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Research Article

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Antifungal activity of synthesized dithiocarbamate derivatives on *Fusarium oxysporum f sp. albedinis* in Algeria

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ABSTRACT

The date palm is threatened both in Morocco and recently in Algeria by a vascular fusariose, called "Bayoud", where the causal agent is a fungus; Fusarium oxysporum f sp. albedinis (Foa). A variety of dithiocarbamic ester (P1-P15) has been prepared by the condensation of lithium dithiocarbamate salt with natural substances, itols and carbohydrates, to test their antifungal activities. The in vitro antifungal activity of these new compounds against this fungus was evaluated by the growth inhibitory effect, at concentrations of 200, 100, 60, 40, 20 and 10 ppm dissolved in DMSO and placed on PDA plates at 22° C. All inhibition observations were normalized as percentage inhibition (pI) compared to the control plates using only DMSO. At 200 ppm most of our products inhibit the growth of Foa with percentages of inhibition varying between 2.16 % (P14) and 64.23 % (P3). The three dithiocarbamic esters P3, P4 and P9 exhibited the highest inhibition toward mycelial growth of Foa (39–64%). Based on these results, large scale testing on date palm of these products is warranted.

Key words: Dithiocarbamates, Carbohydrates, Itols, Antifungal activity, Date palm, *Fusarium oxysporum f sp. albedinis*

INTRODUCTION

The date palm (*Phoenix dactylifera*) is a cultivated tree representing a major economic value for several populations in the arid regions. It is one of the oldest fruit trees in the world. Furthermore, on a commercial scale the Middle East and North Africa are the major date palm producing areas in the world. Date palm trees are essential integral components of farming systems in dry and semi-arid regions and can be produced equally well in small farm units or as larger scale commercial plantation units. The date palm also makes a significant contribution towards the creation of equable microclimates within oasis ecosystems. Plants exist in an environment rich in pathogens including bacteria, fungi and viruses at every stage in their lifecycle. Although plants have no immune system like mammalians to protect them from invading pathogens, the vascular fusariosis commonly named *Bayoud*, caused by *Fusarium oxysporum* f.sp. *albedinis* (Foa) is the most destructive fungal disease of this date palm (Djerbi M. et *al*., 1991). The impact of this disease is most severe in North Africa particularly in Morocco where 2/3 of palm trees have been destroyed so far (Fernandez et *al*., 1997) and recently in Algeria, where data indicate that fungi affect half the date plantations in western Algeria. Plantations in the east of Ghardai'a are still unaffected (Macheix 1992; Brac de la Perrière et *al*., 1995). Various combinations of techniques were proposed by integrated *Bayoud* control, based on more than 20 years of research, essentially in North African laboratories (Freeman and Maymon, 2000). Biotechnology tools of tissue culture and genetic engineering can speed up all of the above processes and in addition chemical treatment can be used to eradicate this fungus at large scale (Jasso de Rodriguez et *al*., 2007). Interestingly, Foa shows a very low genetic diversity (Bendiab et *al*., 1992). Biological control of *Bayoud* disease in date palm has recently been realized by a selection of microorganisms inhibiting the causal agent and inducing defense reactions (El Hadrami et *al*., 1996; El Hassni et *al*., 2007). Other techniques make use of new phenolic compounds to stop

the development of this fungi (Ramos et *al*., 1996; Daayf et *al*., 2003) by the synthesis of low-molecular mass inhibitory compounds such as elanins, tannins or phytoalexins, and the accumulation of some peptides and proteins with ability to inhibit the growth of pathogens (Sitohy et *al*., 2007). Little is known until now about chemical ways to test the synthesized compounds as antifungal activity against the Foa (Loginova et *al*., 2006).

The present work evaluates the antifungal activity of novel synthesized dithiocarbamic esters, derivatives of saccharides and itols, against mycelial growth of Foa by the measurement of the percentages of inhibition.

