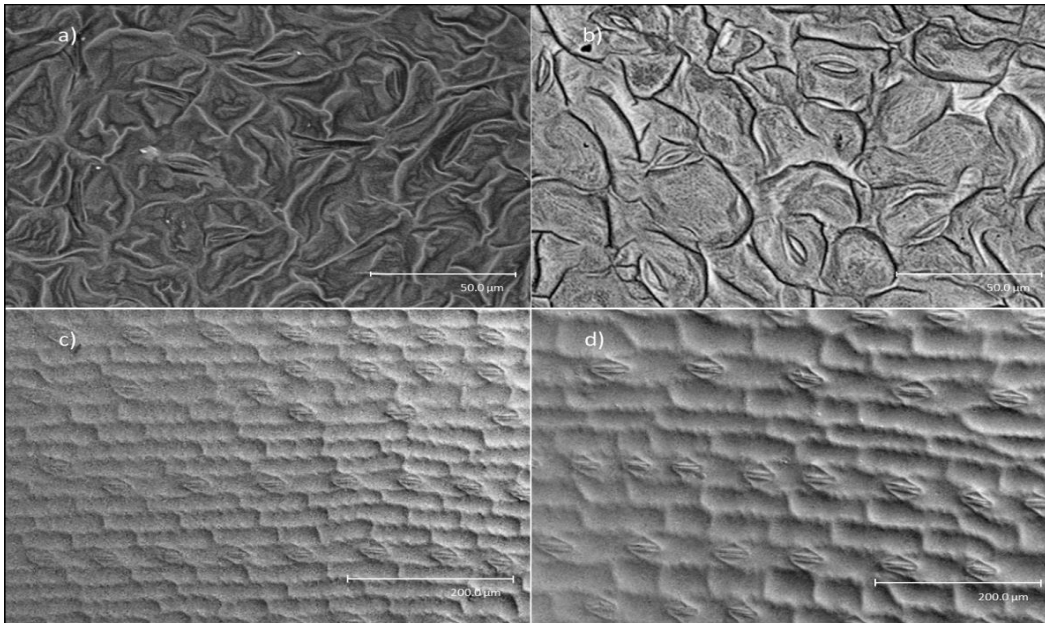


Figure 2 Examples of foliar surfaces under a scanning electron microscope (Hi-SEM S-3400N, Hitachi®). Leaves were taken from a) a direct seeding (DS) corn plot; b) a direct seeding under cover crop (DSCC) corn plot; c) a DS soybean plot; and d) a DSCC soybean plot. Each image is associated with a similar scale for soybean (50 µm) and corn (200 µm).

Sampling

The sampling took place during two campaigns in 2017 where six plants were randomly sampled on June 13th in each plot at V4 stage (BBCH:14) and on July 13th at V10 stage (BBCH:19) in corn plots (Table 1). In the soybean plots, six plants in each plot were sampled on June 29th at V1 (stage BBCH:12) and on July 13th at V3 stage (BBCH:14) (Figure 1). These stages matched with periods before and after the GBH applications in both crops. Fresh above-ground biomass and plant growth were measured on the field excluding the root system during those campaigns.

Analysis of RR cultivars physiological activity

Stomatal conductance was measured with a steady-state diffusion porometer (SC-1 Leaf porometer, Decacon Devices®) using six different leaves similarly arranged in the canopy. All leaves were taken from the sampled plants (see section 2.2). The stomatal conductance was measured at midday between 10:30 a.m. and 3:30 p.m. on both foliar surfaces. The leaves temperature (T) in Celsius was also recorded in order to calculate the corresponding V_{pd} at the leaf level using the August-Roche Magnus

formula, where $V_{pd} = 6.109417.625 \cdot T/T + 243.04$ (Alduchov and Eskridge, 1996; Murray, 1967). The stomatal conductance (G_s) values obtained during both campaigns were normalized between them based on the maximal G_s value (G_{smax}). The normalized values were then used to build a specific stomatal activity model for each WMP.

Foliar traits analysis

The leaves from the second campaign where G_s were measured have also been used to analyze the various foliar traits. These traits are as follows: stomatal density (StoDensity), length (StoLength), width (StoWidth) and total size (StoSize); stomatal index (StoIndex); and leaf dimensions. Because corn and soybean stomata differ in shape, their area (in μm^2) was respectively calculated based on the shape of a trapezium and an ellipse. In every case, stomata were analyzed on both foliar surfaces, adaxial (Ad) and abaxial (Ab).

