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Study of chemical stability for chlorothalonil and their fungicidal effect against *Fusarium solani* and *Botrytis cinerea*.

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ABSTRACT

This study investigated the degradation of two chlorothalonil formulations [Chlorcal 70% Wettable Powder (WP) and Open 72% Suspension Concentrate 72% (SC)]. The tested fungicides were stored at 54 ± 2°C for 14 days. During the different storage periods, samples were taken after 1, 3, 7, and 14 days to determine physical properties, evaluate the chemical stability of the active ingredient: Hexachlorobenzene as impurities by HPLC and fingerprint (GC/MS and IR), as well as the evaluation of their efficacy, by testing them against the mycelial growth of *Fusarium solani* and *Botrytis cinerea* under *in vitro* condition. The results indicated that Open 72% SC was the most effective fungicide against *F. solani* and *B. cinerea* it recorded the lowest EC_{50} value of 28.4 and 46.8 ppm respectively, followed by Chlorcal 70%WP with EC_{50} values of 42.9 and 59.4 ppm respectively. On the other hand, the effectiveness of tested fungicides deteriorated as the result of storage at 54 ± 2 ºC for 14 days with EC_{50} values increased from 28.4 to 130.9 and 46.8 to 278.1 in the case of Open 72% SC against *F. solani* and *B. cinerea* respectively, while the degradation efficiency of Chlorcal 70%WP was decreased at storage with EC_{50} values from 42.9 to 89.4 and 59.4 to 145.3 against these two fungi respectively. Chlorcal 70%WP was more stable at during storage and less effective against fungal pathogens and determination of chemical content (total soluble sugar; total protein; free amino acids) and some enzymes (amylase and catalase) activities of mycelium fungi. Potato dextrose (PD) media treated with chlorothalonil formulations induced a significant decrease in all components in each fungus except the catalase enzyme in the case of *B. cinerea.* The obtained results showed that active ingredients were 69.82%, 71.9% and reached 69.3%, and 70.75% after 14 days of storage at 54 ± 2 ºC for Chlorcal 70% WP, Open 72% SC, respectively. Also calculated half-life ($T_{0.5}$) values for Chlorcal 70% WP, Open 72% SC were 1382.07 days and 614.035 days, respectively. This result refers that Chlorcal 70% WP is more stable than Open 72% SC after storage at 54 ± 2 ºC for 14 days, whereas the amount of hexachlorobenzene was within the FAO limits for all sources before and after storage. Then the other tested GC / MS was used for the degradation of chlorothalonil fungicide, the two major degradation products in chlorothalonil were 2,5,6-trichloro-4- hydroxyisophthalonitrile and 2,4,6-trichloroisophthalonitrile.

Keywords: Fungi, Fungicides, Chlorothalonil, Hexachlorobenzene, IR, GC/MS, Chemical stability, Physical properties, Antifungal activity, Chemical composition, Enzyme.
Article type: Research Article.

INTRODUCTION

Agriculture is one of the main human activities at the economic level. It supplies food for a human (Lazarovits *et al.* 2014; Timothy & Adejumo 2021). Crop diseases are responsible for extreme economic losses, and they may be caused by different types of organisms (Fisher *et al.* 2012; Kudryavtsev *et al.* 2021; El-Sayed *et al.* 2022; Almuhsin Ahmed *et al.* 2022). Indeed, fungi are one of the most important threats to agriculture systems (Doehlemann *et al.* 2017; Cordero-Bueso *et al.* 2017). This is because they possess characteristics and properties

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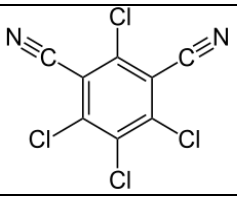
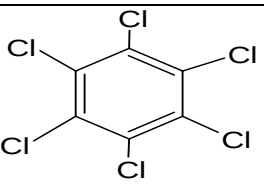
that make them dangerous. Fungi are considered the most outstanding of all plant pathogenic, which cause economic losses of USD 10 to 100 billion worldwide (Hua *et al.* 2018). *Botrytis cinerea* causes a grey mold on more than 240 plant species and it is a major pathogen of cultivated fruits and ornamental flowers. Economic damage attributed to *Botrytis* is enormous. It includes pre-and post-harvest losses in quantity and quality of vegetables (Rocío *et al.* 2021). *Fusarium solani* and *Rhizoctonia solani* are the most important soil-borne fungal pathogens causing the symptoms of damping-off and root rot diseases in a wide range of vegetable and crop plants including tomatoes (Abu-Taleb *et al.* 2011). *F. solani* causes severe root rot in snap beans (*Phaseolus vulgaris* L.). Although this disease has caused great crop loss in the bean-growing areas of the United States for many years (Burke 1972). The application of fungicides is the most convenient and predominant way of disease control. Their use had made it feasible to enhance crop yields and food production. The efficacy of fungicides is influenced by many biological and environmental factors that directly influence the metabolic activities of fungal cells (Reinprecht 2010; Ijaz *et al.* 2015; Peerzada *et al.* 2020). Chlorothalonil, a widely-used chloroisophthalonitrile fungicide, is highly toxic to aquatic organisms and amphibians. It is essential to understand the persistence and fate of chlorothalonil in aquatic environments (Reyna *et al.* 2021; Pei Lv *et al.* 2022). Other metabolite products cited in the literature are the result of reactions leading to the substitution of Cl atoms in chlorothalonil and conversion of the CN functional groups to amides, thiazoles, and acidic groups (Rouchaud *et al.* 1988; Regitano *et al.* 2001; Putnam *et al.* 2003; Kwon & Armbrust 2006). Chlorothalonil (1,3-Benzene dicarbonitrile,2,4,5,6. tetrachloro) is contaminated with carcinogen hexachlorobenzene during its manufacture NTP (2011). It may contain main contaminants, i.e., hexachlorobenzene maximum up to 0.004% according to FAO (2005). Hexachlorobenzene (HCB) is one of the highly toxic and persistent compounds that are released unintentionally through various man-made chemicals. It is considered a member of POPs (persistent organic pollutants), since it is persistent for a long period of time in the environment and found to be hazardous to all living organisms (Mukesh Kumar *et al.* 2013). The present study aimed to evaluate the effect of storage at 54 ± 2 ºC for 14 days on fungicides (Chlorcal 70% WP; Open 72% SC) and determine physical properties. The active ingredient of chlorothalonil; hexachlorobenzene as impurities by HPLC and fingerprint (GC/MS and IR spectra), as well as the evaluation of their efficacy, by testing them against the mycelial growth of *Fusarium solani* and *Botrytis cinerea* under *in vitro* condition and effect of chlorothalonil formulations on chemical content and enzymes activities of mycelium fungi.