EXPERIMENTAL SECTION

Chemicals

A series of sucrodithiocarbamic esters were prepared (Len et *al*., 2000; 2001) to be tested as anti-fungal agents. The monosacharides and itols (Su $(OH)_n$) were protected with isopropylidene to obtain the corresponding Sup_iOH which were actived by iodo at the non protected site. They were subsequently reacted with a lithium *NN*-dialkyldithiocarbamate salt to obtain the correspondents dithiocarbamic esters Sup-S-CS-N(R_1R_2). All the esters were characterized by lH- and ^{13}C-NMR spectra recorded on a Bruker AM 300 (operating at 300.13 MHz for ^{1}H and at 75.47 MHz for ^{13}C) spectrometer, and elementary analysis. These compounds are used as listed in Table 1. All chemical reagents used were grade quality.

Table 1: Listing of the synthesized dithiocarbamic esters and their code

Product code	Nomoclature
P1	Methyl dimethyl dithiocarbamate
P2	Methyl morpholine dithiocarbamate
P3	Methyl piperidine dithiocarbamate
P4	Methyl trimethylpipéridine dithiocarbamate
P5	Glycerol dipropyldithiocarbamate
P6	Glycerol morpholine dithiocarbamate
P7	Glycerol pyrrolidine dithiocarbamate
P8	Glycerol piperidine dithiocarbamate
P9	Diacetone xylitol piperidine dithiocarbamate
P10	Diacetone xylitol diethyl dithiocarbamate
P11	Diacetone xylitol morpholine dithiocarbamate
P12	Solketal pyrrolidine dithiocarbamate
P13	Solketal morpholine dithiocarbamate
P14	Galactose piperidine dithiocarbamate
P15	Galactose pyrrolidine dithiocarbamate

Stock Culture

At stock culture of Foa was obtained from the Biological Laboratory of Bechar university center by Dr. Moussaoui A. The testrun was isolated from a date palm infected by the vascular fusariose, and was maintained on PDA at 18° C in phytotron room. Fungal growth plugs were cut using a 4.5 cm cork borer and transferred from stock plates to fresh agar biweekly to maintain actively growing fungi.

Evaluation of Dithiocarbamates Effect on Foa Mycelial Growth

After purification and characterization of dithiocarbamates series each compound was dissolved with 10% DMSO (Sigma Chemical, France) by stirring for 30 min at 25° C, and then sterilized by filtration using 0.22 μm filter (Whatman, UK).

For incorporation into media and treatment against Foa, synthesized dithiocarbamic esters, at concentrations of 200, 100, 60, 40, 20 and 10 ppm, dissolved in DMSO were added in sterile potato dextrose agar (PDA) and then adjusted to pH 7.4 with 2 N NaOH. The sealed Petri dishes were incubated aerobically at 22° C for 72 hours in a humid chamber. The diameter of the colonies was measured daily (24, 48 and 72 hours) until the leading edge of the fastest-growing colony had reached the edge of the Petri dish. For growth inhibition analysis, colony diameter data taken after 72 hours were used. The inhibitory activity of each treatment was expressed as the percent growth inhibition compared to the negative control (0 %) using the following formula, where DC= diameter of control, and DT = diameter of fungal colony with treatment:

$$\text{growth inhibition}\,(\%) = \left[\frac{DC - DT}{DC}\right] \times 100$$

The dilutions of the compounds (200–10 ppm) were made by dispensing the solutions to the remaining discs.

The results were obtained as inhibition zone diameter (mm) of three measurements, and the data presented here are the average of three experiments.

Thermal Stability Determination

For thermal stability determination, the synthesized dithiocarbamate esters in medium of Foa growth were incubated at different temperatures (22, 37, 45, 55 and 65° C) for 0, 10, 20, 30 and 60 min. After cooling the treated esters on ice for 10 min, the residual antifungal activities were measured as described (Yang et *al*., 2006).

