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Mineralization of Chlormequat chloride (CCC) by different microbial pesticides using different media in grapes

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Abstract
Modern agriculture along with different chemical pesticides uses diverse array plant growth regulators which accumulate in fruits and demonstrated residue effect. The Chlormequat Chloride (CCC) belongs to quaternary ammonium growth regulator. This study deals with degradation effect of different fungal and bacterial biopesticides viz. Trichoderma viride, Trichoderma harzianum, *Beauveria bassiana*, Verticillium lecanii, Penicillium notatum, Bacillus megaterium, Pseudomonas fluorescens, Pseudomonas putida & Bacillus subtilis on solid and liquid media enriched with different concentrations of Chlormequat Chloride. Two fungal biopesticides Trichoderma harzianum and Trichoderma viride significantly indicated growth of 12.20 mm/day 11.48 mm/day average linear growth rates after 3 days of inoculation. These were found to be fast growing biofungicide on Potato dextrose agar and Potato dextrose broth enriched with 2500 mg/l CCC was best growth medium, which was confirmed by increased fresh spore biomass 2.3g/100 & 2.8g/100 ml medium and dry spore biomass 1.25 g & 1.65 g respectively as compared with different growth media. Additionally Bacillus subtilis with 6.366 mm/day & Pseudomonas fluorescens with 1.266 mm/day average colony diameter growth on nutrient agar enriched with 2500 ppm CCC were found to be the fast growing biocontrol agents and it was confirmed by optical density 3.120 and 2.365 respectively, at 560 nm. Remarkable growth of Trichoderma sp., Pseudomonas sp. and Bacillus with CCC indicates that apart from chemical or natural processes microbial degradation proved to be best alternative for seriously pertaining food safety issue.

Keywords: Mycelial, Chlormequat chloride (CCC), average linear growth, inoculation, biofungicide, biocontrol

Introduction
The existing circumstances in developed and developing countries in food habits among people are focused towards organic agricultural produces. Hence, it has become determined obligatory to take the best scientific knowledge and a technological breakthrough which are vitally important to meet today's complex challenges in food safety. Increasing demand for residue free crop protection products expected to boost the demand for bio pesticides in these days worldwide. The development of new bio pesticides with several mode of action against insect pests and bio fertilizers with multi crop growth promoting activities are most important for sustainable global agriculture. To make bio pesticides more successful, accurate plan of action intended to accomplish through IPM. Microbial degradation is a crucial step in the disappearance and detoxification of pesticides. Many soil applied pesticides are degraded more rapidly following repeated applications to the same site (Kauffman A. K., 1987) [6]. Bioremediation proved to be an environmental cleanup process that currently being investigated for use on a wide verity of chemicals (Arun *et al*., 2014) [2]. Organophosphate and Organochlorine class of pesticides and plant growth regulators are most commonly used by farmers in worldwide to increase growth and yield of fruit crops especially in vineyards.
The endeavor of present study was to assess the growth performance of most commonly used five fungal and four bacterial strains as bio control agents against pathogens and insect pest damaging grape berries quality with different concentrations of well-known plant growth regulator Chlormequat Chloride (CCC) at different time intervals *in vitro.* Moreover it is most important to know the compatibility of potential bioagents with Chlormequat Chloride used as growth retardant in grape. The effect of common fungicides, herbicides and insecticides on Trichoderma spp., Beauveria spp., Verticillium spp., Penicillium spp., was reported earlier. But the effect of growth regulators which were used in excess quantity and cause residual effects in fruits was not explored which leads rejection of fruits from European countries. Present study was carried out to understand growth potential of different bioagents in media

supplemented with Chlormequat Chloride (CCC). This provides information about capability of bioagents to degrade the growth regulator as opportunity to get rid from residue effect of CCC.

Materials and Methods

Microbial strains: Five pure fungal strains viz. Trichoderma viride NCIM-1355, Trichoderma harzianum NCIM-1373, Beauveria bassiana NCIM-1216, Verticillium lecanii NCIM-1312, Penicillium notatum NCIM-1206 and four bacterial strains Bacillus megaterium NCIM 2475, Pseudomonas fluorescens NCIM 2390, Pseudomonas putida NCIM 2847, Bacillus subtilis NCIM 5523 which are commonly used as Bio pesticides in agriculture are procured from National Collection of Industrial Microbes- National Chemical Laboratory Pune with all details.

