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Effects of the herbicides acetochlor and metolachlor on antioxidant enzymes in soil bacteria

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ABSTRACT

The aim of this study was to investigate the antioxidant responses of three bacteria (SD1, KD and K9) isolated from soil previously treated with the herbicides metolachlor and acetochlor. By 16S rRNA gene sequencing, we determined that SD1 is phylogenetically related to *Enterobacter asburiae*, while KD and K9 have divergent genomes that more closely resemble that of *Enterobacter amnigenus*. Decreased levels of lipid peroxidation were observed in SD1 and KD following treatment with 34 mM metolachlor or 62 mM acetochlor, respectively, indicating that both bacteria were able to adapt to an increase in ROS production. In the presence of 34 mM metolachlor or 62 mM acetochlor, all bacterial isolates exhibited increases in total catalase (CAT) activity (81% for SD1, 53% for KD and 59% for K9), whereas total SOD activity (assessed based on the profile and intensity of the bands) was slightly reduced when the bacteria were exposed to high concentrations of the herbicides (340 mM metolachlor or 620 mM acetochlor). This effect was due to a specific reduction in SOD IV (K9 and KD isolates) by 45% and 90%, respectively, and SOD V (SD1 isolate) isoenzymes by 60%. The most striking result was obtained in the SD1 isolate, where two novel isoenzymes of glutathione reductase (GR) that responded specifically to metolachlor were identified. In addition, acetochlor was shown to induce the expression of a new 57 kDa protein band in the K9 and KD isolates. The bacteria isolated from the herbicide-contaminated soil exhibited an efficient antioxidant system response at herbicide concentrations of up to 34 mM metolachlor or 62 mM acetochlor. These data suggest a mechanism for tolerance that may include the control of an imbalance in ROS production versus scavenging. The data suggest that specific isoenzymes of CAT and GR could be involved in this herbicide tolerance mechanism.

1. Introduction

Changes in agriculture practices in recent years have resulted in a substantial increase in the worldwide use of pesticides in agriculture [1]. Pesticide contamination of surface and ground water has been well documented [2–4], although an effective way to eliminate environmental contaminants has not yet been found. However, some significant progress in soil bioremediation technology, including microbe-induced, chemical reductive and oxidative technologies, has been made in recent years [5].

The selection pressure applied by herbicides stimulates the soil bacterial community to adjust rapidly to this environmental contamination [6]. Tolerance can be achieved if bacterial metabolism and cellular integrity are maintained by balancing the redox state of the cell [7]. Reactive oxygen species (ROS) are normally produced in the cells of all aerobic organisms; thus, there is a need for mechanisms to protect the cell against the toxic effects of ROS [8–10]. The defense responses to excessive ROS production involve several strategies, including increases in the rates of synthesis of nonenzymatic antioxidants (e.g., reduced glutathione (GSH) and ascorbic acid) and increases in the activity of specific enzymes that are able to metabolize ROS [11]. Gram-negative bacteria such as *Escherichia coli* possess a specific peroxide defense mechanism, which is mediated by the transcriptional activator OxyR, and a superoxide defense mechanism, which is controlled by the two-stage SoxRS system [9]. Enzymes such as superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, 1.11.1.6) play a major role in these protective processes [8]. In addition, the enzyme glutathione reductase (GR, EC 1.6.4.2) has an important function in the activation of the OxyR system and in main-

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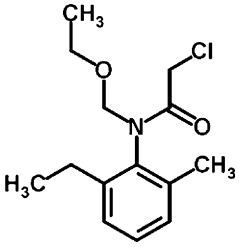
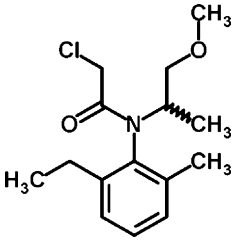
Table 1
Characteristics of the herbicides acetochlor and metolachlor.

	Acetochlor	Metolachlor
Manufacturer	Monsanto	Syngenta
Agrochemical formulation	840 g a.i. L^{-1}, EC	960 g a.i. L^{-1}, EC
Molecular formula	$C_{14}H_{20}ClNO_2$	$C_{15}H_{22}ClNO_2$
Molecular weight	269.77	283.80
Chemical structure		
Vapor pressure	3.4×10^{-8} mm Hg at 25 °C	3.1×10^{-5} mm Hg at 25 °C
K_{ow}	300	794
Water solubility	233 mg L^{-1} at 25 °C	480 mg L^{-1} at 25 °C

a.i., active ingredient; EC, emulsifiable concentrate; K_{ow}, octanol–water partition constant.

taining the levels of GSH, a compound that is key for stress tolerance [12].

Recent studies have focused on the bacterial antioxidant response to soil contamination, which is important for bioremediation [13]. For example, Lü et al. [14] compared the quinclorac-degrading bacteria *Stenotrophomonas maltophilia* WZ2 with *Escherichia coli* K12 and showed that more detailed information on antioxidant properties can be useful for developing strategies for herbicide bioremediation. Other classes of herbicides, such as bipyridyliums and synthetic auxins, can also induce oxidative stress due to blockade of electron flow through the electron transport chain and can directly or indirectly affect the structure and function of membranes [14,15].

In this study, we tested the effects of two chloroacetanilide herbicides, acetochlor and metolachlor, on soil bacteria, paying special attention to antioxidant responses. Both are selective pre-emergence herbicides, which inhibit the elongation of C18 and C16 fatty acids species to form very long-chain fatty acids (VLCFAs) [16]. The persistence of chloroacetanilide herbicides in soil and their tendency to accumulate in the environment have been documented [3,4]. The herbicides tested in this study have been extensively used in agriculture for the cultivation of a wide range of important crops such as soybean, sugarcane, maize and cotton [2–4].

2. Materials and methods

2.1. Herbicides

The relevant characteristics of the herbicides acetochlor (2-chloro-*N*-ethoxymethyl-6′-ethylacet-*o*-toluidide) and S-metolachlor (2-chloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl)acet-*o*-toluidide) are listed in Table 1 [17]. Acetochlor (trade name Kadett) can be applied at 3 L ha^{-1} (2.52 kg ha^{-1}) to control annual grasses and certain annual broad-leaved weeds, was used at 840 g L^{-1}. S-metolachlor (trade name Dual Gold) can be applied at the recommended dose of 2 L ha^{-1} (1.92 kg ha^{-1}) for the cultivation of soybean, cotton, sugarcane and maize crops, was used at 960 g L^{-1}.

2.2. Bacterial isolates and growth conditions

The bacteria used in this work were isolated from two soil samples collected from Capão da Onça School Farm (Fescon) (50°03′W; 25°05′S; average altitude approximately 1000 m) in the district of Ponta Grossa, Paraná State, Brazil. The soils were classified as Oxisol [18] of medium texture and had a history of acetochlor and metolachlor applications for two consecutive years.

