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Cellular Membranes, the Sites for the Antifungal Activity of the Herbicide Sethoxydim

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Abstract: The fungicidal effect of sethoxydim on the canola (*Brassica napus* var. Olifera) white stem rot pathogen (*Sclerotinia sclerotiorum*) encouraged us to conduct a series of studies on the mechanism of the antifungal activity of this herbicide commonly applied in Iranian fields under canola cultivation. Present preliminary studies on the changes in the level of Malondialdehyde (MDA) as the main product generated through peroxidation of polyunsaturated fatty acids indicated the disintegration of the fungal bilayer of plasma membrane as the result of the herbicidal treatment. Also, it was demonstrated that the amount of hydrogen peroxide in the treated samples was higher than the control samples with no herbicidal treatment. Therefore, our present results confirm the disintegration of the plasma membrane as one of the mechanism for the antifungal impact of sethoxydim. As with weed plants, the phytotoxic impact of this herbicide has been attributed to the inhibition of the first enzyme in the lipid biosynthesis pathway, acetyl-CoA carboxylase, therefore, it would be very interesting to study on this subject and the relations between the sensitivity of different fungi and their DNA and protein sequences of acetyl-CoA carboxylase.

Key words: Sethoxydim, acetyl-CoA carboxylase, MDA, fungicidal activity, herbicide

INTRODUCTION

Integrated disease management is a non-separable part of all the eco-friendly stable agricultural programs that seek the high yield production of agricultural products beside the rational application of the present facilities. One of the important points to be paid attention in these programs is to manage the field in a way that the conditions become non-suitable for the growth and development of the main diseases and still favorable or at least rather so for the beneficial microorganisms, among them biological control agents. For example, in 2002, we found the antifungal activity of the herbicide from the family cyclohexandiones, sethoxydim and it potential for the inhibition of the development of the sclerotia in the canola white stem rot pathogen, *Sclerotinia sclerotiorum* (Pakdaman *et al*., 2002). Sclerotia are not only resting over-wintering organs of this pathogenic fungus, but also the beginning foci for the sexual reproduction and genetic diversity of the pathogen through germination to apothecia and therefore, of high importance in the epidemiology of the various diseases caused by this fungus on more than 400 plant species from 75 families most of them are herbaceous plants from the subclass Dicotyledonae of the Angiospermae (Boland and Hall, 1994). Moreover, it has been demonstrated that not only the herbicide is of no much inhibitory effects on the growth of the biological control agent, *Trichoderma* (Pakdaman *et al*., 2002), but also theoretically of positive synergistic effects and therefore can be regarded as a suitable candidate for programs aimed to the integrated control plant diseases (Pakdaman and Mohammadi Goltapeh, 2007). With weed plants, the herbicide exerts its phytotoxic impacts through inhibition of the first enzyme involved in lipid biosynthesis, namely acetyl-CoA carboxylase, however, with fungi it is not still clear that how it imposes its fungicidal effects, hence, we had enough incentives to be

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curious about the mechanisms of the antifungal activity of this herbicide.

MATERIALS AND METHODS

Fungal isolates: The isolate of *Sclerotinia sclerotiorum* was kindly provided by Dr. H. Afshari Azad at Plant Pests and Diseases Research Institute, Tehran, Iran. *Trichoderma* sp. was awarded by Kasra Sharifi from the same institute. This isolate originally isolated from Tabriz, East Azerbaijan, Iran, had shown well in preliminary studies for determination of its biological control potential against *Sclerotinia sclerotiorum* and *Fusarium graminearum* (unpublished data).

***In vitro* studies:** To study the impact of sethoxydim (Nabu®S) on the growth and development of the fungal isolates and on the biological control of *S. sclerotiorum* by *Trichoderma* isolate, Potato Dextrose Agar (PDA) plates with and without sethoxydim were prepared. The studied dose of the herbicide was 5000 ppm that is the least recommended dose for field applications. The incubation temperature was about 15±1°C, a temperature favorable for the growth and development of *S. sclerotiorum*. The discs of 5 mm diameter from 4-day old cultures were applied for inoculations. To study the effect of the herbicide on the growth, was placed in the center of each plate and to study its effect on the biological control, two discs of both fungi were placed oppositely on each agar plate. Incubation and daily records were continued till the full growth in all three control replicates for each fungus.

The experiment was conducted in factorial arrangement in Completely Randomized Design (CRD) with 3 replications and the experimental data were statistically analyzed for a variance using the SAS System (SAS Institute Inc, 1997). When variance analysis showed significant treatment effects, Duncan's Multiple Range test was applied to compare the means at $p<0.05$.