Maintenance of organism (culture): A loopful of inoculum from the pure slants collected from NCIM-NCL Pune of respective organisms were transferred to respective specific growth medium slants and maintained as pure culture and stored at 4 ^{0}C.

Chemicals and Media: Standard formulation of Chlormequat Chloride 50 % SC formulation was purchased from local pesticide market in Sangli (MS) India. Different required concentrations as 500, 1000, 1500, 2000, 2500 mg/l were prepared as per standard dilution procedures with D/W. For fungal cultures different solid and liquid growth mediums such as Potato dextrose agar (PDA), Sabouraud Dextrose Agar (SDA), Czapek Dox Agar (CDA), Molasses Yeast Agar (MYA), Black gram soaked water & Coconut water Agar (BGCA) were used. Moreover Nutrient Agar and Nutrient broth were used for the bacterial.

Plant Growth Regulator CCC evaluation by poison food technique: The poisoned food technique (Shravelle, 1961) was followed to evaluate the efficacy of Chlormequat Chloride for radial mycelial growth inhibiting of fungal bio fungicides viz. Trichoderma viride NCIM-1355, Trichoderma harzianum NCIM-1373, Beauveria bassiana NCIM-1216, Verticillium lecanii NCIM-1312, Penicillium notatum NCIM-1206 on five different growth mediums viz. PDA, SDA, CDA, MYA, BGCA media in both solid and liquid forms. Bacterial biocontrol agents viz. Bacillus megaterium NCIM 2475, Pseudomonas fluorescens NCIM 2390, Pseudomonas putida NCIM 2847, Bacillus subtilis NCIM 5523 were also evaluated on Nutrient agar and Nutrient broth.

Fungal- Solid growth media study: All the fungal media viz. PDA, SDA, CDA, MYA BGCA were prepared, pH maintained and sterilized at 121^{0}C for 20 minutes in a autoclave, and before solidification of growth media, media enrichment with different concentrations of CCC as 500 mg/l, 1000 mg/l, 1500 mg/l, 2000 mg/l and 2500 mg/l. A 5 mm block of the mycelium was inoculated on solidified plates supplemented with CCC as mentioned above with sterile cork borer. Two replicates for each treatment were maintained with variable concentrations of CCC and plate without CCC was maintained as control. The plates were incubated at 27^{0}C ± 2 ^{0}C for 7 days. Average linear growth of the colony in millimeters was calculated and recorded.

Fungal -Liquid growth media (Broth) study: To determine the mycelial mass the liquid media such as PDB, SDB, CDB, MYB, BGCB were prepared, pH was maintained and sterilized at 121^{0}C for 20 minutes. On cooling the respective media were supplemented with 500 mg/l, 1000 mg/l, 1500 mg/l, 2000 mg/l and 2500 mg/l concentrations of CCC. The fungal growth was inoculated in respective flasks keeping media without CCC as control and incubated for 10 days at 28^{0}C ± 2^{0}C. To determine the biomass the mycelium growth was filtered through Whatman No.4 filter paper. After washing with distilled water, fresh weight of each replicate and average fresh biomass was recorded. Dry weight was determined after drying at 60^{0}C for 72 hrs and subsequently cooling in desiccators. Average dry weight of the mycelial mass was recorded as standard value for comparison of growth.

Bacterial- Solid growth medium study: All bacterial strains were investigated for their growth performance on sterilized Nutrient Agar (NA) at 121^{0}C for 20 minutes in autoclave enrichment with different concentrations of CCC viz. 500, 1000, 1500, 2000 and 2500 mg/l. The pure bacterial cultures were individually inoculated and incubated overnight at 37^{0}C ± 2^{0}C and determined their growth performance.

Bacterial -Liquid growth media (Broth) study: To evaluate the biomass increase of bacterial cultures nutrient broth was prepared supplemented with 500, 1000, 1500, 2000 and 2500 mg/l concentrations of CCC per 100 ml separately. Bacterial culture inoculated aseptically and incubated for 24 h at 37^{0}C± 2 ^{0}C in bacteriological chamber. Optical density at 560 nm was recorded for each treatment on spectrophotometer and control was maintained without CCC.