The bacterial isolation was carried out using the plating technique with serial dilution in 0.85% NaCl at concentrations of 10^{-3} and 10^{-5} bacteria inoculated in nutrient agar (Biobrás – Brazil), which contained 5 g peptone, 3 g yeast extract and 15 g agar per liter distilled water (pH 7.0), at 30 °C in the absence or presence of the herbicides. The concentrations of 62 mM acetochlor and 34 mM metolachlor were used based on the recommendations on the spray tank solution for each herbicide (12.6 g L^{-1} for acetochlor and 9.6 g L^{-1} for metolachlor).

The following tolerant bacterial isolates were selected for this study: K9 and KD were taken from soil with a history of acetochlor applications, and the isolate SD1 was taken from soil where metolachlor had been applied. These bacteria exhibited faster growth rates in the presence of the herbicides, and halo formation was observed around the bacterial colony [19], indicating possible herbicide degradation, as noted by Alley and Brown [20].

Selected isolates were identified and subjected to *in vitro* growth tests in the presence of two concentrations of the herbicides, one according to the recommendation for the preparation of the spray tank solution and herbicide application (which is the concentration to which the bacteria developed tolerance) and one that was 10 times higher.

The bacteria were grown aerobically in nutrient agar at 30 °C; acetochlor was added to the K9 and KD cultures at 0 mM, 62 mM and 620 mM, while metolachlor was added at 0 mM, 34 mM and 340 mM to the SD1 culture. The cell extracts were prepared and the assays were performed during the exponential phase (after 12 h of growth) for all treatments.

2.3. Bacterial identification

Bacterial DNA was extracted as previously described by Araújo et al. [21], and a partial sequence of the 16S rRNA gene was amplified with the primers R1387 [22] and P027F [23]. The PCR products were purified and sequenced with primer R1387 for 16S rRNA (MegaBACE 1000). The sequences of three bacterial isolates were retrieved from databases and used for alignment and phylogeny analysis [24,25] with the MEGA 4.0 software package [26] based on the maximum parsimony. The sequences obtained have been deposited in GenBank® under the accession numbers HM229435, HM229434 and HM229436 for the isolates K9, KD and SD1, respectively.

2.4. Growth determination

Bacterial growth was monitored by measuring the colony-forming units mL^{-1}, as described by Sangali and Brandelli [27]. Cultures inoculated with 0.1% of the original (λ = 1.4 at 600 nm) were grown in 250 mL Erlenmeyer flasks containing 50 mL of nutrient medium and incubated in the dark on a rotary shaker (140 rpm) at 30 °C for 12 h. The bacterial suspension was diluted to 10^{-6} in a physiological solution containing 0.85% NaCl and then homogenized. Samples (20 μL) were loaded in triplicate onto nutrient agar plates, which were incubated at 30 °C for 24 h for subsequent counting.

2.5. Lipid peroxidation

Lipid peroxidation was determined by estimating the content of thiobarbituric acid reactive substance as described by Heath and Packer [28]. The malondialdehyde (MDA) concentration was monitored at 535 and 600 nm with a Perkin Elmer Lambda 40 spectrophotometer and the MDA concentration calculated using an extinction coefficient of 155 mM cm^{-1}.

2.6. Enzyme extraction and protein determination

The cultures were centrifuged at 10,000 rpm for 20 min at 4 °C, and the pellets were macerated with liquid nitrogen. The extracts were homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol

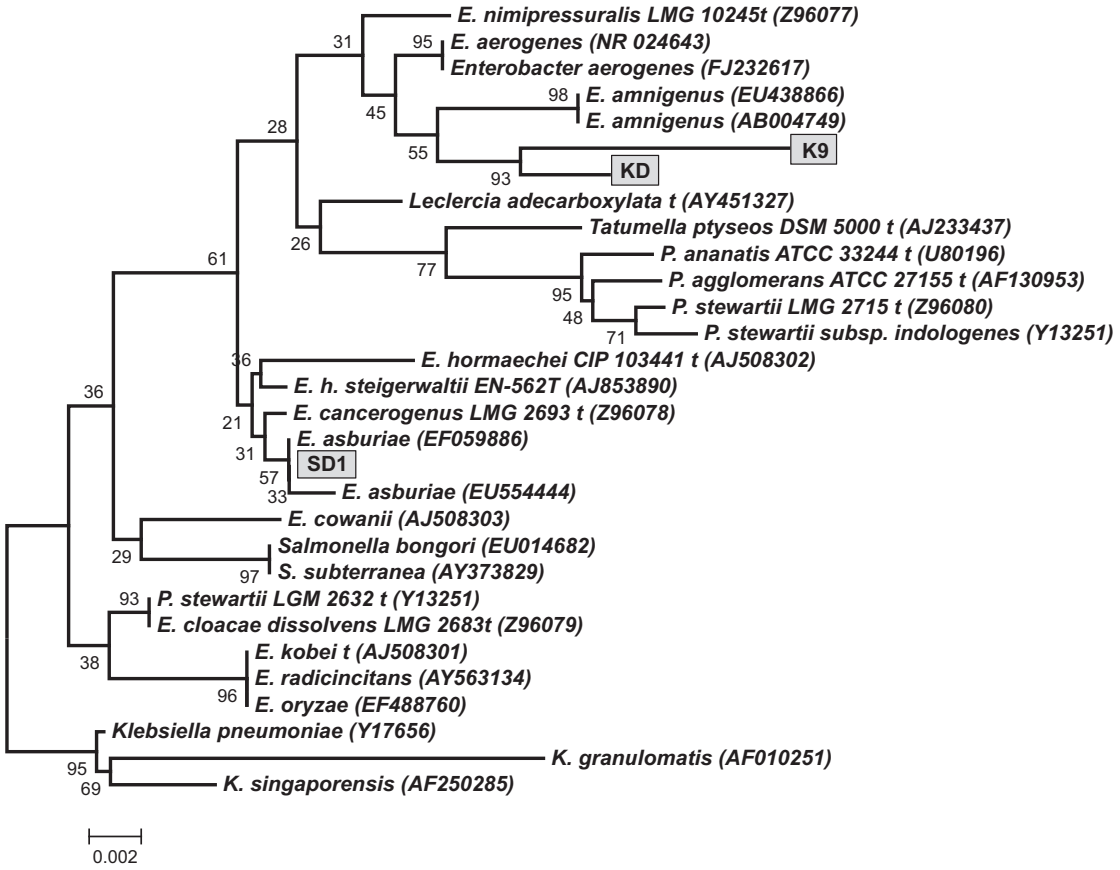


Fig. 1. Maximum-parsimony phylogenetic tree constructed from the 16S rRNA gene. Sequences of 600 bp fragments of the genomes of *Enterobacter* spp. (available at the RDP database) versus *Klebsiella pneumoniae*, *K. granulomatis* and *K. singaporensis* (used as the outgroup). Bars indicate the number of evolutionary steps with diverging sequences. The isolates K9, KD and SD1 are shown inside the boxes.

and 5% (w/w) polyvinylpolypyrrolidone [29]. The homogenates were centrifuged at 10,000 rpm for 30 min, and the supernatants were stored in separate aliquots at −80 °C for further biochemical analyses. Protein concentration was determined by the Bradford method [30] using bovine serum albumin as standard.

