

EFFECT OF DUPONT BENLATE ON
GROWTH PARAMETERS, PROTEIN AND NUCLEIC ACIDS OF
SOME FUNGI

BY

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ABSTRACT

The effect of the fungicide Dupont Benlate (in concentrations ranging from 10 to 1000 ppm a.i.) on spore germination, length of germ-tubes, radial growth, biomass production, total protein production, RNA and DNA contents was studied in Aspergillus fumigatus, Verticillium agaricinum, Helminthosporium oryzae and Fusarium oxysporum f.sp. lycopersici.

The increase in the fungicide level was accompanied by an effective decrease in percentage spore germination and germ-tube lengths in all test fungi. In case of V. agricinum and F.o. lycopersici no germinated conidia could be detected at the level 200 ppm or more.

There was a concomitant decrease in radial growth rate (Kr) with the increase in fungicide concentration. The inhibitory effect of the fungicide on mycelial growth (biomass production) was also clear, as a significant decrease in mycelial dry weight was observed, even at the lowest applied concentration.

The results show that the fungicide induced an increase in total protein production in all fungi except V. agaricium. It also induced a high RNA content in all fungi except H. oryzae which showed an increase in RNA content only at the concentration 10, 100, 200 ppm. On the other hand, DNA content decreased with the increased of fungicide level in all fungi except A. fumigatus where the reverse was true.

INTRODUCTION

In the last decades, fungicides have been applied extensively to control plant pathogenic fungi. This led to the widespread in vitro studies on the effects of many fungicides on germination, growth and physiological activities of these fungi.

Gizi et al. (1985) tested the effect of 17 fungicides, singly and in mixtures, on Phytophthora cactorum and P. cinnamomi. Sharma and Gupta (1985), in an evaluation of fungicides in vitro for the control of Colletotrichum state of Glomerella cingulate, found that 10 out of 15 tested fungicides inhibited spore germination. Sharma

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and Jain (1984) stated that the 9 Fusarium isolates (several spp.) tested against 3 fungicides were more sensitive to the systemic Bavistin and MBC (carbendazims), differing in their relative sensitivity.

Martin et al. (1984) studied the comparative sensitivity of Rhizoctonia solani and Rhizoctonia- like fungi to selected fungicides added to PDA at 0, 1, 10 and 100 mg a.i. for tests of in vitro growth responses (inhibition of linear growth) of the isolates. They reported that R. solani and binucleate Rhizoctonia- like fungi sensitive to benomyl (EC_{50} 10mg/ litre), whereas isolates of R. zeae were tolerant of benomyl (EC_{50} 50 mg/ litre) but sensitive to other fungicides. Also, Radzuhn and Lyr (1984) recorded that the in vitro growth of Mucor mucedo was impaired almost immediately after the addition of the fungicide etridiazole to the medium.

The literature showed variable effects of fungicides on fungi. The objective of the present investigation is to determine in vitro the effect of Dupont Benlate on germination, growth activities, protein and nucleic acids of some fungi.

MATERIALS AND METHODS

Four fungi were selected for the present study :

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Aspergillus fumigatus Fresenius, Verticillium agaricinum Link (Corda), Helminthosporium oryzae Breda de Haan and Fusarium oxysporum f.sp. lycopersici (Sacc.) Snyder & Hansen. The first two fungi were isolated from Egyptian soil and have a high percentage germination of their conidia and a saprophytic nature. These characters were valuable in using these organisms for comparison with the pathogenic fungi. The last two organisms are important plant pathogens in Egypt causing leaf spot of rice and tomato wilt respectively. They were isolated from their infected plant hosts. All fungi are isolates in a culture collection.

The fungicide (Dupont Benlata) used was Benomyl (Methyl 1-(butyl carbamoyl)-2-benzimidazole carbamate) produced by Kafz El-Zayat Company of Biocides and Chemicals. Active ingredient Benomyl was 50 %. For experimental use, a stock solution of concentration 10,000 ppm was prepared and from which the following concentration were prepared: 10, 50, 100, 200, 400, 600, 800 and 1000 ppm. The fungicide was added after autoclaving. Fungal growth was maintained on Czapek-Dox Agar medium (Thom and Raper, 1945).

