In vitro studies on the integrated control of rapeseed white stem rot disease through the application of herbicides and *Trichoderma* species

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The antifungal activity of five herbicides commonly used to control weeds in rapeseed, canola (*Brassica napus* var Olifera) fields in Iran were studied against six isolates of *Trichoderma* species and also the phytopathogenic fungus *Sclerotinia sclerotiorum*. The doses tested were as recommended for applications in canola crops. The dinitroaniline herbicides (trifluralin and ethalfluralin) had the highest anifungal activities *in vitro* on the toxified CDA media preventing mycelium growth. Cycloxydim was toxic against *Trichoderma* spp. and sethoxydim had the least deletrious effects. But, with, *Sclerotinia sclerotiorum*, sethoxydium had the most toxicity together with cycloxydim with an intermediate level of anti-fungal activity between sethoxydim and haloxy fop ethoxy ethyl. This may be the first global report of the antifungal activities of the haloxy fop ethoxy ethyl, ethal fluralin, and cycloxydim.

Key words: *Sclerotinia sclerotiorum*, *Trichoderma*, herbicide, biocontrol, integrated control, rapeseed, canola.

Introduction

Sclerotinia sclerotiorum (Lib.) De Bary is an important plant pathogen that causes substantial losses in crop production worldwide annually. Diseases caused by this pathogen occur in numerous plant hosts. The broad host range of this fungus is important to the control of the disease in agricultural crops because it restricts the number of non-host crops that can be included in crop rotations designed to reduce the concentration of sclerotia in infested soils. Furthermore, inoculum produced on alternative hosts such as dandelions and

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clover in noncultivated areas can attack susceptible crops (Abawi and Grogan, 1975). Little information is available on the relative importance of these sources of inoculum to outbreaks of disease (Boland and Hall, 1994).

Based on an index of plants reported to be susceptible to *S. sclerotiorum*, the fungus infects 42 subspecies or varieties, 408 species, 278 genera, and 75 families, most of them are herbaceous plants from the subclass Dicotyledonae of the Angiospermae but several hosts also occur in the subclass Monocotyledonae, and only one species from the Peridophyta, leather leaf fern [*Rumohra adiantiformis* (G. Forst.) Ching] from the Polypodiaceae is infected by the pathogen. At least four species of Pinaceae, class Gymnospermae have been known as hosts for this fungus (Boland and Hall, 1994).

In Iran, rapeseed (*Brassica napus* var Olifera) is the most commonly cultivated oilseed crop and white stem rot, caused by the *S. sclerotiorum*. The most destructive and harmful disease which is widespread in the most important areas of oilseed production, especially in the northern marginal flats of Caspian sea.

Due to the importance of the disease and the difficulty of its control through the prevalent methods, it seems that an integrated control by a combination of several methods will be useful in disease management. Considering the broad host range of the pathogen and their significant influences on the epidemiological aspects of the disease, it may be possible to use a chemobiologically method including herbicides and biocontrol agents. Especially this method can decrease the volume of agricultural practices causing soil erosion, and reduce several applications of other chemicals of ecological danger. This work is to solve a problem of those associated to the sustainable agriculture. As for effective biocontrol of soil-borne plant pathogens, hyphal growth of *Trichoderma* through soil is important for colony extension and colonization of target propagules after introduction into soil (Dandurand and Knudsen, 1993; Knudsen and Bin, 1990; Knudsen *et al*., 1991), therefore, we focused on the effects of commonly used rapeseed crop prevalent herbicides and their effects on the hyphal growth of *Trichoderma* as the first step.

Materials and Methods

To study the effects of herbicides commonly used in rapeseed (canola) production areas in Iran, five herbicides including trifluralin (Treflan®, EC 48% w/v), ethal fluralin (Sonalan®, EC 33% w/v), sethoxydim (Nabu S®, OEC 12.5% w/v), cycloxydim (Focus®, EC 10% w/v), and haloxy fop ethoxy ethyl (Gallant®, EC 12.5% w/v) were selected. Trifluralin and ethal fluralin are of

soil herbicides, with broad herbicidal range of activities and others are selectively applied in canola fields to control unwanted inter-grown plants.