2.7. Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing polyacrylamide gel electrophoresis was carried out as described by Gratão et al. [29]. For denaturing SDS–PAGE, the gels were rinsed in distilled, deionized water and incubated overnight in 0.05% Coomassie blue R-250 in a water/methanol/acetic acid 45/45/10 (v/v/v) solution and destained by successive washing in the same water/methanol/acetic acid 45/45/10 (v/v/v) solution.

2.8. SOD and CAT activity stainings

Superoxide dismutase activity staining was carried out as described by Medici et al. [31]. After non-denaturing-PAGE separation, the gel was rinsed in distilled, deionized water and incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium and 0.3% N,N,N′,N′-tetramethylethylenediamine. One unit of bovine liver SOD (Sigma, St. Louis, USA) was used as a positive control for activity. After 30 min, the gels were rinsed with distilled-deionized water and then illuminated in water until the development of achromatic bands of SOD activity was visible on a purple-stained gel. To identify SOD isoenzymes from the different bacterial isolates, samples were subjected to non-denaturing PAGE and the SOD bands classified as described by Guelfi et al. [32]. Superoxide dismutase isoenzymes were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide and 5 mM hydrogen peroxide (H_2O_2).

Catalase activity following non-denaturing PAGE was determined as described by Ferreira et al. [33]. Gels were incubated in 0.003% H_2O_2 for 10 min and developed in a 1% (w/v) $FeCl_3$ and 1% (w/v) $K_3Fe(CN_6)$ solution for 10 min. One unit of bovine liver CAT (Sigma, St. Louis, USA) was used as a positive control of activity. The relative intensities of the stained bands were determined by an ImageScanner III (GE Healthcare, Little Chalfont, UK) and the ImageQuant™ TL software (GE Healthcare, Uppsala, Sweden).

2.9. CAT total activity determination

CAT activity was assayed as described previously by Gratão et al. [29] at 25 °C in a reaction mixture containing 1 mL 100 mM potassium phosphate buffer (pH 7.5) with 2.5 μL H_2O_2 (30% solution). The reaction was initiated by the addition of 25 μL of protein extract, and the activity was determined by following the decomposition of H_2O_2 as a change in absorbance at 240 nm.

2.10. GR activity staining

GR activity following non-denaturing PAGE was determined as described by Gomes-Junior et al. [34]. The gels were rinsed in distilled, deionized water and incubated in the dark for 30 min at room temperature. The reaction mixture contained 250 mM Tris (pH 7.5), 0.5 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 0.7 mM 2,6-dichloro-*N*-(4-hydroxylphenyl)-1,4-benzoquinoneimine sodium salt (DPIP), 3.4 mM GSSG (oxidized glutathione) and 0.5 mM NADPH. One unit of bovine liver GR (Sigma, St. Louis, USA) was used as a positive control.

2.11. GR total activity determination

Glutathione reductase activity was assayed as described by Gratão et al. [29] at 30 °C in a mixture consisting of 1.7 mL 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5,5′-dithiozbis(2-nitrobenzoic acid) (DTNB), 1 mM GSSG and 0.1 mM NADPH. The reaction was started by the addition of 50 μL of protein extract. The rate of reduction of oxidized glutathione was followed in a spectrophotometer by monitoring the change in absorbance at 412 nm for 1 min.

2.12. Statistical analysis

Total protein content and enzyme activity determinations were performed on three replicates for each treatment, and the significance of the observed differences was verified using a one-way analysis of variance (ANOVA) followed by Tukey's test. Differences with a p value of <0.05 were considered significant. All statistical analyses were carried out using the SAS program, version 9.1.

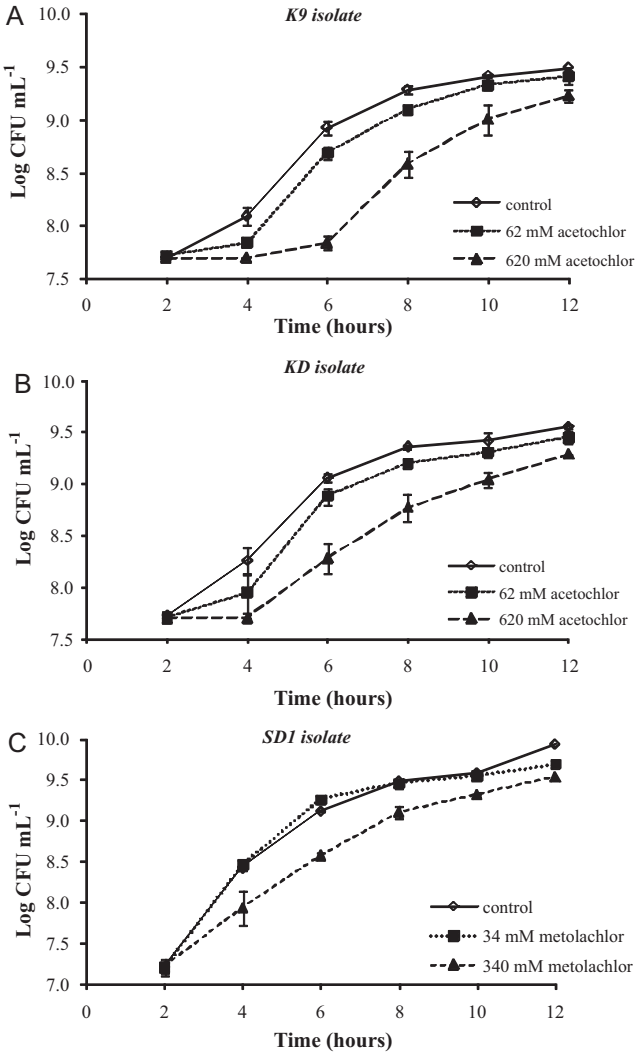


Fig. 2. Bacterial growth at 30 °C in the presence of different concentrations of herbicides. Values represent the means from three experiments ± SEM.

