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Effect of the newly synthesized Pyrazole, And Pyrazolo Pyrimidine derivatives on *Pythium Aphanidermatum* (Edson) Fitzp

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Abstract

Root rot caused by *Pythium aphanidermatum* is one of diseases of olive trees. Three synthetic chemical compounds were tested to assess their inhibitory effect on *Pythium aphanidermatum* Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**), Ethyl 5-amino-7-((3-hydroxy-5-methyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**8**) and Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**9**) were synthesis to study their effect on mycelium growth, zoospore and oospore production of *P. aphanidermatum* isolated from young olive trees cultivated in Ain Seleen, Fayoum governorate, Egypt. Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**), showed high efficiency as growth inhibition of fungal mycelia, followed by (pyrazolopyrimidine 9, and then pyrazolopyridine 8 at 10 mg/ L. Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**), showed its ability to decline the production of zoo-spores and oospores of the fungus at concentration 300 mg/L, compared to other chemicals of Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**9**), Ethyl 5-amino-7-((3-hydroxy-5-methyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**8**). Rate of inhibition in all treatments reached more than 85% in case of the use of Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**). This study offers data for the use of Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**) in controlling fungal disease to olive feeder roots infected by *P. aphanidermatum.*

Keywords: Mycelium growth; Oospores; Pyrazole; Pyrazolopyrimidine; *Pythium aphanidermatum*: Zoospores

INTRODUCTION

The olive tree (*Olea europaea* L.) was considered one of the important crops in the world, which prefer to grow in the Mediterranean Basin, subtropical regions of Australia, southern Africa, and the west northern part of Saudi Arabia **(Al-Sheikh and Abdelzaher, 2012)**. Olive trees were usually attack by several pathogenic soil fungi that causing losses in crops yield **(Sergeeva *et al*., 2005).** Previous studies revealed several soil-born pathogenic fungi such as, *Rhizoctonia solani*, *Fusarium* spp, *Phytophthora* spp. and *Pythium* spp. caused diseases to olive trees **(Sergeeva *et al*., 2005; Mousa *et al*., 2006; El-Morsi *et al*., 2009; Yaseen and D'Onghia, 2012; Abdelzaher, 2013)**.

Pythium spp. survive in soil as oospores, hyphae and sporangia, which it may remain in soil for many years as oospores under unfavorable conditions. *Pythium aphanidermatum* was known as a major destructive parasite survives on residues of decomposed organic materials in most soils. This fungus become very risky pathogen to germinating seeds, seedling and feeder roots of old trees. It has a wide host range that can infected many plants and causes different diseases such as damping-off and root rot. In additions, this pathogen is capable of surviving in soil in absence of their host plants and when weather conditions become unfavorable for disease initiation and development **(Bruehl, 1987)**. This fungus penetrates tissues of the germinating seeds and young seedlings to cause infection. Therefore, many scientists consider that these fungi affect young plants and do not pose a risk for older ones of shrubs and trees. However, it was found that

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these fungi can infect small juicy root hairs that are in the absorption area in roots of trees and shrubs, resulting in negatively affects growth and fruit production **(Elnaghy *et al.*, 2014)**. It is worth mentioning that, *P. aphanidermatum* is one of the most powerful fungal pathogens that cause diseases to many plants especially in moderate, warm and hot environments.

Control of diseases caused by species of *Pythium*, including *P. aphanidermatum*, requires great effort and special fungicides **(Suleiman, 2011; Anusuya and Sathiyabama, 2014)**. It is known scientifically, that biological control methods can be applied before the expected infection process and not after the exacerbation and appearance of the disease **(Maghazy *et al.*, 2008)**. Therefore, many farmers resort to chemical control methods to eliminate the disease. One of the most famous fungicides used in biological control against pathogens, is the chemical compound "metalaxyl", but unfortunately, most of fungal pathogens had acquired immunity to this pesticide **(Abdelzaher *et al.*, 2004)**. It became necessary and important to search for new fungicides to combat these fungal diseases.

Benomyl [methyl 1-(butyl carbamoyl) benzimidazol-2-ylcarbamate], and Metalaxyl [methyl-N-(2, 6-dimethylphenyl) –N-(2-xylyl)-DL-alaninate] were systemic fungicides used in mixture against most fungi, as soil treatments for inhibition of pathogenic fungi specially *Pythium* and *Phytophthora*. **(Abdel-Fattah and Baka, 2000; Abdelzaher *et al.*, 2004)**.

Heterocyclic compounds possess great applications in agrochemical and pharmaceutical products and these compounds exhibit activity towards a specific organism. Heterocyclic system containing pyrazole ring had pharmacological properties and act as biomolecules and also have therapeutic activities **(Steinbach *et al.*, 2000; Uslaner *et al.*, 2009; Friedrich *et al.*, 2002; Hampp *et al.*, 2008, Spitz *et al.*, 1982; Luttinger *et al.*, 1987; Tsutomu and Toshitaks, 1978; García-Lozano *et al.*, 1997)**. Pyrazolopyrimidine were considered heterocyclic bioactive substances which useful as anti-inflammatory **(Quintela *et al.*, 2003)**, antifungal **(Gomha and Hassaneen, 2011)**, antibacterial **(Moukha-Chafiq *et al.*, 2002; Elkanzi *et al.*, 2019)**. Therefore, due to the antifungal activity of heterocyclic compounds, we aimed to synthesis new pyrazolopyridine 7, 8 and pyrazolopyrimidine 9.

Among the most important objectives of this study is the synthesis of chemical compounds that can control the fungus of *P. aphanidermatum*, which was isolated in this study from the root capillaries of the olive plant cultivated in Ain Seleen, Fayoum, Egypt . Effect of the synthetic chemicals (in this study) on all stages of life cycle of the fungus have been investigated.

Material and Methods

Laboratory chemicals were provided by Sigma and Aldrich. Melting points were determined by the open tube capillary method at Fisher Scientific Ltd. The careful purity of the compound was determined by thin layer chromatography (TLC) plates (silica gel G) in the solvent system. Spots were observed by UV light or by exposure to iodine vapors. IR spectra were received by PerkinElmer 1720 FT-IR spectrometer (KBr pellets). ^{1}HNMR and ^{13} CNMR spectra were obtained by Bruker Advance II400 spectrometer in DMSO-d6. The chemical shifts were expressed in ppm relative to TMS as an internal reference. Mass spectra were recorded on 70 eV EI Ms-QP 1000 EX (Shimadzu, Japan)**,** Microanalytical Center, Cairo University; Elemental analysis of the new synthesized compounds was obtained by elemental analyzer at Cairo University Unit, Egypt.

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Ethyl 5-amino-7-((1-cyano-2-ethoxy-2-oxoethyl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**3**).

