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Conservation Study of Benlate Fungicide and its Effect on Cellulases and β- Glusidases of *Fusarium oxysporum* Isolated from Old Documents

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Abstract: Benlate fungicide was tested for its effect on the growth and cellulases and β.glucosidases produced by a strain of *Fusarium oxysporum* isolated from old manuscripts. Results obtained showed that inhibitory effect of benlate against *F. oxysporum* growth and protein content were increased as the level of benlate increased on different substrate cultures under static and shaking conditions. Also, the addition of benlate affected drastically the β.glucosidases and CMC-ase production in the mycelium and culture filtrate of the fungus under the same conditions. On the other hand, doubling the benlate concentration did not double its effect nor on the fungal growth or on enzyme production. The treatment of papyrus and linen-papers with some consolidate materials including carboxymethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose and benlate as fungicide, proved to have a positive effect on the resistance of the bio-deterioration effect of *F. oxysporum* on both types of papers.

Key words: Fusarium oxysporum · Benlate · old documents · cellulases · β.glucosidases · contamination · Papyrus · linen paper · paper conservation

INTRODUCTION

Paper is primarily composed of cellulose and other substances which is susceptible to degrade by cellulolytic microorganisms especially by the fungus *F. oxysporum* [1-3]. This fungus cause not only loss in appearance, smoothness, softness, brightness, glossiness and stretching but also weaken the fiber strength of a basis biopolymer cellulose.

The fungicide benlate has been extesively used both *in-vitro* and *in-vivo* against *Fusarium graminearum, F. moniliforme, F. avenaceum, F. oxysporum* and *F. solani* (the causal organisms of wilt and root rot diseases of some vegetable and crops plants as reported by Paul and Mishra [5], Mardels and Webbers [6], Umechuruba and Madvekue [7], Abou Ellil and F. Sharaf [8], and El- Sayed [9] reported that microbial infection produced unfavorable action on paper quality especially tear resistance and break length. Also Shaba [2] reported that the susceptibility of paper to fungal infection were differed essentially according to paper kind, age and effectual fungus.

Cellulose is the most abundant biological compound on terrestrial earth. This polymer is decomposed by a synergetic action of a multi-enzyme complex known generally as cellulases, which consists of at least, three enzymes that may vary with microorganism [fungi, bacteria, actinomycetes] and/or substrate [10-12]. These systems comprise, minimally, endoglucanases [1,4-β-D-glucan, glucanohydrolase, EC 3.2.1.4;] which cleave internal 1,4-β-linkage of cellulose; exoglucanases [cellobiohydrolase, 1,4-β-D-glucan cellobiohydrolase, EC3.2.1.91] which cleaves cellobiose unit from the non- and or-reducing end of cellulose chains; or exoglucohydrolase, [1,4-β-D-glucan glucohydrolase, EC 3.2.1. 74] which cleave glucose unit from cellulose chains and β-glucosidases (1,4-β-D-glucoside glucohydrolase, EC 3.2.1.21] which hydrolyzes cellobiose to glucose [13].

Fusarium oxysporum is considered one of the most potent fungi for the degradation of the cellulolytic materials [1, 3, 14]. The effect of some fungicides and consolidants to prevent or slow down the fungal activities were researched by Caneva *et al.* [15], Gutierrez and Flores [16] and Gutierrez. and Helio [17].

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The present work was carried out on *F. oxysporum* as one of the most active cellulolytic microorganism isolated from the old manuscripts from the view concerning: the effect of some fungicides and some consolidates to prevent or to slow down the deterioration of old manuscripts and to increase the time of safe storage of these valuable articles.

MATERIALS AND METHODS

Fungal isolate: An isolate of fungi isolated from deteriorated old manuscripts, obtained from the stores of General Egyptian Book Organization [11] and identified as *Fusarium oxysporum* in the Plant Pathology Unit of the National Research Center, Cairo, Egypt, according to Barnett and Hunter [18] and Nelson *et al.* [19] was used. The identified strain was maintained on potato dextrose agar slants (PDA) supplemented with 5% avicel.