Preparation of fungal masses for biochemical studies: To compare the amount of hydrogen peroxide in control and herbicide-treated *Sclerotinia sclerotiorum*, the fungus was grown in Potato Dextrose Broth (PDB) with and without herbicide supplementation (2500 ppm) and incubated at room conditions on a shaker at 80 rpm. After 10 days, the cultures were filtered through Whatman No. 1 filter papers, rinsed three times with sterilized distilled water and desiccated in 50 mL vials through lyofilization. The dried samples were stored in a freezer at -20°C for next steps.

Measurement of hydrogen peroxide: To measure hydrogen peroxide production in the fungal samples, the modified form of the method used by Loreto and Velikova (2001) was applied. 0.1 g samples of each dried fungal masses from control and herbicidal treatment were separately grinded in liquid nitrogen and homogenized in 5 mL aliquots of trichloroacetic acid solution (0.1% W/V). The hemogenates were centrifuged at 12000 g for 15 min. Then, 0.5 mL aliquot of each supernatant was added to a 0.5 mL aliquot of 10 mM potassium phosphate buffer (pH 7.0) in a 2 ml tube. One milliliter aliquots of 1 M potassium iodide solution was added to each of the 2 mL tubes. The level of absorbance of the wave-length 390 nm was determined by a UV-VIS spectrophotometer (CARY, 300 Scan, UV-Visible Spectrophotometer, Varian).

Measurement of malondialdehyde (MDA): The method of Stewart and Bewley (1980) was used to compare the levels of MDA production by *S. sclerotiorum* under control and herbicide treatment conditions. Two separate samples (each of 0.5 g in weight) of the fungus *S. sclerotiorum* were taken from each of the vials for control and treatment and homogenized in 5 mL of 50 mM phosphate buffer (pH 7.0). The hemogenates were centrifuged at 16000 rpm for 30 min and 1 mL of each of the supernatants was separately added to 1 mL of 0.5% (W/V) thiobarbituric acid (TBA) in 20% Trichloroacetic Acid (TCA). The mixture was incubated in a boiling water-bath for 30 min and the reaction was stopped by placing the reaction tubes in ice for 30 min. Then, the reactions were centrifuged at 10000 g for 15 min and level of absorbance by each supernatant was determined at 532 nm, subtracting the value of non-specific absorption at 600 nm. The amount of MDA-TBA complex (red color) was determined based on the extinction coefficient 155 mM^{-1} cm^{-1}.

RESULTS AND DISCUSSION

The *in vitro* studies indicated that sethoxydim imposes a significantly negative impact on the growth of both fungi ($p<0.01$), including the biological control agent *Trichoderma*, however, this effect is more considerable with the growth and development of the pathogenic fungus, *S. sclerotiorum* ($p<0.05$). With addition of agar to two-fold ratio, the superiority of *Trichoderma* against *S. sclerotiorum* was maintained, so that *Trichoderma* isolate T-100 was of the fast growth on PDA, followed by *S. sclerotiorum* on PDA and ranked as the second. T-100 on the sethoxydim-amended PDA medium ranked later as the third and the least growth was found with *S. sclerotiorum* grown on sethoxydim-amended PDA ($p<0.05$). With the latter, *S. sclerotiorum* began to grow lately and developed dark hyphae and sclerotial droplets at late stages (Fig. 1).

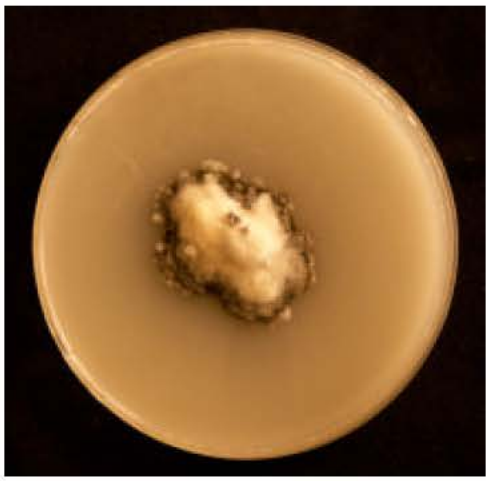

Fig. 1: Effect of sethoxydim on the growth and development of *Sclerotinia sclerotiorum*. The formation of dark hyphae adhered to the medium, as well as sclerotial primordia are notable

Fig. 2: Destructive effect of *Trichoderma* isolate T-100 on the pathogenic *Sclerotinia sclerotiorum* on sethoxydim-amended PDA medium

Also, the results from *in vitro* confrontation of two fungi demonstrated no significant effect on the biological control by *Trichoderma* sp. imposed by the herbicide sethoxydim, so that although the growth of the biological control agent decreases, however, the further effect on the pathogenic partner compensates for this negative impression (Fig. 2).