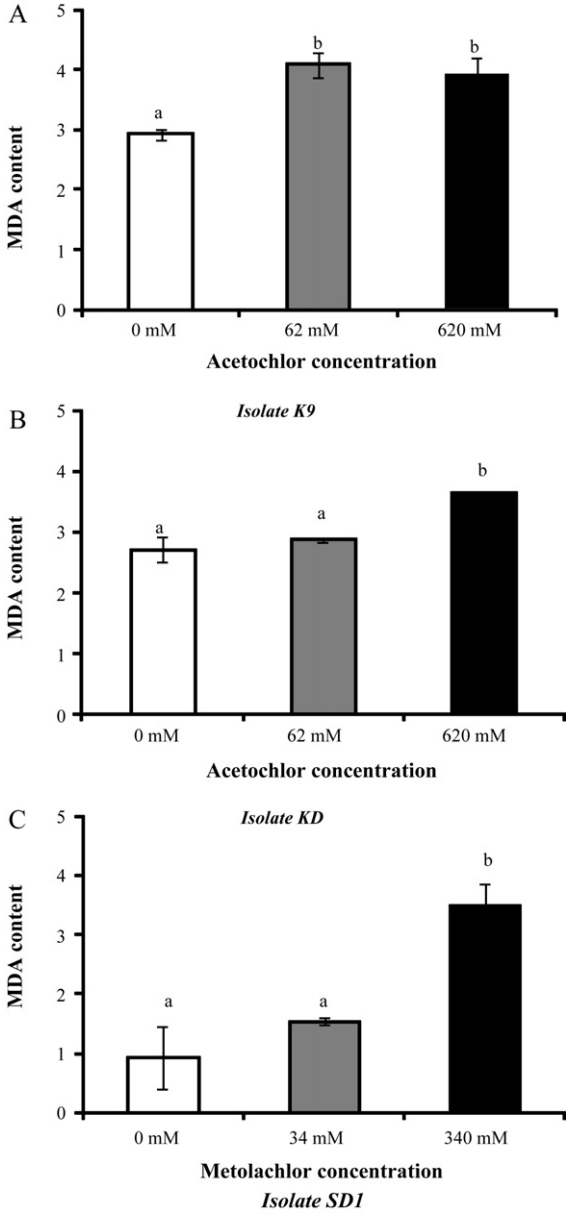


Fig. 3. MDA content (nmol g^{-1} fr. wt) of bacteria grown in different concentrations of the herbicides. Values represent the means from three experiments ± SEM. Means with different letters are significantly different ($P < 0.05$) by one-way analysis of variance (ANOVA) and Tukey test.

3. Results and discussion

3.1. Characterization of herbicide-tolerant isolates

Prior to any biochemical analyses, we initially conducted the identification of the bacteria isolated and the construction of a phylogenetic tree. Youssef et al. [35] reported that fragments encompassing the V6 and V7 regions of the 16S rRNA gene yielded comparable results to those obtained with nearly full-length fragments. However, analysis of some isolates by this method combined with comparison to sequences in GenBank® can result in misclassification. A study of the family *Enterobacteriaceae* emphasized that the *Enterobacter* genus is considered a polyphyletic group [36]. Therefore, more sequences and molecular markers are needed to obtain an encompassing phylogeny of this genus [37]. A phylogenetic tree was built up using the type strains and GenBank® strains that are closely related to the studied isolates; these derive from *Pseudomonas* spp., *Salmonella* spp., *Tatumella* spp. and *Leclercia* spp. As the outgroup, *Klebsiella pneumoniae*, *K. granulomatis* and *K. singaporensis* were used (Fig. 1). By 16S rRNA gene sequencing, the SD1 isolate is phylogenetically related to *Enterobacter asburiae*, while the isolates KD and K9 have different genotypes that are more closely related to that of *Enterobacter amnigenus*. The phylogeny of the *Enterobacter*/*Pantoea* group is incompletely known [37], and previous studies have suggested that a review of this group is necessary for better bacterial identification and definition of the monophyletic groups.

Several studies have reported the identification of bacteria of the *Enterobacteriales* that are capable of degrading contaminants like e.g. as pesticides [38,39]. For instance, Wang et al. [40] reported that two bacterial strains, identified as *Pseudomonas* sp. and *Enterobacter cloacae*, isolated from contaminated soil, have the ability to degrade hexazinone. Thus, bacterial isolates that can degrade pesticides could potentially be used in the bioremediation of areas contaminated with these compounds.

In general, bacterial growth was not dramatically affected after 12 h of growth in the presence of the herbicides at either of the concentrations tested, but there was a difference in growth during the exponential phase (between 4 and 10 h of growth) between bacteria cultured in the control medium (0 mM) and those cultured in media containing higher concentrations of the herbicides (Fig. 2).

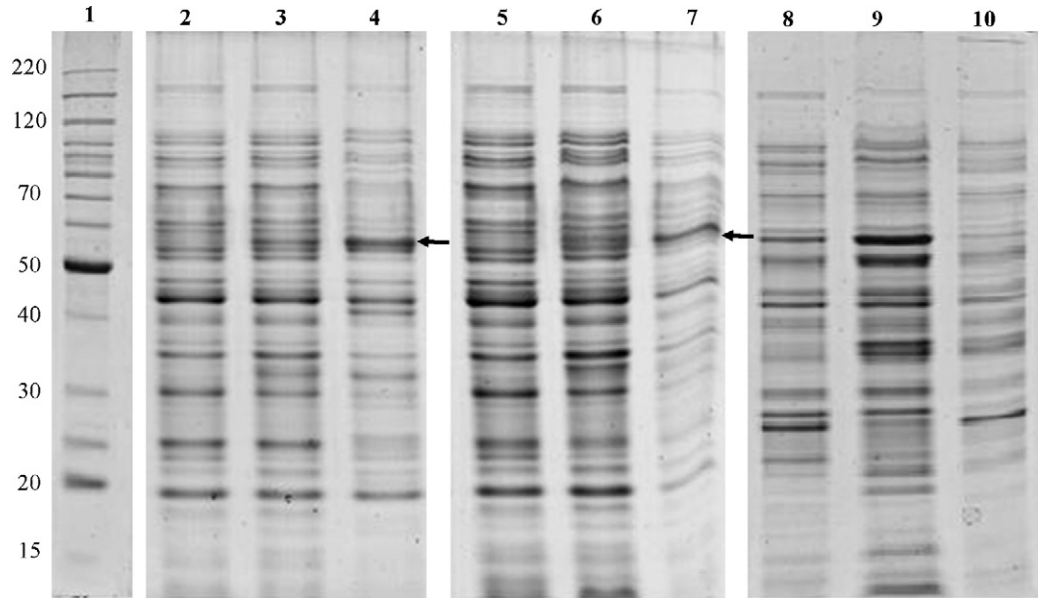


Fig. 4. SDS–PAGE protein profiles of bacteria exposed to the herbicides. Lane 1, protein molecular mass markers (220–15 kDa). Lanes 2, 3 and 4, K9 isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 5, 6 and 7, KD isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 8, 9 and 10, SD1 isolate grown in the presence of 0 mM (control), 34 mM and 340 mM metolachlor, respectively. The two arrows indicate a 57 kDa protein band that is specifically induced in the presence of acetochlor.