Spore germination test

Eighteen Petri-dishes (90 mm diameter) were used for each fungal species, Two plates contained a thin layer

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of Czapek-Dox Agar medium (about 10 ml/ plate) for control (non-treated medium) and the other 16 plates contained medium supplemented with the above-mentioned fungicide concentrations by adding known volumes from the stock preparation to the plates while medium still warm, then mixed well and the plates were left to solidify. After solidification, the medium in each plate was divided into area of approximately 1 cm^2 . On each agar square a drop of spore suspension from 7 days old culture slant was placed. The spore suspension was carefully prepared to give drops containing a moderate density of spores (25-30 spore in a microscopic field of magnification power x 100). The plates were then incubated at 27°C . By the end of the needed incubation period (preliminary trials were made to determine the proper incubation period that allows for 50- 60% spore germination in control samples) two agar blocks from each plate were removed on labelled slides and transferred to a desicator with a vapour of formalin to fix and kill the spores. Four microscopic fields (magnification power x 100) per block were then examined at random for their content of percentage germinated spores and lengths of germ tubes in microns using calibrated ocular micrometer. Further examination, after longer periods of incubation, was performed in order to find out a clear picture of the effect of the fungicide.

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Radial growth

Petri-dishes containing 20 ml Czapek-Dox Agar medium were amended with a range of fungicide concentrations as described previously. A loopful of the spore suspension from 7 days old culture was placed at the centre of each plate. Plates were then incubated at 27 °C and the diameter of the developed colonies was recorded daily for a period of 7 days. The radial growth rates ($K_r = \text{mm/h}$) were then estimated and compared with their corresponding controls.

Production of fungal biomass

For each fungus 27 Erlenmeyer flasks (250 ml), each containing 50 ml Czapek-Dox liquid medium, were used. Triplicate flasks served as controls or experimental; where the media were amended with the previously mentioned concentrations of the fungicide. Each flask was inoculated with 1 ml of the spore suspension obtained from 7 days old cultures of the required fungus. The flasks were then incubated at 27°C for 7 days after which the produced mycelial felts were collected and oven dried at 80°C till constant weight.

Preparation of fungal mats for protein and nucleic acids determinations

The above-mentioned steps for fungal biomass production were again applied here but by the end of the

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incubation period, the mycelial felts were collected by filtration, washed twice with bi-distilled water and then used for the estimation of total protein and nucleic acids.

Protein determination

Ten mg of fresh mycelium were mixed and homogenized with 1 ml phosphate buffer (pH 8.04), then the mixture was left overnight for complete extraction. The extracts were clarified by centrifugation for 10 min at 8000 x g and then analysed for protein as described by Bradford (1976).

Nucleic acids determination

Nucleic acids were extracted by a method cited by Marmur (1961) and modified by Mohamed and Capesius (1980). Determination of DNA was carried out using the method adopted by Dische and Schwartz (1937). RNA was estimated according to the method of Schneider (1957). Determinations were carried out using a single-beam Spectrophotometer SPEKOL (VEB, Carl-Zeiss, Jena,DDR).

Statistical analyses

All the experiments were carried out in duplicates. All means are given as $\bar{x} \pm S.D.$, the standard deviation

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was calculated at 9 % confidence limit. The differences between the means of two samples was tested using T test at 95% c.l.

For percentage spore germination and length of germ tubes, the L.S.D. at 99% and 95% confidence limits were calculated.

RESULTS

Effect of dupont benlate on spore germination & germ tube lengths

Table 1 shows the effect of different concentrations of the fungicide on spore germination. The results indicate that the rate of inhibition increases in all fungi under test with the increase in the fungicide concentration. An effective decrease in spore germination for all fungi as compared to their corresponding controls appeared at the lowest applied concentration (10 ppm). In case of V.agaricinum and F. oxysporum f.sp. lycopersici, no germinated conidia could be detected at the level 200 ppm of the fungicide. On the contrary, the other two fungi, A. fumigatus and H. oryzae showed germinated conidia at the highest fungicide level (1000 ppm). However, at this concentration, the percentage germination was very low (2 %) as compared with their corresponding control treatments.