Statistical Analysis

Analysis of variance was used to evaluate the effects of experiment, carbamic esters concentration, Foa isolate carbamic esters concentration and Foa isolate interactions on colony diameter. Mean colony diameters for the Foa isolate at each carbamic ester concentration and the corresponding standard errors of the mean (SEM) were calculated. $p<0,05$ values were considered as significant.

RESULTS

Stability of Dithiocarbamic esters

In the first set of experience, the synthesized dithiocarbamic esters displayed thermo stability in antifungal activity, retaining 59% activity at 55° C for 1 hour and 79% of activity at 65° C for 20 min. At 22, 37 and 45° C, the antifungal activity toward all esters was above 85% (Figure 1).

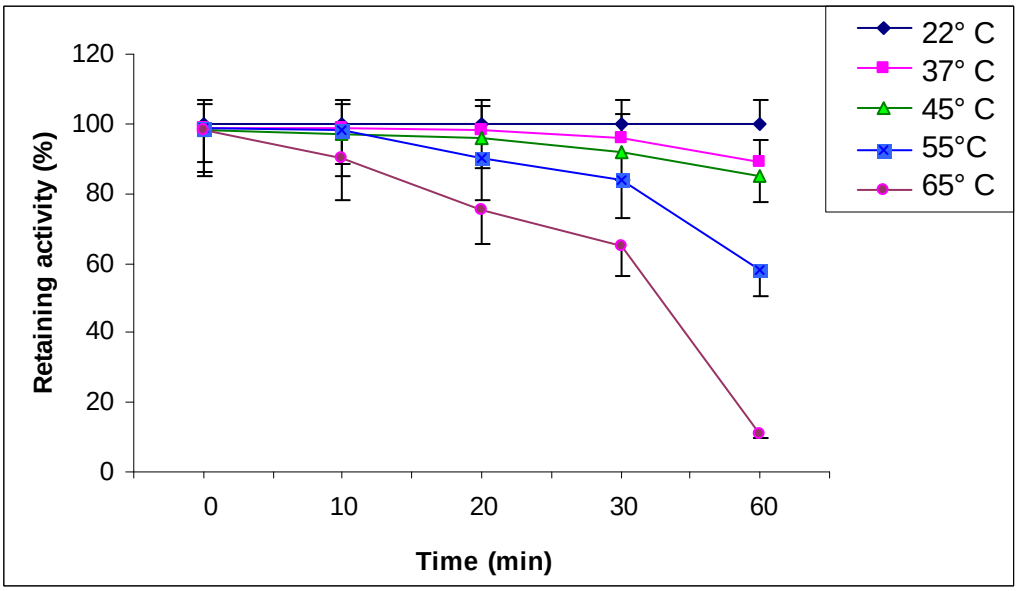

Figure 1: Thermal stability of the dithiocarbamic ester P5. The residual activity of the P5 was measured after 60 min pre-incubation at different temperatures

Data are expressed as averages with standard errors of duplicate measurement

There was no inhibitory activity in the disc plate containing only DMSO 100% solution used as control. As shown in Figure 2, several dithiocarbamic esters, (P5, P6, P11 and P12) showed a minor inhibitory effect against Foa (less than 25% inhibition), whereas P1, P3, P4, P9 and P10 showed a strong inhibition (above the 50%) at 200 ppm. None of the tested concentrations of P2, P7, P8, P14, P13 and P15 were able to inhibit above 8% of the growth, but the highest effect and the most significant growth inhibition (64.23%) were observed with **P3** at 200 ppm (Figure 2).