MATERIAL AND METHODS

Experimental work.

Fungicides Formulations Used

Table 1. The structure of tested fungicides used and its impurities.

Trade name	Common name	Structure of Chlorothalonil	Structure of impurities (Hexachlorobenzene)
Chlorcal 70% WP* Open 72% SC**	Chlorothalonil		

*WP: Wettable Powder. **SC: Suspension Concentrate.

Accelerated storage procedures

Formulations of chlorothalonil (Chlorcal 70%WP and Open 72%SC) were placed in bottles (about 50 mL). These bottles were then exposed to storage at 54 ± 2 ºC for 14 days. During storage period, samples were taken at 1, 3, 7 and 14 days to determine the chemical and physical properties, the toxic impurities content and fingerprint by (GC/MS and IR) according to FAO (2010).

Standard preparation

Standard preparation of the chlorothalonil

Weight 10 mg of Chlorothalonil of known purity analytical content in 25 mL grade (A) flask measurement dissolved and finished methanol.

Standard preparation of hexachlorobenzene as impurities

Weight 10 mg of hexachlorobenzene of known purity analytical content in 25 mL grade (A) flask measurement dissolved and finished methanol.

Sample preparation for tested fungicides

Accurately weighed sufficient samples formulation equivalent to 10 mg of standard in a different 25 mL volumetric flask for each samples, and slowly mixed with methanol.

Sample preparation for impurities

Formulation samples 1 g tested in 25 mL volumetric flask for each samples were prepared and slowly mixed with methanol and the volume was completed with methanol.

Determination of fungicides used by HPLC instrument

Determination of chlorothalonil

The active ingredient percentage for Chlorcal 70%WP and Open 72%SC were determined before and after storage according to a modified method (CIPAC Handbook K, p.13, 2003) for chlorothalonil. Under these conditions, the retention time (RT) of chlorothalonil was 2.23 min. Some modification (Agilent technologies 1260 Infinity II) with used ultraviolet UV-detector and the column Eslips Plus C18, diameter 5 mm and length 4.6 × 2.5 mm. Methanol – Acetonitrile (30:70) v/v was used as a mobile phase, at the rate of 1.3 mL/min, 40 ºC column temperature and wavelength 210 nm.

Determination of hexachlorobenzene

They were determined before and after storage according to this method of CIPAC (1995). At these conditions the retention time (RT) of chlorothalonil was 4.13 min. Some modification (Agilent technologies 1260 Infinity II) with used ultraviolet UV-detector and the column Eslips Plus C18, diameter 5 mm and length 4.6 × 2.5 mm. Methanol – Acetonitrile (30: 70) v/v was used as a mobile phase, at the rate of 1.3 ml/Min, 40ºC column temperature and wavelength 210 nm.

Determination of the main physical properties

Preparation and determination of physical properties of standard water used

According to CIPAC (1995) MT 18.1 Non-CIPAC Standard Waters, 18.3.1 WHO Standard Hard Water, (342 ppm hardness) was used in all tests of physical properties. Calcium chloride ($CaCl_2$; 0.304 g) and magnesium chloride ($MgCl_2$. 6 H_2O; 0.139 g) were dissolved in distilled water and made up to 1000 mL.

Suspensibility test of Chlorcal 70% (WP) and Open 72% (SC) formulations

The test was run according to CIPAC (2003). Weighing accurately sufficient sample of WP and SC (2.5 ×100 /conc. of the sample) after shaking the container. Samples were transferred quantitatively into a 250 mL Stoppard measuring cylinder containing the standard water, the volume was completed to 250 mL with the water, the cylinder was inverted 30 times in one min, and then placed in a water bath maintained at 30°C ± 2°C for 30 min. At the end of this time, the separated materials or precipitation, if any, were measured.