A ethyl 5, 7-diamino-3, 4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate **1** (**Elkanzi N.A.A.2019**) (0.01mol, 2.39g) was diazotized By sodium nitrite and concentrated hydrochloric acid at 0°C, The resulting solution of diazonium salt was then added with continuous stirring to cyanoethylacetate (0.01 mol, 1.13g) to afford the corresponding **3** The precipitated product, formed upon the addition of 10% w/v sodium chloride, was filtered off, washed with 10% brine solution and dried in an oven at 50°C to afford corresponding compound **3** as orange crystals, 68% yield. M.P. 210-212°C; IR (KBr): ν (cm-1), 3100-3400 (N-H, NH_2), 2216(CN),1735-1740 (2C=O ester); ^{1}H-NMR (δ, DMSO-d_6):1,23(t, *J* = 7.53 *Hz*,3H,$CH_2\underline{CH_3}$), 1.27 (t, *J* = 7.51 *Hz* ,3H, $CH_2\underline{CH_3}$), 1.98 (t, 2H, CH_2), 2.5 (m, 2H, CH_2), 3,41(s,1H,CH-CN),3.61(s, 1H, N-H), 4.23(q, *J* = 7.51 *Hz*,2H,$\underline{CH_2}CH_3$),4.48 (q, *J* = 7.52 *Hz*, 2H, $\underline{CH_2}CH_3$), 4.41 (t, 2H, CH_2O), 4.48 (s, 1H, CH-N-H), 5.56 (s, 2H, NH_2); ^{13}C-NMR (DMSO-d6): 15.16 ,15.89($2CH_3$),56.12(CH-CN),61.54, 62.44 (CH_2ester), 21.83 (CH_2), 63.69 (CH_2), 18.38 (CH_2), 56.84 (C-NH_2),111.13(C=N), 115.67(CN),156.77, 83.46, 167.3 (C=O), 104.73 (C-C=O), 59.88 (CH-NH_2); MS (m/z, %): 363 (M+, 35). Anal. Calcd for: $C_{16}H_{21}N_5O_5$ (363.37): C,

52.89; H, 5.83; N, 19.27 %. Found: C, 52.82; H, 5.78; N, 19.20%.

Ethyl 5-amino-7-((2,4-dioxopentan-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(4)

A solution of diazonium salt **2** (0.01mol) was added drop by drop with stirring to acetyl acetone (0.01 mol, 1g) to afford the corresponding **4** The precipitated product, formed upon the addition of 10% w/v sodium chloride, was filtered off, washed with 10% brine solution and dried in an oven at 50°Cto afford corresponding compound **4** as orange crystals, 63% yield. M.P. 225-227°C; IR (KBr): ν (cm^{-1}), 3100-3400 (N-H, NH_2), 1655-1665 (2C=O), 1740 (C=O ester); ^{1}H-NMR (δ, DMSO-d6): 1.28 (t, *J* = 7.54 *Hz* ,3H, $CH_2\underline{CH_3}$), 1.98 (t, 2H, CH_2),2.35(s,6H,2CO$\underline{CH_3}$), 2.5 (m, 2H, CH_2), 3.57(s, 1H, N-H),6.56(s,1H,CH-N=N), 4.47 (q, *J* = 7.53 *Hz* ,2H, $\underline{CH_2}CH_3$), 4.49 (s, 1H, CH-N-H), 4.41 (t, 2H, $\underline{CH_2}O$), 5.56 (s, 2H, NH_2); ^{13}C-NMR (DMSO-d_6): 15.12 ($CH_2\underline{CH_3}$), 27.21(2CO$\underline{CH_3}$),62.44 ($\underline{CH_2}$C=O ester), 21.83 (CH_2), 63.69 ($\underline{CH_2}O$), 18.38 (CH_2), 90.23(CH-N=N), 156.77, 83.46, 167.3 (C=C,C=O_{ester}), 104.73 (C-C=O), 59.89 (C-NH_2); MS (m/z, %): 350 (M+, 45). Anal. Calcd for: $C_{16}H_{22}N_4O_5$ (350.37): C, 54.85; H, 6.33; N, 15.99; Found; C, 54.82; H, 6.27; N, 15.93.

Ethyl 5-amino-7-((1-ethoxy-1,3-dioxobutan-2-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(5)

A solution of diazonium salt **2** (0.01mol) was added drop by drop with stirring to ethyl acetoacetate (0.01 mol, 113g) to afford the corresponding **5** The precipitated product, formed upon the addition of 10% w/v sodium chloride, was filtered off, washed with 10% brine solution and dried in an oven at 50°Cto afford corresponding compound **5** as orange crystals, 64% yield. M.P. 264–266°C; IR (KBr): ν (cm^{-1}), 3100-3400 (N-H, NH_2),1655-1665(C=O), 1737-1740 (2C=O ester); ^{1}H-NMR (δ, DMSO-d6): 1.24(t, *J* = 7.57 *Hz* ,3H,$CH_2\underline{CH_3}$),1.28 (t, 3H, $CH_2\underline{CH_3}$), 1.98 (t, 2H, CH_2), 2.5 (m, 2H, CH_2), 2.61(s,3H,CH_3),3.57(s, 1H, NH), 3.35(s,1H,$\underline{CH}$-N=N), 4.43(q, *J* = 7.55 *Hz* ,2H,$\underline{CH_2}CH_3$),4.47 (q, 2H, $\underline{CH_2}CH_3$), 4.49 (s, 1H, $\underline{CH}$-NH), 4.41 (t, 2H, $\underline{CH_2}O$), 5.56 (s, 2H, NH_2); ^{13}C-NMR (DMSO-d_6): 14.32,15.12 (2$\underline{CH_3}CH_2$), 27.24(CH_3),61.14,62.44 (2$\underline{CH_2}CH_3$ester), 21.83 (CH_2), 67.69 (CH_2), 23.97 (CH_2), 82.21(CH-N=N),165.88, 86.46, 1661.23,164.53 (C=C,C=O), 104.73 (C-C=O), 115.36,141.27 (C=C-NH_2); MS (m/z, %): 380 (M+, 25). Anal. Calcd for: $C_{17}H_{24}N_4O_6$ (380.40); C, 53.68; H, 6.36; N, 14.73; Found: C, 53.72; H, 6.38; N, 14.78.

General procedure for synthesis of compounds 6, 7, 8 and 9

Hydrazine hydrate (0.05mol) was added to a neutral solution of **3, 4, and 5**(0.01 mole). The reaction mixture was heated under reflux for 3-4 h, then cooled to room temperature and the precipitated dye intermediate that isolated by the addition of sodium chloride (5% w/v) was filtered off, and dried in a vacuum oven at 50°C to give dye intermediate **6**, **7, 8**

Ethyl 5-amino-7-((5-amino-3-hydroxy-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(6)

Red crystals, 69% yield ,M.P. 230–232°C; IR (KBr): ν (cm^{-1}),3450(O-H), 3100-3400 (2N-H, $2NH_2$), 1740 (C=O ester),1550(N=N); ^{1}H-NMR (δ, DMSO-d_6): 1.28 (t, 3H, $\underline{CH_3}CH_2$), 1.98 (t, *J* = 7.53 *Hz*, 2H, CH_2), 2.5 (m, 2H, CH_2), 3.57(s, 1H, NH), 4.47 (q, *J* = 7.56 *Hz* ,2H, $\underline{CH_2}CH_3$), 4.49 (s, 1H, CH-NH), 4.41 (t, 2H, CH_2O), 5.56 (s, 2H, NH_2), 9.58(s,2H,NH_2),11.78(s,1H,O-H),12.23(s,1H,N-H); ^{13}C-NMR (DMSO-d_6): 14.36, (CH_3), 62.41 ($\underline{CH_2}CH_3$ester), 21.83 (CH_2), 67.69 (CH_2), 23.97 (CH_2), 160.37(C=N), 111..22,115.45,141.11, 150.33, 152.72, 88, 86 (C=C) , 165.22 (C=O); MS (m/z, %): 349 (M+, 16). Anal. Calcd for: $C_{14}H_{19}N_7O_4$(349.35); C, 48.13; H, 5.48; N, 28.07%; Found ; C, 48.18; H, 5.52; N, 28.11%**.**

Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (7)

green crystals, 72% yield ,M.P. 230–232°C; IR (KBr): ν (cm^{-1}), 3100-3400 (2N-H, NH_2), 1740 (C=O ester).1555(N=N); ^{1}H-NMR (δ, DMSO-d_6): 1.28 (t, *J* = 7.56 *Hz* ,3H, $CH_2\underline{CH_3}$), 1.98 (t, 2H, CH_2), 2.36(s,6H,CH_3), 2.5 (m, 2H, CH_2), 3.57(s, 1H, N-H), 4.47 (q, *J* = 7.58 *Hz* 2H, $\underline{CH_2}CH_3$), 4.49 (s, 1H, CH-NH), 4.41 (t, 2H, CH_2O), 9.66 (s, 2H, NH_2), 12.03(s,1H,N-H); ^{13}C-NMR (DMSO-d_6): 13.56(2CH_3),14.36, ($\underline{CH_3}CH_2$), 62.41 ($CH_3\underline{CH_2}$ester), 21.83 (CH_2), 67.69 (CH_2), 23.97 (CH_2), 144.65(C=N), 87.62(CH-O),102. 33,111.21, 145.25, 141.13, 150.36, 152.72 (C=C), 165.25 (C=O); MS (m/z, %): 346 (M+, 20). Anal. Calcd for: $C_{16}H_{22}N_6O_3$ (346.38); C, 55.48; H, 6.40; N, 24.26%; Found; C, 55.51; H, 6.46; N, 24.30%.

