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Dynamics of Soil Fungal Population in Herbicide Resistant Canola Fields

Ravneet Kaur[1], Harvinder S. Syan[1], Suha Jabaji[1], Denis Pageau[2] and Jaswinder Singh[1*]

[1]Department of Plant Science, McGill University, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada.
[2]SCRDC of Agriculture and Agri-food Canada, Normandin, Quebec, Canada.

Authors' contributions

This work was carried out in collaboration between all authors. Author RK conducted molecular experiments, analysed qPCR data and wrote the first draft of the manuscript. Author HSS conducted soil sampling from LODs center McGill site, soil sample analysis, literature searches, wrote the 2nd draft. Author SJ provided expertise in the analysis of soil microbes. Author DP collected and organized soil samples in replicated trials and author JS defined the protocols, managed the experimental process, and refined the writing. All authors read and approved the final manuscript.

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ABSTRACT

High yielding and stress tolerant crops are vital for food security needs of a growing population. New approaches are being devised to develop genetically modified (GM) plants suitable for changing climate, resistance to pests and herbicides and enhanced nutrition. Despite having great future promises, the general public and scientific community are concerned about the influence of growing GM plants on the ecosystem and human health. Herbicide-tolerant (HT) crops are among such GM crops which are considered to modify soil milieu by introducing herbicides compounds. Using quantitative polymerase chain reaction (qPCR), we examined the effect of herbicides, glyphosate, glufosinate ammonium sulphate and imazethapyr ammonium, on soil fungal population in the HT canola fields over two consecutive growing seasons (2010 and 2011) at two different locations in Quebec, Canada. Our data indicate that although slight increase in copy number of fungal population was observed after glyphosate treatments, this change was statistically

**Corresponding author: E-mail: jaswinder.singh@mcgill.ca;*

non-significant, even in case of glufosinate ammonium sulphate and imazethapyr ammonium. Thus herbicides in study had no adverse effects on fungal population.

Keywords: GM canola; herbicide; glyphosate; glufosinate ammonium sulphate; imazethapyr ammonium; fungal population.

1. INTRODUCTION

In last few decades, canola (*Brassica napus* L.) has become one of the most profitable commodities for farmers, mainly in Canada, Australia, USA, China, and India. It is developed by Canadian plant breeders through conventional breeding of *Brassica* sp. having low erucic acid and glucosinolate contents. Since, canola is often seeded during spring or early summer, so it faces strong competition from weeds most particularly, wild mustard (*Sinapis arvensis*) and quack grass (*Elymus repens*) which significantly reduce canola yield. Seed contamination of the canola especially with wild species could lead to reduced quality including commodity price. Hence, canola seed industry is largely focused on development and use of herbicide tolerant (HT) varieties. These varieties facilitate low or no tillage cultural practices and better management of weeds. Several reports indicate that herbicide resistant crops attributed to grower's satisfaction with better weed control [1].

In Canada, mainly glyphosate and glufosinate tolerant canola varieties are grown for commercial purposes. Glyphosate (N-phosphonomethylglycine) is one of the most commonly used broad-spectrum herbicide, which is relatively effective against a wide variety of weeds and predominantly applied to agricultural fields. Glyphosate is also among widely used herbicide in forestry because of its better efficacy in controlling several understory plant species [2,3] and fast degradation in soil [4-6] However, the potential non-target effects of glyphosate on soil microflora and their nutrient cycling and maintenance of soil structure, are of profound concern. Glyphosate obstructs protein synthesis in bacteria and fungi via the shikimic acid pathway [7] and polyoxyethylene tallow amine, one of its surfactants, exhibit toxic effects against bacteria and protozoa [8]. However, glyphosate has generally been found to be innocuous to soil microorganisms in field studies and laboratory bioassays at recommended doses [9-11]. High rate applications of glyphosate have direct effect on microbial respiration [10-12], and adversely affect nutrient cycling [13-15].