Gas-Chromatography-Mass spectrometry analysis of chlorothalonil (Chlorcal 70% WP and Open 72% SC)

Apparatus Agilent 7890 B, 5977 A MSD gas chromatography equipped with an Agilent mass spectrometric detector, with a direct capillary interface and fused silica capillary column (30 m × 0.025 mm HP-5-0.25 micron -60 to 325/325ºC) was used. Samples were injected under the following conditions: Helium was used as carrier gas at approximately 1 mL min^{-1}, pulsed split mode, split ratio (10:1), split flow 10 mL min^{-1}. The solvent delay was 4 min and the injection size was 1 µL; oven temperature program, 50 °C for 0.5 min, then 10 °C/min ramp to 190 °C followed by a 10 °C min^{-1} ramp to 210 °C for 1 min followed by a 10 °C min^{-1} ramp to 300 °C and held for 2 min (total run time: 34 min) the injector temperature was set at 280 °C. W9N11 mass spectral database was used in the identification of the separated peaks.

The absorbance of chlorothalonil (Chlorcal 70% WP and Open 72% SC) in infrared (IR spectra)

The Fourier transform infrared (Avtra 330 Thermo Nicolet) was used to analyse, with some alteration, the effect of storage on the absorption of feature groups and the fingerprint of formulations of chlorothalonil. The samples

were prepared by homogenizing 0.01 g of the sample with 0.1 g of dry potassium bromide (KBr) using an agate mortar and pestle, and then 0.03 g of the above mixture was transferred with forceps to a clean stainless steel slide and put in the piston to form a clear and thin film of a sample.

Kinetic study

The rate of degradation of the tested active ingredient and half-life periods ($T_{0.5}$) for the tested pesticides were calculated according to equation presented by Moye *et al.* (1987).

$T_{0.5}$ = ln 2/ K= 0.6932/K and K = $\frac{1}{TX}$ ln $\frac{a}{bx}$

where K = rate of decomposition; a = initial residue; tx = time in days of hours; bx = residue at x time

Fungal strains used

Pure cultures of *Fusarium solani* and *Botrytis cinerea* were supplied from the Bactericide & Fungicide and Nematicide Research Department, Central Agricultural Pesticide Lab. (CAPL), Agricultural Research Centre (ARC), Dokki, Giza, Egypt.

Antifungal assay

Efficacy tested of chlorothalonil formulations against *F. solani* and *B. cinerea in vitro*

Used to two different fungicides (Open 72%SC and Chlorocal 70%WP) were tested at concentrations of 1000, 500, 250, 125 and 62.5 ppm before and after storage at 54 ± 2 ºC for 14 days and they were evaluated on radial growth of test fungus by Poisoned Food Technique (Mohanty *et al.* 2012 and Moreno-Gavira *et al.* 2020). Stock solutions of the fungicides were prepared in sterile distilled water and added separately to get the required concentrations thin mixed with 50 mL sterilized PDA medium and transferred equally into three Petri dishes. The media were allowed solidifying. The plates prepared without fungicides served as control. These plates were inoculated with a 5-mm disc of 7 days old culture of the test fungi. All dishes were incubated at 27 ± 2 ºC for 7 days and radial growth of the colony was measured when the mycelia of control completely with the colonization of the fungi. Each treatment was performed in triplicate. The fungal growth inhibition, which calculated due to treatment against the control using the following formula (Satya *et al.* 2014):

Inhibition of growth (%) = R-r/R *100

where R is the radial growth of fungal mycelia in the control plate.
r is the radial growth of fungal mycelia in the treated plate.

Statistical Analysis

The concentration inhibition regression lines were drawn according to the method of Finney (1971).

Effect of chlorothalonil formulations (EC_{50}) on total soluble sugar, total protein, free amino acids and amylase; catalase enzymes produced by *F. solani* and *B. cinerea*

Open 72%SC and Chlorocal 70%WP at EC_{50} of each fungus were added to 50 mL sterilized (PD) medium inoculated with 3 discs (5mm) of any fungus *F. solani* or *B. cinerea.* Three flasks were used as replicates. All flasks were incubated at 27 ± 2 ºC. When the mycelial growth covered the surface, in the untreated flask (check). The mycelial matrix was excluded by filtration and mycelial mates were homogenized and were carried out.

Determination of the chemical content of mycelium fungi

Determination of total soluble sugar

Total soluble sugars of fungi were determined according to Shaffer-Somogi micro method described in A.O.A.C. (1995).

Determination of total protein

A ratio of 1: 2.5 (w/v) of each fungus to extraction buffer (0.125M Tris-borat, pH 8.9) was used. The soluble protein concentration was spectroscopically determined by referring to a calibration curve relating to the concentration of authentically albumin bovine at 546nm according to Lowery *et al.* (1951).

Quantitative assay of free amino acids as lysine

Samples were prepared by extracting 0.5g of each fungus by 25 mL ethanol 80% (Jayaraman 1985). A standard solution of lysine was prepared by dissolving 0.02 g lysine in 100 mL of 80% ethanol. The colour developed was measured using a spectrophotometer at wavelength of 570 nm. The concentrations of free amino acids were calculated as lysine.

Determination of total amylase activity

One gram of each fungus was homogenized in a mortar with 4 mL 0.01M Tris-HCL buffer pH8.0 containing 0.02 M NaCl and $CaCl_2$. The supernatant was used for total amylase activity according to the method described by Dewez *et al.* (2005). The total amylase activity is expressed as mg starch consumed/ 15min/1g fungus.

Determination of catalase activity

One gram of each fungus was homogenized two times with 0.01 M phosphate buffer (pH 7.0). Catalase activity was determined according to the method described by Dewez *et al.* (2005). The enzyme was determined as the change in absorbance at 240 nm per one-gram fungus/1min.

Statistical analysis

of the data was carried out by analysis of variance (ANOVA) with SAS software as stated by SAS Institute, (1982). The $p < 0.05$ (low), $p < 0.01$ (moderate), $p < 0.001$(high) were accepted as the level of probability and comparison of means by tukey's studentized range test.