Ethyl 5-amino-7-((3-hydroxy-5-methyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(8)

Yellow crystals, 70% yield ,M.P. 255–257°C; IR (KBr): ν (cm^{-1}),3460(O-H), 3100-3400

(2N-H, NH_2), 1740 (C=O ester).1553(N=N); ^{1}H-NMR (δ, DMSO-d_6): 1.28 (t, *J* = 7.52 *Hz* ,3H, $\underline{CH_3}CH_2$), 1.98 (t, 2H, CH_2), 2.36(s,3H,CH_3), 2.5 (m, 2H, CH_2), 3.57(s, 1H, N-H), 4.47 (q, *J* = 7.51 *Hz* ,2H, $\underline{CH_2}CH_3$), 4.49 (s, 1H, CH-NH), 4.41 (t, 2H, CH_2O), 9.66 (s, 2H, NH_2),11.65(s,1H,O-H),, 12.04(s,1H,N-H); ^{13}C-NMR (DMSO-d_6): 13.56(CH_3),14.36, ($\underline{CH_3}CH_2$), 62.41 ($CH_3\underline{CH_2}$ester), 21.83 (CH_2), 67.69 (CH_2), 23.97 (CH_2), 164.34(C=N), 87.66(CH-O),102. 33, 111.21, 143.46, 141.13, 150.36, 152.72 (C=C), 165.25 (C=O); MS (m/z, %): 348 (M+, 25). Anal. Calcd for: $C_{15}H_{20}N_6O_4$ (348.36); C, 51.72; H, 5.79; N, 24.12; %; Found; C, 51.76; H, 5.82; N, 24.17; %

Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(9)

To a neutral solution of compound **6** (0.01mol, 3.49 g), ethylcyanoacetate (0.01 mole , 1.13g) was added. The reaction mixture was stirred under reflux for 4 h, then cooled to room temperature and the product was precipitated using sodium chloride (10%w/v), filtered off and dried in an oven at 50°C to give the corresponding **9**.

pall Yellow crystals, 64% yield ,M.P. 275–277°C; IR (KBr): ν (cm^{-1}),3400-3500(2O-H), 3100-3400 (N-H, $2NH_2$), 1740 (C=O ester).1556(N=N); ^{1}H-NMR (δ, DMSO-d_6): 1.28 (t, *J* = 7.53 *Hz* ,3H, $\underline{CH_3}CH_2$), 1.98 (t, 2H, CH_2), 2.5 (m, 2H, CH_2), 3.57(s, 1H, N-H), 4.47 (q, *J* = 7.51 *Hz* ,2H, $\underline{CH_2}CH_3$), 4.49 (s, 1H, CH-N-H), 4.41 (t, 2H, CH_2O),5.23(s,1H,CH), 6.87(s,2H,NH_2),9.66 (s, 2H, NH_2),11.69,11.71(s,2H,2O-H); ^{13}C-NMR (DMSO-d_6): 14.36, ($\underline{CH_3}CH_2$), 62.41 ($CH_3\underline{CH_2}$ester), 21.83 (CH_2), 67.69 (CH_2), 23.97 (CH_2), 161.76, 162.14 (2C=N), 87.66(CH-O),110.56,116.03,140.61, 149.87,101.05,148.71,99.57,147.52 (C=C), 165.25 (C=O); MS (m/z, %): 416 (M+, 17). Anal. Calcd for: $C_{17}H_{20}N_8O_5$ (416.39); C, 49.04; H, 4.84; N, 26.91%; Found; C, 49.07; H, 4.88; N, 26.96%.

Fungal isolation (*Pythium* spp.)

Feeder roots of olive trees were collected from Ain Seleen, Fayoum, Egypt, in which symptoms of root rot disease was appeared. Roots were washed from the adhered soil and then sterilized with $NaClO_2$ (2%) solution for 2 minutes, after that washed twice with sterile distilled H_20. Pieces of 0.5 cm from each end of the roots were transferred by sterile forceps to Petri-dishes containing sterile NARM medium [nystatin (10 mg/ L^{-1}), ampicillin (250 mg /L^{-1}), rifampicin (10 mg/ L^{-1}) and miconazole (1 mg/ L^{-1}) in cornmeal agar (CMA)] for isolation of *Pythium* species, selectively **(Senda *et al.*, 2009)**. Subsequently, incubation was done at 28ºC ± 2 for 3 - 7 days. Small pieces of agar medium having mycelium growth were inoculating on WA [20 g/ L^{-1} in 1000 mL distilled water] medium (2.5%). After hyphal growth appeared, medium in Petri-dish has been exchanged upside-down in the same Petri-dish and incubated until the hyphal growth appeared.

Identification of *Pythium* spp. was done using keys of **(Waterhouse, 1968; Plaats-Niterink, 1981; Dick, 1990 and Abdelzaher, 1999)**. **(Figure 4)**

Effect of the synthetic compounds on mycelial growth of *P. aphanidermatum* on solid cultural media (Figure 5, 6)

Steralized warm PDA medium was prepared, and poured in sterile Petri dish supplemented with different concentration of the tested chemical compounds (0.5%, 1%, 2% , 3% and 4%). Agar discs (1 cm diameter) were taken from the margins colony grown on water agar, and then placed in the center of each Petri dish. Petri-dishes were then incubated in the dark at 28ºC. Radial fungal mycelial growth was measured by determined colony diameters in each dishes, after 24 hours of incubation.

Effect of the tested synthetic compounds on zoospore formation of *P. aphanidermatum* (Figure 7)

P. aphanidermatum was inoculated in Petri dishes containing 2.5% water agar until mycelial growth reached about 6 cm diam. Autoclaved pieces of *Zea mays* leaves (10 x 5 mm) were placed upon mycelia and incubated at 25º C. After 24h of incubation, part of the colonized *Zea mays* leaves were taken and putted in Petri-dishes having 10 mL of sterilized distilled water (control), and other *Zea mays* leaves were placed in petri dishes containing 10 mL of sterilized and distilled water with different concentration of the three tested chemical compound (0.5%, 1%, 2% ,3% and 4%), and incubated at 28º C for 24 h. Number of zoosporangial vesicles were counted under the microscope. Three dishes of each treatment were used. The experiment was repetitive three times.

Effect of the tested synthetic compounds on oospore production of *P. aphanidermatum* (Figure 8)

In Erlenmeyer flasks (100 mL) containing 10 mL of corn meal broth medium, *P. aphanidermatum* has

been inoculated with different concentration of the three tested chemical compound (0.5%, 1%, 2% ,3% and 4%) at 27ºC. Fifteen days later, oospores suspensions have been obtained by shredding mycelium mats in a homogenizer at high speed for 3 min. The producing suspension was filtered through a suitable diameter of sieve (its pores were less than diameters of oospores) followed by counting number of mature oospores.

