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Genomic analysis of the *Bacillus megaterium* Mes11: new insights into nitroreductase genes associated with the degradation of mesotrione.

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Highlights

The complete genome of the *Bacillus megaterium* Mes11 was sequenced

The strain transformed mesotrione alone or in herbicide mixtures

The NfrA2 nitroreductase played a key physiological role in mesotrione transformation

Abstract

Mesotrione is among the most recently marketed herbicides belonging to the triketone family and applied on maize crops. A *Bacillus megaterium* Mes11 strain isolated from an agricultural soil was able to completely transform 1 mM of mesotrione, alone or in a mixture with 1 mM of nicosulfuron and/or S-metolachlor herbicides, in 5 h. Its complete genome contains one circular chromosome (5,191,612 bp) and nine plasmids (from 45,372 to 169,684 bp). Genome analysis revealed that 10 genes encoded oxygen-insensitive nitroreductases of which only one (NfrA2) was shown to be involved in the first step of mesotrione transformation « *in cellulo* ». Indeed, quantitative reverse-transcription PCR analysis demonstrated that the NfrA2 expression was concomitant with mesotrione transformation, showing a maximum 6-fold increase after a 4 h exposure to this single molecule. As opposed to nicosulfuron, the presence of S-metolachlor led to a significant 12 to 24-fold increase of NfrA2 expression after the same time of exposure, in the binary and ternary mixtures, respectively. These results highlight, for the first time, the physiological role of NfrA2 in mesotrione transformation « *in cellulo* » and the environmental role of *B. megaterium* Mes11 for bioremediation of mesotrione-contaminated ecosystems.

Keywords: *Bacillus megaterium* Mes11; complete genome sequence; herbicide degradation; mixtures; nitroreductases

1. Introduction

Mesotrione (i.e. 2-(4-methylsulfonyl-2-nitrobenzoyl) cyclohexane-1,3-dione) is one of the new generation pesticides, chemically derived from a natural phytotoxin (leptospermone), and now becoming a major commercial herbicide (Casida and Durkin, 2017). Targeting a key enzyme involved in the plant carotenoid biosynthesis, the 4-hydroxyphenylpyruvate dioxygenase, by inhibition, its success was due not only to its high efficiency towards a wide range of broad-leaved and grass weeds, in particular those resistant to other herbicides, but also to its rather low recommended agronomic dose (applied at a rate of 150 g active ingredient ha⁻¹) conforming to the Integrated Pest Management (IPM), aiming to minimize pesticide use. It was also one of the first atrazine replacement herbicides, widely used worldwide (Mitchell et al., 2001). Belonging to the triketone family, mesotrione is a systemic post-emergence herbicide, typically used to protect maize crops.

Mesotrione fate has already been extensively studied in various matrices such as soil, water and crops (Barchanska, 2012; Dumas et al., 2017). Highly soluble, with a K_d value range from 0.13 to 5.0 L.kg⁻¹, this herbicide is classified as relatively mobile and bioavailable in soils, making biotransformation processes quite possible (Barchanska et al., 2016; Chaabane et al., 2008; Dyson et al., 2002; Gámiz et al., 2019).

The chemical mesotrione is distinguished by the presence of a nitro group on the benzoyl moiety. Indeed, a detailed analysis of the structure-activity relationships showed that an electron-withdrawing substituent at the 2-position was required for herbicidal activity, -NO₂ being one of the strongest and mesotrione the most efficient herbicide compared to other triketone derivatives (Beaudegnies et al., 2009; Lee et al., 1998). Nevertheless, this nitro group also constitutes a potential site of attack for its (bio)degradation, by reduction into hydroxylamino and then amino group.

Various bacterial strains able to transform mesotrione have already been isolated (Dumas et al., 2017). Although several mesotrione metabolic pathways have been observed for a few of the isolated strains (Dobrzanski et al., 2018; Olchanheski et al., 2014; Pileggi et al., 2012; Romdhane et al., 2016), they are most frequently well conserved and common to the majority of mesotrione-transforming strains (Dumas et al., 2017). Not surprisingly, the major pathway generally involves reduction of the nitro group of mesotrione into intermediate free or cyclized hydroxylamino derivatives, these being subsequently transformed to the final metabolite, 2-amino-4-methylsulfonylbenzoic acid (AMBA) after the hydrolysis of the β-diketone bond. AMBA then accumulates in the medium and has also been detected in the environment (Alferness and Wiebe, 2002; Dumas et al., 2017).