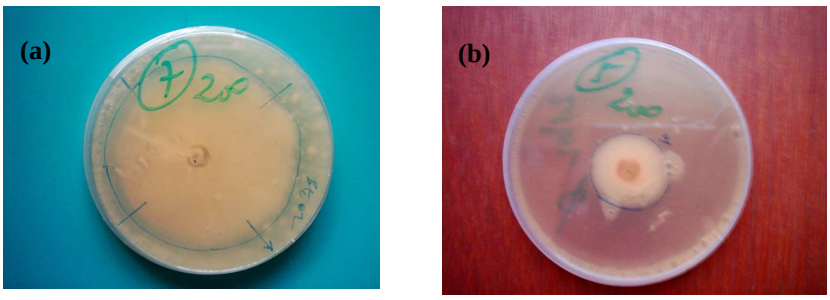

Figure 2. Colony diameter of isolate of *Fusarium oxysporum* f. sp. *albidinis* on media PDA amended with different concentrations of carbamic esters: (a) P5 with pI =19.12% and (b) P3 with pI =64.23%

In order to understand the structure-activity relationships of the analogue series of dithiocarbamic esters, we have divided them in groups according to their carbohydrates: galactose, glycerol, methyl, xylitol and solketal. At a concentration of 200 ppm, compounds P1, P3, and P4 derived from methyl, showed the highest mycelial growth inhibition of the phytopathogenic fungus Foa (PI= 35.58, 64.23 and 47.89%, respectively). They have as alkyl substrates, dimethyl, piperidine and trimethylpiperidine, respectively. Compound P2 (PI= -3.22 %) differed by having morpholine as alkyl substituent. This difference was significantly relative to the fungicide Ziram (zinc dimethyldithiocarbamate) (PI = 21.6%; p< 0.01) used as a reference (Len et *al*., 1997a).

The nature of a carbohydrate moiety derivatives of glycerol and it protected form by isopropylidene, the solketal, and D-galactose in the compounds P5, 6, 7, 8, 12, 13, 14 and P15 was not significant in inhibiting mycelial growth of Foa at 200 ppm, resulting in lower than 25% growth inhibition, what ever the type of alkyl (Figure 3). All these compounds were as efficient as Ziram. The diisopropylidene-xylitol moiety was important as vector on the antifungal activity of the dithiocarbamates, P9 and P10 with piperidine and diethyl as alkyls, respectively. They had a higher activity against Foa mycelium growth (pI= 55.9 and 45.2%, respectively), whereas, the P11 derived from the same vector but with different alkyl, morpholine exhibited a lower antifungal efficacy (pI= 12.70%). This was also observed *in vitro* studies conducted with others fungal pathogens (Len et *al*., 1997b).

These results showed that the type of carbohydrate moieties was important for the biological activity since the P1, P3, P4, P9 and P10 have selective antifungal activities at different stages of the fungal growth.