Statistical analysis

Data was statistically analyzed using GraphPad Prism 2.01 program, results were expressed as mean values ± standard error. Significant variance of synthetic compounds effects were assessed using One-way ANOVA.

RESULTS AND DISCUSSION

Chemistry

The primary amino group of Ethyl 5, 7-diamino-3, 4, 8, 8a-tetrahydro-2H-pyrano [2, 3-b] pyridine-6-carboxylate (**1**) **(Elkanzi *et al.*, 2019)** was converted via diazotation to its respective diazonium salt (**2**)(Rakhit S.1979) after treatment with HNO_2 generated in situ from $NaNO_2$/HCl at 0-5ºC **(Norman and Coxon, 2009**, **Yusuf Y. Lams 2014)**. The reactions of diazonium salt (**2**) with ethylcyanoacetate acetyle acetone, and ethyl acetoacetate in mixtures of sodium acetate and ethanol solutions afforded pyrano pyridine (**3**, **4** and 5) as crystal powders. Formation of compound (**3**, **4** and **5**) is presented in Scheme 1. Purity of the monoazo dyes (3, 4 and 5) was checked by TLC using ethylacetate-acetic acid (3:1), Formation of monoazodyes (3, 4 and 5) was confirmed by spectral analysis (FTIR,^{1}HNMR,^{13}CNMR and Mass spectra). We take compound **3** for illustrate their spectra as an example of mono azodyes **.**

Formation of Ethyl 5-amino-7-((1-cyano-2-ethoxy-2-oxoethyl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(3) was established by its spectral analysis ,the IR spectra indicate the presence of signals at 3100-3400 , 2216,1735-1740 cm^{-1} assigned for N-H, NH_2, CN, 2C=O groups respectively ,The ^{1}H-NMR spectra revealed the presence of triplet signals at δ1.23, 1.27 assigned for ($2CH_2\underline{CH_3}$), quartet signal at 4.23,4.48 assigned for ($2\underline{CH_2}CH_3$) ,The mass spectra of compound **3** equal 363 is agreement with structure of **3** . The structure of compound **3** was established also by ^{13}CNMR spectra (see experimental section).on the other hand

The structures of compounds **(6, 7)** and **(8)** were established chemically by the reaction of (**3, 4 and 5**) with hydrazine hydrate in ethanol respectively Scheme **1**.

Pureness of compounds (**6, 7** and **8**) was determined by thin layer chromatography using ethylacetate-acetic acid (3:1) solvent system. Infrared spectra of compound (**6**) displayed a band at 3450 assigned for (O-H), also a band appearing at 1740 cm^{-1} as concern with C=O of ester group, also band appear at 3100-3400 assigned for 2N-H, $2NH_2$groups.

The IR spectra of Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**) revealed the existence of bands in the frequency of3100-3400 , 1740 cm^{-1} ,and1555 cm^{-1} corresponding to(2N-H, NH_2, C=O ester, and N=N groups respectively (**Figure 1**). ^{1}H NMR spectra confirmed the structure of compounds (**7**) where spectrum of (**7**) showed signals varying from 1.28 and 4.47 ppm corresponding to CH_3 and CH_2 groups and signals at 9.66,and 12.03 assigned for NH_2 and NH respectively .

The IR spectra of compound (**8**) showed bands at 3460, 3100-3400, 1740, and 1553cm^{-1} corresponding to O-H, N-H, NH_2, C=O ester, and N=N respectively (**Figure2**).

The ^{1}H NMR spectrums of compounds **(8)** revealed signals at9.66, 11.65, 12.04ppm forNH_2, O-H), N-H respectively.

Reaction of compound (**6**) with ethylcyanoacetate provide the corresponding Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**9**)in 64% yield and the spectral data (IR, 1H-NMR, ^{13}C-NMR) of the obtained product (**9**) were in full agreement with the proposed structure. IR spectra of compound (**9**) revealed the existence of bands in the frequency of 3400-3500,3100-3400, 1740 cm^{-1} and 1556cm^{-1} corresponding to (O-H, N-H, NH_2, C=Oester) and N=N groups respectively (**Figure 3**).

^{1}H-NMR spectra of pyrimidine (**9**) depicted signals at 6.87,9.66 ,11.69,and 11.71ppm corresponding to the $2NH_2$and 2O-H protons.

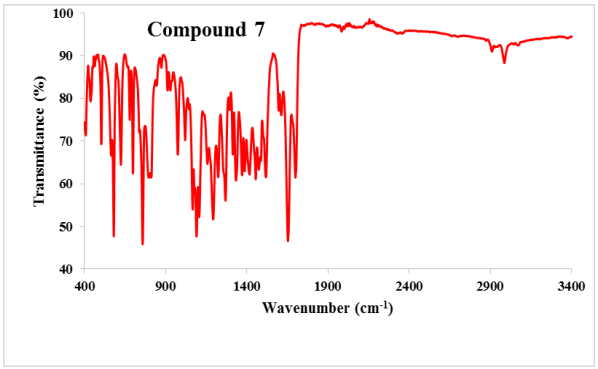


Fig.1: FTIR spectra of compound **7**

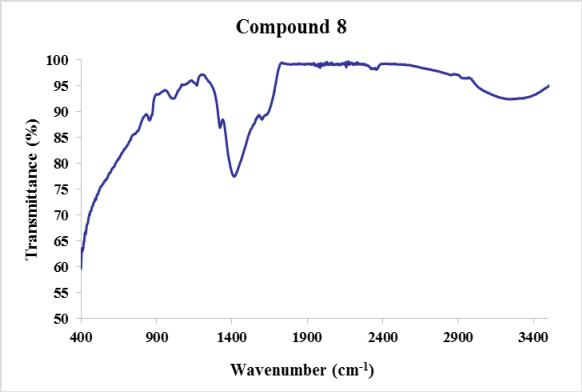


Fig.2: FTIR spectra of compound **8**

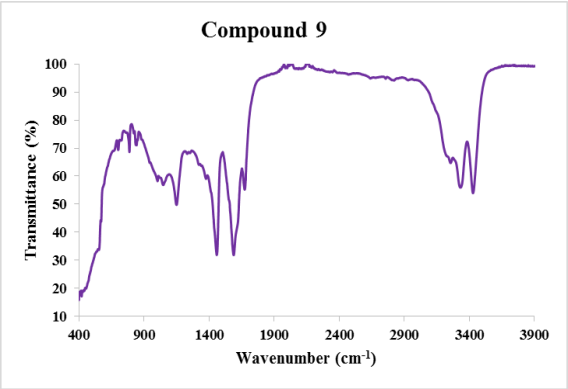
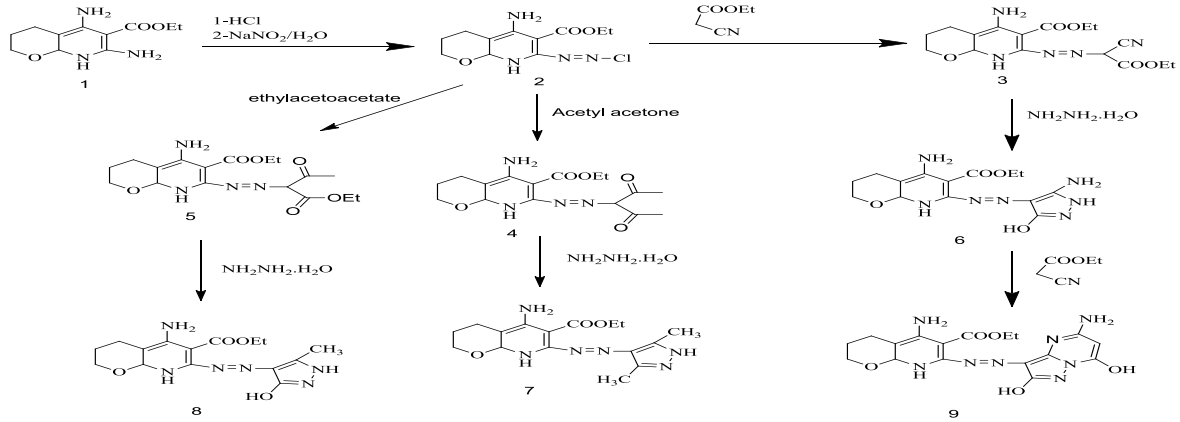


Fig.3: FTIR spectra of compound **9.**



Scheme 1: Synthesis of new pyrazole 7, 8 and pyrazolo pyrimidine 9.