Type II and type I nitroreductases are the enzymes involved in such transformations of nitroaromatic compounds, such as mesotrione, by reduction *via* one or two-electron transfer, respectively (Bryant et al., 1981; Peterson et al., 1979). The type II nitroreductases, whose activity is oxygen-sensitive, catalyse the one-electron reduction of a substrate, using FMN or FAD as cofactor and forming a nitro anion radical. This radical undergoes futile cycling in the presence of oxygen giving super-oxide anions and regenerating the parent nitro-compound (Mason and Holtzman, 1975). The oxygen-insensitive type I nitroreductases are NAD(P)H-dependent, FMN binding proteins. These enzymes are responsible for catalytic reduction of the nitro group formally by addition of two electrons to give the nitroso intermediate. This, in turn is reduced further to hydroxylamino and amino intermediates accepting pairs of electrons from NAD(P)H (Bryant et al., 1981; Peterson et al., 1979). Therefore, nitroreductases are good candidates for bioremediation of nitroaromatic compounds and several enzymes have been characterized as able to degrade the toxic 2,4,6-trinitrotoluene (TNT) (Gumuscu and Tekinay, 2013; Hannink et al., 2001; Ramya Sree et al., 2019).

A *Bacillus megaterium* Mes11, among all the degrading-strains, was isolated from an agricultural soil and is able to completely and rapidly biotransform mesotrione in aerobic conditions (Batisson et al., 2009). The fact that nitroreductase is involved in this

biotransformation by *B. megaterium* Mes11 was first evidenced by a network analysis of mesotrione-adaptative proteins which emphasizes a direct link with this type of enzyme (Bardot et al., 2015). More recently, Carles et al. (2016) confirmed that two oxygen-insensitive nitroreductases of the *B. megaterium* Mes11 strain, NfrA1 and NfrA2 (Genbank accession nos. KT008921 and KT008916), expressed in a heterologous *E. coli* system and purified, were involved in the first step of mesotrione transformation when exposed to this sole molecule.

To further investigate the role of nitroreductases in mesotrione transformation, a study of their « *in cellulo* » expression was carried out by sequencing and analysing the whole genome of the *Bacillus megaterium* Mes11. We also compiled an inventory of all the nitroreductase-coding genes potentially involved in mesotrione-transformation. Moreover, as mesotrione is usually applied agriculturally in mixture with the herbicides S-metolachlor (chloroacetanilide) and nicosulfuron (sulfonylurea) in order to improve its efficiency and the range of weeds treated, a focus was made on the impact of such mixtures on the nitroreductase expression. We have already shown that when mixed, any of these herbicides could have an impact on their individual toxicity and on the transformation kinetics of the others (Carles et al., 2018; Joly et al., 2013). In this manuscript, the physiological activity of the various nitroreductases found in *B. megaterium* to be involved in mesotrione-transformation was determined under multi-contamination conditions to account for agricultural mixture exposure more realistically.

2. Materials and Methods

2.1. Bacterium, chemicals and media

Bacillus megaterium Mes11 was isolated from soil (Batisson et al., 2009). The analytical grade herbicides (Mesotrione, nicosulfuron, S-metolachlor (Pestanal) and the Tryptic Soy broth (TS) medium were purchased from Sigma Aldrich (France). The composition of Glucose-mineral Salt Medium (GSM), pH 6.7, was previously reported by Carles et al. (2018).

2.2. Whole genome sequencing of *B. megaterium* Mes11 and annotation.

Pac-Bio RS technology (GATC Biotech) was used to sequence the *B. megaterium* Mes11 genome. The draft genome was annotated using RAST version 4.0 (1) and the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP; <http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html>).

The *B. megaterium* Mes11 genome sequences have been deposited in GenBank under accessing numbers: CP048883 to CP048892.

2.3. Expression and purification of the plasmidic nitroreductase in *E. coli*

The PCR amplification was carried out using the primers NRK forward (5'-ATGAATAATACAATTGAATTACTTTCT-3') and NRK reverse (5'-TTTTTTAGCAAACCCTTGTTTGTTTAA-3'). The amplicon was cloned into the expression vector pEXP5-CT/TOPO (Invitrogen). The protein expression and mesotrione biotransformation tests were carried out as described by Carles et al. (2016).