RESULTS AND DISCUSSION

Effect of storage on the stability of chlorothalonil (Chlorcal 70% WP and Open 72% SC) and their impurities

Table 2 shows the effect of storage at 54 ± 2 ºC for 14 days on the stability of the commercial chlorothalonil formulation under the trade names Chlorcal 70%WP and Open 72%SC. chlorothalonil active ingredient were 69.82%, 71.9% and reached 69.3%, 70.75% after 14 days of storage at 54 ± 2 ºC for Chlorcal 70% WP and Open 72% SC, respectively. In addition, percentage loss reached 0.74 and 1.59% after 14 days of storage for Chlorcal and Open respectively, while calculated half-life ($T_{0.5}$) values were 1382.07 and 614.035 days respectively. This result refers that Chlorcal 70% WP is more stable than Open 72% SC. The data in Table 2 shows the effect of storage on the amount of hexachlorobenzene (HCB) at 54 ± 2 ºC for the various study intervals. The amount of hexachlorobenzene was within the FAO limits for all sources before and after storage in chlorothalonil content. In addition, the amount of hexachlorobenzene before storage was 0.00383% and 0.00383%, which became 0.00754% and 0.00549% after 14 days of storage at 54 ± 2 ºC in Chlorcal 70%WP and Open 72%SC, respectively for to hexachlorobenzene. This level is decreased than the maximum level (0.04 g kg^{-1}) defined by FAO (2005). Hexachlorobenzene (HCB) is a persistent, non-degradable, chlorinated hydrocarbon that was first introduced as a fungicide in 1945 for seed treatment. It is also found as an unintentional by-product in the manufacture of chlorinated solvents such as carbon tetrachloride, perchlorethylene, trichloroethylene, and pentachlorbenzene (Mukesh Kumar *et al.* 2013). Hexachlorobenzene (HCBz) is an environmentally-persistent organic pollutant but is widely applied as a pesticide and raw material for pesticide manufacture or other chemical synthesis (Selli *et al.* 2008).

Table 2. Effect of storage of chlorothalonil (Chlorcal 70% WP and Open 72% SC) and their impurities.

Storage Periods (Days)	Chlorcal 70%WP			Open 72%SC		
	A.I 70%	Loss%	Hexachlorobenzene	A.I 72%	Loss%	Hexachlorobenzene
0	69.82	0.00	0.00383	71.9	0.00	0.00383
1	69.79	0.043	0.00410	71.82	0.11	0.00395
3	69.70	0.17	0.00463	71.66	0.33	0.00419
7	69.58	0.34	0.00569	71.33	0.79	0.00466
14	69. 3	0.74	0.00754	70.75	1.59	0.00549
$T_{0.5}$ (days)	1382.07			614.035		

Note: Zero: one hour before storage; AI: active ingredient of Chlorothalonil; $T_{0.5}$ = half life

Effect of storage at 54 ± 2 °C on suspensibility of Chlorcal 70% WP and Open 72%SC.

The data presented in Table 3 depicts a suspensibility test for Chlorcal 70% WP and Open 72% SC stored at 54 ± 2 °C for 14 days. Data revealed that no sediment was formed during storage period in initial, 3 and 7 days for these fungicides, but a little of sediment was appeared for them in 14 days from storage. According to FAO specifications, data showed that the samples conform this specification.

Table 3. Effect of storage at 54 ± 2 ºC on suspensibility of Chlorcal 70% WP and Open 72% SC.

Storage Periods (Days)	Chlorcal 70%WP	Open 72% SC
0	-	-
1	-	-
3	-	-
7	-	-
14	*	*

Note: (-) means no sediment; (*) means a little sediment.

Effect of chlorothalonil formulations before and after storage at 54 ± 2 ºC for 14 days on the mycelial growth of *F. solani* and *B. cinerea in vitro*

Data in Table 4 indicated that there is relationship between the tested concentration of Chlorothalonil formulations (Chlorcal 70%WP and Open 72%SC) before storage and their inhibition effect against *F. solani* and *B. cinerea* under laboratory conditions. The results showed that the Open 72% SC was the most effective fungicide against *F. solani* and *B. cinerea,* recording the lowest EC_{50} value of 28.4 and 46.8 ppm respectively, whereas the highest EC_{50} value was found by Chlorcal 70%WP. Notably, the increased fungicidal activity of Open 72% SC may be due to the wetting and the depressing agents that are used in the formulation. On the other hand, the effectiveness of tested fungicides deteriorated as the result of storage at 54 ± 2 ºC for 14 days with EC_{50} values increased from 28.4 to 130.9 and 46.8 to 278.1 in the case of Open 72% SC against *F. solani* and *B. cinerea* respectively, while dry fungicide formulations such as Chlorcal 70%WP was more stable during storage and less effective against *F. solani* and *B. cinerea,* since it recorded the highest EC_{50} value of 42.9 and 59.4 ppm respectively. The degradation efficiency of Chlorcal 70%WP was decreased at storage by EC_{50} values from 42.9 to 89.4 and 59.4 to 145.3 against *F. solani* and *B. cinerea* respectively.