Glufosinate ammonium sulphate, a post-emergence herbicide, is the active ingredient of the broad spectrum herbicide Basta and is used along with glufosinate tolerant, or Liberty-Link® crops. Glufosinate is a phosphinic acid analog of L-glutamate, hence, acts as a potent inhibitor of Gln synthetase, a key enzyme of nitrogen metabolism in plants and is devastating to plant survival [16]. Glufosinate tolerance in plants is achieved by incorporation of either the *pat* gene (phosphinothricin-acetyltransferase) or the *bar* gene (bialaphos resistance), whose protein product inactivates glufosinate by acetylation [17]. Glufosinate showed higher activity on annual weeds than perennial weeds, whereas glyphosate, a slower acting herbicide, had lower activity on annual species as compared to perennial species. Although glufosinate was considered a microbial toxin, however, reports on the sensitivity of the soil microorganisms to glufosinate are scarce and inconclusive [18,19].

Despite the benefits of growing HT canola, the contribution of HT canola is still controversial as some argue the environmental impact of genetically modified crops in general [20]. Keeping in view the major concern of growing herbicide tolerant GM canola on fungal population, we assessed the effect of different herbicides on increase/decrease of soil fungal population in the HT canola fields over two growing seasons at two different locations in Quebec, Canada.

2. MATERIALS AND METHODS

2.1 Experimental Design and Field Sampling

The experiments were performed during two growing seasons (2010 and 2011) and at two locations; the LODS Agronomy center of McGill University, Ste Anne de Bellevue, QC (45°24'14"N,73°57'9"W) and SCRDC, Agriculture and Agri food Canada, Normandin, Quebec (48° 49' 60.00" N, 72° 31' 60.00" W). The canola crop was planted in May for both years. Three herbicide tolerant canola cultivars viz. Roundup ready® (varieties: 45H28 and 45H29; GM;

resistant to glyphosate; Monsanto), Liberty link® (varieties: 5030 and 5040; GM; resistant to glufosinate ammonium sulphate; Bayer Crop science), Pursuit Smart® (variety: 45H73; resistant to imazethapyr ammonium; Pioneer Hi-bred; developed by conventional breeding) were grown, at both locations in four replications and during the two subsequent years 2010 and 2011. Herbicide application schedules on crops were chosen based on the common practices of Quebec farmers. Herbicides were applied according to manufacturer's instructions. Glyphosate (450 g ai ha^{-1}), glufosinate (400 g ai ha^{-1}) and imazethapyr ammonium (80 g ai ha^{-1}) herbicides were sprayed as post-emergent herbicides. Each treatment was replicated four times at both locations. Field plots were 6 m × 2 m size with 0.18 m row spacing. Soil samples were collected from two depths (0-15 cm and 15-30 cm) from all the four replications. Sampling was done with two augers, one for each soil depth, and these were thoroughly cleaned between each sampling activity. Samples were collected from five different points from each treatment and then mixed together in a bag to get a representative sample from each depth. Soil samples were placed into Ziploc™ bags and stored at -20°C until analysis. The time between soil sampling and storage varied between 6 and 8 hours. Soil samples were collected on a day prior to herbicide application (0 day) and 20 days after herbicide application. To minimize cross-contamination, the experimental units were separated by border (6 m x 2 m) around each plot.

2.2 DNA Extraction

DNA was extracted from each soil sample (500 mg) using the Fast Soil DNA Isolation Kit (MP bio, Canada) following the manufacturer's instructions. DNA was checked on gel electrophoresis whereas for quantitation, Nanodrop Spectrophotometer was used. Equal amount of DNA was used as template for qPCR analysis.