Substrates: Different types of the substrates were used individually in these research including: avicel, papers of papyrus and linen. The used papers were cut into small pieces and were used in medium without further pretreatments.

Growth conditions: One ml spore suspension ($1x10^6$ conidia) of *F. oxysporum* was transferred to 250 ml Erlenmeyer flask containing 50 ml of sterilized [6] medium at pH 5 and incubated in a rotatory shaker (180 rpm) at 30°C for three days before use as inoculum. The culture flasks, which contained 50 ml of the above medium was supplemented with different substrates (0.5 g/flask) and inoculated with 5% inoculum (v/v). Unless otherwise stated the culture flasks in all the experiments were incubated at 30°C for 20 days in a rotatory shaker (180 rpm) for shake culture or placed in an incubator for static cultivation [11].

Enzymes Preparation: Extracellular enzymes were prepared by filtering the culture through Whatman No.1 filter paper. Intracellular enzymes were obtained by grinding the washed, cold mycelium with sand in a minimum volume of citrate-phosphate buffer (0.05 M, pH 4.8). The mixture was then centrifuged and the supernatant was used as the enzyme solution.

Enzyme assay: Endoglucanase (1,4, β-D-glucandydrolase) was assayed as carboxymethyl cellulase (CMC-ase) according to the method of Mandels and Webbers [6]. The resulting reducing sugar, in both case, was measured by, Somogyi reagent using glucose as standard.

Cellobiase (1,4-β-glucosidase) was measured by a modification of the method of Bergham and Petterisson [20] where 0.5 ml of enzyme solution was incubated with 0.5 ml of 0.4% cellobiose in 0.05 M citrate- phosphate buffer at pH 4.8 for 30 min at 50°C. The reaction was stopped by heating the reaction mixture in a boiling water bath for 5 min. The enzyme activity was determined by measuring the concentration of the released glucose using glucose-oxidase kit (Bioanalytical laboratories-Palm City U.S.A.).Enzyme assays were performed in duplicate at the condition specified. Filtrate and substrate controls were included in all assays. One enzyme unit was considered as the amount of enzyme necessary to liberate one μ mol of the reducing sugar under assays conditions specified above.

The protein contents of both culture filtrate and intercellular solution were estimated calorimetrically according to the method of Lowry *et al.* [21] using bovine serum albumin as standard.

Effect of benlate fungicide on the growth and enzyme production: Benlate as fungicide was added in the cultures of avicel; papyrus and linen- papers at two different concentration (6.25 and 12.5 ppm) to test its effect on fungal growth and enzyme production.

Effect of some consolidates on paper fungal infection: Specimens of papyrus and linen- papers were treated with some consolidated materials, including: carboxymethyl cellulose, (CMC, 2.5%); hydroxyethyl cellulose, (HEC, 1%) hydroxypropyl cellulose, (HPC, 1%) and benlate as fungicide (12.5 ppm). These specimens were sprayed with the spore suspension of *F. oxysporum* and incubated for one month in a dessicator with 100% relative humidity at 28°C. At the end of the incubation period, all paper specimens were carefully washed with distilled water, air dried, then evaluated their resistance to the deterioration effect of *F. oxysporum* in terms of tensile strength and α-cellulose percentage.The tensile strength was tested in Paper Lab., Chemistry Administration, Ministry of Industry and Technology. Each experiment was performed in triplicate; the average was taken as data.

RESULTS AND DISCUSSIONS

Effect of benlate on F. oxysporum growth

Mycelium dry weight: Data in Table 1 showed that both concentrations of benlate (6.25 and 12.5 ppm) suppressed the mycelium dry weight of *F. oxysporum* grown on different substrate cultures under the two types of culture conditions (shaken and static). Data also showed

Table 1: Effect of benlate fungicide on the mycelium dry weight of *F.* oxysporum on different substrate cultures under shaking and static conditions

	Cultivation conditions					
	Shaking			Static		
Benlate (ppm)	Papyrus	Linen	aviceil	papyrus	linen	aviceil
0.0	0.164	0.496	0.5798	0.196	0.469	0.449
6.25	0.155	0.433	0.470	0.167	0.449	0.428
12.5	0.133	0.416	0.419	0.161	0.439	0.401
Mean	0.151	0.448	0.489	0.175	0.452	0.426

that when avicel was added to the medium contain different concentrations of benlate under shaking conditions gave higher mycelium dry weight than paperus and linen papers. While, under static condition, higher dry weight was obtained with linen. These results are totally in agreement with those of Wahid *et al.* [22], Zeidan [23]and Nawar [24], they reported that benlate at 10 ppm in a synthetic nutrient agar medium inhibited growth of *F. moniliforme*. The difference between results may be due to the differences in tested fungal isolates.