The fungi chosen for study were three isolates of *Trichoderma* spp. isolated from the soils (T-5, T-7, and T-35), three other *Trichoderma* isolates from the plant shoots (T-26, T-94, P-24) and one isolate of *S. sclerotiorum*, pathogenic on the canola plants.

The experiment was set up in two groups; one including terrestrial isolates of *Trichoderma* spp, *S. sclerotiorum* and soil herbicides; and the other including shoot isolates of *Trichoderma* spp., *S. sclerotiorum* and selective herbicides.

Cultures of the fungi on Czapek's dox agar (CDA), incubated in dark at 25°C for 5 days were used. The studied doses of individual herbicides were those recommended for canola crop. The studied doses were as Treflan® (2000, 3000 and 4000 ppm), Sonalan® (6000, and 6500 ppm), Nabu S® (1500, 5000, 7500 and 10000 ppm), Focus® (3000, 6500, 10000 and 16000 ppm), and Gallant® (1500, 6500 and 10000 ppm). No herbicide was added to the media considered for checks.

The total volume considered for each plate was 20 ml of the final medium. The experiment was performed in triplicates for each isolate and herbicides. The fungi were cultured by plating a 5 mm disc of the active culture per plate. The incubation in the case of the first part of the experiment performed under dark conditions at 25±1°C, however, with the second experiment, it was carried out in light conditions at the room temperature 25±1°C.

The results were recorded when the fungal (F) colony diameter had reached to 90 mm in control plates recorded. This time for *Trichoderma* spp. was 3 days after culture, but for *S. sclerotiorum* was 10 days. To compensate the significant difference, daily growth rate (mm/d) was considered. Also, the cultures of *S. sclerotiorum* were inspected from the viewpoint of sclerotia development. The data were statistically analysed based on the completely random design, and the comparison of the means were carried out through Duncan's test using SAS software.

Results and Discussion

The decrease in disease after herbicide application due to reduced density of the pathogen has been suggested earlier (Altman and Campbell, 1977). All the herbicides studied here, have been officially recommended for applications in rapeseed (canola) crop in Iran (Razavi, 1995). Therefore, the post-

emergence herbicides used in this study are not phytotoxic under usual conditions recommended for field applications.

No fungal isolation could grow on the media with soil herbicides trifluralin and ethal fluralin belonged to the group dinitroanilines. Infact, these herbicides had shown really the high antifungal activities and did not permit any growth of fungi on CDA media under incubation conditions. Even after several additional days, no fungus could begin to grow. These results are in contrast to the results obtained by other workers (Tyunyaeva *et al*., 1974; Fontana *et al*. 1976) but in agreement with the results of Grinstein *et al*. (1976), and Makawi *et al*. (1979). Anicuta (1985) reported the favored growth of both *Fusarium oxysporum* f. *phaseoli* and *F. solani* f. *phaseoli* using Treflan® in bean (*Phaseolus vulgaris*) cultivation. It has been reported that Treflan® is utilized by *Penicillium waksmani* and *Alternaria alternata* as sole C and N source (Abushady *et al*., 1983). However, Grinstein *et al*. (1981) have reported the effect of trifulralin as a sensitizer for *Fusarium* resistance in tomatoes.

Although trifluralin and ethalfluralin aer strong against the germlings and active mycelia of white stem rot pathogen in soil, they can never impose their supressive effect on *S. sclerotiorum* when it is active inside weed plants or plant debris, because as members of dinitroaniline herbicides, they can not move inside the plant (Gunsolus and Curran, 1999). The action target of these herbicides is tubulin protein involved in plant cell division; however, it is not clear if the same mechanism of action prevents fungal growth.

Johnson (1994) has propsed a model of dose-response relationship, stating that the degree of disease control obtained with a biological agent depends on the density of the agent, the density of the pathogen, how efficiently individual units of the agent render units of the pathogen ineffective, and on the proportion of the pathogen population potentially affected by the agent. The reduction of *S. sclerotiorum* biocontrol efficacy of *Trichoderma* due to increased interactions between *Trichoderma* and soil microorganisms, and the favored shift from hyphal growth to sporulation because of the microbial competition in soil has been indicated most recently (Bae and Knudsen, 2005). The application of soil herbicides may help to control diseases in the field in soils that have had a previous history of disease occurrence. Additionally, as these herbicides are of a broad range, therefore they can undubtedly create a partial biological vaccum in the soil and favor the establishment of certain exogenously introduced or indigenous *Trichoderma* isolates, so that diseases may be suppressed (Baker, 1981; Papavizas, 1985). However, it seems that the antifungal activity of these herbicides is still a controversial subject, and there are different texts in literature (Anicuta, 1985; Abushady *et al*., 1983; Makawi

et al., 1979; Fontana *et al*., 1976; Tyunyaeva *et al*., 1974) dependant on the kind and species of the microorganisms and conditions.

