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Biological Evaluation of 1, 4-Disubstituted 1,2,3-Triazole Derivatives as Plant Pathogenic Fungus Inhibitors

KEYWORDS: 1,2,3-triazoles; Verticillium dahliae Kleb; Fusarium oxysporum f. sp albedinis; Antifungal activity.

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ABSTRACT *In order to create novel potent antifungal agents, the antifungal effects of 1,4-dissubstitued 1,2,3-triazole derivatives on plant pathogenic fungi were evaluated for bioactivity against two of the most devastating fungal Verticillium dahliae Klep(Vd) and Fusarium oxysporum f.sp. albedinis (Foa) using the mycelia growth rate method and sporulation. Some of the compounds showed definite activities in vitro against the tested fungi at 20 µg/ml. For instance, Compound 2h can be used as a good starting point for further optimization.*

1. Introduction.

Verticillium wilt, caused by Verticillium dahliae Kleb., is one of the most serious diseases affecting olive tree world-wide. It causes severe losses to olive plantations in the Mediterranean region (Heale, 2000). Many factors may account for the spread of the disease, including the dissemination by seed; this factor has been documented in several crop species (Toit et al., 2005; Valled et al., 2005). Another important fungal organism of cultivated soils are Fusarium oxysporum species (Jaiti et al., 2008 ; Yahyi et al., 2007 ; Bouizgarne et al., 2006).The number of pathogen forms of F. oxysporum is estimated at 80 forms, some of them were capable of producing a plant disease for more than one plant family such as F. oxysporum f.sp. albedinis (Sedra et al., 1998 ; Bautisa et al., 2006 ; Daboussi et al., 2003). The impact of this disease is so serious in North Africa where losses are increasing and may be a threat for palm-groves around the world

1,2,3-Triazole and its derivatives are an important class of nitrogen-containing aromatic heterocyclic compounds, and have attracted a great deal of interest due to their diverse biological activities such as fungicidal (Yu et al., 2009 ; Wang et al., 2010)insecticidal (Cudworth et al., 2007; Chai et al.,2003) herbicidal (Ma et al., 2006) and bactericidal (Shi et al., 2011; Kaplan et al., 2008). It may also serve as a plant growth regulatory agent (Liu et al., 2006) and has excellent potential in the pesticide field. Since the discovery of triadimefon by Bayer in 1976, triazole has been used as fungicide for around 30 years. It gained an importance in the protection of various crops, representing a significant progress in the chemical control of fungal diseases.

Encouraged by these biological properties, and there is an urgent need to discover efficacious new antifungal agents, we reported herein the fungicidal effect of a new synthetic serie of 1,4-dissubstitued 1,2,3-triazoles derivatives Figure 1 against Verticillium dahliae Kleb and Fusarium oxysporum f. sp albedinis.

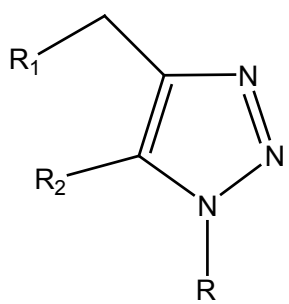

FigurE 1. General Structure of compounds 1 a-d , 2 a-h ; 3 a-h.

ENTRY	R	R_1	R_2
1a	benzyl	CO2Et	H
1b	benzyl	CO2Et	CO2Et
1c	TBRB *	CO2Et	H
1d	TBRB *	CO2Et	CO2Et
2a	benzyl	uracil	H
2b	benzyl	thymine	H
2c	benzyl	azauracil	H
2d	benzyl	5-F-uracil	H
2e	benzyl	5-Cl-uracil	H
2f	benzyl	5-I-uracil	H
2g	benzyl	5-Br-uracil	H
2h	benzyl	adenine	H
3a	TBRB *	uracil	H
3b	TBRB *	thymine	H
3c	TBRB *	azauracil	H
3d	TBRB *	5-F-uracil	H
3e	TBRB *	5-Cl-uracil	H
3f	TBRB *	5-I-uracil	H
3g	TBRB *	5-Br-uracil	H
3h	TBRB *	adenine	H

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With 2',3',5'-tri-O-benzoylribofuranose (TBRB*).