2.4. Culture of *B. megaterium* Mes11 with herbicides

Bacillus megaterium Mes11 was grown in 750 mL-TS broth supplemented with 0.1 mM mesotrione, under agitation (150 rpm) at 28 °C. After an overnight culture, the cells were centrifuged (8,000 g, 5 min, 4 °C) and washed with a solution of NaCl (0.8 %). The pellet was then resuspended in GSM (150 mL) supplemented with 1 mM of mesotrione, nicosulfuron and S-metolachlor, either alone, in binary or ternary mixtures. Abiotic controls (medium without inoculation and inoculation without herbicide) were also realized. For each condition, the cultures were carried out in triplicate and incubated in the dark (28 °C, 150 rpm). Samples (1 mL) were regularly taken and centrifuged (12,500 g, 5 min). HPLC analyses were performed on the supernatants and total RNA was recovered from the corresponding pellets (see below). The herbicide biosorption was measured from a 1 mL culture pellet, stirred vigorously overnight in 500 µL ethanol at room temperature before being centrifuged (12,500 g, 5 min). The extraction process was repeated twice.

2.5. Analysis of the herbicides and their metabolites

The herbicide concentrations were measured by HPLC as described previously by Carles et al. (2018). The mesotrione metabolites were identified and quantified by LC-MS and ¹H NMR, respectively, as previously described by Carles et al. (2018).

2.6. Reverse transcription- quantitative PCR (RT-qPCR) analysis

Total RNA was extracted at T0, 2, 4 and 8 h as described by Dugat-Bony et al. (2011). The DNase treatments (twice) and reverse transcription were performed with the DNaseI kit (Ambion) and the SuperScript™ III Reverse Transcriptase kit (Invitrogen) using Random Primers (Invitrogen), respectively. The qPCR reactions were performed in a total reaction volume of 15 µL containing 7.5 µL of MESA GREEN qPCR MasterMix Plus for SYBR® Assay Low ROX kit (Eurogentec), 0.3 µM of each primer and 1 µL of 0.1 X cDNA. The primers used for *NfrA1* and *NfrA2* nitroreductase genes amplification were reported by Carles et al. (2016). The qPCR was carried out in an Realplex2 egradient S Mastercycler (Eppendorf) as follows: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 57 °C for 15 s and elongation at 72 °C for 20 s. The 16S rRNA gene was used to normalize the results using primers described by Borrel et al. (2012).

2.7. Statistical analysis

R (v3.5.0) and RStudio (v1.1.456) were used for statistical analyses. Differences in *NfrA2* expression fold-change were assessed in triplicate experiments using two-way ANOVA followed by separate post hoc comparisons (Tukey's test, $P < 0.05$). The two-way ANOVA includes the factors treatment (mesotrione; mesotrione + nicosulfuron; mesotrione + S-metolachlor; mesotrione + nicosulfuron + S-metolachlor) and time (0, 2, 4 and 8 h). The normality and homogeneity of variance were checked prior to ANOVA tests (Shapiro's and Levene's tests, respectively, for $P < 0.05$).

3. Results

3.1. Analysis of the *B. megaterium* genome

The PacBio sequencing of the *B. megaterium* Mes11 genome generated ca. 1,15 Gbp of nucleotides and the average subread length was 12,649 bp. A total of 90,915 reads were sequenced with an N50 of ca. 16,970 kb. After the *de novo* assembly, 10 contigs were produced

and the genome size of *B. megaterium* Mes11 was predicted to be 5.2 Mbp. It includes one chromosome and 9 plasmids (pBMG1 to pBMG9). Their general features are summarized in Fig. 1 and Table 1.

In our previous study, *in silico* analysis of the three available *B. megaterium* genomes highlighted 9 chromosome encoded oxygen-insensitive nitroreductase genes also detected in the Mes11 genome (Carles et al., 2016; Genbank accession nos. KT008914 – KT008922). The analysis of the whole genome of *B. megaterium* Mes11 also revealed an additional nitroreductase gene encoded by the pBMG7 plasmid (accession no. BMG_6397).

3.2. Mesotrione transformation by *B. megaterium* Mes11

The *B. megaterium* strain showed similar rapid mesotrione transformation kinetics whether used alone or in binary or ternary mixtures with other herbicides (nicosulfuron and S-metolachlor) (Fig. 2). A complete dissipation of the herbicide was observed within 5 h of culture, whereas none was noted without the strain (abiotic control). Concomitantly to the mesotrione dissipation, two transitory metabolites, the hydroxylamino derivative in equilibrium with its cyclized form isoxazolol, appeared after 2 h of incubation and reached their maximum concentration when there was no mesotrione left (Table 2). They were formed quantitatively by the reduction of the nitro group of mesotrione before being transformed into AMBA. Mass balance analyses showed that no loss of compound occurred during the mesotrione transformation since values ranged from 86 % to 108 % at the end of the experiment (Table 2). This is in agreement with the absence of significant mesotrione and metabolite adsorption observed on bacterial cells (data not shown). The two other herbicides, nicosulfuron and S-metolachlor, were not degraded during the 8 h of incubation monitored under these conditions.