Sethoxydim, cycloxydim, and haloxy fop ethoxy ethyl had shown different levels of antifungal actitvities with three *Trichoderma* isolates and an isolate of *Sclerotinia sclerotiorum* tested (Fig. 1). The daily growth mean was significatly influenced by treatments (herbicide-dose components), and the fungal factor (isolate or species). Additionally, it was under the meaningful interactive effect of treatment and fungal factors.

The first two herbicides are of cyclohexanediones (DIMs) and the latter is of propionic acid derivatives, aryloxyphenoxypropionates (FOPs), (http:www.weedresearch.com/summary/chemfamilySum.asp?lstActive=29&btnSub1=Go&lstHRAC=).

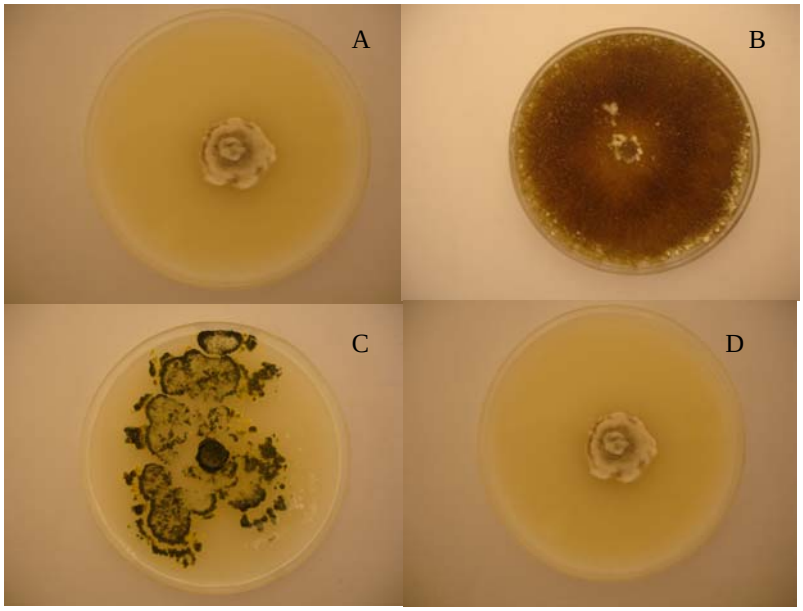

Fig. 1. Effect of sethoxydim (Nabu S) herbicide on the growth of *Sclerotinia sclerotiorum* and *Trichoderma* isolate *in vitro*: (A) suppressed growth of *Scl. sclerotiorum* on potato dextrose agar (PDA) medium including the herbicide (5000 ppm); (B) growth of *S. sclerotiorum* on PDA medium as control; (C) Growth of *Trichoderma* on the PDA medium with sethoxydim compared with it's growth on PDA medium (control) (D).

In the case of sethoxydim, the isolate P-24 had more growth rate than other isolates of *Trichoderma* on the CDA media amended with this herbicide (Table 1). There was a significant difference among the fungal isolates tested and the isolate P-24 was of the most fast daily growth rate. All three

Tichoderma isolates were of more daily growth rates compared to the isolate of *S. sclerotiorum* (Table 1).

Table 1. Comparison of fungal isolates based on their daily growth means (mm) *in vitro*.

Fungal Isolate	Daily Growth mean (mm)	Duncan's Group
P-24	11.1285	A
T-94	8.4031	B
T-26	7.7010	C
Scl	2.4567	D

In the case of haloxy fop ethoxy ethyl, with all doses tested, the isolate P-24 had more growth rate than other *Trichoderma* isolates studied (Table 2).

Finally, with cycloxydim, no isolate of *Trichoderma* could grow on the media, which contained higher doses of the herbicide (10000, and 16500 ppm), but in the case of two lower doses (3000 and 6500 ppm), the isolate P-24 reached the highest growth compared to other *Trichderma* isolates investigated.