2. Experimental

General

For all the synthesized compounds, we prepared a solution Under aseptic and sterilized condition; In 250 ml Erlenmeyer flask , 20 µg/ml of each compound were dissolved in 0,1% dimethyl sulfoxide (DMSO), added to 100 ml of CZAPECK solid medium (sucrose 30 g, sodium nitrate 2g, dipotassium phosphate 1g, magnesium sulphate 0.5 g, potassium chloride 0.5g, ferrous sulphate 0.010g, agar 15 g, and final pH (at 25°C) is 7.3±0.2). Suspended 49.01 grams in 1000 ml distilled water and heated the mixture to boiling in the aim to dissolve the medium completely and sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes). and then poured it into Petri dishes.. Control set was made using the 0,1% dimethyl sulfoxide (DMSO without any of the tested materials and used as a negative control. The fungicide standard, Pelt which contains 70% methyl thiophanate (Trademark: PROCIDA GROUPS ROUSSEL UCLAF) was used as a positive control. Mycelial discs of 5 mm diameter, from young (5-days-old) grown cultures of Verticillium dahliae and Fusarium oxysporum f. sp. Albedinis which were provided by CRRA Marrakech city Morocco (Centre Regional de Recherche Agronomique) and maintained on CZAPECK medium were placed in the center of the petri dishes. Incubation took place at 28 ° C under continuous light (Bouslim ,1996). The experiment was carried out in five replicates. Mycelial growth of colonies was estimated every 2 days for eight days of incubation by the average of two perpendicular diameters.

For the evaluation of sporulation, we used tubes containing 5 mm of slices diameter taken along the diameter of the colony of Verticillium dahliae or Fusarium oxysporum f. sp. Albedinis (8 days) in 100 ml of distilled water, two dilutions were prepared 10^{-5} and 10^{-6}, the fungal suspension was then stirred using a vortex for 20 seconds to release the spores conidiophores. For each dilution we took 1ml of the solution being distributed in a Petri dish containing CZAPECK medium. These experiments were repeated five times.

Evaluation of mycelial growth

The inhibition rate (%) of mycelial growth fungi tested is calculated using the following formula (Leroux et Credet, 1978 ; De Corato et al., 2014).

Growth inhibition rate (%) = ((Dco-Dt) / Dco) x100

Where: Dco= average colony diameter of the control (water). Dt= average colony diameter in plates treated with each compounds at the mentioned concentration

2.1. Evaluation of sporulation.

We proceeded to count the total number of spores using a Malassez cell. Values are expressed as the number of spores per unit area (mm^2). The inhibition rate (%) of the sporulation is calculated using the following formula (Leroux et Credet, 1978 ; De Corato et al., 2014).

Sporulation inhibition rate (%) = ((Nco-Nt) / Nco) x100

Where: Nco =estimated number of germinated spores of the control (water). Nt = estimated number of germinated spores of the test plate.

Statistical analysis

The trial was established as a completely randomized experimental design with five replicates. Data were subjected to analyze of variance using SPSS software V17.0. The mean values among treatments were compared by Duncan's multiple range test at a 5% (p ¼ 0.05) level to determine significant difference between the inhibition rates of various compounds at the same concentration

3. Results and Discussion

Screening of Antifungal Activity in Vitro.

Compounds 1 a-d, 2 a-h, 3 a-h were obtained by chemical synthesis (El ayadi et al., 2012 ; El ayadi et al., 2014) and their structures and substituent model are shown in Figure 1 and Table 1. 1 a-d, 2 a-h, 3 a-h were screened for antifungal activities in vitro against two phytopathogenic fungi (Verticillium dahliae Kleb and Fusarium oxysporum f. sp albedinis) at 20µg/ml; by using the mycelia linear growth rate method and sporulation method . Pelt, a Systemic Fungicide which contains 70% methyl thiophanate is used as positive control. Mean inhibition rate of all the tested compounds against the two fungi were compared by Ducan's multiple test. The results are enrolled in Table 1.

Table 1: Antifungal activities of compounds 1a-d, 2a-h, 3a-h at 20µg/ml against Verticillium dahliae Kleb and Fusarium oxysporum f. sp albedinis

Compounds	Linear growth inhibitory rates (means %)**	
	Foa	Vd
1a	12 k	4,36 g
1b	14,69 j	9,15 f
1c	45,92 e	28,19 d
1d	50,47 d	30,43 c
2a	22,84 h	15,43 e
2b	10,14 l	0,64 i
2c	10,14 l	0,43 i
2d	60,85 c	0 j
2e	10,61 l	0,43 i
2f	12,12 k	0,64 i
2g	8,51 m	0,64 i
2h	77,51 b	40,64 b
3a	40,48 f	-0,43 kl
3b	10,02 l	1,28 h
3c	8,12 m	-0,21 k
3d	51,76 d	0,43 i
3e	17,45 i	-0,21 k
3f	34,18 j	-0,64 l
3g	-1,33 n	0 j
3h	-5,21 o	-0,43 kl
Pelt	96,97 a	88,52 a

* TBRB:.,2',3',5'-tri-O-benzoylribofuranose; Vd: Verticillium dahliae Kleb; Foa.: Fusarium oxysporum f. sp albedinis

** The differences between data with the different lowercase letters within a column are significant for the same tested fungus ($p < 0.05$).