Determination of Average linear growth rate (ALGR): The mycelial growth of each treatment was measured 3 days after inoculation (DAI) with digital Vernier caliper. Average liner growth rate (ALGR) was calculated by formula (Aneja, 1993 and Elad *et al*, 1981) [1, 3]

ALGR (mm/ day) = (C3 – C1) / 3

Where,

C3 = Colony diameter in mm after three days.

C1 = Colony diameter in mm after one days.

Results and Discussion

In vitro investigation was conducted to observe the impact of different growth media supplemented with different concentrations of Chlormequat Chloride on the growth and sporulation of the different fungal biopesticides and determine the linear hyphal growth rate. The results revealed that average linear growth rate after 3 DAI of fungal biopesticides in different media supplemented with different concentration of Chlormequat Chloride (CCC) varied significantly (Table 1). The maximum mycelial growth and biomass production was recorded in fast growing Trichoderma harzianum 12.20 mm (Table 1) and fresh biomass 9.8 g, dry biomass 2.8 g (Table 2) followed by Trichoderma viride 11.48 mm and fresh biomass 9.62 g, dry biomass 2.3 g respectively and proved to be excellent bio degrader. Among the media Potato dextrose agar enriched with 2500 mg/l CCC was found to be best growth medium. Beauveria bassiana with 2.68 mm in Czapek Dox agar enriched with 2500 mg/l with 8.14 g and 1.33 g fresh and dry biomass respectively. Verticillium lecanii with 6.8 mm in Molasses Yeast Agar enriched with 2500 mg/l with 7.84 g and 2.36 g fresh and dry biomass respectively and Penicillium notatum with 8.24 mm in Czapek Dox agar enriched with 2500 mg/l with 8.55 g and 2.65 g fresh and dry biomass respectively. Black gram soaked coconut water agar media found to be less significant as compared to other growth media. Among all Potato Dextrose Agar medium was found best for all Trichoderma spp. to utilize the different culture media with difference in their contents (Wang *et al*. 1999) [16].

Table 1: Growth rate of fungal bio pesticides on different culture medium enriched with CCC.

Bio pesticide Fungi	CCC concentration mg/l	Average linear growth (mm/day)				
		PDA	SAB	CZA	MYA	BGCA
Trichoderma viride NCIM- 1355	500	7.10	4.32	4.19	6.58	1.10
	1000	7.51	5.10	4.90	7.31	1.10
	1500	10.10	5.34	5.12	7.80	1.10
	2000	11.17	7.35	7.11	9.35	1.12
	2500	11.48	9.75	9.20	10.56	1.40
	Control	5.11	3.98	3.49	4.95	1.76
Trichoderma harzianum NCIM-1373	500	7.15	6.42	6.10	7.10	1.0
	1000	8.32	7.13	7.10	7.75	1.0
	1500	11.18	9.35	8.21	9.35	1.12
	2000	12.04	10.68	9.51	10.56	1.24
	2500	12.20	11.60	11.10	11.40	1.50
	Control	5.24	4.12	3.96	5.10	1.96
Beauveria bassiana NCIM-1216	500	0.638	0.540	0.240	1.21	0.54
	1000	0.73	0.75	0.59	1.35	0.82
	1500	0.63	0.84	0.74	1.64	1.21
	2000	0.85	1.12	1.35	1.20	1.84
	2500	0.33	1.14	2.68	1.80	1.47
	Control	10.89	10.45	9.45	8.41	10.23
Verticillium lecanii NCIM-1312	500	3.70	3.42	2.56	3.65	1.25
	1000	4.11	4.52	3.98	3.98	3.56
	1500	4.08	4.86	4.60	4.85	4.10
	2000	4.71	5.10	4.82	6.30	4.65
	2500	4.59	5.30	5.64	6.80	5.10
	Control	7.94	7.32	6.98	7.45	5.90
Penicillium notatum NCIM-1206	500	4.61	3.21	2.56	3.96	1.10
	1000	5.24	4.36	2.98	5.32	2.13
	1500	7.98	6.98	3.14	6.98	2.96
	2000	6.01	7.20	3.58	7.23	3.14
	2500	4.40	5.26	8.24	4.10	4.17
	Control	9.22	8.40	5.90	9.10	6.50

CCC- Chlormequat chloride, PDA-Potato dextrose agar, SAB- Sabouraud Dextrose agar, CZA-Czapek Dox agar, MYA-Molasses yeast agar, and BGCA- Black gram soaked water & Coconut water agar.