In order to calculate StoDensity, the leaves collected in the field were observed under a scanning electron microscope (Semerdjieva *et al.* (2015) (Hi-SEM S-3400N, Hitachi®) (Figure 2). The number of stomata was counted three times on similar foliar surfaces ($\pm 1 \text{ cm}^2$) on the middle part

Table 2. Stomatal conductance (Gs) values of the coordinates associated with points of interest (MaxFit and Pol) as a function of the vapor pressure deficit (Vpd).

Parameters	Corn			Soybean		
	DS1	DS2	DSCC	DS1	DS2	DSCC
<i>Adaxial surface (Ad)</i>						
GS _{MaxFit}	226	224	208	803	472	570
Vpd _{MaxFit}	51	50	45	0	25	12
GS _{POI}	70	58	48	455	336	377
Vpd _{POI}	32	42	40	22	37	29
GS _{Average}	180	161	166	355	349	318
Std. Err. GS _{Average}	43	35	44	72	72	75
<i>Abaxial surface (Ab)</i>						
GS _{MaxFit}	280	235	334	524	507	781
Vpd _{MaxFit}	46	50	53	26	33	28
GS _{POI}	74	88	65	360	418	466
Vpd _{POI}	49	39	11	42	40	46
GS _{Average}	265	232	235	514	517	523
Std. Err. GS _{Average}	22	22	25	122	113	213

These values were calculated using the functions derived from the GLM for corn and soybean. Mean conductance (GS_{Average}) in mmol*m⁻²*s⁻¹ was obtained using a variance analysis of the Gs values for every WMP (non-significant, $p > 0.05$).

of the leaves, at the same place where Gs was measured. These foliar surfaces were carefully chosen to avoid any foliar vein. The number of stomata on each foliar surface was measured by μm^2 , then extrapolated by mm^2 . A picture of the stomata in each zone was taken to calculate StoDensity and measure StoWidth and StoLength using the ImageJ 1.0 (NIH) software. The stomatal index was then calculated by multiplying the size of the stomata with their density (by μm^2).

Statistical analysis

The GLM development was based on the methodological approach recommended by Krober and Bruelheide (2014). The normalized values (Gs/Gs_{max}) were integrated into a statistical software (JMP13, SAS®) to obtain a generalized linear model (GLM) with binomial distribution (with Logits) as a function of the corresponding Vpd. The resulting curve of each model follows a function of the type: $\ln(\text{Gs}/\text{Gs}_{\text{max}}) = a\text{Vpd}^2 + b\text{Vpd} + c$. The optimal values (MaxFit) and points of inflexion (Pol) of each curve were obtained by calculating the first derivative of the functions. The interesting facts to observe on the curve are those associated with the coordinates (Vpd_{MaxFit}; Gs_{MaxFit}) that represent MaxFit, the probable optimal condition for gas exchanges, as well as the coordinates (Vpd_{POI}; Gs_{POI}) that represent the point of inflexion (Pol)

associated with the Vpd value where the plant closes its stomata.

All foliar traits data obtained during the second campaign as well as the data on the plant characteristics (gains in plant height and fresh above-ground biomass, represented by the difference between the values of the second and the first campaign) were analyzed using JMP13 (SAS®) in order to compare the results for each WMP. An analysis of variance (ANOVA) was used for data following a normal distribution. A Wilcoxon test was used where a nonparametric test was necessary, namely with the soybean StoDensity. A Student's t-test and a Wilcoxon test were then used to respectively compare the paired means of parametric and nonparametric data. The tolerance limit for these tests was set at 95%, and differences were considered significant when $p < 0.05$.

RESULTS

Soybean stomatal traits

The results obtained between the first and the second campaign showed several differences between the adaxial (Ad) and abaxial (Ab) foliar surfaces. A difference was observed in the stomatal activity based on the stomatal conductance (Gs) ($p = 0.0004$), where the Gs values on Ad were 34% lower than on Ab (Table 2, Figure 3 and 4)).