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For the length of germ tubes, the same was almost true, as the fungicide evenly retarded growth of the developed germ tubes of the tested fungi with the exception of H. oryzae at the low concentration (100 ppm).

Effect on radial growth rate (K_r)

The results in table 2 show a concomitant decrease in radial growth and consequently the growth rate with the increase in fungicide concentration. At the level 600 ppm of the fungicide, the non-pathogenic fungi, A. fumigatus and V. agaricinum showed radial growth rates less 15 % than their corresponding controls. On the other hand, the radial growth rates of the pathogenic fungi H. oryzae and F. oxysporum f.sp. lycopersici were greater 40 % than their corresponding controls at the same fungicide level. No radial growth was detected at levels higher than 600 ppm except in case of H. oryzae which gave radial growth rate 0.09 mm/h compared to 0.5 mm/h of the control.

Effect on biomass production

The inhibitory effect of the fungicide on the mycelial growth in liquid culture was clear as shown in table 3. A significant decrease in dry weight was observed after application of the fungicide at almost all concentrations. Mycelial growth decreases with the increase in fungicide

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concentration. Reduction in biomass production continued in A. fumigatus up to the level 400 ppm, while in the other three fungi, reduction continued up to the fungicide concentration 800 ppm.

A marked decrease in the mycelial growth compared to the corresponding controls detected in both A. fumigatus and H. oryzae began at the lowest concentration of the fungicide. No growth was detected at the highest level 1000 ppm of the fungicide by the end of the incubation period.

Effect on total protein

The results in table 4 show the mean values of the total protein calculated as mg/ g fresh weight of the fungal mycelium at different levels of the fungicide (10-1000 ppm) as well as the control treatment.

The amount of protein was found to increase in cultures of A. fumigatus grown in presence of the fungicide. Such increase was remarkable at the level 50 ppm as the amount produced was 48.3 mg compared to 21.4 mg of control and reached 54.6 mg at the concentration 400 ppm. In the two pathogenic fungi H. oryzae and F. oxysporum f.sp. lycopersici, the induction of total protein was clear at the

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lower concentration 10 ppm and evenly continued as the fungicide concentration increased. The rate of increase in both pathogens was lower than that of A. fumigatus (255 %), as the total protein reached 59.4 mg at the level 600 ppm compared to 40.1 mg in the control of H. oryzae (148 %), while it reached 32.9 mg at the level 200 ppm compared to the control 23.9 mg in case of F. oxysporum f.sp lycopersici (138 %).

V. agaricinum behaved differently as there was a significant increase in total protein up to the concentration 50 ppm (29.4 mg compared to 24.2 mg in the control) and then a significant decrease was detected at higher concentration up to 400 ppm.

Effect of nucleic acids

Table 4 shows also the mean values of DNA and RNA calculated as mg/ g fresh weight of the fungal mycelium at different levels of the fungicide. The results indicate variable responses of the tested fungi towards different concentrations of the fungicide. DNA and RNA contents of A. fumigatus increased evenly with the increase in the fungicide level. At the level 400 ppm, the increase in RNA content was almost 3 times the amount of the control, while the increase in DNA at the same level was 142 % as compared to the control.

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In case of V. agaricum, the increase in RNA content reached its maximum at the concentration 50 ppm where it was 47.4 mg and then decreased to reach 42.8 mg at the concentration 400 ppm but still significantly higher than its corresponding control (39.1 mg). The content of DNA was inhibited with the increase in the fungicide concentration to reach 31.2 mg at 400 ppm with the exception of the lowest level (10 ppm) at which the amount of DNA was 39.1 mg compared to 37.6 of the control.