3.3. “*In cellulo*” expression of the mesotrione-transforming nitroreductase

Since the newly-discovered plasmid encoded nitroreductase was unable to transform mesotrione after heterologous expression and purification, reverse transcription - quantitative PCR using the 16S rRNA gene for normalization (Borrel et al., 2012) was focused on the only two nitroreductases previously described to do it *in vitro* (Carles et al., 2016) in order to confirm their function « *in cellulo* » and in various multi-contamination contexts. Their transcription level was determined during mesotrione biodegradation by *Bacillus megaterium* Mes11.

The qPCR results showed no induction of the NfrA1 expression but an induction of NfrA2 by mesotrione in the *B. megaterium* Mes11, whatever the herbicide exposure conditions. No significant induction of its expression was observed when *B. megaterium* was exposed to nicosulfuron alone (data not shown). Conversely, in the presence of mesotrione, alone or with the other herbicides, the induction of NfrA2 expression was observed concomitantly to the dissipation of mesotrione (Fig. 2). In comparison to the control culture (without any herbicide), a NfrA2 expression was detected as soon as T0, showing similar values for all treatments (Table 2). It reached its maximum 4 h after induction before a decrease, probably due to the complete dissipation of mesotrione after 5 h of culture (Fig. 2 and Table 2).

An effect of time (taking all the treatments together), treatments (taking all the times together) and an interaction time*treatment for a given time (ANOVA, $P < 0.001$) was observed on NfrA2 expression level. Thus, compared to the value at T0, the six-fold expression obtained after 4 h of culture in the presence of mesotrione alone is unaffected by the addition of nicosulfuron.

At the same time, when S-metolachlor was present in the mixture, a greatly increased induction was, nevertheless, observed compared to the T0 expression (around 12 and 24 fold for binary and ternary mixtures, respectively), highlighting a two- to four-fold stimulation compared to mesotrione applied alone or with nicosulfuron. A slight stimulation of NfrA2 expression was also observed in the *B. megaterium* strain when the culture was exposed to the

S-metolachlor alone (about 4-fold at T4 h compared to the T0 expression) or in mixture with nicosulfuron (about 2.5 at T8 h compared to the T0 expression) (data not shown). The up-regulation of the *NfrA2* gene observed in the presence of S-metolachlor was synergized by the presence of mesotrione with a 5 fold and 8.5 fold increase of the *NfrA2* expression with binary and ternary herbicide mixtures, respectively.

4. Discussion

The genome analysis of *Bacillus megaterium* Mes11 revealed one circular chromosome and 9 indigenous plasmids (Fig. 1). The high number of plasmids of this strain is within the range of that already described for other *Bacillus megaterium* strains, harboring a highly variable number of plasmids, from zero (DSM319 strain; Stahl et Esser, 1983; Eppinger et al., 2011) to 10 (strain NTC-2; Wang et al., 2020). The GC % of the *B. megaterium* Mes11 plasmids, varying between 33.5 and 35.5 %, was lower than that of the chromosome (38.2 %) (Table 1). This is in agreement with other studies which also revealed a lower GC content for plasmids in several *B. megaterium* strains (Eppinger et al., 2011; Liu et al., 2011; Wang et al., 2020). Among the 4055 genes with predicted functions detected on the chromosome and the 520 genes harbored by the nine plasmids (Table 1), 9 and 1 oxygen-insensitive nitroreductase were detected, respectively. This plasmid-encoded nitroreductase blasted only with *Bacillus* genomes. Unless already detected in the chromosome of several *Bacillus* strains (e.g. *B. subtilis* (McIntyre et al., 2019), *B. intestinalis* (Xu et al., 2015), *B. pumilus* (Zhang et al., 2019)), this nitroreductase was only recently detected in the chromosome of a *B. megaterium* strain (strain Ni2-3; Cai et al., 2019) and on a plasmid of *B. megaterium* strain KNU-01 (Jo et al., unpublished).