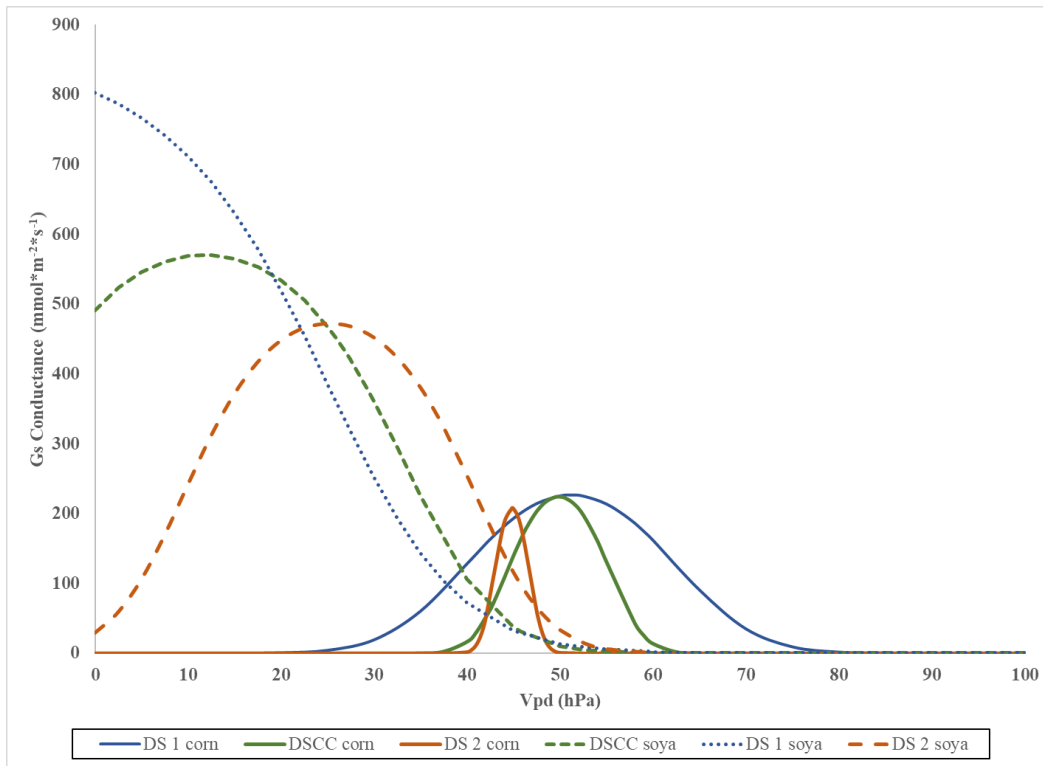


Figure 3. Generalized linear model used to predict the stomatal conductance (G_s) values for adaxial (Ad) foliar surfaces in soybean ($R^2=0.882$) and corn ($R^2=0.153$) from direct seeding systems (DS) and direct seeding on cover crops system (DSCC) as a function of the vapor pressure deficit (Vpd).

Regardless of the WMP, the stomatal development measured using the stomatal length (StoLength) ($p < 0.0001$), width (StoWidth) ($p < 0.0001$), total area (StoArea) ($p < 0.0001$), density (StoDensity) ($p < 0.0001$) and index (StoIndex) ($p < 0.0001$) was significantly higher on the Ab surfaces than on the Ad surfaces. The StoDensity Ad values accounted for 40.7% of the StoDensity Ab values (Table 3a).

The stomatal traits measured in adaxial (Ad) and abaxial (Ab) foliar surfaces were stomatal width (StoWidth), length (StoLength), total size (StoSize), density (StoDensity) and index (StoIndex). Gain in plant height and fresh above-ground biomass as well as foliar areas are represented by the difference between the values of the second and the first campaign. P-values are significant ($< 0,05$) with an ANOVA* or a Wilcoxon test**.

The highest G_s values were observed in the DS1 plots ($905 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) on the Ad foliar surfaces and in the DSCC plots ($932 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) on the Ab foliar surfaces. The curves representing G_s as a function of the Vpd values obtained from the generalized linear models (GLM) are shown in Figure 3 (Ad surfaces) and Figure 4 (Ab

surfaces). The values of the MaxFit and Pol points as well as their associated coordinates (VpdMaxFit;GsMaxFit and VpdPol;GsPol) were calculated using the curves for each practice and are shown in Table 2. When comparing Gsmax with GsMaxFit, it is observed that the values of the latter are much lower in each practice, regardless of the foliar surface. However, the order of importance of the practices is similar for Gsmax and GsMaxFit, where the highest values of GsMaxFit on the Ab surface have been observed in the DSCC plots ($741.82 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

The foliar traits analysis showed significant differences between the practices. These results are shown in Table 3a. StoWidth and StoArea on the Ad and Ab surfaces of the plants in the DSCC plots were higher in comparison to other practices. Significant differences in StoDensity were only observed on the Ad surface, with the lowest values being associated with the DS1 plots. Differences in StoWidth on both foliar surfaces were observed between DS1 (Ad: $1.630 \mu\text{m}$, Ab: $2.142 \mu\text{m}$) and DS2 (Ad: $2.102 \mu\text{m}$, Ab: $2.631 \mu\text{m}$), whereas a significant difference in StoArea was only observed on the Ad surface between the same practices (Table 3a).

Table 3. Means and Standard error on means of stomatal traits and plants parameters between the different treatments in a) corn and b) soybean.