2.3 Quantitative PCR

To assess the soil fungal population, LSU ribosomal DNA specific primers were designed (forward; QFL1-5′ ATGCAGCTCAAAATGGGT GGT 3′ and reverse; QRL1-5′ ATCCCACAC AACGCGACTGAC 3′) to amplify a fragment size of 165 bp. Quantitative assessment of fungal population was carried out by real-time PCR (Stratagene Fast7500P, USA). The reference standard for qPCR, PCR was done with QFL1 and QRL1 primers in a reaction mix containing 1 µl of each primers (10 nm), 5 µl of MP bio mastermix (MP Bio, Canada) and soil DNA (50 ng) as a template in a final volume of 25 µl. Cycling conditions used were: initial denaturation 3 min at 95°C followed by 40 cycles of 30 s at 95°C, 40 s at 60°C and 1 min at 72°C [21]. PCR amplified product was purified from gel using gel extraction kit (Qiagen, Germany) and ligated into TOPO TA pCR 2.1 cloning vector kit (Invitrogen, USA) followed by transformation into *E. coli* DH5α competent cells. Recombinant plasmid was purified using Plasmid Mini kit (Qiagen, Germany) and sequenced. The cloned circular plasmid was quantified by spectrophotometer and used as template to prepare reference standard curve for quantification purposes. qPCR reaction contained equal amount of DNA (50 ng) from soil samples, Brilliant III UltraFast SYBR® Green QPCR MasterMix, Primers and Ref. Dye (Agilent Technologies, USA) in a reaction volume of 20 µl. Conditions for amplifications included 5 min at 95°C followed by 40 cycles of 10 s at 94°C, 20 s at 62°C and 30 s at 72°C. The critical threshold values and baseline subtracted PCR raw data for each real-time qPCR were exported to MS Excel for further analysis. The actual copy number\s of target amplicon was calculated by relating the *Ct* value to a standard curve and a strong linear correlation (R^2 = 0.992) was obtained from the standard curve (Fig. 1).

2.4 Data Analysis

Data from real-time PCR was analyzed using Mean and Standard error for crop, herbicide and depth as the fixed factors and block as a random factor [22]. Four data points from each experimental plot were averaged and the mean values were used for further calculations by using proc T-test in SAS.

3. RESULTS AND DISCUSSION

The study was focussed to characterize the effects of major herbicides (glyphosate, glufosinate ammonium sulphate and imazethapyr ammonium) on soil microbes by monitoring the fungal population in all treatments at two different soil depths. The soil chemical analysis was done to assess the difference due to pH, soil texture and other parameters in fungal population. Experiments were performed in four replications at each location to overrule the effect of environment and location. Analysis by qPCR and t-test indicated that after 20 days of application, all three herbicides showed varied effects on fungal population as discussed below.