The RNA content in H. oryza increased at first with the increase in fungicide concentration and reached its maximum (42.1 mg) at the level 10 ppm; then decreased to 30.1 mg at the level 600 ppm while the control was 36.5 mg. On the other hand, the amount of DNA decreased with increase in fungicide concentration with an exception of its increase at 10 ppm where it reached 30 mg as compared to 26.4 mg of the control.

In case of F. o. lycopersici, the RNA content increased with the increase in the fungicide concentration to reach 175 % of the control at the level 200 ppm. DNA content decreased significantly to reach 30.9 mg at the same level of the fungicide with one exception at the level 10 ppm as it reached 52.4 mg while the control was 46.5 mg

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DISCUSSION

Application of the fungicide Dupont Benlate in the present investigation showed variable results depending on the concentration and the fungus under test.

An effective decrease in spore germination as well as germ tube length for all fungi was detected at the lowest fungicide concentration (10 ppm). While no germinated conidia were observed in V. agaricinum and F. oxysporum f.sp. lycopersici at the level 200 ppm, the other two fungi A. fumigatus and H. oryzae showed germinated conidia at the highest fungicide level (1000 ppm) even the percentage germination was very low (2 %) as compared to their corresponding controls. The length of germ tubes was almost coinciding with the percentage spore germination i.e decrease with the increase in the fungicide concentration with the exception of H. oryzae at concentrations lower than 100 ppm. Sharma and Verma (1985) stated that sclerotia of Corticium rolfsii were inhibited by all concentrations of PCNB (quintozene) tested in vitro, although 50 ppm was less effective than 100, 500, or 1000 ppm, and Bavistin (Carbendazim) was ineffective. On the other hand, Hashimoto et al. (1986) concluded that the fungicide triflumizole did not inhibit spore germination of several kinds of fungi even at 100 ppm, although the germ tubes of treated spores were swollen, abnormally branched and shortend. In another direction

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of research, Molnar et al. (1985) determined the high level of benomyl tolerance in Fusarium oxysporum by the synergistic action of two genes.

A concomitant decrease in radial growth rate was coinciding with the increase in the fungicide concentration. At the level 600 ppm of the fungicide, the non-pathogenic fungi showed radial growth rates less than 15 % of their corresponding controls, while K_r of pathogenic ones were greater than 40 % at the same fungicide level. No radial growth was observed in almost all fungi at concentrations greater than 600 ppm. Kesavan (1984) deduced that mycelial growth of Rhizoctonia solani was effectively controlled with benomyl and other fungicides, all at the concentration 10 ppm. On the other hand, Saracchi and Lorenzini (1985) in in vitro tests, stated that sensitivity of strains of Botrytis cinerea to benzimidazoles of mycelium (in terms of ED_{50} value for growth on agar medium) was not correlated to the sensitivity of conidia (ED_{50} values for germination), or to growth, conidial and sclerotial production on nonemended agar medium or growth on carrot silces.

Again, a significant decrease in mycelial dry weight was observed after application of the fungicide at almost all concentrations. The results of Rodriguez- Kabana et al. (1967) are in agreement with that of the present study.

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Also, Hickish et al. (1987) have stated that in liquid medium, applying herbicides even when added in very low quantities, have an effect on mycelial mass formation of most species, mostly in terms of a more or less distinct inhibition.

Total protein, DNA and RNA contents either stimulated by low concentrations of the fungicide and then declined after certain limit depending on the fungus itself, or an induction process achieved only after treatment with the fungicide in higher rates. Such variations may be due to the effect of the fungicide on certain enzyme systems in the metabolic pathway. Coghe et al. (1984) reported that at the sublethal concentrations of the fungicide in the culture medium, reduced mycelial growth was accompanied by increase in the DNA and RNA content of the mycelium. However, when expressed in ug/ total dry mycelium, the DNA and RNA contents were markedly reduced in relation to the untreated controls.

In general, Dupont Benlate is of significant inhibitory effect on germination, radial growth rate and biomass production. The effect on germination is reflected on the following growth stages and accordingly radial growth retarded and mycelial growth in liquid cultures decreased. variable degrees of responses to the fungicide lead to suggest that fungicides effects are dependable on their chemical

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structure, their concentration and the characteristics of the individual organisms.