Deng *et al.* (2015) reported the effect of temperature and pH on the chlorothalonil degradation by the strain BJ1, indicating that strain BJ1 and the degradation efficiency of chlorothalonil were decreased at a temperature lower than 25 °C. Barak & Edgington (1984) reported that chlorothalonil and MDR were perused a field resistance and other plant pathogenic fungi, such as *B. cinerea*. From these results, it could be concluded that additives in the Open 72% SC decreased the effectiveness of active ingredients against *F. solani* and *B. cinerea* and increased its degradation under storage conditions, while Chlorcal 70% WP was more stable during storage and less effective against *F. solani* and *B. cinerea*.

Effect of chlorothalonil formulations (EC_{50}) on chemical content and enzymes activities of mycelium fungi

According to data in Tables 5 and 6, the PD liquid media treated with Chlorcal 70%WP or Open 72%SC showed a significant decrease in total soluble sugars for *F. solani* and *B. cinerea* compared to untreated media. The reduction of total soluble sugars was moderately significant ($p < 0.01$) in the case of *B. cinerea,* while it was low ($p < 0.05$) in that of *F. solani*. Data revealed that the reduction of free amino acids for the fungi was observed due to treatment of PD media with both tested chlorothalonil formulations. The reduction of free amino acid showed significant reduction being 3.81 ± 0.1 and 3.32 ± 0.1 respectively in *B. cinerea* and moderated (41.8 ± 5.2 and 39.9 ± 5.3 mg g^{-1}) in *F. solani.* Data also showed the inhibition of total protein for the two fungi in PD media treated with chlorothalonil formulations.

The inhibition of total protein was highly significant in the case of *B. cinerea*, while moderate in *F. solani,* compared to the untreated media. Amylase activity was moderately inhibited in both *F. solani* (1.6 ± 0.01 and 1.1 ± 0.01 respectively) and *B. cinerea* (10.6 ± 3.2 and 10.0 ± 3.2 mg g^{-1} respectively) compared to untreated media (3.7 ± 0.1 and 14.7 ± 2.2 mg g^{-1} respectively).

Table 4. Effect of chlorothalonil formulations before and after storage at 54 ± 2ºC for 14 days on the mycelial growth of *F. solani* and *B. cinerea in vitro*.

Concentrations (ppm)	Fungicides			
	Mycelial growth inhibition percentages			
	Before storage		After storage	
	Chlorcal 70% WP	Open 72% SC	Chlorcal 70% WP	Open 72% SC
	F. solani			
1000	84.3	89.7	68.7	65.3
500	71.1	70.6	57.5	58.0
250	67.7	52.7	47.2	45.7
125	54.7	38.4	30.3	37.0
62.5	46.3	28.0	21.0	25.6
Control	0.0	0.0	0.0	0.0
EC_{50}	42.9	28.4	89.4	130.9
EC_{90}	168.2	104.5	324.3	352.4
Slope	0.25 ± 0.14	0.42 ± 0.14	0.39 ± 0.133	0.24 ± 0.13
	B. cinerea			
1000	75.6	82.6	65.0	62.3
500	67.4	67.0	52.0	53.6
250	57.0	58.3	43.0	48.0
125	43.6	50.0	38.6	40.2
62.5	37.6	38.3	30.3	30.0
Control*	0.0	0.0	0.0	0.0
EC_{50}	59.4	46.8	145.3	278.1
EC_{90}	259.4	187.9	441.9	586.5
Slope	0.32±0.14	0.21±0.14	0.69±0.13	0.25±0.13

Note: Each number represents the mean of 3 replicates; *Control without active ingredient (medium free and discs were cut from the pathogen only on PDA).

Table 5. Effects of chlorothalonil formulations at EC_{50} on total soluble sugars; free amino acids; total protein, as well as the amylase and catalase activities produced by *Fusarium solani*.

Components/ Parameters	Treated with Chlorcal 70%WP	Treated with Open 72%SC	Untreated (control)
Total soluble sugars (g/1g fungus)	0.015 ± 0.001*	0.012 ± 0.001*	0.025 ± 3.8
Free amino acids ($g\ g^{-1}$ fungus)	41.8 ± 5.2**	39.9 ± 5.3**	50.5 ± 3.5
Total protein ($g\ g^{-1}$ fungus)	4.7 ± 1.3**	4.0 ± 1.3**	5.7 ± 1.4
Amylase activity (mg starch/ 1g)	1.6 ± 0.01**	1.1 ± 0.01**	3.7 ± 0.1
Catalase activity (OD/1min)	0.81 ± 0.03	0.80 ± 0.2	0.84 ± 0.01

Note: * = significant at $p < 0.05$; ** = at $p < 0.01$; *** at $p < 0.001$.

According to data in Tables 5 and 6, the treatment with chlorothalonil formulations exhibited non-significant effect on catalase activity for *B. cinerea* (0.65 ± 0.02 OD/1 min) compared to untreated media (0.55 ± 0.02 OD/1 min).

These results are in agreement with Petit *et al.* (2012) and Ijaz *et al.* (2015) who illustrated that pesticides applied to protect plants against fungal diseases contain one or more active substances, and their action in fungal cells includes: arrest of mitosis and cell division, inhibition of respiration, synthesis of nucleic acids, amino acids and proteins, lipids and cell membranes, as well as cell wall melanin, along with inhibition of sterol and cell wall biosynthesis, and signal transduction. Shi *et al.* (2011) found that chlorothalonil affects inhibitory fungus cells, leading to the inhibition of enzymes responsible for cellular respiration, also affecting soil biochemical properties and proving to be an inhibitor of acid phosphatase, catalase and dehydrogenase activities.