Antifungal activity of the synthetic compounds on the tested *P. aphanidermatum*

Table (1) shows the antifungal influence of different synthetic chemical compounds Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**), Ethyl 5-amino-7-((3-hydroxy-5-methyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**8**), and Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**9**) on *P. aphanidermatum* mycelial growth on PDA medium after 6 days of incubation at 28ºC.

Results reveled that (pyrazolo pyridine (7, 8) and pyrazolopyrimidine (9) significantly reduced mycelial growth of *P. aphanidermatum* at different concentrations (0.5, 1, 2,3 and 4%) compared to control ($p \leq 0.001$).

As shown in Figures (5, 6) efficacy of Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate (**7**), was greater than of compounds Ethyl 5-amino-7-((3-hydroxy-5-methyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(8) and Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(**9**) in *P. aphanidermatum* growth inhibition. Percentage of inhibition of (pyrazolo pyridine (7, 8), and pyrazolopyrimidine (9) were (70.5%, 60.3% and 67.37%) at concentration 3% and 4%, respectively.

Results in Table 2 show that zoospore production of *P. aphanidermatum* was high after 24h incubation at 20ºC in the control trial. Compounds (pyrazolo pyridine (7, 8), pyrazolopyrimidine (9) significantly, inhibited the production of zoospores of the fungus at different concentrations compared to control ($p \leq 0.001$). Sterilized distilled water supplemented with synthetic chemical compounds inhibited production of zoospores, which the highest effect was observed in 3% of (pyrazolo pyridine (7) was (68.3%), followed by pyrazolopyrimidine (9) was (64.2%) and pyrazolo pyridine (8) had a less effect of three chemical compounds was (59.7%), Fig (8).

Results in figure (7) reveal that pyrazolo pyridine (7, 8) and pyrazolopyrimidine (9) significantly, inhibited the formation of oospores of *P. aphanidermatum* at different concentration

compared to control ($p \leq 0.001$). Highly observed formation of oospore of *P. aphanidermatum* was appeared after 15 days of incubation at 27ºC grown in corn meal broth medium with (3%) of pyrazolo pyridine (7) highly reduced oospore formation than pyrazolo pyridine (8) and pyrazolopyrimidine (9) (Table 3).

Table (1): Comparison of inhibitory activity of synthetic chemical compounds (pyrazolo pyridine (7, 8) and pyrazolopyrimidine (9) on mycelial radial growth of *P. aphanidermatum* grown on PDA medium after 6 days at 25ºC in the dark.

Concentration of the chemical compound	Metalaxyl	Pyrazolo pyridine (7)	Pyrazolo pyridine (8)	Pyrazolopyrimidine (9)
	Average of Radial growth rate (mm)/day	Average of Radial growth rate (mm)/day	Average of Radial growth rate (mm)/day	Average of Radial growth rate (mm)/day
Control (sterilized distal water)	100.7 ± 0.3			
0.5%	72* ± 0.18	78.6 ± 0.13	92 ± 0.1	88 ± 0.08
1%	56 ± 0.08	61.67 ± 0.18	84 ± 0.14	76.6 ± 0.13
2%	39 ± 0.14	41 ± 0.08	80 ± 0.1	61.6 ± 0.14
3%	26 ± 0.13	30 ± 0.1	59.3 ± 0.2	42.3 ± 0.3
4%	26 ± 0.13	30 ± 0.1	59.3 ± 0.2	42.3 ± 0.3

*Values are means of three measurements ± standard error

Table (2): Effect of synthetic chemical compounds (pyrazolo pyridine (7, 8), and pyrazolopyrimidine (9) on zoospore production of *P. aphanidermatum* after 24 h at 20ºC, in the dark.

Concentration of the chemical compound	Metalaxyl	Pyrazolo pyridine (7)	Pyrazolo pyridine (8)	Pyrazolopyrimidine (**9**)
	Average no .of zoosporangia vesicles (mm)	Average no .of zoosporangia vesicles (mm)	Average no.of zoosporangia vesicles (mm)	Average no.of zoosporangia vesicles (mm)
Control (sterilized distal water)	4±0.12			
0.5%	0.48* ± 0.05	0.52 ± 0.05	0.60 ± 0.08	0.55 ± 0.08
1%	0.32 ± 0.01	0.49 ± 0.03	0.56 ± 0.03	0.51 ± 0.01
2%	0.29 ± 0.03	0.41± 0.06	0.52 ± 0.03	0.48 ± 0.08
3%	0.28 ± 0.01	0.3 ± 0.03	0.51 ± 0.08	0.45 ± 0.05
4%	0.28 ± 0.01	0.3 ± 0.03	0.51 ± 0.08	0.45 ± 0.05

*Values are means of three measurements ± standard error.

Table (3): Effect of synthetic chemical compounds (pyrazolo pyridine (7, 8) and pyrazolopyrimidine (9) on oospore formation of *P. aphanidermatum* after 15 days of incubation at 27ºC, in corn meal broth medium.

Concentration of the chemical compound	Metalaxyl	Pyrazolo pyridine (7)	Pyrazolo pyridine (8)	Pyrazolopyrimidine (9)
	Average no. of oospores (mm)	Average no. of oospores (mm)	Average no. of oospores (mm)	Average no. of oospores (mm)
Control (corn meal broth)	4200 ± 1.3			
0.5%	1080* ± 0.3	2001±1.3	3560 ± 1.45	3020 ± 0.3
1%	860 ± 1.1	1020 ± 1.15	3004 ± 2.6	2400 ± 0.8
2%	410 ± 1.4	518 ±1.3	790 ±0.3	607 ± 1.4
3%	200 ± 0.3	310 ± 0.3	697 ± 0.3	430 ± .08
4%	200 ± 0.3	310 ± 0.3	697 ± 0.3	430 ± .08

*Values are means of three measurements ± standard error

Previous studies revealed that, several species of *Pythium* were associated with root rot of olive trees **(Sánchez Hernández *et al*., 1998; El-Morsi *et al*., 2009; Elnaghy *et al*., 2014; Hair *et al* ., 2017)**. Symptoms of root rot and wilt diseases on olive transplants was previously reported by **Sanei and Razavi (2011)**. Results of the current study showed that *P. aphanidermatum* was isolated from rotted feeder roots of olive trees cultivated in Ain Seleen, Fayaum, Egypt.

It was very common to use metalaxel, to control pathogenic *Pythium* spp. but unfortunately, the majority of these fungi have gained resistance against this fungicide. Results here appeared that the synthetic chemical compound of pyrazolo pyridine (7) inhibited all growth and reproduction units including (mycelial growth and production of zoo- and oospores) of *P. aphanidermatum* more than the other synthetic compound of pyrazolo pyrimidine (9) and pyrazolo pyridine (8). The highest antifungal activity observed in pyrazolo pyridine (7) is probably due to the presence of methyl group.