Protein content: Data in Table 2 show that protein content of *F. oxysporum* was decreased in different substrate cultures and the two types of cultural conditions. Data also clearly indicate that the protein content of the mycelium extract was always higher than the corresponding figures of the filtrate culture.

In general, protein content of *F. oxysporum* in culture filtrate and mycelium extract of different substrate cultures was higher in shaking than static cultivations.

1,4-β-glucosidase: Data in Table 2 show that addition of benlate to different substrate culture medium led to reduction of *F. oxysporum* 1,4-β-glucosidase in the mycelium and culture filtrate under static and shaking cultivations except, benlate when used in media applied with linen under static condition which did not affect β-glucosidase production.

The production of β-glucosidase in the mycelium of *F. oxysporum* of the control and benlate treatment was higher than those recorded in culture filtrate under static and shaking conditions. Generally, the β-glucosidase production in the presence of benlate was high in the mycelium and culture filtrate of *F. oxysporum* under static and shaking conditions growing on mineral medium in the

Table 2: Effect of benlate concentrations on protein content and enzyme production in filtrate and mycelium of *F. oxysporium* on different substrate cultures under shaking and static conditions

	Shaking culture						Static culture					
Benlate (ppm)	papyrus		linen		avicel		papyrus		linen		avicel	
Sub.	Filt.	Myc	Filt.	Myc	Filt.	Myc	Filt.	Myc	Filt.	Myc	Filt.	Myc
a- protein content												
0.00	0.92	34.09	0.58	23.71	0.64	49.38	0.38	38.27	0.29	9.90	0.38	11.27
6.25	0.84	30.94	0.41	17.22	0.34	15.96	0.31	31.01	0.27	8.22	0.33	11.06
12.50	0.80	21.62	0.36	7.91	0.28	13.02	0.30	29.04	0.25	7.97	0.32	9.94
Mean	0.85	28.89	0.45	16.28	0.42	26.12	0.33	32.77	0.27	8.69	0.34	10.76
b- 1,4-β-glucosidase												
0.00	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
6.25	12.94	13.77	3.61	27.5	11.28	12.33	52.48	60.16	90.91	100.0	86.67	53.92
12.50	12.22	10.76	3.16	12.39	9.86	11.23	49.84	53.71	86.36	10.0	61.33	40.00
Mean	41.72	41.51	35.59	46.63	40.38	41.19	67.44	71.29	92.42	100.0	82.67	64.64
c- Carboxymethyl cellulase (CMC-ase)												
0.00	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
6.25	6.61	21.01	0.0	61.61	3.48	12.14	56.46	N.D.	45.10	62.50	57.50	58.04
12.50	6.31	N.D.	0.0	25.0	2.03	5.77	42.76	N.D.	35.29	42.50	52.50	N.D.
Mean	37.64	60.51	33.33	62.20	35.17	39.30	66.40	100.0	60.13	68.33	70.00	79.02

*Filt= Filtrate

**Myc.= Mycelium

Table 3: The biodeterioration effect of *F. oxysporum* on papyrus and linen papers pretreated with benlate and some consoldate materials