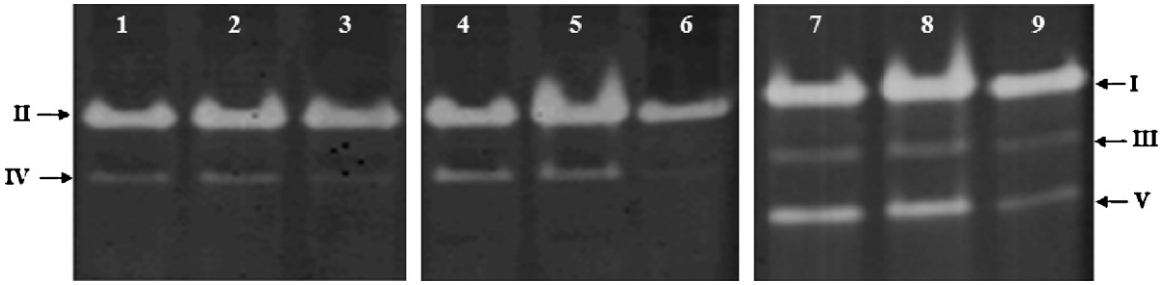


Fig. 5. SOD activity staining following non-denaturing PAGE of cultured bacteria cell extracts. Lanes 1, 2 and 3, K9 isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 4, 5 and 6, KD isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 7, 8 and 9, SD1 isolate grown in the presence of 0 mM (control), 34 mM and 340 mM metolachlor, respectively. Arrows indicate sequentially numbered SOD bands (I–V) that are independent of the bacterium isolate.

3.2. *Effect of the herbicides on lipid peroxidation*

Herbicides have been shown to cause oxidative stress in bacteria [41,42]. During dechlorination, the early step of the degradation of chloroacetanilide herbicides, ROS can be produced [43,44]. In addition, the mode of action of the chloroacetanilide class of herbicides involves inhibition of the elongation of C18 and C16 fatty-acids species to VLCFAs [16], which results in damage to cell membranes [44]. Oxidative stress was clearly established when the highest concentrations of the herbicides were added to the cultures because lipid peroxidation (based on MDA production) was increased significantly in each of the isolates (Fig. 3). Lipid peroxidation is one of the best, most widely used indicators of oxidative stress [11,45]. The K9 isolate exhibited significant increases of 39% and 34% in lipid peroxidation for the 62 mM and 620 mM acetochlor treatments, respectively (Fig. 3A), whereas the KD and SD1 isolates exhibited increases (5% and 33%, respectively) in lipid peroxidation only at the higher concentrations of the herbicides (Fig. 3B and C). These results suggest that the observed increases in lipid peroxidation may be associated with oxidative stress in these bacteria. High levels of MDA were also observed in *Escherichia coli* strains exposed to the herbicide 2,4-dichlorophenoxyacetic acid [46]. According to Balagué et al. [46], bacterial cells modify their membrane lipid molecules to avoid the toxic effects of the herbicide. This study demonstrated that bacteria exposed to 2,4-D may reduce membrane fluidity to withstand chemical injury; because of lipid–protein interactions, the transport processes of molecules may be diminished. Işık et al. [15] also reported an increase in MDA levels in *Streptomyces* sp. M3004 treated with H_2O_2 and the herbicide paraquat, further suggesting that lipid peroxidation is a marker of membrane damage. In addition, the control SOD and CAT enzyme activities of *Streptomyces* spp. M3004 exhibited a negative correlation with membrane MDA levels, which suggests that these enzymes work cooperatively to protect the membrane against ROS [15].

In addition, the KD and SD1 isolates may also prevent lipid peroxidation by alteration of fatty acid biosynthetic pathways, which are targets of chloroacetanilide herbicides. Differences among VLCFA contents, such as an increase in saturated fatty acids [16], can result in changes in the composition of the cell membrane, which could diminish lipid peroxidation, thus compensating for the presence of the herbicide. A more pronounced increase in lipid peroxidation level was observed for the SD1 isolate at 340 mM metolachlor (264%) than for the KD isolate (Fig. 3C), indicating a greater ability of the KD isolate to cope with additional stress. This suggests that an efficient response by the bacterial

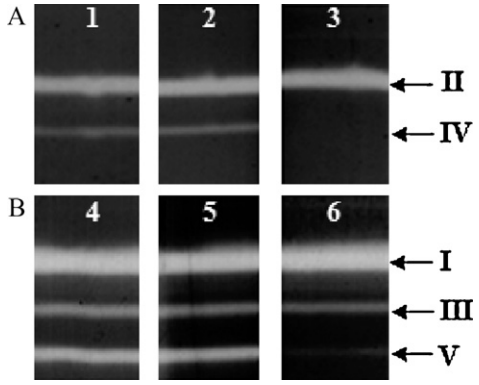


Fig. 6. SOD activity staining following non-denaturing PAGE of KD and K9 isolates (A) and SD1 isolate (B), used for classification of SOD isoenzymes. Lanes 1 and 4, control SOD activity. Lanes 2 and 5, SOD activity upon with 2 mM potassium cyanide treatment; lanes 3 and 6, 5 mM H_2O_2 treatment. Arrows indicate SOD bands that are sequentially numbered (I–V) according to Fig. 5.

antioxidant system comprises part of the mechanism of herbicide tolerance.

3.3. SDS–PAGE protein profile

Analysis of protein profiles following denaturing SDS–PAGE revealed that at the high concentration of both herbicides, all three bacterial isolates exhibited an overall reduction in protein concentration, as indicated by a general reduction in intensity of the majority of the protein bands (Fig. 4). Some of the protein bands disappeared altogether, but these changes could not account for the observed reduction in the total protein concentration.

A 57 kDa protein band present in both the KD and K9 bacterial isolates appears to be specifically induced in response to acetochlor because this band was only present when the bacterial isolates were exposed to either concentration of this herbicide (Fig. 4). We also observed an overall increase in protein concentration in response to the lowest metolachlor concentration used (34 mM; Fig. 4, lane 9); however, at the high concentration, the total protein level was reduced (Fig. 4, lanes 4, 7 and 10).