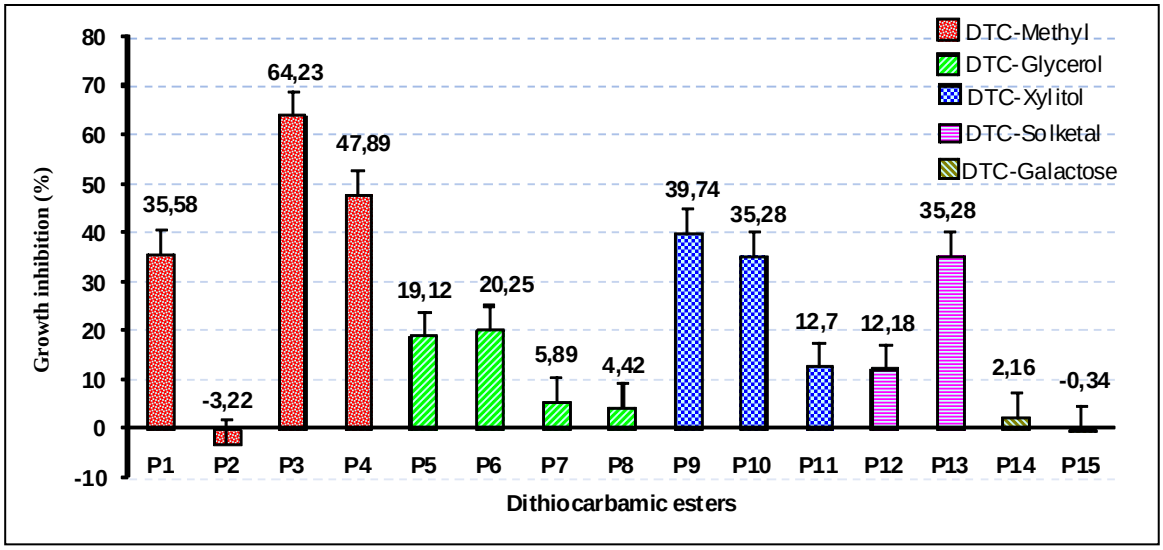

Figure 3. Mycelial growth inhibition percentage of Foa on media amended with 200 ppm concentration of each dithiocarbamic ester
Data are expressed as averages with standard errors of triplicate measurement

To examine the minimum dithiocarbamic esters concentrations required for the maximum inhibition of radial macroscopic growth of the mycelium *Foa*, dose-response curves were measured for these fungi *in vitro* disc assays. As shown in Figure 4, the inhibitory activity of P1, P3, P4, P9 and P10 compounds against the tested fungi varied significantly. The lowest concentration (< 80 ppm) of all compounds showed a moderate inhibition, with less than 25% inhibition.

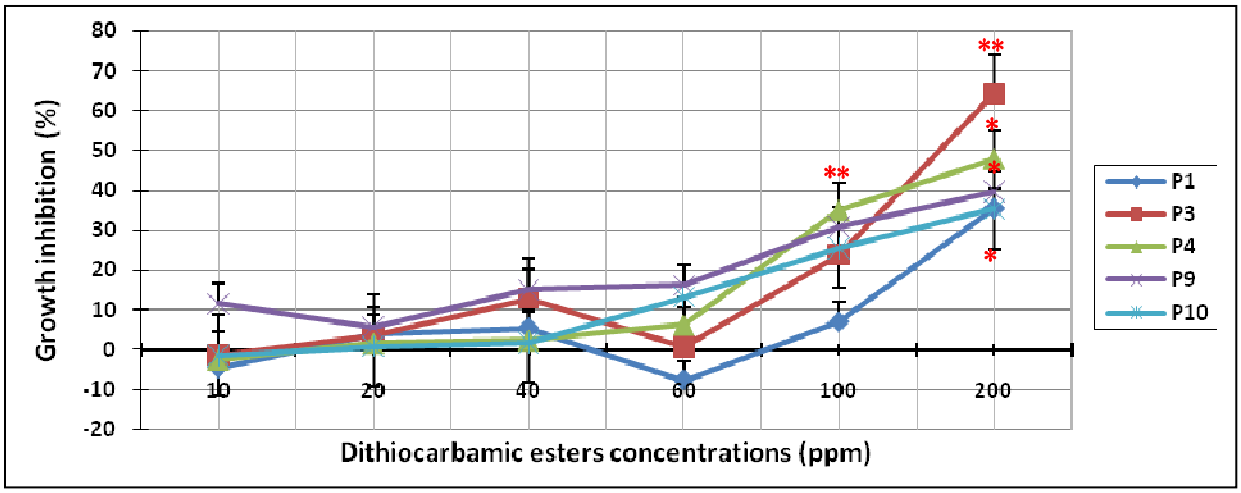

Figure 4: Dose-dependent effect of the bioactive dithiocarbamates *in vitro* as antifungal compounds against Foa fungal growth
Results were evaluated after incubation for 72 h at 22 ° C. Data are expressed as averages with standard errors of triplicate measurement
** P<0,05 ; ** P<0,001*

The inhibition of the Foa isolate *in vitro* by dithiocarbamate esters was also tested at 100 ppm concentration during five days in order to determine the time to stop its development. The role of these compounds as an active inhibitor of fungal growth is little documented. A time of 72 hours was selected to determine the final inhibition of Foa after treatment with dithiocarbamate derivatives P1, P3, P4, P9 and P10 (Figure 5).