A) Means and Standard error on means for treatments in Soybean				
Parameter	Treatments			p-value
	DS 1	DS 2	DSCC	
<i>Leaf traits</i>				
StoLength Ab (um)	12.17 ± 0.38	12.01 ± 0.32	13.10 ± 0.41	0.1040
StoLength Ad(um)	10.95 ± 0.43	11.32 ± 0.33	11.54 ± 0.37	0.5525
StoWidth Ab (um)	2.14 ± 0.07 ^C	2.63 ± 0.15 ^B	3.02 ± 0.21 ^A	0.0001*
StoWidth Ad (um)	1.63 ± 0.05 ^C	2.10 ± 0.15 ^B	2.58 ± 0.13 ^A	<0.0001*
StoSize Ab (um ²)	20.75 ± 1.05 ^B	25.25 ± 1.70 ^B	33.43 ± 2.52 ^A	0.0001*
StoSize Ad (um ²)	14.03 ± 0.64 ^C	19.06 ± 1.84 ^B	23.69 ± 1.59 ^A	0.0002*
StoDensity Ab	352.62 ± 17.29	373.33 ± 20.08	332.02 ± 23.72	0.976
StoDensity Ad	124.44 ± 11.23 ^B	165.96 ± 11.23 ^A	145.19 ± 8.01 ^A	0.0081**
StoIndex Ab	0.0071 ± 0.0003 ^B	0.0092 ± 0.0009 ^{AB}	0.0114 ± 0.0011 ^A	0.0041*
StoIndex Ad	0.0017 ± 0.0002 ^B	0.0030 ± 0.0003 ^{AB}	0.0035 ± 0.0003 ^A	<0.0001*
<i>Plant growth</i>				
Gain height (cm)	13.09 ± 0.88	13.59 ± 2.29	13.35 ± 0.80	0.988
Gain aerial biomass (g)	0.448 ± 0.171	0.741 ± 0.215	0.429 ± 0.126	0.311
Leaf size (cm ²)	11.26 ± 0.61 ^A	12.47 ± 1.66 ^{AB}	9.34 ± 0.51 ^B	0.082
B) Means and Standard error on means for treatments in Corn				
Parameter	Treatments			p-value
	DS 1	DS 2	DSCC	
<i>Leaf traits</i>				
StoLength Ab (um)	34.21 ± 1.01	35.26 ± 0.950	36.33 ± 1.48	0.4488
StoLength Ad(um)	36.57 ± 0.85 ^B	36.92 ± 1.42 ^B	41.57 ± 1.41 ^A	0.0131*
StoWidth Ab (um)	23.33 ± 0.92 ^B	25.47 ± 0.85 ^B	31.07 ± 1.80 ^A	0.0004*
StoWidth Ad (um)	24.91 ± 0.88 ^B	26.66 ± 0.72 ^B	29.11 ± 0.70 ^A	0.0021*
StoSize Ab (um ²)	415.64 ± 15.31 ^B	455.89 ± 10.82 ^B	578.36 ± 35.68 ^A	<0.0001*
StoSize Ad (um ²)	441.65 ± 13.89 ^B	499.94 ± 23.19 ^C	614.84 ± 19.66 ^A	<0.0001*

Table 3B. Contd

StoDensity Ab (mm ⁻²)	91.82 ± 3.86 ^A	56.38 ± 4.26 ^C	72.67 ± 5.17 ^B	<0.0001*
StoDensity Ad (mm ⁻²)	111.43 ± 3.37 ^A	79.14 ± 3.46 ^B	82.27 ± 4.73 ^B	<0.0001*
StoIndex Ab	0.0378 ± 0.0014 ^A	0.0258 ± 0.0021 ^B	0.0409 ± 0.0026 ^A	<0.0001*
StoIndex Ad	0.0489 ± 0.0014 ^A	0.0393 ± 0.0020 ^B	0.0504 ± 0.0030 ^A	0.0022*
<i>Plant growth</i>				
Gain height (cm)	122.44 ± 2.04 ^A	116.57 ± 1.77 ^A	103,14 ± 5.46 ^B	0.0028*
Gain aerial biomass (g)	295.52 ± 9.16 ^A	302.97 ± 20.49 ^A	215.02 ± 27.07 ^B	0.0101*
Leaf size (cm ²)	87.26 ± 9.16 ^B	95.27 ± 3.37 ^B	125.69 ± 11.61 ^A	0.0132*

When comparing StoIndex, which represents the proportion of a leaf allocated to stomata, significantly lower values were observed on the Ad surface in DS1 plots (0.17%) in comparison with DSCC (0.35%) and DS2 (0.3%) plots (Table 3a). On the Ab surface, even though the StoIndex value in the DS1 plot (0.71%) is even lower than that of the DSCC plots (1.14%), no significant difference was observed with that of the DS2 plot (0.92%). There was no difference between the practices in terms of height gain ($p = 0.9880$) and fresh above-ground biomass gain ($p = 0.3110$). However, at the time of the second campaign, the leaves in the DSCC plots had the smallest foliar area (9.344 cm²) when compared with the leaves in the DS1 plot (11.264 cm²).