3.4. Superoxide dismutase (SOD) activity

The bacterial isolates did not exhibit any major changes in total SOD activity following treatment with the herbicides, which suggests excess superoxide radicals are not being produced. Following non-denaturing PAGE, SOD activity staining revealed the presence of at least two SOD isoenzymes in KD and K9 isolates and three SOD isoenzymes in the SD1 isolate (Fig. 5). Based on the profile and intensity of the bands (ImageQuant™ TL software), the total SOD activity was only slightly reduced when the bacteria isolates were exposed to the highest concentrations of the herbicides. This was due to specific reductions in SOD IV activity in the K9 and KD isolates (by 45% and 90%, respectively) and SOD V activity in the SD1 isolate (by 60%; Fig. 5).

Superoxide dismutase isoenzymes present in the isolates were classified as Mn-SOD (bands I and II) and Fe-SOD (bands III, IV and V); the presence of Cu/Zn-SOD isoenzymes was not detected (Fig. 6). Distinct types of SOD isoenzymes, which can be classified according to dependence on metal cofactors such as Mn-SOD, Fe-SOD, Ni-SOD or Cu/Zn-SOD, have been detected in various organisms [47–49]. For instance, in bacteria, SodA and SodB are cytoplasmic SODs that contain Mn^{2+} (MnSOD) and Fe^{3+} (FeSOD), respectively, while SodC is a periplasmic Cu^{2+}- and Zn^{2+}-containing SOD (Cu/ZnSOD) [48,50].

The bacterial isolates used in this study expressed only Fe-SOD and Mn-SOD isoenzymes and not Cu/Zn-SODs; however, none

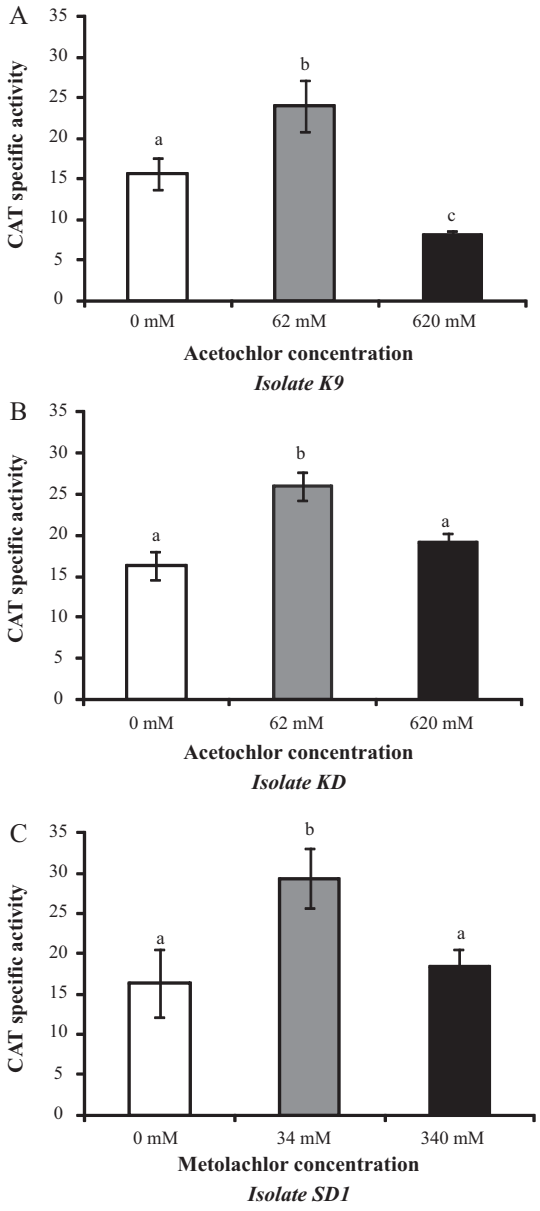


Fig. 7. Specific CAT activity, expressed as μmol min^{-1} mg^{-1} protein. Values represent the means from three experiments ± SEM. Means with different letters are significantly different ($P < 0.05$) by one-way analysis of variance (ANOVA) and Tukey's test.

of these enzymes exhibited any specific response that could be clearly attributed to the herbicides. Under controlled conditions, some studies have shown that the Cu/Zn-SOD isoenzymes are not required for bacterial growth in the laboratory because they do not appear to have a major role in the catabolism of superoxide anions [47,49].

3.5. Changes in CAT activity in response to herbicides

The specific activity of CAT was shown to increase by 59% (K9), 53% (KD) and 81% (SD1) when the bacterial isolates were exposed to the lowest concentration of the herbicides (Fig. 7). On the other hand, when the bacteria were grown in the presence of the high concentrations of the herbicides, CAT activity was shown to be equal to the control levels for KD (Fig. 7B) and SD1 (Fig. 7C); the K9 isolate exhibited only 49% of the CAT activity of the corresponding control (Fig. 7A). CAT activity staining following non-denaturing

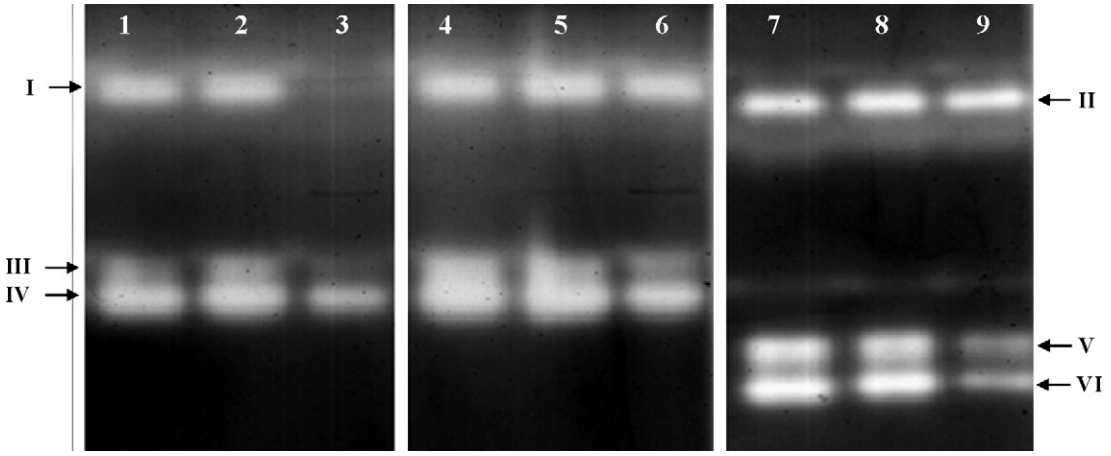


Fig. 8. CAT activity staining following non-denaturing PAGE of cultured bacteria cell extracts. Lanes 1, 2 and 3, K9 isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 4, 5 and 6, KD isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 7, 8 and 9, SD1 isolate grown in the presence of 0 mM (control), 34 mM and 340 mM metolachlor, respectively. Arrows indicate sequentially numbered CAT bands (I–V) that are independent of the bacterium isolate.