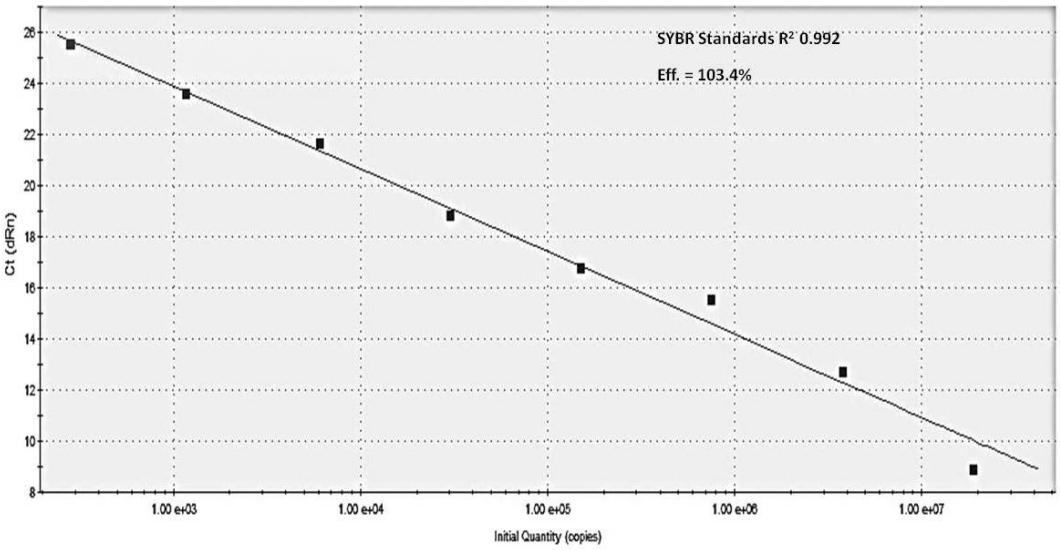

Fig. 1. Standard for fungal copy number

3.1 Effect of Glyphosate

Both Normandin and LODS center soil samples treated with glyphosate showed slightly higher fungal population (Figs. 2a-d). The copy number increased from 2.29×10^6 to 4.26×10^7 in year 2010 and $2.41x10^6$ to $3.39x10^7$ during year 2011 at LODS center at 0-15 cm soil depth. Similarly, samples from SCRDC of Agriculture and Agri food Canada, Normandin, QC also showed slight increase in copy number from $1.53x10^6$ to $3.46x10^7$ in 2010 and $2.25x10^6$ pre-treatment to $4.82x10^7$ post-treatment in 2011 at 0-15 cm soil depth. Although the difference was not statistically significant, a similar trend was observed in all replications of both locations during two growing seasons. It is probable that degraded glyphosate is available to the microbes as a carbon source and these compounds may boost microbial growth rate. This effect was only observed in 0-15 cm soil depth but at depth 15-30 cm, interestingly in 2010 growing season at both locations copy number of fungal population decreases unlike in 2011 growing season. Stimulation in fungal population in the 0-15 cm of soil surface is observed which may be due to the fact that glyphosate binds to top soil firmly and does not leach to deeper layers and fungi are the main microbial degraders of glyphosate.

3.2 Effect of Glufosinate

Effect of glufosinate on fungal populations is shown in Figs. 2 (a-d). In order to identify the effect of glufosinate on fungal population, the samples were collected pre-spray (0 day) and 20 DAT. Soil fungal population increased from 3.55×10^6 to 4.67×10^6 in year 2010 at LODS center and $2.68x10^6$ to $8.29x10^6$ during year 2011 at soil depth of 0-15 cm. Similarly, samples from SCRDC of Agriculture and Agri-food Canada, Normandin, QC also showed slight increase in fungal population from $3.79x10^6$ to $4.65x10^7$ in 2010 and $2.66x10^6$ pre-treatment to $3.13x10^6$ post-treatment in 2011 at soil depth of 0-15 cm. Unlike at soil depth 0-15 cm fungal population decreases at 15-30 cm soil depth from the samples of Normandin site. However these differences were non-significant at both the locations, during two subsequent years in glufosinate treated soil. Thus, concluding that the glufosinate does not affect fungal population.

3.3 Effect of Imazethapyr

The fungal population was reduced at 20 DAT by imazathapyr as compared to the control (0 day) at both the depths in 2010 and 2011 at LODS Centre of McGill University but this difference was non-significant as in Figs. 2 (a-d). However at Normandin site fungal population reduced at 0-15 cm soil depth and increases in 15-30 cm soil depth. The response of soil fungal population to imazethapyr treatment was almost similar on both locations in both years except slight increase in number at 15-30 cm depth at Normandin site, indicated that this herbicide has slight negative effect on fungal population.

Commercialization and widespread cultivation of HT canola has changed management practices in modern agriculture. Widespread use of herbicide resistant cropping systems has raised concerns that are developed over potential impacts of these herbicides on microbial community in soil and subsequent effects on crop productivity. There are concerns that herbicides applied to the plant and soil surface may be transported rapidly to the groundwater [23,24]. HT canola varieties resistant to glyphosate, glufosinate ammonium sulphate and imazethapyr ammonium were grown for two seasons (2010-11) at two different locations (LODS Agronomy center of McGill University, Ste Anne de Bellevue, QC and SCRDC of Agriculture and Agri food Canada, Normandin, QC). The physical and chemical properties of soils from both the locations are shown in Table 1.