Corn stomatal traits

Differences were also observed between the adaxial and abaxial foliar surfaces of the corn plants. Regardless of the practices, the stomatal development was significantly higher on the Ab surfaces (Table 3b). This has been observed using measures of various stomatal traits: StoLength ($p = 0.0043$), StoDensity ($p = 0.0006$) and StoIndex ($p < 0.0001$). In fact, the Ad StoDensity accounted for 80.95% of the Ab StoDensity. Results also showed that Gs rose from 31% on the Ab surface between the first and the second campaign ($p = 0.0060$).

When comparing all WMP, the highest Gs values were recorded in the DS2 plots (614 mmol m⁻² s⁻¹) for the Ad surfaces and in the DS1 plots (430 mmol m⁻² s⁻¹) for the Ab surfaces (Table 2). The GLM curves representing the Gs as a function of the Vpd, shown in Figure 3 and Figure 4 for the adaxial and abaxial surfaces respectively, also showed differences between the practices. When comparing the Gsmax and GsMaxFit it can still be

observed that the latter are much lower in every practice. It was also noted that the highest GsMaxFit value on the Ab surface has been observed in the DSCC plots (334,13 mmol m⁻² s⁻¹) (Table 2).

The stomatal trait analysis revealed significant differences between the practices. These differences were observed among all the variables studied and are shown in Table 3a. On the Ad surface, the stomata of the cultivars in the DSCC plots were significantly larger ($p < 0.0001$). However, foliar surfaces in the DSCC plots had significantly lower StoDensity ($p < 0.0001$) compared with the DS plots. Similarly, it has been observed that the stomata on the Ab surface in the DSCC plots were larger and had a smaller density in comparison with the DS plots. However, results showed that StoDensity was significantly higher on the foliar surfaces in the DS1 plots (Ad: 91.88 mm⁻², Ab: 111.43 mm⁻²) compared with the DS2 plots (Ad: 56.37 mm⁻², Ab: 79.14 mm⁻²). StoIndex values for the DS2 plots were lower compared with the other practices, both on the Ad (2.58%) and Ab (3.93%) surfaces.

DISCUSSION

In this study, stomata of plants grown in DSCC systems seem to be capable to perform gas exchanges at higher Vpd as suggested by the VpdPOI values from corn and soybean abaxial stomata (Table 2). Higher values were observed on the abaxial surface of the plants in the DSCC system in comparison with both variants of the DS systems (Table 2). GsMaxFit values in the DSCC system were 19% higher in corn and 49% higher in soybean in comparison with the DS1 system (Table 2). Stomata in both crops grown in DSCC plots were also bigger compared to those from plants in DS plots. We also observed that StoWidth is

