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# Physiological and leaf ultrastructural characteristics of perennial ryegrass (*Lolium perenne* L.) biotypes from Tunisia under sulfonylurea herbicide application

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ABSTRACT

During the last decade, reduced efficacy and failure to control perennial ryegrass (*Lolium perenne* L.) with labeled rates of Acetolactate Synthase (ALS) inhibitor herbicide (MSM-IDS) were reported. In this context, the effects of MSM-IDS (Sulfonylurea) on suspected resistant (Mateur) and susceptible (Tinja) ryegrass biotypes, prospected in Tunisian wheat fields, were investigated in comparison with those of Pinoxaden, an Acetyl CoA Carboxylase (ACCase) inhibitor herbicide, based on physiological and ultrastructural studies. At their labeled rates, both herbicides significantly affected biomass and gas exchange of Tinja biotype. In Mateur biotype, similar responses as control were observed after ALS inhibitor application. At the molecular level, the most common mutations (P197 and W574) of the ALS gene were not identified in Mateur biotype. Microscopically, a marked collapse of Tinja biotype mesophyll cells was observed by Scanning Electron Microscopy (SEM) after both herbicide applications. However, Mateur biotype mesophyll surface exhibited abundant epicuticular wax deposits and was not affected by ALS inhibitor herbicide. By Transmission Electron Microscopy (TEM), cuticle in Mateur biotype presented a continuous thick lamellate region, while in Tinja biotype, the lamellate region was discontinuous indicating an easy diffusion of MSM-IDS through the cuticle. MSM-IDS resistant Tunisian Mateur ryegrass biotype seems to have developed efficient micromorphological and ultrastructural barriers against sulfonylurea absorption, which in turn allowed a photosynthetic capacity similar to control.



## 1. Introduction

Modern agriculture relies on a massive use of herbicides. Often they are the most effective and least expensive tool in weeds control (Powles and Shaner, 2001). One of the negative sides of this efficient tool is the appearance of resistant weeds, mainly after the introduction of selective herbicides with specific metabolic targets (Heap, 1997) such as ALS inhibitors. Herbicide resistant weeds (144 dicots and 103 monocots) have evolved resistance to 22 of the 25 known herbicide sites of action and to 157 different herbicides and have been reported in 86 crops in 66 countries worldwide (www.weedscience.org 2015). Among resistant biotypes one of the largest and most important group are the monocots resistant to graminicides, especially *Alopecurus myosuroides* L., *Avena fatua* L. and *Lolium rigidum* Goud. (Saja et al., 2013).

In Tunisia, cereal cultivation represents a strategic choice for the country's economy. Indeed, cereals cover 1.5 million hectares, of which, 70% is wheat. However, the expected returns were never achieved. Weeds represent one of the major constraints limiting wheat production (Aubry et al., 1994), due to the emergence of resistance following the massive use of specific herbicides. Reliance on a single herbicide mode of action in wheat has been associated with most cases of resistance (Saari et al., 1994). The prevalence of no-tillage in conservation agriculture, particularly in N/W wheat producing regions, allowed the emergence of several perennial and weak seed dormancy species, especially where the same herbicides were repetitively applied by farmers. Chauhan et al. (2012) reported in this context that perennial weeds may become more challenging in the absence of tillage. This consequently led to herbicide treatment failures of several weed species such as ryegrass (*Lolium sp.*), which seemed to develop resistance to ACCase and ALS

*Abbreviations:* MSM-IDS, mesosulfuron methyl-iodosulfuron; ALS, acetolactate synthase; ACCase, acetyl-CoA carboxylase; EH, equivalent humidity; $P_N$, net assimilation of $CO_2$; Ci, internal $CO_2$ concentration; gs, stomatal conductance; E, transpiration; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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inhibitors as reported by farmers, especially in N/W wheat fields. The main objectives of this study were as follows:

1 To compare the responses of two perennial ryegrass (*Lolium perenne*) biotypes to the most commonly applied herbicides (ALS and ACCase inhibitors) in Tunisian wheat fields at the physiological, molecular and ultrastructural levels
2 To investigate the morphological barriers involved in the resistance in the ALS inhibitor-resistant biotype.

## 2. Materials and methods

### 2.1. Plant material and experimental conditions

Perennial ryegrass (*L. perenne* L.) seeds were collected in June 2012 from two wheat fields in N/W Tunisia (sub-humid bioclimatic range), referred to as Tinja and Mateur, where unsatisfactory ryegrass control was reported following application of ALS inhibitors from the sulfonylurea herbicide family. Within both ryegrass biotypes, Tinja was presumably susceptible while Mateur was suspected as resistant. Seeds representing both biotypes were soaked in a $KNO_3$ (20 mM) solution in petri dishes for a one-week stratification period at 4 °C under darkness. They were then incubated in a controlled room at 18–22 °C (night/day) under a 16 h photoperiod and 70% EH till germination. Fifteen germinated seeds were consequently transferred to 20 cm diameter pots containing a natural sand-clay soil, regularly irrigated (once a week) to field capacity and grown under controlled greenhouse conditions (T:18–22 °C, EH: 60%). At 2–3 leaf stage, the Tinja and Mateur ryegrass biotypes were sprayed with two different herbicides at their respective label rates (Table 1), using a backpack sprayer equipped with a Tee-Jet 800 L flat-fan nozzle delivering 200 L $ha^{-1}$. An ALS inhibitor (MSM-IDS) and ACCase inhibitor (Pinoxaden) herbicide was applied at the 2–4 leaf growth stage for each rigid ryegrass biotype and untreated checks were included. At 2 and 3 weeks after treatments, plants from both ryegrass biotypes were evaluated for visual injury symptoms.