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Table 1 : Effect of Benlate on percentage germination of conidia (%G) and length of germ tubes (l.g.t. μ) in Aspergillus fumigatus, Verticillium agaricinum, Helminthosporium oryzae and Fusarium oxysporum f.sp. lycopersici.

Concentration (ppm)	A. fumigatus		V. agaricinum		H. oryzae		F. oxysporum	
	% G	l.g.t.	% G	l.g.t.	% G	l.g.t.	% G	l.g.t. (μ)
0	81.8	44.0	88.1	28.5	80.2	17.3	84.7	26.7
10	48.8	23.3	34.9	12.9	59.6	17.3	13.2	5.2
50	44.7	10.4	18.8	7.8	61.7	15.5	9.2	5.2
100	33.8	8.6	12.2	9.5	40.3	11.2	11.1	6.0
200	28.5	9.5	0.0	-	36.7	10.4	0.0	-
400	11.5	6.9	0.0	-	17.1	6.9	0.0	-
600	10.8	6.0	0.0	-	20.4	8.6	0.0	-
800	2.9	3.5	0.0	-	7.8	2.2	0.0	-
1000	2.8	1.7	0.0	-	7.7	2.2	0.0	-
L.S.D. at	%	%	% germination			Length of germ tube		
			7.6			5.6		
			4.9			3.2		

Table 2 : Effect of the fungicide Benlate on the radial growth rate (K_r -mm/h) of Aspergillus fumigatus, Verticillium agaricinum, Helminthosporium oryzae and Fusarium oxysporum f.sp. lycopersici.

Concentration (ppm)	A.fumigatus	V.agaricinum	H.oryzae	F.oxysporum
0	1.0	1.48	0.5	1.25
10	0.5	1.23	0.41	1.15
50	0.39	0.9	0.38	1.1
100	0.32	0.49	0.31	0.95
200	0.27	0.35	0.28	0.9
400	0.22	0.32	0.22	0.75
600	0.11	0.23	0.21	0.65
800	-	-	0.09	-
1000	-	-	-	-

Table 3 : Effect of the fungicide Benlate on biomass production (mg mycelial dry weight/50 ml medium) of Aspergillus fumigatus, Verticillium agaricinum, Helminthosporium oryzae and Fusarium oxysporum f.sp. lycopersici.

Concentration (ppm)	A. fumigatus	V. agaricinum	H. oryzae	F.oxysporum
0	354 ±12	201 ±8	210 ±10	165 ±15
10	227 ±5	184 ±6	148 ±9.5	161 ±12.5 [@]
50	126 ±4	146 ±6	131 ±8	107 ±10.6
100	108 ±4	127 ±5	118 ±6	75 ±6.8
200	53 ±3.5	51 ±3	97 ±5.5	61 ±5
400	23 ±1.7	46 ±3.2	81 ±3.4	27 ±4
600	-	30 ±2	75 ±4	21 ±2.4
800	-	8 ±1.6	21 ±2.5	21 ±2.4
1000	-	-	-	-

@ No significant difference with controls.

- No growth by the end of incubation period.

Table 4 : Effect of the fungicide Benlate on total protein, RNA and DNA (mg/g fresh weight) of Aspergillus fumigatus, Verticillium agaricinum, Helminthosporium oryzae and Fusarium oxysporum f.sp. lycopersici.