PAGE revealed the presence of three CAT isoenzymes (CAT I, III and IV) in the K9 and KD bacterial isolates and three CAT isoenzymes (CAT II, V and VI) in the SD1 isolate (Fig. 8). The changes observed following gel staining were for the most part in agreement with those measured by spectrophotometry (Fig. 7). Staining for activity also revealed that the reduction in CAT activity in K9 following treatment with 620 mM acetochlor (Fig. 7A) was partially due to a reduction in CAT III isoenzyme activity but mostly due to the total disappearance of CAT I activity (Fig. 8). In the SD1 isolate, the reduction in CAT activity observed at 340 mM metolachlor was due to reduced activities by 41% and 36% of the isoenzymes V and VI, respectively (Fig. 8).

These results, in combination with the lipid peroxidation ones, suggest that the isolated bacteria appear to be tolerant to the herbicides at the low concentrations used in this study, but not entirely to the extremely elevated herbicide concentrations, which were used to guarantee an oxidative response. This could be due to the increased levels of lipid peroxidation observed at the highest herbicide concentrations tolerated by the bacteria. It should also be noted that all three isolates exhibited reduced growth during the log phase, though this reduction was not deleterious (because the growth returned nearly to the control levels after 12 h) (Fig. 1). This may also be an indication that the antioxidant response was efficient at the highest concentrations of the herbicides, because after 12 h, there was hardly any growth inhibition when compared to the controls (Fig. 1).

A key result obtained in this report is the specific induction of a 57 kDa protein in the K9 and KD isolates in response to acetochlor, independent of herbicide concentration. This result indicates a potential involvement of this protein in the bacterial response to the oxidative stress caused by this particular herbicide. Furthermore, it cannot be ruled out that this protein may well be part of another metabolic route that might have been affected by the herbicide. Further investigation by 2D-PAGE, isolation and protein sequencing must be pursued to identify this protein and connect its function to the effect of acetochlor on K9 and KD.

Lü et al. [42] reported that quinclorac, an herbicide that stimulates the induction of 1-aminocyclopropane-1-carboxylic acid synthase activity, in turn promoting ethylene biosynthesis, induced an increase in both CAT and SOD activities. These authors suggested that both CAT and SOD are involved in the mechanism of tolerance to the herbicide. In another study with these enzymes, acetamiprid induced oxidative stress to a lesser extent in the insecticide degrading *Pseudomonas* FH2 than in the non-degrading *Escherichia coli* K12 bacteria [41].

3.6. GR isoenzymes appeared to behave differently in different isolates

During oxidative stress in bacteria, the activation of OxyR induces the expression of a number of genes, including the *gor* gene, which encodes a GR enzyme [12]. GR is a member of the flavin-containing enzyme family and catalyzes the transfer of reducing equivalents from NADPH to GSSG; this process seems to play a crucial role in the redox balance of the cell [51]. Similar to their effects on CAT activity, treatments with the highest concentrations of both herbicides (620 mM acetochlor and 340 mM metolachlor) resulted in drastic reductions in the specific activity of GR in the K9 (87%), KD (71%) and SD1 (85%) isolates (Fig. 9). Furthermore, GR activity staining revealed the presence of up to 9 distinct isoenzymes among the three bacterial isolates, consisting of four GR isoenzymes (GR II, IV, V and IX) in the K9 and KD isolates and five GR isoenzymes (GR I, III, VI, VII and VIII) in the SD1 isolate (Fig. 10). Overall, the GR isoenzyme activity changes observed agreed with the results obtained for total specific GR activity. The reductions in total specific GR activity (Fig. 9) at both concentrations of acetochlor are due to a reduction in GR II isoenzyme activity, which accounts for the majority of the total GR activity in K9 and KD isolates and almost completely disappeared after 620 mM acetochlor treatment (Fig. 10). Although GR activity at 34 mM metolachlor was not statistically different from the control GR activity in SD1, non-denaturing PAGE revealed that the herbicide induced the appearance of two new GR isoenzymes (GR VII and VIII) and an increase in GR I activity (46%) (Fig. 10).

Glutathione reductase activities were at or below the control level depending on the concentration of the herbicide. However, many functions of the thiol–redox system involving the GR enzyme can be carried out by enzymes with a redundant function [52,53]. For instance, the dimeric flavoenzyme thioredoxin reductase, catalyzes the NADPH-dependent reduction of thioredoxin, contributing to the balance between thioredoxin and glutathione levels [53]. Furthermore, glutathione is the most important component of the redox balance in gram-negative bacteria [51]. Oxidized glutathione is reduced to GSH by GR; however, GSH concentrations are maintained in *E. coli* mutants that lack GR, likely via the

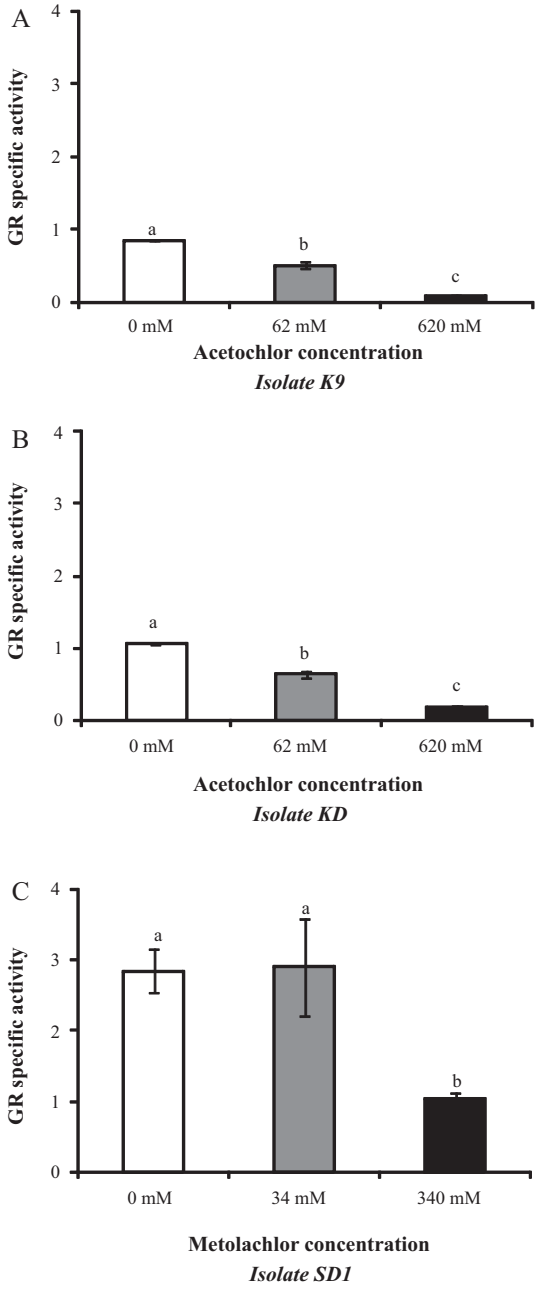


Fig. 9. Specific GR activity, expressed in μmol min^{-1} mg^{-1} protein. Values represent the means from three experiments ± SEM. Means with different letters are significantly different ($P < 0.05$) by one-way analysis of variance (ANOVA) and Tukey's test.