		α-cellulose		Tensil strength kg/15mm	
Paper type	Treatment	Value %	Change %	In machine direction	In cross direction
*Papyrus	Control (uninfected or treated)	80.46	100.0	N.d	N.d
	Infected paper	55.73	-30.73	N.d	N.d
	Infected paper and treated with:Benlate	70.44	-12.45	N.d	N.d
	CMC	64.01	-20.45	N.d	N.d
	HEC	60.71	-24.55	N.d	N.d
	HPC	66.04	-17.92	N.d	N.d
Linen	Control (uninfected or treated)	91.90	100.0	20.5	12.1
	Infected paper	76.8	-16.43	9.6	5.7
	Infected paper and treated with: Benlate	90.84	-1.15	12.9	6.5
	CMC	85.74	-6.70	10.2	6.5
	HEC	83.49	-9.15	9.8	6.1
	HPC	90.7	-1.31	12.1	7.3

*:Du to the nature of papyrus, its tensile strength can not be measured N.d: Not determined CMC:carboxymethylcellulose. HEC: hydroxyethyl cellulose. HPE: hydroxypropyl cellulose.

presence of linen than the corresponding figures of papyrus and aviceil substrates.

Carboxymethyl cellulase (CMC-ase): It is clear from Table 2 that addition of benlate affected drastically the *carboxymethyl cellulase (CMC-ase)* production in the mycelium and culture filtrate of *Fusarium oxysporum* of different substrate cultures under the two types of cultural conditions. Also, the CMC ase content of the mycelium extract was always higher than in cultures filtrates in all substrates and cultural conditions.

In general, *carboxymethyl cellulase (CMC-ase)* produced by *F. oxysporum* in culture filtrate and mycelium extract of different substrates was higher in shaking culture than static conditions.

Results also in general, indicated that the effect of benlate on the fungal growth and enzymes production depend on the benlate concentrations and the types of the substrate in the media putting in consideration the cultivation conditions. As the effect of benlate on the production of *CMC-ase* was more than its effect on the production of β-glucosidase of *F. oxysporum* for all types of the substrate.

Although, benlate fungicide at the two concentrations used had a great effect on the production of the two enzymes, it can even prevent their formation extracellularly. On the other hand, doubling the benlate concentration did not double its effect nor on the fungal growth or on enzyme production. The protein contents,generally were lower than the control which maybe taken as evidence for the effect of benlate on fungal growth.

Effect of benlate and some consolidates on paper fungal infection

Effect o benlate fungicide: The treated paper were prepared by impregnated paper specimens in benlate at 25 ppm ; 2.5% carboxy methyl cellulose, 2% hydroxyl methylcellulose and 2% hydroxyl propyl cellulose, sprayed with spore suspension of *F. oxysporum* then incubated for one month at 28°C in a desiccators with 100% relative humidity. The results in Table 3 showed that *F. oxysporum* has a deteriorating effect on linen and papyrus papers.

The result showed that *F. oxysporum* has a deteriorating effect on linen and papyrus papers. The observed decrease in α-cellulose in Table 3 is related to the breakdown of the long cellulose chains to small units, due to the effect of fungal growth and the enzymes production. So it becomes more susceptible for solubilization in NaOH (17.5%). The positive effect of the treatment on both types of papers was in the order: 25 ppm of benlate > HPC>CMC>HEC.

The results also showed that benlate fungicide has a positive effect on α-cellulose value and tensile strength, i.e., the deterioration of the paper is retarded or prevented. The same results were reported by Shaba [2] and Ismail *et al*. [25].

In respect to the consolidated polymers, all materials gave positive effects for decreasing the deterioration effect of *F. oxysporum*. The uses of the above consolidated polymers for conservation of different types of papers had been reported by Shaba [2], De-Graaff [26] and Bicchieri and Mucci [27]. Paper conservators can use the information from these

experiments to help protect manuscripts against deterioration *by F.oxysperium*.

In conclusion, the isolated strain of *F. oxysporum* from old manuscripts,proved to have the ability to deteriorate cellulosic materials depending on the substrate and cultivation conditions. Although the hydrolysis of the cellulosic materials produced di and mono sugars which may cause a decrease in the enzymes activities by what is called product inhibition, this may not be true for this microorganism where this strain have the ability to grow and produce a considerable level of the cellulosic enzymes on mono-and di-sugars, this means that the deterioration effect was increases all the time. However, the treatment o the cellulosic materials by benlate or some conservators can slow down the biodeterioration effect caused by *F. oxysporum.*

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