Table 6. Effects of chlorothalonil formulations at EC_{50} on total soluble sugars, free amino acids, total protein, along with the amylase and catalase activities produced by *Botrytis cinerea.*

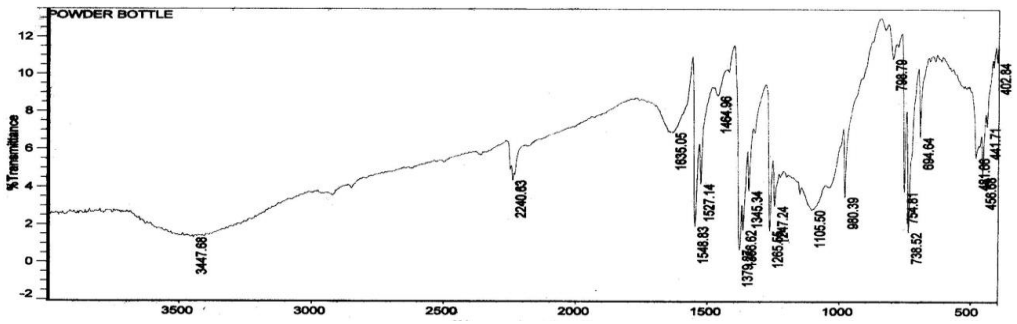
Components/ Parameters	Treated with Chlorcal 70% WP	Treated with Open 72% SC	Untreated (control)
Total soluble sugars (g g^{-1} fungus)	0.05±0.01**	0.04 ± 0.01**	0.06 ± 0.001
Free amino acids (g g^{-1} fungus)	3.81±0.1*	3.32 ± 0.1**	5.8 ± 0.02
Total protein (g g^{-1} fungus)	11.5 ± 2.6***	10.8 ± 2.7**	15.8 ± 1.3
Amylase activity (mg starch/ 1g)	10.6 ± 3.2**	10.0 ± 3.2**	14.7 ± 2.2
Catalase activity (OD/1min)	0.65 ± 0.02	0.55 ± 0.02	0.77 ± 0.02

*significant at $p < 0.05$ **$p < 0.01$ *** $p < 0.001$.

Identification of chlorothalonil in infrared.

The data in Table 7 and Figs. 2-3 reveal all possible conformations of chlorothalonil (Chlorcal 70% WP and Open 72% SC) before and after storage. The tangential C–C stretching in benzene derivatives found broad, strong bands at 3434–3460 cm^{-1}. Also, Table 7 depicts that the benzene molecule has two out-of-plane skeletal vibrations, such as modes 4 and 6, the former is also said to be C–C–C puckering. In the substituted benzenes, C–C–C puckering is expected normally in the region of 694–695 cm^{-1}. Generally, stretching and bending vibrations of C–Cl bonds lie in the low wavenumber region of the spectrum defined by Doddamani *et al.* (2007). In the vibrational studies of 3-chloro, 4-chloro, and 5-chloro-2-methyl phenyl isocyanates, C–Cl stretching vibration gives rise to strong absorption in the range of 755–694 cm^{-1}. The C–Cl bond energy of chlorothalonil has a great influence on the selection of degradation path, and this bond with minimum dissociation energy is the easiest to break during the free radical reaction. It is very difficult to measure the chlorothalonil C–Cl dissociation energy experimentally. Therefore, many researchers have been trying to calculate it theoretically with the quantum chemistry method. The data shown in Table 7 also proves again the principle that the lower the dissociation energy the easier for the bond to break. On the other hand, the C=C stretching vibration heteroaromatic compound was observed in the region of 1467-1458 cm^{-1}. The frequency of C≡N vibration is useful for estimating the degree of intramolecular charge transfer since it is highly sensitive to the electron density borne by the CN triple bond. Aromatic nitriles with an electron-donating substituent on the ring have a much more intense C≡N stretching vibration than those with electron-accepting groups. The C≡N stretching vibration is expected to be between 2220 and 2242 cm^{-1}.

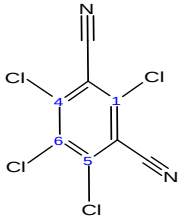
The C≡N stretching vibration is expected to be at 2240 cm^{-1}. The C–C≡N in-plane bending vibration is observed as a very weak band with mode 3. In the infrared spectrum, the stretching of C–(CN) is assigned to 979–980 cm^{-1}, which is a medium-to-weakly intense band. Finally, we note that there is no difference in chlorothalonil concentrations measured by IR under different environmental conditions.



Wavenumber (cm^{-1})

Fig. 1. Infrared spectrum of Chlorothalonil degradation.

Table 7. Identification of chlorothalonil using the IR spectrum.

Product Chlorothalonil	bonds	Chlorcal 70%WP		Open 72%SC	
		Before storage	After storage	Before storage	After storage
	C–C	3474	3447	3466	3430
	C–C–C	695	694	694	695
	C=C	1436	1464	1434	1458
	C–Cl 1	755	755	754	755
	C–Cl 4	738	738	738	738
	C–Cl 5	738	738	738	738
	C–Cl 6	695	694	694	695
	C≡N	2240	2240	2240	2240
	C–C≡N	980	980	979	890

Identification of chlorothalonil by chemical ionization GC/MS spectroscopy.