Fungus	Concentration(ppm)							
	0	10	50	100	200	400	500	800
	mg/g fresh wt.							
A. fumigatus	Total protein	21.4 ±1.2	22.2 ±1.3	48.3 ±1.4	54.1 ±1.9	52.2 ±2.2	54.6 ±2.1	-
	RNA	7.4 ±0.9	8.3 ±0.8	13.1 ±1.1	15.8 ±1.2	19.2 ±0.9	21.7 ±1.1	-
	DNA	28.5 ±1.1	27.5 ±1.2	30.1 ±1.5	33.4 ±1.4	34.1 ±0.8	40.6 ±1.8	-
V. agaricinum	Total protein	24.2 ±1.1	26.1 ±1.2	29.4 ±1.1	20.0 ±0.8	21.4 ±0.9	21.7 ±1.0	-
	RNA	39.1 ±1.3	45.2 ±1.7	47.4 ±1.9	46.9 ±1.4	43.2 ±1.7	42.8 ±2.1	-
	DNA	37.6 ±1.4	39.1 ±1.6	32.9 ±1.5	30.4 ±1.4	29.9 ±1.2	31.2 ±1.9	-
H. oryzae	Total protein	40.1 ±0.9	43.2 ±1.4	51.7 ±2.1	55.1 ±1.9	57.3 ±2.1	60.9 ±2.4	59.4 ±2.3
	RNA	36.5 ±1.3	42.1 ±1.5	31.6 ±1.8	39.9 ±1.7	38.4 ±1.8	33.5 ±1.7	30.1 ±1.4
	DNA	26.4 ±1.1	30.0 ±1.2	21.1 ±1.1	25.2 ±0.9	22.3 ±1.2	22.4 ±1.2	25.9 ±1.3
F. oxysporum	Total protein	23.9 ±0.8	27.2 ±0.9	30.3 ±0.9	29.1 ±1.2	32.9 ±1.1	-	-
	RNA	20.2 ±0.7	24.4 ±0.6	22.9 ±0.8	30.1 ±0.9	35.4 ±1.2	-	-
	DNA	46.5 ±1.3	52.4 ±1.3	37.2 ±1.1	33.3 ±1.0	30.9 ±1.2	-	-

- = Very low or no growth appeared in the flasks.

تأثير المبيد الفطرى دييونت نبلات على مظاهر النمو والبروتينات والاحماض النووية لبعض الفطريات

اسماعيل محمد كامل ، تهانى محمد على ، محمد عثمان* ، عصمت على

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مصر

تم دراسة تأثير المبيد الفطرى دييونت نبلات (فى تركيبات تتراوح من ١٠-١٠٠٠ جزء فى المليون) على نمو الجراثيم وطول انابيب الانبات والنمو الخطى ونتاج الغزل الفطرى والمحتوى البروتينى وكذلك الاحماض النووية فى الفطريات التالية : اسبيرجيللى فيوميغاتس ، فيرتيسيلليم اجاريسينم ، هيلجينيثوسيوريم اورريزى ، قيوزاريوم اوكسيسبورم (ليكوبيرس) .

وقد بينت الدراسة ان الزيادة فى تركيز المبيد الفطرى تؤدى الى نقص واضح فى نسبة الانبات وكذلك فى طول انابيب الانبات فى كل الفطريات المختبره . وفى حالة فطرى فيرتيسيلليم اجاريسينم وفيوزاريوم اوكسيسبورم (ليكوبيرس) فان الجراثيم لم تكن لها القدره على الانبات عند تركيز ٢٠٠ جزء فى المليون او اعلى من ذلك .

وقد تبين من الدراسة ايضا ان معدل النمو قد تناقص مع زيادة تركيز المبيد ونفس الشيء قد حدث مع كتلة النمو الفطرى المنتج حتى عند استخدام المبيد فى تركيبات منخفضة .

اما بالنسبة للمحتوى البروتينى فقد ادى وجود المبيد الفطرى الى زيادة المحتوى البروتينى فى كل من الفطريات ما عدا فطره فيرتيسيلليم اجاريسينم .

اما بالنسبة للحمض النووى RNA فقد زاد المحتوى فى وجود المبيد الفطرى فيما عدا فطره هيلمينثوسيوريم اورينى حيث ان المحتوى قد ازداد فقط فى التركيزات ١٠، ١٠٠، ٢٠٠ جزء فى المليون . اما الحمض النووى DNA فقد نقص محتواه فى وجود المبيد الفطرى ما عدا فى حالة فطره اسبيرجيللى فيوميغاتس .