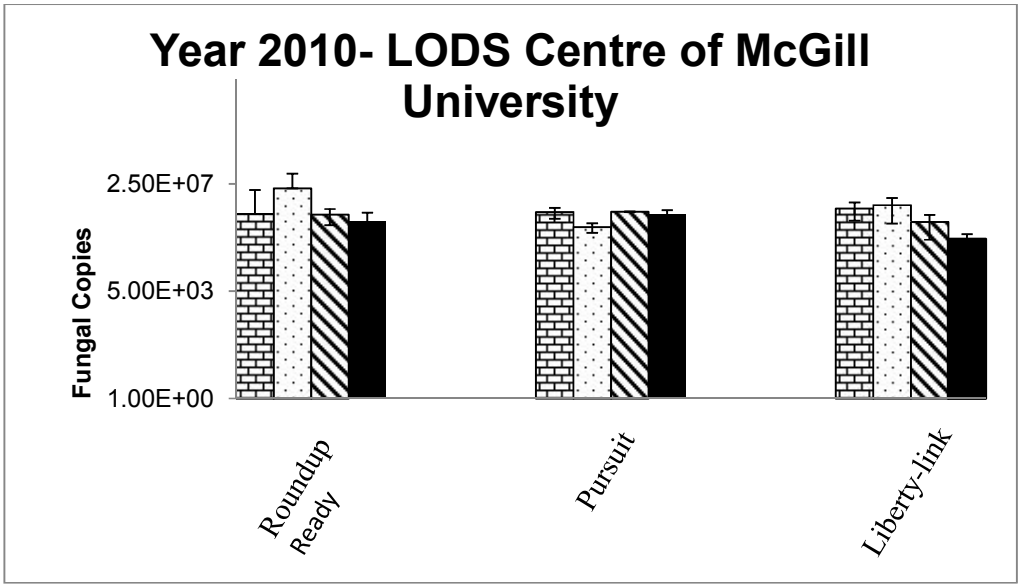

Fig. 2a.

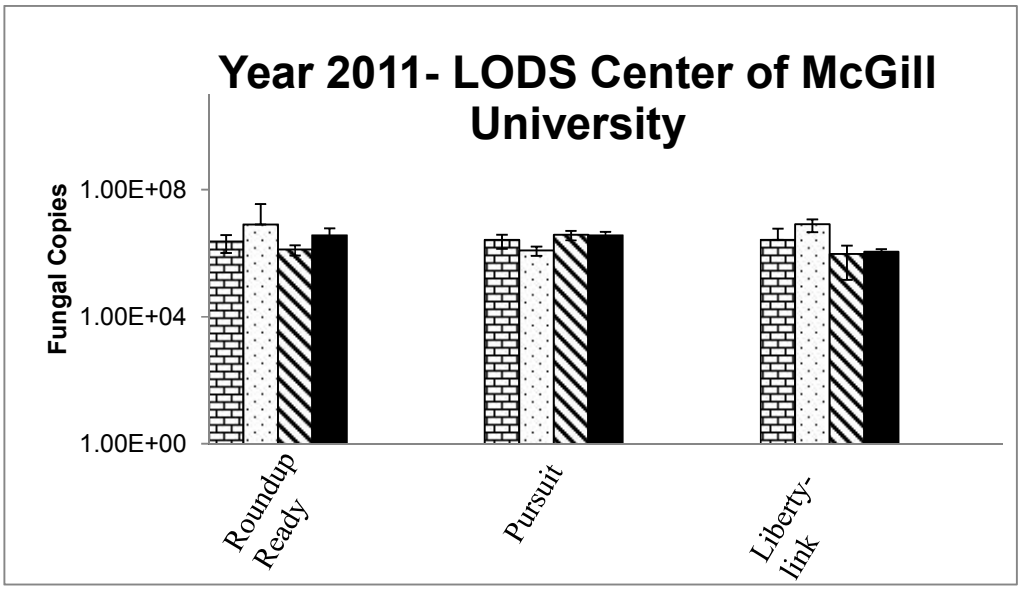

Fig. 2b.

