Pest Management in Horticultural Ecosystems
Vol. 25, No.1 pp 109-115 (2019)

Dissipation of chlorothalonil in leafy vegetables accelerated by use of bio-fertilizer

JYOTHI V DIVAKARA[1,2] and DEBI SHARMA[1*]

[1] ICAR - Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bengaluru-560089,India
[2]Research Scholar,Centre of Post Graduate Studies, Jain University, Bengaluru, India

***E-mail:** debisharma@gmail.com

ABSTRACT: Persistence of the fungicide, chlorothalonil, in spinach and green onions was studied following application of the fungicide along with or without co-application of a bio fertilizer *viz* Arka microbial consortium. Samples of these leafy vegetables drawn periodically at 0 (1h), 1, 3, 5, 7, 10,15 and 20 days after application of chlorothalonil were analysed by GC-ECD. The residues of chlorothalonil in spinach as well as green onions persisted longer in bio fertilizer untreated crop than in treated crop, following application of the fungicide at recommended as well as double the recommended doses. Increase in biomass by 11 to 15 per cent in spinach and green onions resulting from application of the bio fertilizer seemed to have resulted in faster dissipation of chlorothalonil in the produce. The waiting period recommended for safe harvest of these leafy vegetables were reduced by 0.1 to 2.6 days in bio fertilizer treated produce. Waiting period reduced significantly in case of spinach following both treatments mentioned above but following only the higher dose treatment in green onion. The bio fertilizer used in this study increased the yield without harming the environment, thereby, also, accelerating the reduction in chlorothalonil residues in the produce.

Keywords: bio fertilizer;chlorothalonil; green onion;pesticide residues; spinach

INTRODUCTION

Chlorothalonil (IUPAC name- 2,4,5,6-tetrachloroisophthalonitrile, Fig. 1) is a common broad spectrum fungicide used for controlling various diseases of vegetable crops. It is non systemic, protectant fungicide effective against many diseases which damage vegetables *viz*. rusts, anthracnose, downy mildews, leaf spots, soft rot, leaf blight, scab,early blight, late blight, pink rot, powdery mildew, etc.Chlorothalonil is commonly used in green onions mainly for control of purple blotch (Miller et al. 1986). Spinach-beet or Palak (Beta vulgaris L) can be infected by several fungi that cause leaf spot diseases (Correll et al. 1994). Fuentes-Davila (1988) reported that leaf spot caused by *C. variabile* can be controlled by chlorothalonil alone or in combination. Spinach and green onions are among popular leafy greens that are an important part of Indian diet. Such leafy vegetables offer a cheap but rich source of pro-vitamin A, vitamin C, folic acid and minerals like calcium, iron, phosphorus, sodium and potassium. Leafy vegetables also contain antioxidants necessary for neutralizing free radicals which result in many diseases in humans (Ashok Kumar et al. 2013). Since leafy vegetables are grown with little or no spacing they grow densely,often have larger surface area, and, therefore,are likely to contain higher initial deposit of any pesticide following its foliar spray. Spinach and green onions are harvested at several pickings and are consumed fresh, sometimes raw. So there is a high risk of these leafy vegetables being contaminated with chlorothalonil residues.Thus, it is important that persistence of chlorothalonil residues in spinach and green onions be studied and waiting period determined. This is even more important due to the fact that there is no label claim for chlorothalonil use in any leafy vegetable in India so the residue persistence data and risk assessment data can be used for fixing maximum residue limits of this fungicide in these crops.

Under ideal conditions, any pesticide applied on a crop should persist for as long as it is required for it to control the target pest(s) and quickly dissipate thereafter, so that pesticide residues in the harvested crop,are below permissible levels and the produce is safe for consumption. It is well known that faster the growth of vegetables, faster is the reduction in the amount of pesticide residues on the crop due to “residue dilution” or decrease in the concentration of residues resulting from increase in crop weight. A microbial bio fertilizer developed at the Indian Institute of Horticultural Research, Bangalore, India, viz. Arka microbial consortium, consisting of N fixing, P and Zn solubilizing and plant growth promoting microbes in single carrier has shown excellent results in

Table 1. Recovery of chlorothalonil residues from spinach, green onions and soil at various spiked levels.