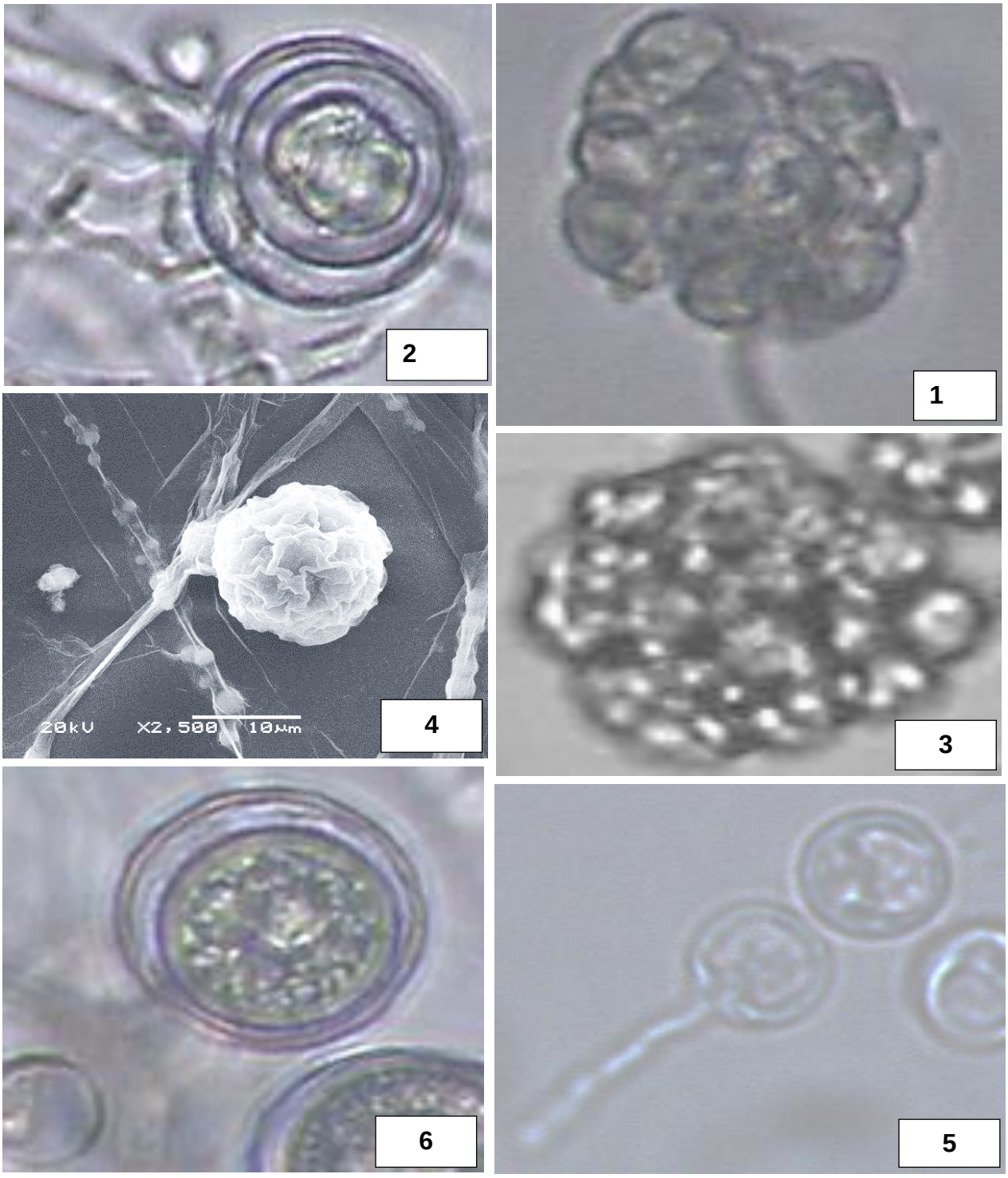


Figure 4: (1) Zoospores of *Pythium aphanidermatum* inside vesicles. (3) Liberated zoospores. (5) germinated zoospores. (2, 6) Oospore of *P. aphanidermatum* under compound microscope. (4) Oospore under electron microscope.

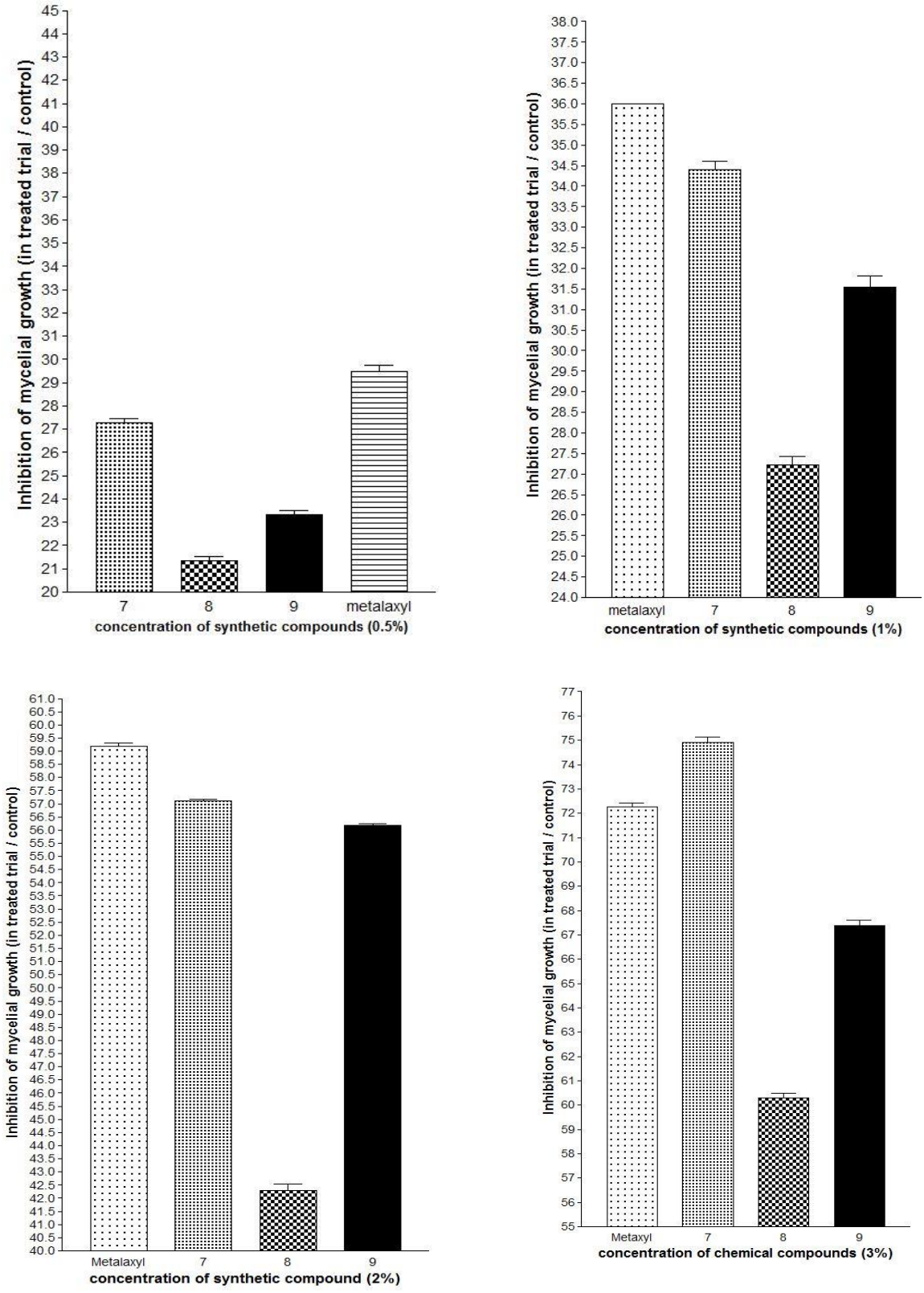


Figure (5): Inhibitory effect of synthetic compounds (pyrazolo puridine (7,8) and pyrazolopyrimidine (9) on radial mycelial growth of *P. aphanidermatum* grown on PDA medium after 6 days at 25ºC in the dark. All the results are the mean of three replications and Bars on each column represents standard deviations**.**