action of thioredoxin reductase [53]. In these mutants, the ratio of reduced to oxidized glutathione does not appear to change, indicating that *E. coli* may have alternate sources of GR activity. Curiously, two GR isoenzymes (GR VII and GR VIII) induced specifically by metolachlor were observed in SD1 bacteria exposed to 34 mM of metolachlor (Fig. 10), suggesting that these two enzymes may have a more specific role in the stress response to the herbicide and be involved in tolerance to metolachlor. This result is doubly important because total GR activity was not significantly different from the control (Fig. 9), suggesting that although total activity was unchanged, some of the GR isoenzymes responded differently to the herbicides. Similar types of responses have been demonstrated in plant cells, specifically coffee cell suspension cultures subjected to heavy metal-induced oxidative stress [34,54,55]. In coffee, induction of one new GR isoenzyme was observed in cell cultures subjected to selenium, cadmium and nickel treatment and can be used as a marker for stress in this species [34,54,55].

In the present study, 340 mM metolachlor and 620 mM acetochlor were shown to change the rate of growth during the exponential phase, but without changing the final total growth (Fig. 2). However, the lipid peroxidation data indicate that these higher concentrations were toxic to the bacteria, thus resulting in an antioxidant stress response, which was only partially effective at helping the bacteria tolerate the herbicides. Moreover, at the lower concentrations of herbicides used (34 mM metolachlor and 62 mM acetochlor), the oxidative stress induced could be dealt by CAT and specific GR isoenzymes. It is possible that other antioxidant enzymes or even non-enzymatic antioxidants may be involved in this process. Nevertheless, the information reported is a step forward in our understanding of the bacterial stress responses to two important herbicides, because previous reports have shown distinct responses [56,57].

Mongkolsuk et al. [57] suggested that *Xanthomonas*, a soil bacterium, can respond to the oxidative stress caused by environmental pollutants that have the ability to generate strong oxidants, such as herbicides and metals, through physiological adaptation to H_2O_2. This process involves not only CAT but also the alkyl hydroperoxide reductase and thiol peroxidase enzymes. It appears that the stress response varies depending on the bacterial species and the type, concentration and perhaps mode of action of the herbicide used. For instance, in contrast to our observations, Lü et al. [42] reported that CAT had only a minor role in the defense against oxidative stress induced by herbicides, whereas SOD was critical. In such study, the authors investigated a different herbicide (quinclorac) and different bacteria (*Escherichia coli*, *Bacillus subtilis* and *Burkholderia cepacia*).

Moreover, the hormesis hypothesis, in which low doses of toxins have been suggested to activate defense mechanisms, leading to 'overcompensation' [58], i.e., a highly induced oxidative response, may also partially explain the responses observed. Drawing on the hormesis hypothesis, Işik et al. [15] observed that *Streptomyces* sp. M3004 exhibited higher CAT activity levels in the presence of 1 mM of the herbicide paraquat compared with 3 mM paraquat.

In conclusion, we have demonstrated that specific CAT and GR isoenzymes may be involved in tolerance of various bacteria to the herbicides metolachlor and acetochlor. The role of antioxidant enzymes such as CAT, SOD and GR in bacterial herbicide tolerance is not fully understood in such mechanism. Nevertheless, we hypothesize that the function of these enzymes is related to avoidance of an imbalance in ROS production versus scavenging and the protection of membrane integrity, eventually leading to tolerance. Our group is currently conducting a comprehensive test of this hypothesis, in which other antioxidant enzymes, such as thioredoxin reductase, glutathione S-transferase and antioxidant compounds such as glutathione and glutaredoxin are being considered. These results, together with the increase in lipid peroxidation levels observed at the high herbicide concentrations, provide new insight into the antioxidant properties of metolachlor/acetochlor-degrading bacterial isolates, which could be very useful in future herbicide bioremediation studies. The systematic and continuous use of herbicides and other chemicals in agriculture in the search for higher productivity will result in further contamination of the environment and a major impact on weeds and non-targeted bacteria, which makes this study also relevant from an environmental point of view. Further characterization by 2D-PAGE is also being conducted to more precisely identify specific changes in soluble proteins in response to herbicide-induced oxidative stress that could potentially correlate with herbicide degradation.

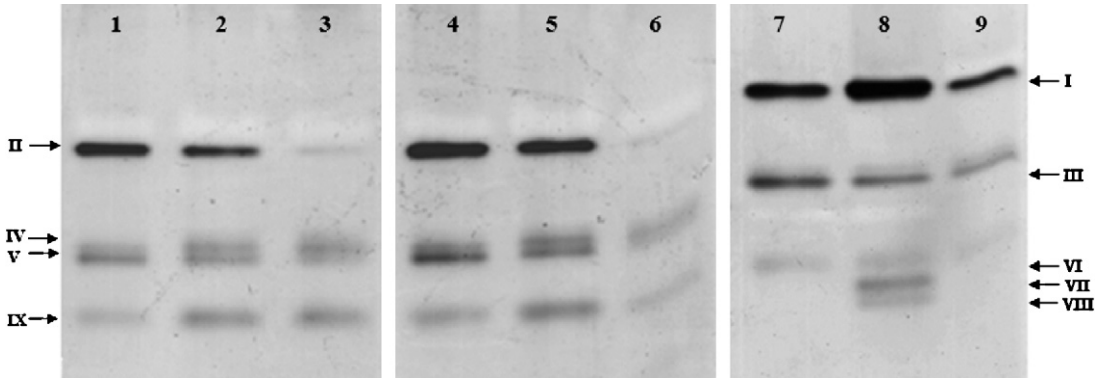


Fig. 10. GR activity staining following non-denaturing PAGE of cultured bacteria cell extracts. Lanes 1, 2 and 3, K9 isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 4, 5 and 6, KD isolate grown in the presence of 0 mM (control), 62 mM and 620 mM acetochlor, respectively. Lanes 7, 8 and 9, SD1 isolate grown in the presence of 0 mM (control), 34 mM and 340 mM metolachlor, respectively. Arrows indicate sequentially numbered GR bands (I–IX) that are independent of the bacterium isolate.

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