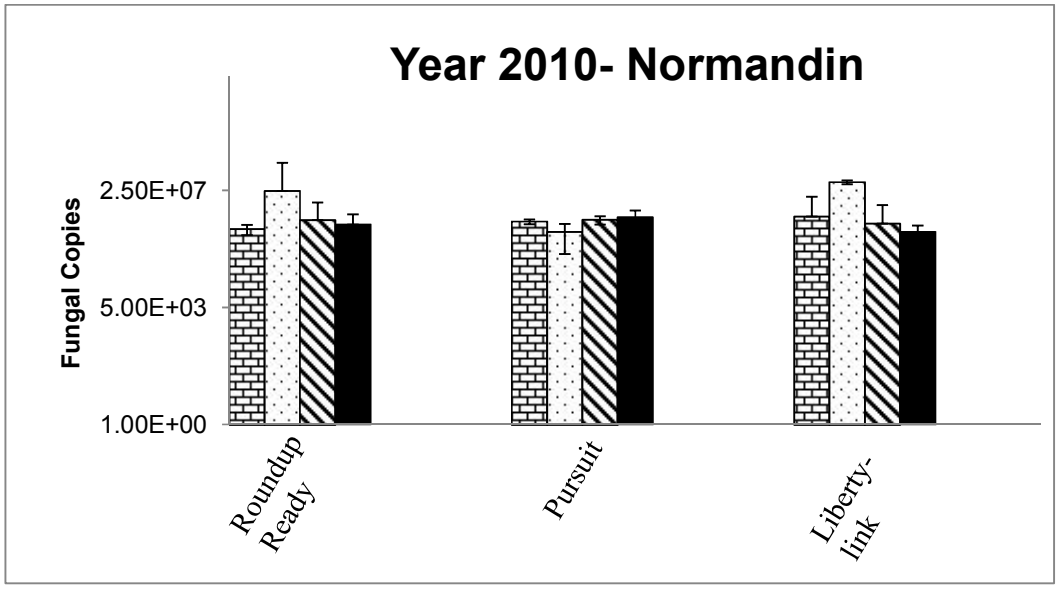

Fig. 2c.

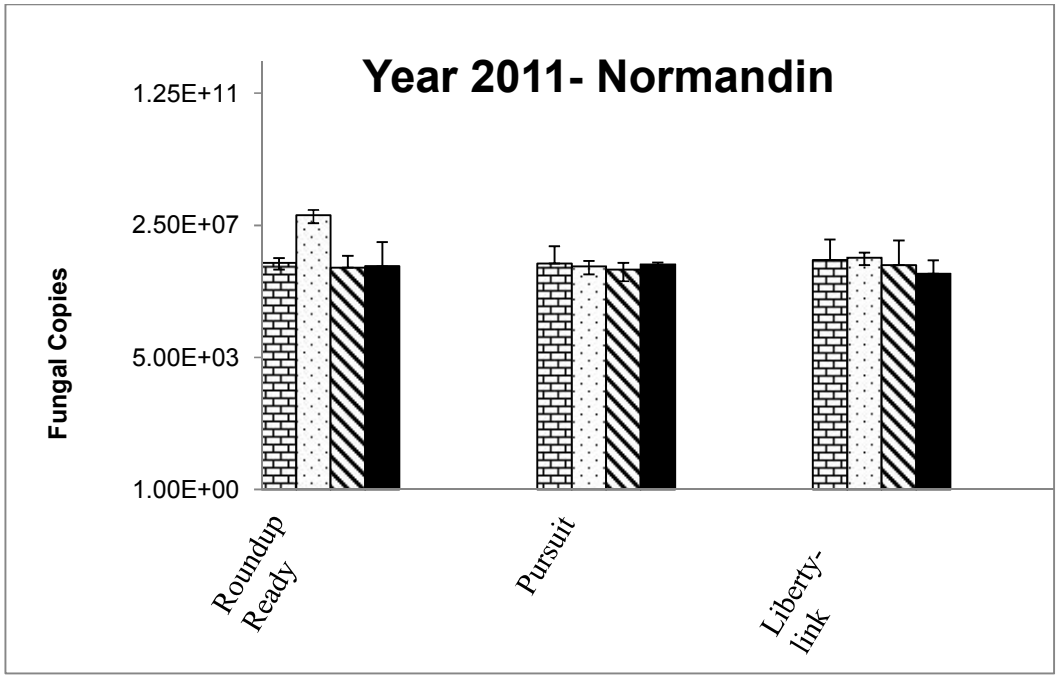

Fig. 2d.

Fig. 2(a-d). Comparison of copy number of fungal population at 0 days control soil sample with soil collected after 20 days of treatment with herbicides, Roundup Ready, Liberty Link and Pursuit Smart. Samples were collected from two depths ranging between 0-15 cm and 15-30 cm. (a) Represents all samples of LODS center in 2010; (b) All samples of LODS center in 2011; (c) All samples from Normandin in 2010; (d) All samples from Normandin in 2011. Data represent mean of four plots, where 2 independent replicates were analyzed in each plot