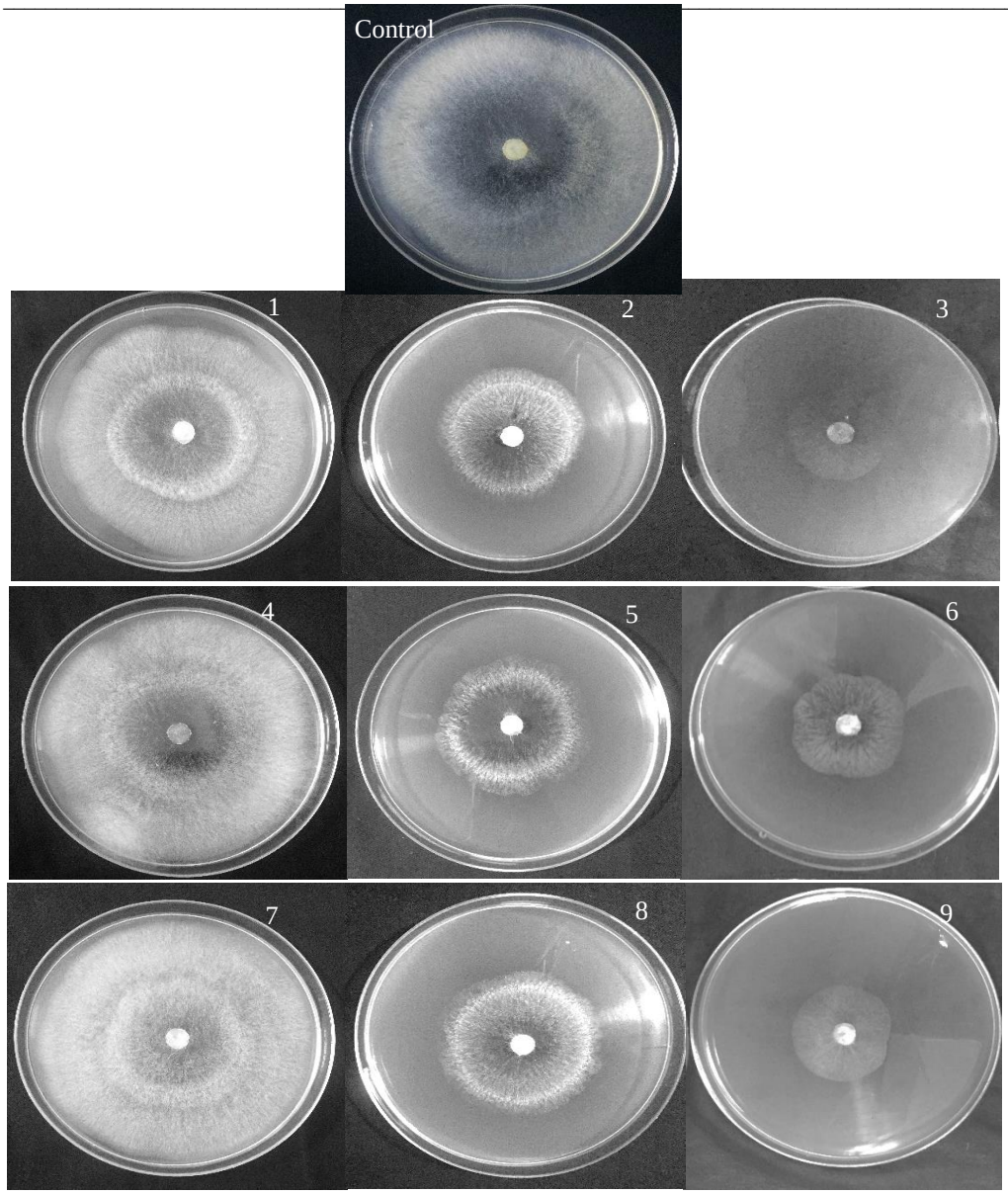


Fig (6): Colony dimensions of *P. aphanidermatum* grown on PDA medium at 25ºC in the dark, after 6 days. (Control) medium without the synthetic compound, (1-3): media with addition of 0.5%, 2% and 4% of synthetic compound (7), respectively. (4-6): media with addition of 0.5%, 2% and 4% of synthetic compound (8), respectively. (7-9): media with addition of 0.5%, 2% and 4% of synthetic compound (9), respectively.