Carles et al. (2016) tested the capacity of these nitroreductases to transform mesotrione *in vitro* under a wide range of experimental conditions (pH, temperature, types of cofactor, concentration of the cofactor). This study showed that after heterologous expression and purification of the 9 chromosomal-encoded ones, only two of them (*NfrA1* and *NfrA2*) were involved in the transformation of mesotrione. The plasmid encoded nitroreductase now highlighted by the analysis of the complete *B. megaterium* Mes11 genome was also tested for its capacity to transform mesotrione but not seen to do so for the various *in vitro* experimental conditions tested. Thus, among the 10 nitroreductases found in the genome of *B. megaterium* Mes11, we were only able to show the mesotrione-transformation capacity of the two chromosomal-encoded enzymes *NfrA1* and *NfrA2* *in vitro* in our experimental conditions.

Moreover, comparing between these two nitroreductases, we showed that *NfrA1* was not involved in the first step of mesotrione transformation “*in cellulo*” and that only *NfrA2* played a key physiological role in *B. megaterium* Mes11. The mesotrione transformation kinetics by this strain and that of metabolite production (Table 2) were similar to those previously observed with purified nitroreductases (Carles et al., 2016).

Furthermore, a previous study has demonstrated the mesotrione transformation capacity of the *B. megaterium* Mes11 strain in a multi-contamination context (Carles et al., 2018). Our results corroborated this capacity “*in cellulo*”. Indeed, we showed that the *NfrA2* expression was strongly increased during the mesotrione transformation and even more in the presence of S-metolachlor coupled or not with nicosulfuron (Fig. 2 and Table 2). The 2 to 4-fold *NfrA2* overexpression when S-metolachlor is present compared to mesotrione alone could not only be attributed to its involvement in the mesotrione degradation since the mesotrione transformation kinetics was similar in all conditions tested. We could thus stipulate that the stimulation of the *NfrA2* expression by S-metolachlor could also be explained by the induction of oxidative stress caused by this molecule which leads to its toxic effect for cells including lipid peroxidation and the excessive production of reactive oxygen species (ROS) (Martins et al., 2011; Nykiel-Szymańska et al., 2019; Machado and Soares, 2021). Defense mechanisms must be set up to protect the cells from oxidative stress. These responses involve specific enzymes such as

disulfide reductase, superoxide dismutase, catalase or glutathione peroxidase. Nitroreductases were shown to also harbour these enzymatic activities (Bang et al., 2012; de Oliveira et al., 2009; Liochev et al., 1999; Mermoud et al., 2010; Paterson et al., 2002; Streker et al., 2005; Tam et al., 2006), thus contributing to the cell defense by an up-regulation of the corresponding genes (Mostertz, 2004; Roldán et al., 2008). The higher NfrA2 expression in presence of the three herbicides studied could be explained by the synergistic action of the pesticides (Cedergreen, 2014; Carles et al., 2018). To our knowledge, this is the first time that the involvement of a nitroreductase has been demonstrated “*in cellulo*” during the degradation of a pesticide, also revealing the influence of other molecules on the nitroreductase physiological activity in a multi-contamination context.

Hence, our results provide new insight into the mesotrione transformation by *Bacillus megaterium* Mes11 strain and highlighted the key physiological role of the NfrA2 enzyme in this process. Nitroreductases being involved in reduction of various nitro-aromatic compounds such as pesticides and antibiotics (Caballero et al., 2005; Symons and Bruce, 2006; Roldán et al., 2008; Carles et al., 2016; El-Hossary et al., 2017; Green et al., 2019), these enzymes (or their genes) could be used as biomarkers to predict the capacity of ecosystems to transform such molecules.

5. Conclusion

In this study, we showed, for the first time, that the NfrA2 nitroreductase of *Bacillus megaterium* Mes11 played a key physiological role in the mesotrione transformation and that it is still active in a realistic agricultural context of herbicide mixture exposure or even overexpressed. The *Bacillus megaterium* Mes11 could thus be used for mesotrione bioremediation and its active nitroreductase could have potential biotechnological applications in the environmental domain.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure captions:

Fig. 1. Circular genome map of *Bacillus megaterium* Mes11. From the innermost to the outermost circle : the inner black circle shows the scale line in Mbps ; circles (1) and (2) represent the G + C content and G + C skew; circles (3) and (4) represent the predicted coding sequences on the forward and backward strands, colored by the COG functional categories.