Generally with all herbicides, the higher the dose tested, and lower the growth rate (Table 2). Collectively, the growth rate of the most tolerant isolate of *Trichoderma*, P-24 was the highest on the media with sethoxydim, and higher on the media involved haloxy fop ethoxy ethyl compared to the media contained cycloxydim (Table 2).

At the dose of 10000 ppm, three herbicides were comparable considering their antifungal activities, so that cycloxydim had shown the most antifungal activity on *Trichoderma* isolates, and sethoxydim had shown the least antifungal influence on these fungi of importance in biological control of plant diseases (Table 2). With cycloxydim and haloxy fop ethoxy ethyl, the levels of antifungal activities were confirmed considering their suppressive effects at the dose of 6500 ppm (Table 2 and 3).

With *S. sclerotiorum*, sethoxydim had the highest antifungal activity, and haloxy fop ethoxy ethyl had the least activity against the fungus. Also, while haloxy fop ethoxy ethyl and cycloxydim induced the formation of sclerotia, sethoxydim prevented the development of these resistant bodies in all the doses tested. The induction of sclerotium formation by Haloxy fop ethoxy ethyl was observed with all of the doses, however, cycloxydim had a suppressive effect on the sclerotium development when it was used at the rate of 16500 ppm.

In an experimental program of the IOBC/WPRS working group, Hassan *et al*. (1994) found that sethoxydim is one of the herbicides more toxic for some of 25 species of beneficial organisms including 3 entomogenous fungi, and concluded that more work should be carried out with this herbicide in semi-field and field experiments.

Our current findings are promising that application of sethoxydim will eliminate weeds in canola fields, and omit their harmful competitive effects and their roles in disease epidemiology; meanwhile, will impose a preventive effect on the growth of *S. sclerotiorum* with less deleterious effects on the growth of *Trichoderma* spp. with saprophytic competition abilities more than those of *S. sclerotiorum*, and thus able to take advantage from the dead weeds residues to raise their own populations and control the disease. Sethoxydim as a herbicide from cyclohexanediones belongs to lipid synthesis inhibitors, i. e. it prevents the enzyme acetyl coenzyme A carboxylase (ACCase) involved in the formation of fatty acids which are the essential components for the *in planta* production of lipids. Lipids are vital to the integrity of cell membranes and to a new plant growth. The lipid synthesis inhibitor herbicides inhibit a single key enzyme involved in fatty acid biosynthesis. These herbicides are taken up by the foliage and move in the phloem to the new growth areas (Gunsolus and Curran, 1999), therefore, sethoxydim might be potentially much effective on the control of rapessed white stem rot disease, as it may have its controlling effect against the pathogen even inside the treated weed plants infected by *S. sclerotiorum.*

Also, as a post-emergent herbicide, its persistence might be lengthened by the shading of the soil surface underneath the plant canopy, which would reduce herbicide volatility and photo degradation (Klingman, 1961). Such an increased persistence has been explained as the reason for the sufficiency of a single post emergence application of the dinitrophenol herbicides for the suppression of Sclerotinia blight as effectively as multiple applications (Porter and Rud, 1980).

On the other hand, if the same mechanism of action is involved in the antifungal activity of sethoxydim against the pathogen like what occurs *in planta*, it may act synergistically together with *Trichoderma* antibiotic peptides, peptaibiotics, or peptaibols. This is expectable as the antimicrobial activity of peptaibols arise from their membrane activity and their ability to form pores in lipid membranes. The pores so formed are able to conduct ionic species; this conductance leads to the loss of osmotic balance and cell death (Chugh and Wallace, 2001; El Hajji *et al*., 1989; Le Doan *et al*., 1986; Molle *et al*., 1987). The last promising point with sethoxydim can be regarded here, is its suppressive impression on the development of sclerotia as observed in this

study. Indeed, the inhibitory effect of sethoxydim on the *S. sclerotiorum* acetyl-CoA carboxylase might be the true reason for its effect on the fungus, as with *Aspergillus fumigatus*, the enzyme has been found essential for survival. Essential genes are those required for growth (metabolism, division, or reproduction) and survival of an organism. It is known that fungal biosynthesis of fatty acids takes place in the cytosol and starts with carboxylation of acetyl-CoA to malonyl-CoA. From this malonyl-CoA consecutive C2 units are added to acetyl-CoA or the growing fatty-CoA ester chain by an intericate fatty acid synthase complex harboring seven different enzymatic activities (http://www.wipo.int/cgi-pct/guest/getbykey5?KEY=00/ 39287.000706&ELEMENT_SET=DECL).