Table 1. Physical and chemical properties of soils at LODS and Normandin sites

Soil Parameters	LODS Site	Normandin Site
Sand (%)	11	59
Silt (%)	64	33
Clay (%)	25	8
SOM (%)	3.97	4.70
pH	5.19	5.51
P (mg kg^{-1})	80.1	47.8
K (mg kg^{-1})	82	166
Ca (mg kg^{-1})	840	1450
Mg (mg kg^{-1})	100	173
Al (mg kg^{-1})	1260	1310
CEC (cmol kg^{-1})	6.06	9.18

Fluctuation in fungal community analyzed by qPCR indicated that glyphosate increased fungal population in both years at McGill University LODS centre (Figs. 2a-b) and similar effects were observed in soil samples of SCRDC, Agriculture and Agri-food Canada, Normandin, QC (Figs. 2c-d). This effect was only observed in 0-15 cm soil depth but not at depth 15-30 cm. Stimulation in fungal population in the 0-15 cm of soil surface is observed which may be due to the fact that fungi are the main microbial degraders of glyphosate [25]. Degradation of glyphosate in the soil is primarily a biological process, performed by microorganisms [26]. Microbial activity is an essential factor to detect the behavior of glyphosate in the soil. Soils with high microbial activity favor the fast biodegradation of glyphosate [27]. Glyphosate has been found to slightly decrease culturable bacterial population, while fungi and actinomycetes populations were significantly increased [28]. Previous studies have shown that glyphosate use is associated with an increase in *Fusarium* and *Pythium* [27-31]. Glyphosate in root exudates has a dual effect on selected fungi: it serves as a nutrient source, and it may also stimulate propagule germination and early growth [32]. Various reports on pesticide monitoring in groundwater provide information on the occurrence of glyphosate in groundwater. This information varies in nature between detailed reports on concentration levels, soil depth and the number of samples about the occurrence or non-occurrence of glyphosate. Glyphosate exhibited vertical mobility in the treated soils [33]. Because of these concerns we decided to study the effect of herbicides at two depths D1 (0-15 cm) D2 (15-30 cm). We observed that glyphosate has stimulatory effect on fungal population in upper layer (0-15 cm) but this effect is not recorded in at lower depths (15-30 cm). These results may indicate that possibly fungi uses glyphosate as a nutrient and energy source. We also observed that glyphosate traces were not found in lower depth (15-30 cm) in the same field [34]. This persistence of glyphosate in top soil may be due to its fast and complete degradation in soil and surface water prevents movement of residues into groundwater.

Our results demonstrated that application of imazethapyr affect the soil fungi as small reduction in number of organisms was observed. Similar effect was observed from soil samples obtained from SCRDC of Agriculture and Agrifood Canada, Normandin, QC (Figs. 1c-d). Our studies are in agreement with previous finding where adding the herbicide imazethapyr tended to decrease the microbial community [35]. Decreased fungi in the herbicide treated soils may suggest that fungi present in the soil were sensitive to the imazethapyr herbicide [36]. The effect of glufosinate ammonium sulphate was similar to glyphosate at 0-15 cm soil depth (Figs. 1a-d) in all the replicates at both locations over two years. With regard to herbicides, the two-carbon phosphorus bond (C–P–C) of glufosinate is difficult to cleave, and although glufosinate is metabolized in soils, it is not clear if the C–P–C bond is broken [37].

Weak antibacterial activity of glufosinate was reported in previous studies [38,39] however, no significant effects on the microbial activity were observed. In some reports application of glufosinate caused minor shifts in the soil microbial community structures that could be due to the enrichment of microbes by herbicide degradation. Microbial community shifts were observed by 16S rRNA-based population analysis of eubacteria and pseudomonas [40]. Soil acidity may have effect on fungal population as indicated by reports that acidic pH of soil could enhance fungal population [41], however

later studies dismissed this claim and fungal growth was found unaffected by pH [42].

4. CONCLUSION

Herbicide exposure on canola fields at two locations in Quebec indicated small influence on the microbial population. Decrease in fungal population was observed after application of imezathapyr, a herbicide used on non-GM canola fields. However, glyphosate, an herbicide applied in GM canola slightly increased the fungal population. This may be due to its quick degradation, thus became available to microbes as a nutrient source. Glufosinate showed similar effect on soil fungal population, it may be due to the fact that glufosinate also degraded faster as glyphosate. Our studies support the claim that recommended dose of herbicides on GM canola fields has no adverse effect on soil microbes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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