Each ryegrass biotype was represented by 105 plants distributed over 7 pots (15 plants per pot per biotype). Each herbicide was applied over three pots while 15 plants were used as control without any herbicide application.

### 2.2. Biomass and leaf area

Three weeks after herbicide application, control and treated plants from each ryegrass biotype were harvested. Shoots and roots were immediately and individually weighed for fresh weight determination using a precision balance (*Mettler Type PJ600*). Leaf area per plant was then measured using a portable leaf area meter (*LI-COR P LI-3000A*). Shoots and roots were then oven-dried at 60 °C for 7 days to obtain the dry weights. Biomass measurements were monitored for 15 control and 45 herbicide treated plants per biotype.

Data are expressed as percentages of the untreated control to standardize comparisons between both biotypes. Treatments were arranged in a randomized complete block with three replications.

### 2.3. Leaf gas exchange

Net assimilation of $CO_2$ ($P_N$), ($\mu mol\, m^{-2}\, s^{-1}$), stomatal conductance (gs) ($mol\, m^{-2}\, s^{-1}$), transpiration (E) ($mmol\, m^{-2}\, s^{-1}$) and internal $CO_2$ concentration (Ci) ($\mu mol\, m^{-2}\, s^{-1}$) of single leaves were measured at the end of the experiment with a portable photosynthesis system *LI-COR 6200* (Li-Cor, Lincoln, NE, USA) equipped with a 250 $cm^3$-cuvette. Three mature leaves from five control and herbicide-sprayed plants per biotype were evaluated. All measurements were made from 10:30 to 11:30 h.

### 2.4. Molecular study

Individual leaf samples were collected from plants of Mateur ryegrass biotype that survived the ALS-inhibitor herbicide treatment after greenhouse pot assays and were investigated for target site resistances using molecular detection tools. The assays targeting ALS were based on the derived cleaved polymorphic amplified sequence d (CAPS) technique (Neff et al., 1998; Délye et al., 2002). DNA was extracted as described by Délye et al. (2002) and kept at −20 °C prior to sequencing or genotyping. PCR mixes and cycling programs were as described by Délye et al. (2002). Primers used in dCAPS analysis were (1) RG574F and ALV7R for target codon Trp574 and (2) RG197F and RG197R for target codon Pro197. Digestions were performed at 37 °C during 3 h using 1 μL of the PCR mixes and 5 U enzyme (*Fermentas,* Vilnius, Lithuania), 1.5 μL enzyme buffer and 7 μL water. dCAPS patterns were visualised by electrophoresis on 3% agarose gels.

### 2.5. Electron microscopy

#### 2.5.1. Environmental scanning electron microscopy (ESEM)

Fresh leaf samples (5 × 5 mm) were excised from the mid-laminar region of both control and herbicide-treated plants of both ryegrass biotypes and immediately mounted on aluminum stubs with a double-sided adhesive tape and examined using an Environmental Scanning Electron microscope (*QUANTA 200 FEI*), suitable for biological material observation. Secondary electron images were taken at an acceleration voltage of (12.5–30 kV) and a vapor pressure of 5.99 Torr (Ben Salem-Fnayou et al., 2011). Observations focused on the epidermis surface details.

#### 2.5.2. Transmission electron microscopy (TEM)

Several leaf blade samples (2 × 2 $mm^2$) were excised from the mid-laminar region of ALS inhibitor herbicide-treated plants of both ryegrass biotypes using a razor blade. The samples were fixed for 2 h in a mixture of glutaraldehyde, cacodylate buffer (pH 7.4), and distilled water (1:4:3) then rinsed in the cacodylate buffer, postfixed in osmium tetroxyde (1%) for 2 h, dehydrated through a gradient of ethanol series (70, 95, and 100), and finally embedded in *Epon* resin. After polymerization, the blocks were first cut into 1 μm semi-thin then 70 nm ultra-thin sections using an ultramicrotome (*Reichert-Jung Ultracute*). After staining the sections with uranyl acetate and lead citrate, cell ultrastructure was observed and photographed using a transmission electron microscope (JEOL-JEM 1010) operated at 60 Kv (Ben Salem-Fnayou et al., 2011). TEM observations focused on outer epidermis layer ultrastructural details.

### 2.6. Statistical analysis

Data were analyzed using STATISTICA software 2012. Mean comparisons were done based on ANOVA post-hoc test: the Duncan's multiple range test at $\alpha = 5\%$ significance level.

## 3. Results

### 3.1. Symptoms and visual rating

When sprayed with ACCase inhibitor herbicide (Pinoxaden), the new leaves of the Tinja ryegrass biotype turned pale or yellow within 10 days; a reddish-blue pigmentation was also observed on the stem sheath prior to desiccation during the 4th week, displaying 100% injury. In the same biotype, the ALS inhibitor herbicide