Fig. 2. Biotransformation kinetics of mesotrione, alone or in mixtures, by *B. megaterium* Mes11 and NfrA2 expression fold-change compared to a control culture without mesotrione. The values are the mean $\pm$ standard errors (n = 3) of experimental data. Statistical differences were indicated between the four conditions at each time by lowercase letters (Tukey's test; a < b, P < 0.05) and between each time and T0 (ANOVA, * P < 0.001). Meso: mesotrione; Nico: nicosulfuron; S-meto: S-metolachlor.

Table 1: General features of *Bacillus megaterium* Mes11 genome.

Table 2: Concentration of metabolites produced during the mesotrione (1 mM) degradation by *B. megaterium* Mes11 and fold change in NrfA2 expression. Values are expressed as means (n = 3) $\pm$ standard error.

666 Figure 2

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669 **Table 1:** General features of *Bacillus megaterium* Mes11 genome.

Features	Genomic size (bp)	CG content (%)	Total genes	Genes with predicted functions	tRNA genes	rRNA operon
Chromosome	5,191,612	38.2	5458	4055	121	11
pBMG1	169,684	34.2	162	104	2	-
pBMG2	122,563	33.7	83	32	-	-
pBMG3	157,203	33.8	175	100	5	-
pBMG4	139,476	33.6	128	64	-	-
pBMG5	87,511	35.1	100	37	-	-
pBMG6	105,079	33.7	109	63	-	-
pBMG7	75,956	33.5	72	42	-	-
pBMG8	70,948	35.1	91	47	17	1
pBMG9	45,372	35.5	66	31	18	1

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Table 2: Concentration of metabolites produced during the mesotrione (1 mM) degradation by *B. megaterium* Mes11 and fold change in NrfA2 expression. Values are expressed as means (n = 3) ± standard error.

Treatment	Mesotrione (mM)	Hydroxylamino derivative (mM)	Isoxazolol (mM)	AMBA (mM)	Mass balance (% initial dose)	NrfA2 fold change (compared to control ^a)
T0						
Meso	0.98 ± 0.02	0	0	0	100%	1.44 ± 0.34
Meso + Nico	0.95 ± 0.01	0	0	0	100%	1.77 ± 0.78
Meso + S-meto	0.96 ± 0.01	0	0	0	100%	1.46 ± 0.69
Meso + Nico + S-meto	0.96 ± 0.01	0	0	0	100%	1.30 ± 0.38
T2						
Meso	0.63 ± 0.05	0.16 ± 0.02	0.14 ± 0.03	0	95%	2.68 ± 1.35
Meso + Nico	0.56 ± 0.02	0.13 ± 0.01	0.11 ± 0.02	0.02 ± 0.02	86%	4.02 ± 1.02
Meso + S-meto	0.66 ± 0.02	0.10 ± 0.01	0.09 ± 0.01	0	89%	14.69 ± 0.49
Meso + Nico + S-meto	0.64 ± 0.01	0.10 ± 0.00	0.07 ± 0.01	0.02 ± 0.02	86%	23 ± 6.35
T4						
Meso	0.09 ± 0.00	0.29 ± 0.01	0.41 ± 0.10	0.12 ± 0.02	93%	8.64 ± 2.32
Meso + Nico	0.09 ± 0.01	0.24 ± 0.02	0.38 ± 0.02	0.17 ± 0.02	92%	9.84 ± 2.32
Meso + S-meto	0.09 ± 0.01	0.22 ± 0.01	0.37 ± 0.02	0.20 ± 0.02	92%	18.63 ± 3.78
Meso + Nico + S-meto	0.10 ± 0.00	0.19 ± 0.00	0.34 ± 0.01	0.22 ± 0.01	89%	32 ± 1.75
T8						
Meso	0	0.33 ± 0.01	0.53 ± 0.01	0.17 ± 0.01	105%	5.71 ± 0.19
Meso + Nico	0	0.32 ± 0.01	0.49 ± 0.00	0.19 ± 0.05	105%	6.58 ± 0.53
Meso + S-meto	0	0.25 ± 0.00	0.54 ± 0.01	0.25 ± 0.01	108%	10.36 ± 1.56
Meso + Nico + S-meto	0	0.25 ± 0.00	0.53 ± 0.00	0.26 ± 0.01	108%	7.24 ± 0.86

Meso: mesotrione; Nico: nicosulfuron; S-meto: S-metolachlor.

^a: the culture control was realized in GSM without herbicide.