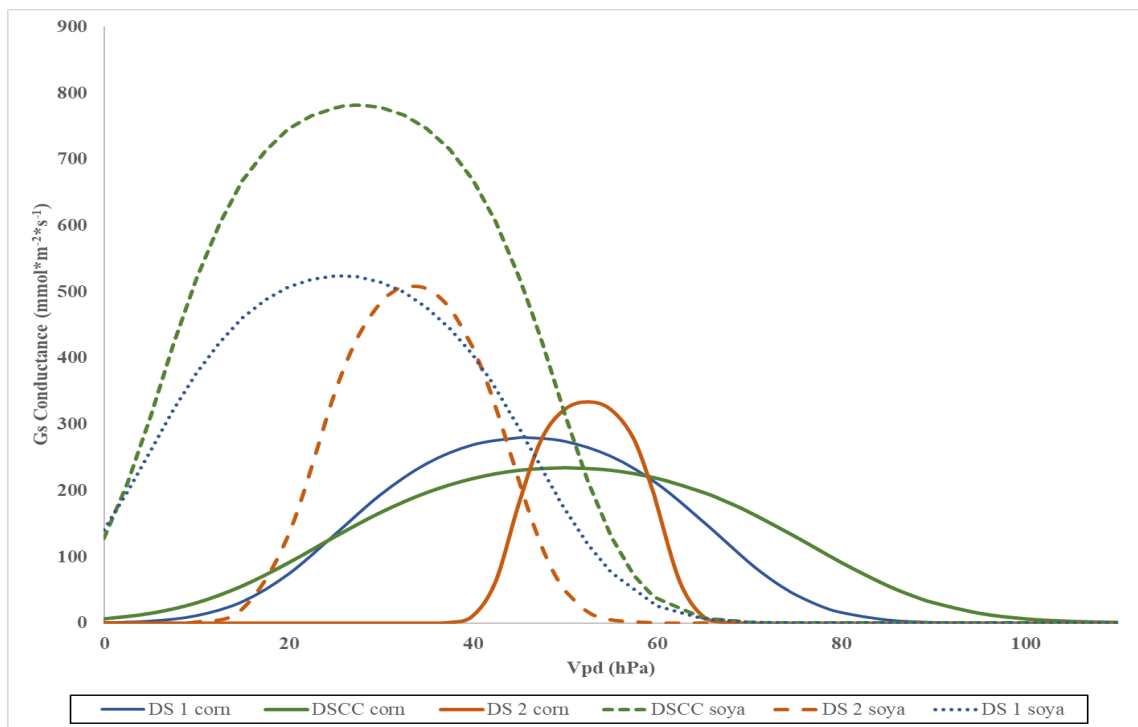


Figure 4. Generalized linear model used to predict the stomatal conductance (G_s) values for abaxial (Ab) foliar surfaces in soybean ($R^2= 0.415$) and corn ($R^2= 0.100$) as a function of the vapor pressure deficit (Vpd) for the direct seeding systems (DS) and direct seeding on cover crops system (DSCC).

the factor with a positive relation with G_s ($R^2 = 0.2585$) in corn and soybean ($R^2 = 0.1668$). This observation reinforces the idea that *StoWidth* is a good indicator for the potential stomata opening in order to execute more important gas exchange. The possibility for greater gas exchanges can be a real benefit for plants in terms of carbon assimilation, development, and potential for competitiveness. Following Aasamaa *et al.* (2001), small stomata generally act as a more fine-tuning management due to their greater sensibility and faster response to environmental changes. At midday, as Vpd increases with temperature in a positive nonlinear regression, the plant response to Vpd is more like a pop and down relationship where the stomata open rapidly as Vpd increases and then close completely at higher Vpd values (Grossiord *et al.*, 2020). Theoretically, when a threshold Vpd (Vpd_{Pol}) is reached, the plant closes its stomata to stop excessive water loss through transpiration and avoiding turgor loss. The curves in Figure 4 can be interpreted as the sensibility to risen Vpd where more abrupt slope represents faster antagonism response (stomata opening and closing) for a less tolerant plant to Vpd rise. Based on the generalized linear models (GLM) built in this study, it was observed that soybean plants are much more sensitive than corn plants to a Vpd rise (Figure 3 and Figure 4), which lead to Vpd_{Pol} values in soybean that are up to 52% lower depending on the foliar surface. According to Ocheltree *et al.* (2014), the Vpd creates a force on the

leaves that stimulates the ascension of water through the xylems. Even though it was predictable that sensitivity to Vpd would vary among the functional groups, it still suggests that soybean plants will probably be more sensitive and influenced more directly by water stress. In this context, the presence of cover crops should lessen the soil evaporation rate during warmer period and reduce the soybean sensibility and mitigate the transpiration demand when Vpd rise. Robertson *et al.* (2014) have also observed that cover crops serve as a buffer zone that slows water evaporation from soils during high temperature and drought episodes, which helps keeping soils hydrated and oxygenated. Even if the aerial part of cover crops may die after herbicides applications, their root systems are still present and may influence the soil function (Ogilvie *et al.*, 2021; Bowsher *et al.*, 2018; Liu *et al.*, 2005). However, the influence of Vpd on G_s seems less striking on corn where the positive relation is less important compared to soybean (see R^2 on Figures 3 and 4). This suggests that other factors (e.g. water and nitrate content in soil and light intensity) may have acted more strongly on stomatal activity in maize. This therefore allows plants to develop larger stomata or to keep them more open which, in both cases, leaves the possibility of achieving greater gas exchange (Whitehead, 1998). The *StoSize* can also help predict the ability of a cultivar to manage water based on the quantity of water available at a given time that could be used later on to maintain productivity at a more advanced

growth stages (Sinclair *et al.*, 2005). Following this idea, bigger stomata can also be interpreted by a positive response of the plant developed in a better soil-plant-atmosphere continuum environment.