Therefore, our results prove that ACCase can be regarded as a new target for the production of a new generation of antimycotics and fungicides that will be of more importance considering the medical problems encountered with the control of the fungal diseases caused by the strains resistant to the applied fungicides and antimycotics. However, it should not be ignored that such a persuptive generation of fungicides and antimycotics shall be applied in a well-planned manner, as because of their single target site of antifungal effect, the probabilty of fungicide resistance development is expected high. The results from our experiment confirm that there are at least some fungal species that are naturally more resistant.

Table 2. The effect of different herbicide-dose components on the daily growth means of *Trichoderma* isolates (P24, T94, T26) and an isolate of *Sclerotinia sclerotiorum* (Scl).

Fungus-Treatment (Herbicide and dose in ppm)	Daily Growth Mean (mm)	Duncan's Group
P24-Control	30.0000	A
T94-Control	30.0000	A
T26-Control	30.0000	A
P24-Nabu S 5000	14.7767	B
P24-Nabu S 7500	13.5567	C
P24-Gallant 1500	13.0000	C
P24-Gallant 3000	12.7767	C
P24-Gallant 6500	11.6667	D
P24-Nabu S 10000	11.5567	D
T94-Gallant 1500	10.6700	DE
T94-Nabu S 5000	10.6667	DE
T94-Gallant 3000	10.2233	E
T94-Nabu S 7500	10.2233	E
P24-Nabu S 15000	10.1100	E
P24-Gallant 10000	10.0000	EF
T26-Nabu S 5000	9.1100	FG
Scl-Control	9.0000	G
T26-Gallant 1500	8.5567	G
T26-Gallant 3000	8.2200	G
T94-Nabu S 10000	7.0000	H
T26-Nabu S 10000	7.0000	H
T26-Nabu S 7500	6.8867	H
T94-Focus 3000	6.7800	H
T26-Gallant 6500	6.7767	H
P24-Focus 3000	6.7767	H
T26-Focus 3000	6.1100	H
P24-Focus 6500	5.1100	I
T94-Gallant 6500	4.7767	IJ
T94-Nabu S 15000	4.5567	IJ
T26-Gallant 10000	4.5567	IJ
T94-Focus 6500	4.5567	IJ
T94-Gallant 10000	4.4467	IJ
T26-Focus 6500	3.7800	JK
T26-Nabu S 15000	3.7767	JK
Scl-Gallant 3000	2.8767	KL
T26-Focus 16500	2.6700	LM
T26-Focus 10000	2.6700	LM
T94-Focus 10000	2.6700	LM
T94-Focus 16500	2.6700	LM
P24-Focus 16500	2.6700	LM
P24-Focus 10000	2.6700	LM
Scl-Gallant 1500	2.6133	LM
Scl-Gallant 6500	2.5833	LM
Scl-Gallant 10000	2.4333	LMN
Scl-Focus 3000	2.1433	LMNO
Scl-Nabu S 5000	1.9900	LMNOP
Scl-Focus 6500	1.8767	LMNOP
Scl-Nabu S 7500	1.6000	MNOP
Scl-Focus 10000	1.5000	NOP
Scl-Nabu S 10000	1.3100	OP
Scl-Nabu S 15000	1.0433	P
Scl-Focus 16500	0.9667	P

Table 3. The total effect of different herbicide-dose treatments on the fungal daily growth mean

Herbicide (ppm)	Daily Growth Mean (mm)	Duncan's Group
No herbicide (Control)	24.750	A
Nabu S (5000)	9.1358	B
Gallant (1500)	8.7100	BC
Gallant (3000)	8.5242	CD
Nabu S (7500)	8.0667	D
Nabu S (10000)	6.7167	E
Gallant (6500)	6.4508	E
Focus (3000)	5.4525	F
Gallant (10000)	5.3592	F
Nabu S (15000)	4.8717	G
Focus (6500)	3.8308	H
Focus (10000)	2.3775	I
Focus (16500)	2.2442	I

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