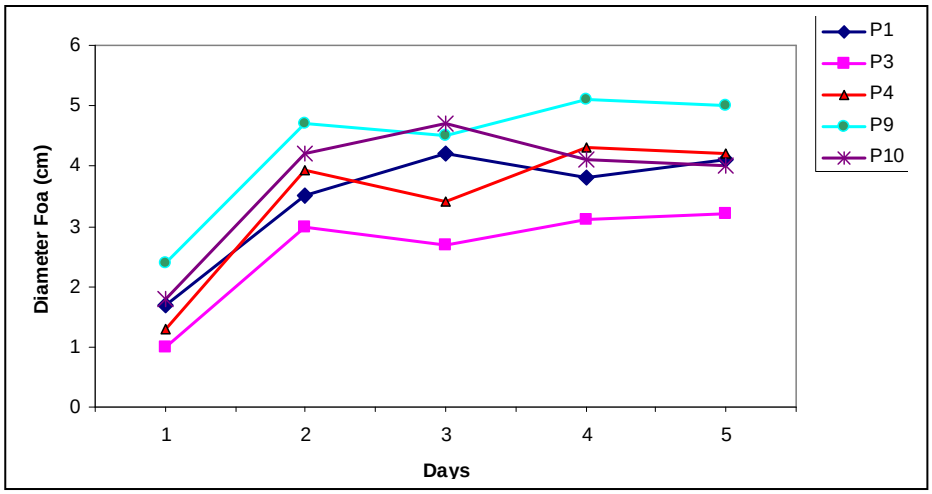

Figure 5: Time course of Foa diameters after treatment with dithiocarbamate derivatives P1, P3, P4, P9 and P10. at concentration of 100 ppm during five days

DISCUSSION

Most antifungal studies of plant extract have been carried out on various plant pathologic funguses (Woldmichael and wink, 2001; Escalante et *al*., 2002; Iorizzi et *al*., 2002). Dithiocarbamates are the most heavily used organic fungicides in terms of tonnages (Corbaz, 1990). They are widely used for the treatment of soil, seeds, and foliar and postharvest diseases affecting several types of crops. Little resistance of these polyvalent fungicides has been observed due to their multisite mode action (Lepoivre, 1989).

The present study developed within this context attempts to test the antifungal effects of several compounds, such as dithiocarbamic esters possessing a carbohydrate moiety derived from D-galactose, glycerol and xylitol with *N,N*-dimethyl, -dipropyl, -morpholine, -piperidine, -pyrolidine, or -trimethylpiperidine carbamoyl groups against Foa, for more environmentally and toxicologically safe and more effective efficacious antifungal. We have compared the antifungal potency of these different esters to commercial dithiocarbamate compound, Ziram (zinc dimethyldithiocarbamate) which used as reference compound (Len et *al*., 2000; 2001)

To our knowledge, this research study is the first report about the antifungal compounds against date pathogenic fungi. Further research on the synthetic of the active principle(s) of dithiocarbamic esters is in progressive process in our laboratory in order to discover new and potent antifungal compound(s).

The success of a biological control agent in turning-on plant defense mechanisms against pathogens depends of their ability to establish metabolically active populations that could mediate host protection and/or compete directly or indirectly with the pathogens for nutrient resources. Yet, the use of chemical agents such as our carbamic esters with natural subtracts (sugar or itols), which have no toxicity to enhance the plant defense mechanisms against pathogens represents an ecologically friendly alternative to ordinary pesticides repeatedly used to control plant diseases.

This strategy has more significance against *Bayoud* on date palm at large scale. The deployment of such strategies should have an impact taking into account the perennial aspect of the crop, the socio-economical and ecological issues to use these esters on date palm groves and the long-and large term solution.

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