The use of GBH is another factor that may have influenced the stomatal development on the adaxial surface in our study. Due to a general horizontal arrangement of the leaves, stomata on the adaxial (upper) surface are more exposed than those on the abaxial (under) to different abiotic (e.g. light intensity, relative humidity and air temperature), biotic (e.g. pathogens) and xenobiotic (e.g. pesticides application and heavy metals) factors. Exposure to foliar herbicide application is greater on that surface compared to the abaxial surface (Dorr *et al.*, 2016). Generally, the quantity, adhesion and persistence of herbicides are highest on the upper leaf surface and this may explain why it is more common to observe signs of toxicity (e.g. yellow flashing) on the adaxial leaf surface. Many researchers only look at stomatal activity on the abaxial surface when comparing cultivars or practices. Nevertheless, stomatal activity on the adaxial surface does contribute to the overall plant gas exchange and plays a crucial role in the foliar trait transmission between mature and new leaves following different exposition conditions (Driesen *et al.*, 2020). In this study, considering both foliar surfaces can give substantial information about adaxial stomatal behaviour facing herbicide application and other stresses. The case of corn production is interesting because the DSCC system required a single GBH application, whereas the DS system required two. Other studies showed that herbicides can influence the stomatal development but none of them so far have considered GBH and GR cultivars before (Semerdjieva *et al.*, 2015; Anastasov, 2010a; Anastasov, 2010b). However, some studies on stomatal activity (Krenchinski *et al.*, 2017; Albrecht *et al.*, 2014; Zobiolo *et al.*, 2010) showed that the use of glyphosate on GR cultivars is not without impact. Zobiolo *et al.* (2010) and Albrecht *et al.* (2014) observed a significant decrease in stomatal conductance following GBH application at different doses at early vegetative stages. The aminomethylphosphonic acid, the main metabolite of glyphosate, could even be toxic for guard and mesophyll cells of GR plants (Gomes and Juneau, 2016; Gomes *et al.*, 2014; Reddy *et al.*, 2004). The oxidative stress pathway and reactive oxygen species (ROS) production involved after GBH exposition are important pathways in plants response to stress with a strong occurrence in their tissues and cellular compartments like chloroplasts (Gomes and Juneau, 2016; Gomes *et al.*, 2014). Repeated use of GBH induces a chronic toxicity in GR plants, which can increase the stress level and impact the recovery time after every exposure (Reddy *et al.*, 2004). However, the plant response to GBH application seems to differ between studies and it is still not clear how ROS following herbicide application can influence the stomatal behaviour. ROS can play a double role on stomatal activity by inducing stomatal

opening and closure at a high and low concentrations in the guard and mesophyll cells (Chen *et al.*, 2016). Surprisingly, both ROS roles have been observed on stomatal activity when GR soybean plants are treated with 4.5 kg glyphosate ha⁻¹ and when turnover role of ROS occurs following the increasing amounts of ROS in the leaves over time (Smedbol *et al.*, 2019). The exposition of glyphosate during the recovery period decreases the stomatal conductance, and a negative correlation was established between the stomatal conductance and the successive increase in the quantity of GBH applied (Zobiolo *et al.*, 2010). Moreover, these effects cumulated with a water stress episode exacerbate the sensitivity of the cultivar (Petter *et al.*, 2016) during the recovery period, which will be longer and thus will impair its competitiveness (Reddy *et al.*, 2004). In other studies, GBH application induced an increasing stomatal conductance on plants exposed to GBH in comparison with non-treated plants (Smedbol *et al.*, 2019; Chen *et al.*, 2016). Although these authors obtained diverging results, they all concluded that the presence of AMPA can induce a stress response in plants and somehow influence the stomatal activity and other important physiological processes for plants development and reproduction stages. The stage of growth and the period of vulnerability of the plants have a stronger repercussion on these processes (Krenchinski *et al.*, 2017; Albrecht *et al.*, 2014; Albrecht *et al.*, 2011; Zobiolo *et al.*, 2010; Reddy *et al.*, 2004).

While StomDensity is closely linked to the plant gas exchange potential and performance, a close relationship was observed in this study between abaxial stomatal size and density, and plant dry matter production (Tables 3a and 3b). In corn, DS plants treated twice with GBH seem to have adopted a strategy focussed on biomass production mainly expressed through a gain in plant height compared to DSCC plants. It has been observed in other studies that plants treated with GBH could have a greater stomatal conductance following sequential application and the outcome may be related to the plants response to GBH toxicity (Smedbol *et al.*, 2019; Chen *et al.*, 2016). Other observations made on corn and soybean plants reported that cultivars with a higher stomatal density were associated with higher values of fresh biomass (Waqas *et al.*, 2021; Sun *et al.*, 2014; Tanaka *et al.*, 2010). To compensate for the alteration of stomatal activity caused by stress, plants may have stimulated the production of functional stomata in order to maintain metabolic demand. In this study, another potential influence of the GBH on stomatal behaviour may come from the salt form of glyphosate in the applied formulation. Among various formulations, the main active ingredient, glyphosate (N-(phosphonomethyl) glycine), is present in different salt form (e.g. potassium, isopropylamine, ammonium and trimesium salt) in order to optimize the herbicide absorption by the plants (Travlos *et al.*, 2017). The Roundup formulation (WeatherMax®) used in this study contains potassium salt of glyphosate and it is well known