These results are in accordance with our previous findings (Pakdaman *et al*., 2002) and expectations.

Measurements made with hydrogen peroxide (H_2O_2) implicated to an oxidative stress occurred under culture conditions in PDB supplemented with sethoxydim. *S. sclerotiorum* had produced more hydrogen peroxide in the presence of sethoxydim (3.8 μg g^{-1}) compared to in its absence (1.9 μg g^{-1}).

Measurements with Malondialdehyde (MDA) also indicated the hazardous effect of the oxidative stress on the lipid membrane bilayers (plasma membrane and membranous organelles) as the consequence of the chemical stress imposed by sethoxydim.

The herbicide-treated samples had significantly higher levels of MDA than did the samples from control cultures (36.704 mM g^{-1} compared with 20.58 mM g^{-1}). MDA is a major cytotoxic product of lipid peroxidation and has been used extensively as an indicator of free radical production (Kunert and Ederer, 1985). Peroxides of polyunsaturated fatty acids generate malondialdehyde on decomposition and in many cases, MDA is the most abundant individual aldehydic lipid breakdown product (Davey *et al*., 2005).

With weeds, sethoxydim exerts its phytotoxic effect through inhibition of acetyl-CoA Carboxylase (ACC), an enzyme involved in the biosynthesis of lipids (Zhang *et al*., 2004b). ACCs are crucial for the metabolism of fatty acids, making these enzymes important targets for development of therapeutics (Abu-Elheiga *et al*., 2001; Alberts and Vagelos, 1972; Cronan and Waldrop, 2002; Harwood *et al*., 2003; Wakil *et al*., 1983; Zhang *et al*., 2003, 2004a, b). They catalyze the carboxylation of acetyl-CoA in two steps: ATP-dependant carboxylation of a biotin group covalently linked to a lysine residue in the Biotin Carboxyl Carrier Protein (BCCP) and then the transfer of this activated carboxyl group to acetyl-CoA. In mammals, yeast and most other eukaryotes, ACC is a large, multi-domain enzyme, with a Biotin Carboxylase (BC) domain that catalyzes the first step and a Carboxyl Transferase (CT) domain that catalyzes the second step of the reaction. Biotin is linked covalently to the BCCP domain in the enzyme (Abu-Elheiga *et al*., 2001; Lenhard and Gottschalk, 2002; Wakil *et al*., 1983; Zhang *et al*., 2004b). CT domain is the action site of two differential classes of widely used commercial herbicides (Gronwald, 1991; Devine and Shukla, 2000; Zagnitko *et al*., 2001; Delye *et al*., 2003), as represented by haloxyfop and diclofop (FOPs) and sethoxydim (DIMs). These compounds are potent inhibitors of ACCs from sensitive plants and kill them by shutting down fatty acid biosynthesis, indicating that an inhibitor of the CT domain is sufficient to block ACC function and this fact establishes this domain as a valid target for the development of inhibitors against these enzymes. The CT domain contains about 800 residues (90 kDa) and constitutes approximately the C-terminal one-third of the

eukaryotic, multi-domain ACCs. The amino acid sequences of this domain are highly conserved; for example, there is 52% sequence identity between the CT domains of yeast ACC and human ACC2 (one of two mammalian isoforms of ACC, associated with the mitochondrial membrane) (Zhang *et al*., 2004b). ACC2 is involved in the production of malonyl-CoA, a potent allosteric inhibitor of carnitine palmitoyl transferase I (CPT-I), which transports long-chain fatty acids into the mitochondria for oxidation (Ruderman *et al*., 1999), so that the inhibition of ACC is an important component in the stimulation of fatty acid oxidation (Chen *et al*., 2000). Therefore, the same mechanism might be true with the sethoxydim-treated fungi. As the raised level of MDA is considered as the main indicator of the peroxidation damage in membrane lipid bi-layer, hence, plasma membrane and organelles membranes might be regarded as the main target sites of the herbicide sethoxydim in fungi. The synergistic importance of membranes destroy in the pathogenic fungus like *S. sclerotiorum* and in the collaboration with the peptaibols and hydrolytic enzymes secreted by the biological control agent, *Trichoderma* has been discussed before (Pakdaman and Mohammadi Goltapeh, 2007).

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