Fortification Level mgkg^{-1}	Mean recovery* (%) ±SD		
Spinach	Green onions	Soil	
0.01	85.5 ± 0.66	90.2 ± 1.93	91.2 ±3.05
0.05	87.5 ± 1.05	92.9 ± 0.86	90.3 ± 0.76
0.1	90.7 ± 0.90	91.7 ± 0.66	97.2± 2.04

*Average of five replicates

Table 2. Dissipation of Residues of Chlorothalonil in Spinach

Days after application	Chlorothalonil residues mgkg^{-1}			
	T1		T2	
	W/O BF* 2.0g/L	BF** 2.0g/L	W/O BF 4.0g/L	BF 4.0g/L
0	111.98±1.64	116.84±3.38	192.93±2.63	191.23 ± 2.29
1	107.57±2.91 (3.94)#	95.95±1.55 (17.88)	161.06± 4.91 (16.52)	157.57 ± 2.05 (17.61)
3	98.86±0.49 (11.72)	60.33±2.02 (48.37)	125.61±1.53 (34.89)	102.18 ± 4.37 (46.57)
5	86.22 ± 0.24 (23.01)	45.27±3.29 (61.25)	103.66±0.25 (46.27)	84.80 ± 1.79 (55.66)
7	40.59±0.99 (63.75)	8.31± 0.03 (92.89)	44.08±0.99 (77.15)	15.99 ± 0.03 (91.64)
10	4.82±0.28 (95.70)	0.24 ± 0.0 (99.79)	5.51±0.09 (97.14)	0.25± 0.0 (99.87)
15	0.02±0.0 (99.99)	BDL##	0.04 ± 0.00 (99.98)	BDL
20	BDL	BDL	BDL	BDL
$t_{1/2}$ (days)	1.27	1.19	1.28	1.14
T_{tol}(days)	19.46	17.47	20.21	17.62

*W/O BF - without bio fertilizer application, **BF- with bio fertilizer application
##BDL - Below Quantifiable Limit, Limit of Quantification = 0.01mgkg^{-1}
#Figures in Parenthesis represent percent dissipation of residues from 0 day values.

increasing vegetative growth of vegetables, and also increased yield by 5 to 15 per cent (Panneerselvam *et al*. 2012). Thus,the hypothesis that the same can be used to reduce the residues of chlorothalonil in leafy vegetables by residue dilution.

The present study therefore aimed at determining the persistence of chlorothalonil residues in spinach beet and green onions grown using bio-fertilizer and also to find whether such bio fertilizer use can accelerate the dissipation of the residues in these vegetables and if so, the stage of bio fertilizer application, thereof.

MATERIALS AND METHODS

Chemicals and bio fertilizer

Chlorothalonil (Kavach 75 WP) was obtained from local market. Analytical standard of chlorothalonil (98%) was procured from M/S Sigma Chemical Co., USA. All chemicals and reagents used were AR grade, from E. Merck (India) Ltd. Arka microbial consortium (AMC) was prepared at the Indian Institute of Horticultural Research, Bangalore, India,

Field Experiments

Field trials were conducted in the experimental field of the Indian Institute of Horticultural Research, Bangalore, India (13.13° N, 77.49° E) as per good agricultural practices.Spinach Beet (cv. Arka Anupama) was grown with and without bio fertilizer treatment and chlorothalonil application. Four sets of 6 plots, each individual plot measuring 5m x 5m with randomized block design were directly sown with spinach beet seeds at the same time. Each set of plots corresponded to one treatment, *viz*. no bio fertilizer control, bio fertilizer control, chlorotalonil treatment alone, bio fertilizer + chlorothalonil treatment. The bio fertilizer i.e. AMC was applied immediately after germination of seeds (6 days after sowing) and again one month thereafter. Two kilograms of AMC was applied to the crop mixed in 100 litres of water. The mixture was sieved through a muslin cloth before spraying. Chlorothalonil was sprayed 2 days after second treatment of AMC at the recommended dose of 2g/L and double the recommended dose of 4g/L. Spray volume used was 500L/ha. Spinach leaf samples were collected at 0(1hour), 1, 3, 5, 7, 10, 15 and 20 days after chlorothalonil application and analysed for its residues. Soil samples were collected on last date of sampling and analysed for residues. Extraction and clean-up of chlorothalonil in spinach as well as green onion was carried out as per standard protocol of Kurz *et al*. (2008). Rose onion (cv. Arka Bindu) was also grown in the same manner as spinach beet. The seeds were broadcast on the plots and the same treatments imposed as above. Green onions were harvested at regular intervals and residues of chlorothalonil determined.Weather parameters *viz*. temperature, relative humidity and rainfall during the period of study were recorded.