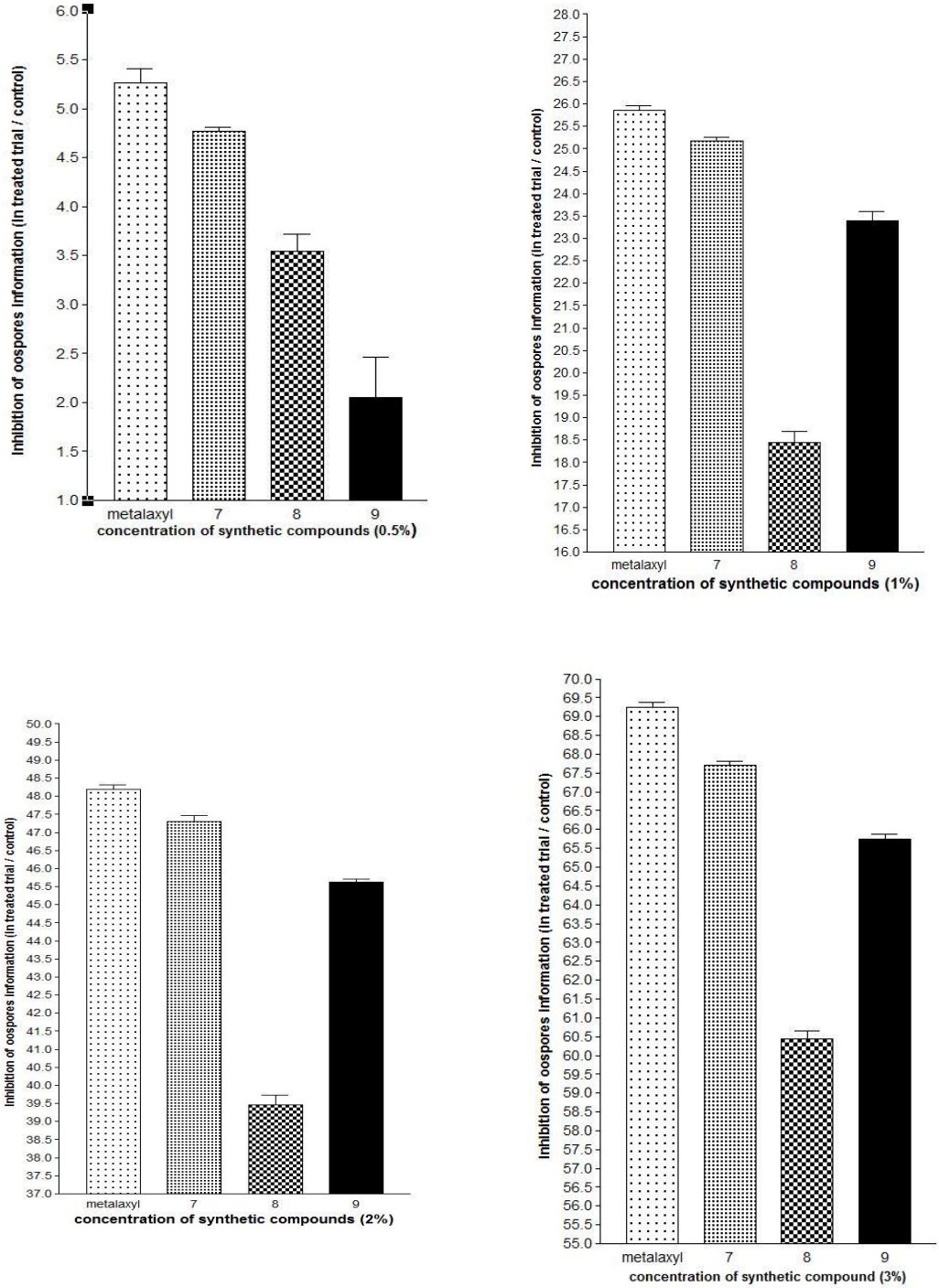


Fig (7): inhibitory effect of synthetic compounds ((Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(7), Ethyl 5-amino-7-((3-hydroxy-5-methyl-1H-pyrazol-4-yl) diazenyl)-3, 4, 8, 8a-tetrahydro-2H-pyrano [2, 3-b] pyridine-6-carboxylate(8) and Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate(9))) on oospore formation by *P. aphanidermatum.* All the results are the mean of three replications and Bars on each column represents the standard deviations.

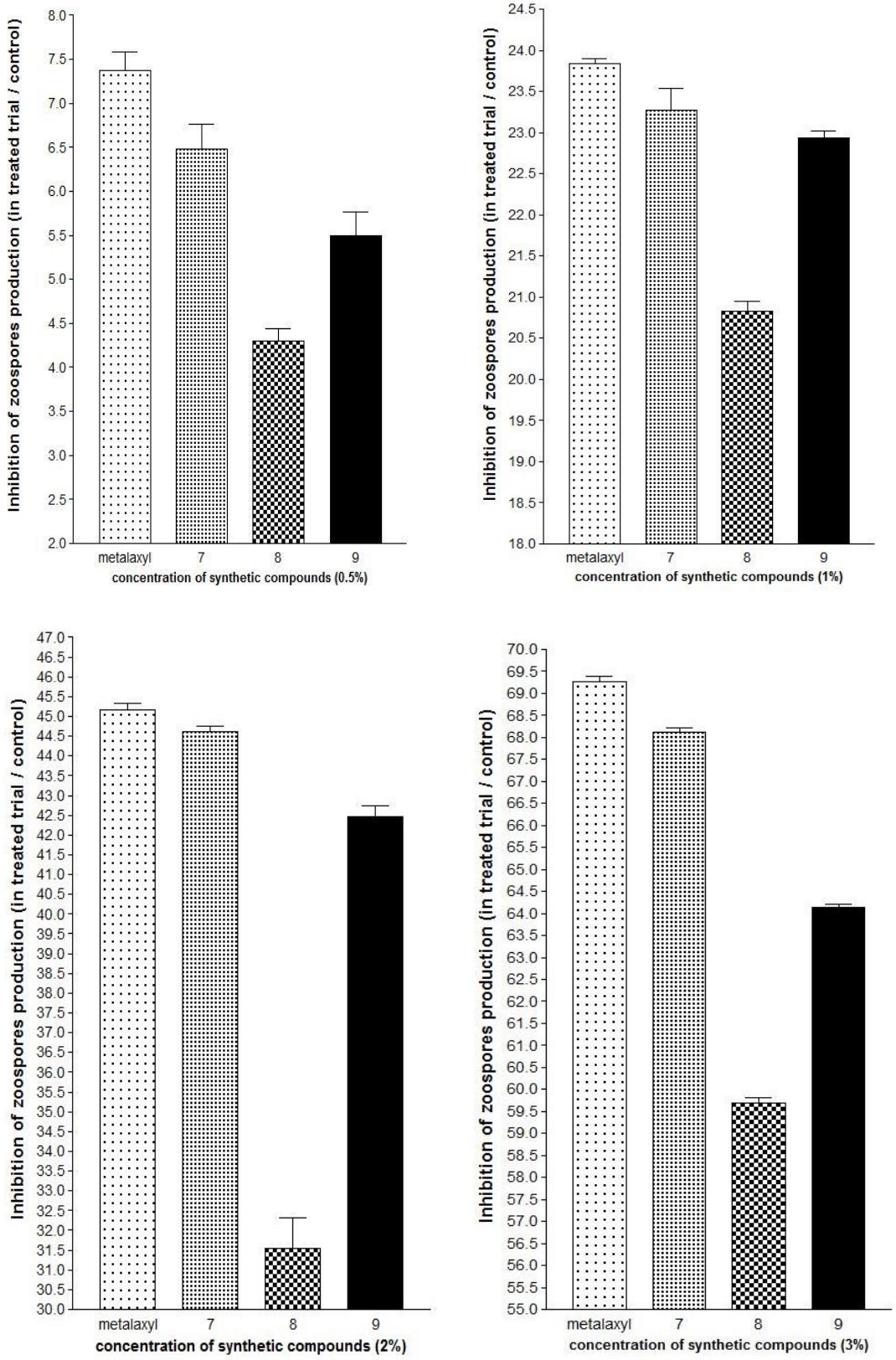


Fig (8): inhibitory effect of synthetic compounds (Ethyl 5-amino-7-((3,5-dimethyl-1H-pyrazol-4-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate, Ethyl 5-amino-7-((3-hydroxy-5-methyl-1H-pyrazol-4-yl) diazenyl)-3, 4, 8, 8a-tetrahydro-2H-pyrano [2, 3-b] pyridine-6-carboxylate and Ethyl 5-amino-7-((5-amino-2,7-dihydroxypyrazolo[1,5-a]pyrimidin-3-yl)diazenyl)-3,4,8,8a-tetrahydro-2H-pyrano[2,3-b]pyridine-6-carboxylate) on zoospore production by *P. aphanidermatum.* All the results are the mean of three replications and Bars on each column represents the standard deviations.

CONCLUSION

P. aphanidermatum were isolated from Feeder roots of olive trees cultivated in Ain Seleen, Fayoum governorate, Egypt. Pyrazolo pyridine (7) showed high efficiency as growth inhibition of fungal mycelia, followed by (pyrazolo pyrimidine (9), and then pyrazolo pyridine (8)at 10 mg/ L. pyrazolo pyridine (7) showed its ability to decline the production of zoo-spores and oospores of the fungus at concentration 300 mg/L. Rate of inhibition in all treatments reached more than 85% in case of the use of pyrazolo pyridine (7). This study offers data for the use of pyrazolo pyridine (7) in controlling fungal disease to olive feeder roots infected by *P. aphanidermatum*.

Among the recommendations emanating from this study, can be summarized as:

- Toxicity of these compounds should be evaluated and measured on plants and organisms in the olive surrounded environment on the extent of residual of those chemicals within the plant tissues.
- Effectiveness of pyrazolo pyridine (7) should be tested on all pathogenic *Pythium* spp. that cause diseases to all other crop plants.

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RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS

This research don't involved Human Participants and/or Animals.

INFORMED CONSENT

We agree to Published manuscript in this respected Journal.

Drs. S.M.N Moustafa and N.A.A.ElKanzi

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