Similar findings were reported regarding the growth and morphological features of Trichoderma spp. on different culture media by Elad *et al*., 1981 [3]; Shalini *et al*. 2006; Harman *et al*. 1991 [5]. Mustafa and Co-workers 2009 studied the growth of Trichoderma spp. on five semi synthetic media including PDA and found PDA as the best medium. Nusrat et al. 2013 [10] reported that PDA was more effective for average linear growth rate of Trichoderma spp. Singh *et al*., 2011 reported that PDA media was best for growth and sporulation of Trichoderma atroviride. Shahid *et al*., 2011 [11] also reported that PDA media was best for growth and sporulation of Trichoderma *longibrachiatum*.

Table 2: Fresh and dry biomass of different fungal bioagents grown in different broth enriched with CCC

Bio pesticide microbe	CCC mg/l	PDB		SAB		CZB		MYB		BGCB	
		F	D	F	D	F	D	F	D	F	D
Trichoderma viride NCIM- 1355	500	4.43	1.06	4.20	0.98	3.51	0.78	6.20	1.54	2.78	0.65
	1000	5.65	1.35	5.65	1.32	4.32	0.95	6.69	1.66	3.68	0.86
	1500	7.44	1.78	6.68	1.56	5.44	1.21	7.98	1.98	4.79	1.12
	2000	8.28	1.98	7.67	1.79	8.58	1.91	8.50	2.11	6.38	1.67
	2500	9.62	2.3	8.87	2.07	9.67	2.15	4.99	2.26	8.08	1.89
	Control	5.65	1.35	4.20	0.98	4.86	1.08	5.12	1.27	3.21	0.75
Trichoderma harzianum NCIM-1373	500	6.00	1.71	3.13	0.98	5.35	1.45	4.94	1.51	4.35	0.94
	1000	6.60	1.88	4.13	1.47	5.65	1.53	5.01	1.68	4.53	0.98
	1500	7.62	2.17	3.90	1.67	6.02	1.63	4.52	1.83	5.23	1.13
	2000	8.00	2.3	4.10	2.01	6.87	1.86	4.75	2.21	7.22	1.56
	2500	9.83	2.8	9.80	2.3	8.09	2.19	9.43	2.54	8.56	1.85
	Control	5.90	1.68	4.70	1.10	5.10	1.38	5.46	1.47	3.75	0.81
Beauveria bassiana NCIM-1216	500	4.65	0.23	0.54	0.11	3.42	0.56	3.22	0.98	0.54	0.11
	1000	5.36	0.49	1.58	0.32	4.77	0.78	3.68	1.12	1.01	0.32
	1500	3.38	0.87	3.04	0.76	6.0	0.98	4.70	1.43	2.12	0.67
	2000	2.82	1.04	5.34	1.34	7.40	1.21	5.85	1.78	2.79	0.88
	2500	1.59	1.65	5.81	1.45	8.14	1.33	6.21	1.89	3.33	1.05
	Control	4.65	1.43	4.21	1.05	3.98	0.65	4.34	1.32	3.21	1.01
Verticillium lecanii NCIM-1312	500	6.11	1.89	6.33	1.98	4.21	1.27	5.37	1.56	3.37	1.34
	1000	6.34	1.95	6.53	2.04	5.11	1.54	6.44	1.87	4.65	1.67
	1500	6.95	2.15	7.23	2.26	6.21	1.87	6.65	1.93	5.54	1.99
	2000	7.67	2.37	8.13	2.54	7.34	2.21	7.06	2.05	5.87	2.11

	2500	7.76	2.40	8.57	2.68	7.27	2.11	7.84	2.36	5.71	2.05
	Control	5.34	1.65	6.21	1.94	4.12	1.24	5.10	1.48	3.12	1.12
Penicillium notatum NCIM-1206	500	4.30	1.45	5.82	1.65	5.03	1.56	3.96	1.32	2.59	0.98
	1000	4.89	1.66	6.12	1.97	6.09	1.89	4.35	1.45	3.06	1.16
	1500	5.60	1.89	7.12	2.23	6.58	2.03	5.34	1.78	3.83	1.45
	2000	6.08	2.05	7.42	2.34	7.55	2.34	6.30	2.10	5.58	2.11
	2500	6.55	2.21	8.22	2.76	8.55	2.65	6.90	2.30	5.89	2.23
	Control	3.65	1.23	4.54	1.42	4.13	1.28	4.68	1.56	2.46	0.93

F- Fresh weight (g), D- Dry weight (g), CCC- Chlormequat chloride, PDB-Potato –dextrose broth, SAB-Sabouraud Dextrose broth, CZA-Czapek Dox broth, MYA-Molasses yeast broth, and BGCA- Black gram soaked water & Coconut water broth.