that potassium (K) is an essential mineral nutrient involved in many physiological processes in the plants (Anschütz *et al.*, 2014; Hasanuzzaman *et al.*, 2018; Zörb *et al.*, 2014). Normally, K accumulation in guard cells promotes the water uptake necessary for their turgor as well as stomata opening. When GBH is applied on the foliar surface, glyphosate can be absorbed by the plants through the stomata where K salt may interact with the surrounded guard cells. Waqas *et al.* (2021) have demonstrated that exogenous K salt application enhances stomatal conductance, overall gas exchange and biomass production. Higher stomatal densities have also been observed on plants with a higher K regime (Sekiya and Yano, 2008; Sun *et al.*, 2014) which is consistent with our observations. It is also well known that K can help reduce abiotic and biotic stress impacts on plants (Gouveia Lana *et al.*, 2021; Zörb *et al.*, 2014). In our case, the presence of K may have attenuated the stress induced by the presence of AMPA considering its involvement in the stress-redox processes of the plant. AMPA can have a negative impact on non-stomatal process like affecting the photosystem and the electron transport chain and may lead to the production of ROS (Gomes and Juneau, 2016; Gomes *et al.*, 2014; Smedbol *et al.*, 2019). In that case, the K upregulation can be beneficial by limiting the ROS generation in plants after GBH exposure.

The presence of cover crops in DSCC plots may have also contributed to reduce the total glyphosate/AMPA content on the soil. The first explanation for this is that cover crops can capture some of the amount of GBH applied before the herbicide reaches the soil (Khalill *et al.*, 2018; Locke *et al.*, 2005; Locke *et al.*, 2008). Secondly, cover crops promote the diversity and density of the roots system which can favorize glyphosate uptake by non-crops plants (Amsili and Kaye, 2021). Soil microorganisms can degrade glyphosate through the cleavage of the carbon-phosphorus bond (C-P lyase pathway) or the cleavage of the carbon-hydrogen bond known as glyphosate-oxidative reductase (GOX pathway) (Kanissery *et al.*, 2019). The GOX pathway are the predominant way of glyphosate degradation and the latter results in AMPA production which can accumulate in soils (Grandcoin *et al.*, 2017; Kanissery *et al.*, 2019). Higher glyphosate or AMPA uptake by non-crop plants may reduce the uptake of these chemicals by crop plants. Even if GR plants are capable to mitigate the effect of glyphosate through resistance mechanism (e.g. GR genes, enhanced metabolism, compensation and protection by glyphosate sequestration in cell vacuoles), glyphosate can accumulate and have a strong persistency in plant tissues which can turn to be toxic on the long term (Délye *et al.*, 2013; Gomes *et al.*, 2014; Smedbol *et al.*, 2019). Moreover, due metal chelating properties of glyphosate, higher content of this chemical in soils can reduce the bioavailability of magnesium (Mg) and manganese (Mn) for plants (Duke *et al.*, 2012; Mertens *et al.*, 2018). Mg and Mn are both involved in the proper cell functions.

Symptoms of Mg and Mn deficiencies are noticeable on

foliar surfaces with negative impacts on enzymes production, gas exchange and photosynthesis (Taiz and Zeiger, 2010; Khalill *et al.*, 2018; Locke *et al.*, 2005; Locke *et al.*, 2008). This may also explain why DS plants were able to counteract the effects of reduced stomatal conductance by compensating with higher numbers of stomata.

CONCLUSIONS

The analysis of the stomatal development and activity was consistent with the fact that plants grown in a DSCC system seem more capable to execute gas exchange, which results on bigger stomata in corn and soybean. Knowing that soybean crops will probably be greatly affected by water stress and higher values of Vpd induced by climate changes, DSCC system could be a convenient alternative for this type of crop production. Moreover, this study showed that higher StoDen was observed on corn crops that were more exposed to GBH. GBH application may have an impact by several means on guard cells (stomatal process) and mesophyll cells (non-stomatal process) which ultimately influence stomatal activity and plants development. In our case, an enhanced stomatal development could be due to the presence of K salt in the applied formulation. Plants more exposed to GBH seems to be more sensitive at higher Vpd values, which affected StoSize. The presence of cover crops can also mitigate the impacts of GBH on crops in different ways such as more important GBH interception and roots uptake, which could reduce the glyphosate/AMPA content on soils and their toxicity on crops. To this day, the use of DSCC system in Quebec's field crops is still marginal in comparison with direct seeding. Further understanding of its applicability in a northern climate and on its potential to initiate a transition towards weed management practices, requiring less GBH is needed.

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