The results in Table 1 showed that all the prepared compounds 1a-d, 2a-h, 3a-h displayed activities in varying de-

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grees against each of the tested fungi at 20 µg/ml. For instance, compounds 2h, 2d, 1d and 3d ($p < 0.05$).showed the greater inhibition rates (77.51%, 60.85%, 50,47%, and 51.76%) against Fusarium oxysporum f. sp albedinis. On the other hand compounds 3h and 3g increased the mycelia growth ($p < 0.05$) with (-5.21% and -1,33%). Whereas, compounds 2h and 1d showed an average activity ($p < 0.05$) (40.64% and 30.43%) against Verticillium dahliae K. We can deduce that the prepared compounds are more effective against the Fusarium oxysporum f. sp albedinis than the Verticillium dahliae Kleb.

Structure-Activity Relationship

Qualitative structure-activity relationship was established by comparison of both the activities and structures of various test compounds 1a-d, 2a-h, 3a-h. On each fungus (Table 1), it was clearly seen that the introduction of various substituents to 1,2,3-triazol had significant effects on the activity. The result indicated that substituent R_1= Adenine and R=Benzyl 2h remarkably enhanced the activity than R_1= substituted pyrimidine (2a-g and 3a-g). Moreover the installation of the halogen group (F, Br, I, Cl) at position 5 of the uracil 2a and 3a pointed out that compounds with F at 5 position (2d and 3d) were found to have higher inhibitory activity against f. sp albedinis (60.85% and 51.76%). Furthermore the use of the protected sugar (R= TBRB) 1c, 1d, 3a-h instead of (R=benzyl) 1a, 1d, 2a-h group led to a decrease of the activity in most cases for the two fungus.

Sporulation

In order to more fully understand the antifungal activities of the compounds, we evaluated the inhibition of their sporulation (spores germination) to conclude whether the product has a fungicidal or fungistatic effect. Table 2

Table 2: Inhibition of spores germination using compounds 1a-d, 2a-h, 3a-h at 20µg/ml against Verticillium dahliae Kleb and Fusarium oxysporum f. sp albedinis

Compounds	sporulation inhibitory rates (means %)**	
	Foa	Vd
1a	-41,68 n	-15,04 j
1b	-9,98 k	-0,67 h
1c	10,41 i	14,26 e
1d	15,17 g	19,82 c
2a	21,36 d	-52,07 o
2b	-41,68 n	-81,3 r
2c	-9,95 k	-59,16 p
2d	-81,3 q	12,72 f
2e	18,7 f	19,82 c
2f	19,19 f	14,07 e
2g	-17,26 l	-65,12 q
2h	77,76 b	57,55 b
3a	19,82 e	-56,2 p
3b	-30,9 m	-8,09 i
3c	-65,12 p	15,67 d
3d	-52,07 o	-29,73 l
3e	14,07 h	-44,97 n
3f	66,55 c	10,44 g
3g	10,41 i	-37,75 m
3h	-8,09 j	-21,51 k
Pelt	100 a	100 a

** The differences between data with the different lowercase letters within a column are significant for the same tested fungus ($p < 0.05$).

Like the linear growth inhibition rate; all the tested compounds showed a different activity degree against both of the fungus (Table 2). it was clearly seen that compound 2h had also the highest sporulation inhibition rate against Foa and Vd with 77.76% and 57.55% respectively, therefore, compound 2h has a fungicidal effect. The compound 3f was found to have a low inhibitory effect on the growth against Foa but in germination assay it showed a high inhibitory (66.55%). The antifungal assay results also showed that even if the compounds 2d and 3d were found to have an effect on the mycilial growth of Foa ($p < 0.05$); these compounds were found to promote sporulation. (-81.3% and -52.07 %), thus, they have fungistatic effect. (Table 2).

4. Conclusion.

We studied the fungicidal effect of a new synthetic serie of 1,4-dissubstitued 1,2,3-triazoles derivatives against Verticillium dahliae Kleb and Fusarium oxysporum f. sp albedinis. using the mycelia growth rate method and sporulation. Some of the compounds showed definite activities in vitro against the tested fungi at 20 µg/ml. For instance, Compound 2h can be a model compound for further studies to develop new antifungal agent.

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