Results from the GC-MS study are shown in Table 8 and Fig. 2 classifying the degradation products of chlorothalonil before and after the effect of storage. We found dechlorination of the main pathway for all levels of degradation. The following degradation products were observed, i.e., chlorothalonil m/z = 265.9. The pathways for the degradation of chlorothalonil is typically dependent on the several mechanisms that have been reported: displacement of the 4-chlorine atom by a hydroxyl group (via hydrolytic dechlorination) to generate 4-hydroxy-2,5,6-trichloroisophthalonitrile. Formation of corresponding thiazoles, acidic and amide groups from the oxidation/hydration of the cyano group includes substitution of 4- chlorine atom by a methoxy or methylthio group to produce 2,5,6-trichloro-4-(methoxy)isophthalonitrile or 2,5,6-trichloro-4-(methyl-thio) isophthalonitrile, respectively, in addition to substitution of chlorine atoms by hydrogen atoms through a reductive dechlorination process; formation of 4-sulfydryl-2,5,6-trichloroisophthalonitrile and also formation of 5-cyano-4,6,7-trichloro-2H-1,2-benzisothiazol-3- one and sulfoxide under the aerobic conditions of marine environments These results are in line with Guangli Wang *et al.* (2011) and Hu *et al.* (2020).

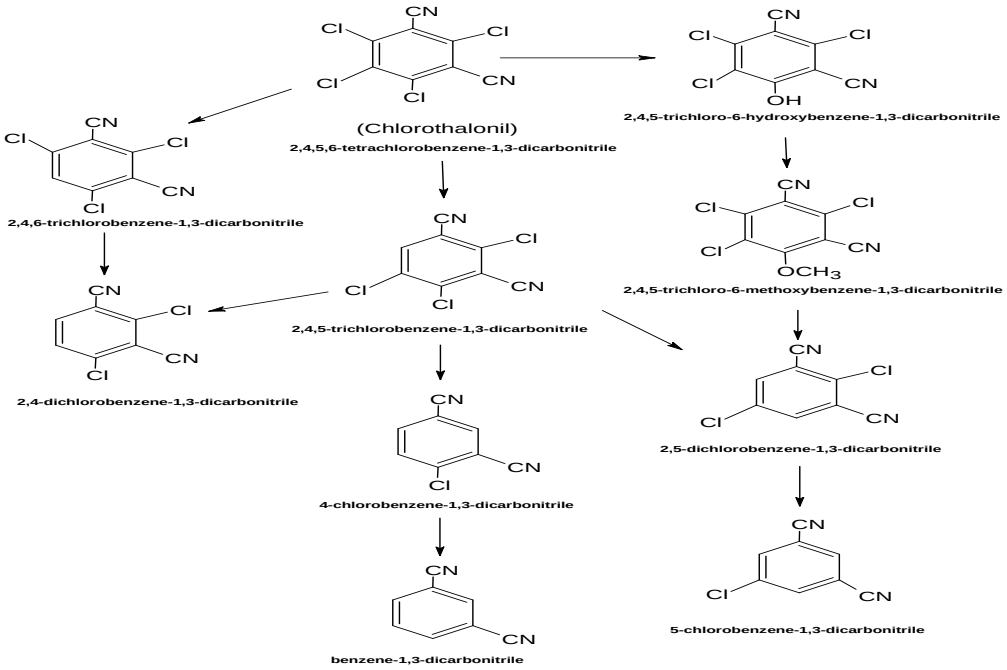


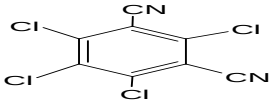
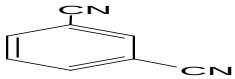
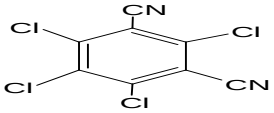
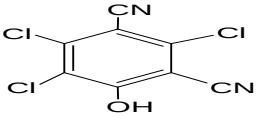
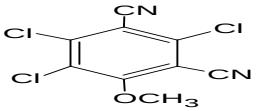
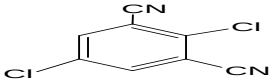
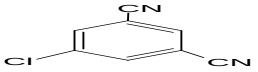
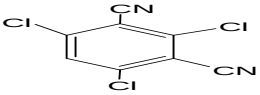
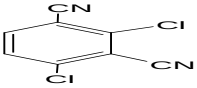
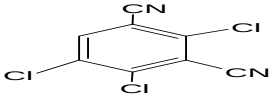
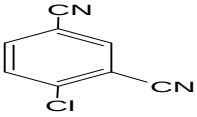
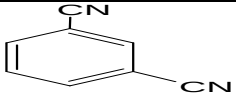
Fig. 2. Degradation pathway of chlorothalonil.

However, Katayama *et al.* (1997) suggested that the first step of chlorothalonil degradation was the substitution of chlorine with hydrogen, methylthio or hydroxyl group. According to the GC-MS analysis and based on the identified intermediates, the possible degradation pathway of chlorothalonil via PAL and ultrasound treatment was thus proposed (Fig. 2). At first, the chemical bonds (C−Cl and C–N) present in the chlorothalonil structure was in Table 8. Data also in Table 8 show that retention time (RT) of a breakdown product of chlorothalonil before