As reported by Engelkes CA, Nuclo RL, Fravel DR, 1997 [4] Carbon is the major component and the molecules of carbon also contribute to oxygen and hydrogen which can be utilized as nutrient sources for the growth and development of microorganisms. Thus our results suggests that different growth media enriched with Chlormequat chloride (CCC) was acts as a good growth regulator to screen microbial biopesticides which are exploited as biocontrol agents in vineyards.

The utilization of CCC as a source of carbon and nitrogen for growth by bacterial strains was shown in Table. 3. It was revealed that the maximum colony diameter was recorded for Bacillus subtilis 6.366 mm in NA enriched with 2500 mg/l CCC and confirmed with optical density 3.120 at 560 nm followed by Pseudomonas fluorescens 1.266 mm, Pseudomonas putida 1.142 mm, Bacillus megaterium 1.00 mm and confirmed by optical density 2.365, 1.398 and 1.350 at 560 nm, respectively.

Table 3: Growth rate of Bacterial bioagents on NA & NB culture medium enriched with CCC

Bio pesticide microbe	CCC concentration mg/l	Average colony diameter on NA (mm/day)	Optical density At 560 nm
Bacillus megaterium NCIM-2475	500	0.948	0.921
	1000	0.480	0.732
	1500	0.218	0.510
	2000	0.961	0.980
	2500	1.00	1.350
	Control	0.485	0.780
Pseudomonas fluorescens NCIM-2390	500	0.932	1.102
	1000	1.160	1.236
	1500	1.120	1.10
	2000	1.200	2.210
	2500	1.266	2.365
	Control	0.485	0.780
Pseudomonas putida NCIM-2847	500	0.512	0.721
	1000	0.694	0.986
	1500	0.738	1.154
	2000	1.120	1.256
	2500	1.142	1.398
	Control	0.485	0.780
Bacillus subtilis NCIM-5523	500	1.016	1.102
	1000	1.056	1.232
	1500	1.390	2.345
	2000	4.067	2.365
	2500	6.366	3.120
	Control	0.485	0.780

NA- Nutrient Agar, NB- Nutrient Broth

Similar findings were documented by Nishiyama N, Toshima Y, Ikeda Y 1995 [9], Van Ginkel CG, Van Dijk JB, Kroon AGM 1992 [15] for biodegradation of alkyl tri-methyl ammonium salts in activated sludge. Chlormequat chloride (CCC) is quaternary ammonium compounds which degrade through different pathways N- dealkylation involving monooxygenase activity with production of TMA and alkyl residue is one pathway among others. When Pseudomonas putida ATCC 12633 grown with TTAB induces NADH or NAD(P)H- dependent TTAB monooxygenase activity, TMA and tetradecanoic acid is responsible for the first step in the degradation of quaternary ammonium compound (Liffourrena AS and *et al*., 2008) [7]. Thus Pseudomonas putida ATCC 12633 is an organism capable of completely mineralizing TTAB in presence of Al^{3+} and efficiently biological removal of similar quaternary ammonium compound (Liffourrena AS and *et al*., 2010).Thus our result suggests that Nutrient broth enriched with Chlormequat chloride (CCC) was acts as a good growth regulator for the bacterial microbes and utilize the CCC as food source.

Conclusion

From the present experiments it was revealed that different growth media for biocontrol agents supplemented with different concentrations of Chlormequat chloride differentially influenced the linear mycelial growth and sporulation of fungal microbes. Out of five fungal isolates grown on five different growth mediums, Trichoderma genera on PDA enriched with 2500 mg/l CCC found to be better agent for utilization of CCC as a carbon source. Two bacterial isolates of Bacillus and Pseudomonas genera found to be having efficient utilization capability of Chlormequat

Chloride (CCC). Remarkable degradation of CCC by Trichoderma harzianum and Bacillus subtilis indicates that apart from chemical or natural processes microbial degradation is better option to get rid from residue issue for food safety.

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