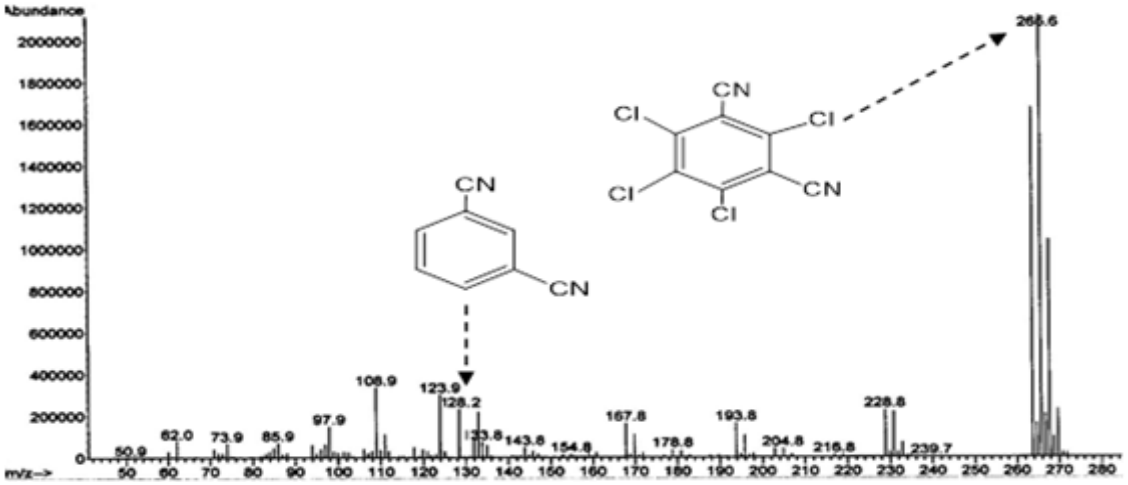
storage was 16.29 minutes while after 14 days of storage was 16.27 minutes and was easily degrade into benzene-1,3-dicarbonitrile that RT before storage was 10.44 minutes, while after 14 days of storage was 10.33 minutes.

From the above results, it can be indicated that hexachlorobenzene was not a degradation product of chlorothalonil and they were produced during at manufactured process. These results are in agreement with UNEP-ILO-WHO (1996) and EPA (1999).

Table 8. Identification of the degradation products of chlorothalonil by GC-MS.

storages periods	Characteristic ions (m/z)	Retention time(min)	Structure and Formula
Initial	chlorothalonil m/z = 265.9	16.29	MF: C_8Cl_4N2 
	benzene-1,3-dicarbonitrile m/z = 128.1	10.44	MF: $C_8H_4N_2$ 
After Storage	chlorothalonil m/z = 265.9	16.27	MF: $C_8Cl_4N_2$ 
	2,4,5-trichloro-6-hydroxybenzene-1,3-dicarbonitrile m/z = 247.4	16.5	MF: $C_8HCl_3N_2O$ 
	2,4,5-trichloro-6-methoxybenzene-1,3-dicarbonitrile m/z = 261.4	16.7	MF: $C_9H_3Cl_3N_2O$ 
	2,5-dichlorobenzene-1,3-dicarbonitrile m/z = 197	16.72	MF: $C_8H_2Cl_2N_2$ 
	5-chlorobenzene-1,3-dicarbonitrile m/z = 162.5	12.15	M.F: $C_8H_3ClN_2$ 
	2,4,6-trichlorobenzene-1,3-dicarbonitrile m/z = 231.4	14.86	MF: $C_8HCl_3N_2$ 
	2,4-dichlorobenzene-1,3-dicarbonitrile m/z = 197	14.36	MF: $C_8H_2Cl_2N_2$ 
	2,4,5-trichlorobenzene-1,3-dicarbonitrile m/z = 231.4	13.26	MF: $C_8HCl_3N_2$ 
	4-chlorobenzene-1,3-dicarbonitrile m/z = 162.5	10.77	MF: C8H3ClN2 
	benzene-1,3-dicarbonitrile m/z = 128.1	10.33	 MF: $C_8H_4N_2$

Note: MF = Molecular Formula; Initial: One hour before storage; RT: Retention time (min).



Wavenumber (cm^{-1})

Fig. 3. GC/MS chromatogram of fragmentation to chlorothalonil.

CONCLUSION

Generally, we evaluated the effects of storage at 54 ± 2 ºC for 14 days on fungicides (Chlorcal 70% WP and Open 72%SC) and determining physical properties as well as the active ingredient for chlorothalonil and Hexachlorobenzene as impurities by HPLC and fingerprint (GC/MS and IR spectra), along with the evaluation of their efficacy, by testing them against the mycelial growth of *Fusarium solani* and *Botrytis cinerea* under *in vitro* condition. The present results indicated that the half-life ($T_{0.5}$) values for Chlorcal 70% WP and Open 72% SC were 1382.07 and 614.035 days, respectively. GC / MS used for the degradation of chlorothalonil fungicide, the two major degradation products in chlorothalonil were 2,5,6-trichloro- 4- hydroxyisophthalonitrile and 2,4,6-trichloroisophthalonitrile. In addition, chlorothalonil formulations were effective against fungal pathogens. Open 72%SC was the most effective fungicide against *F. solani* and *B. cinerea* followed by Chlorcal 70% WP. The increased fungicidal activity of Open 72% SC may be due to the wetting and depressing agents that are used in the formulation. On the other hand, the effectiveness of tested fungicides deteriorated as a result of their storage at 54 ± 2 ºC for 14 days, while dry fungicide formulation such as Chlorcal 70%WP was more stable during storage and less effective against *F. solani* and *B. cinerea*. In addition, the degradation efficiency of Chlorcal 70% WP in each fungus except the catalase in the case of *B. cinerea* was decreased at storage. Moreover, we determined the chemical contents of total soluble sugar, total protein and free amino acids along with some enzymes (amylase and catalase) activities of mycelium fungi. Potato dextrose (PD) media treated with chlorothalonil formulations induced a significant decrease in all components in each fungus except the catalase enzyme in the case of *B. cinerea.*

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