Sample Preparation

Extraction

A 50g portion of representative spinach leaf sample/ green onions sample was homogenized in a Waring blender (Model 7011G, Cole Parmer India) with 100mL hexane + acetone (60 +40) mixture and filtered under vacuum through a Buchner funnel.The filter cake was re-extracted twice with 150mL of hexane + acetone (60 +40) mixture.The filtered extract was combined and concentrated under rotary vacuum evaporator to remove this organic mixture.

Clean up

The aqueous extract thus obtained was transferred into the separatoryfunnel and 200ml distilled water along with 12gm of sodium chloride was added and partitioned with hexane (3x50ml).The organic layer was passed through anhydrous sodium sulphate and combined.The combined hexane extract was concentrated to near dryness using rotary vacuum evaporator(IKA Model RV10) at 40^0C. The residues were re-dissolved in distilled acetone and final volume made up to 5ml in graduated test tube for analysis by gas liquid chromatography (GLC).

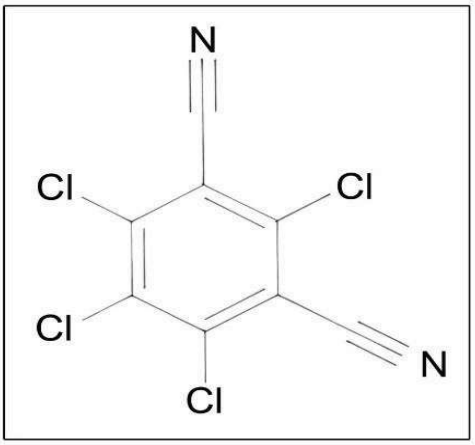


Fig.1 Chemical Structure of Chlorothalonil.

Estimation of increase in biomass

To estimate the increase in biomass due to bio fertilizer application weight of spinach and green onions grown in $1m^2$ area with and without bio fertilizer application was recorded from 5 different plots of each treatment type. The dry weight of the leafy part above ground was also recorded in each case.

Table 3. Dissipation of Residues of Chlorothalonil in Green Onions

Days after application	Chlorothalonil residues mgkg^{-1}			
	T1		T2	
	W/O BF* 2.0g/L	BF** 2.0g/L	W/O BF 4.0g/L	BF 4.0g/L
0	9.63±0.18	9.64±0.79	16.89±0.18	16.68±0.27
1	7.83±0.51 (3.94)#	7.06±0.25 (17.88)	12.68 ± 0.20 (24.97)	11.69 ± 0.25 (29.93)
3	5.09±0.20 (11.72)	4.00±2.09 (48.37)	8.83 ± 0.17 (47.69)	6.56 ± 0.00 (60.62)
5	2.91±0.26 (23.01)	1.07±0.12 (61.25)	5.55 ± 0.26 (67.16)	1.03 ± 0.01 (93.82)
7	1.89±0.42 (63.75)	0.26± 0.07 (92.89)	2.77 ± 0.99 (83.57)	0.17 ± 0.00 (98.98)
10	0.02±0.00 (95.70)	BDL##	5.51 ± 0.14 (99.84)	BDL
15	0.01 ± 0.00 (99.89)	BDL	0.03 ± 0.00 (99.90)	BDL
20	BDL	BDL	BDL	BDL
Half Life(days)	1.37	0.90	1.22	1.06
T_{tol}(days)	1.12	1.01	2.22	1.34

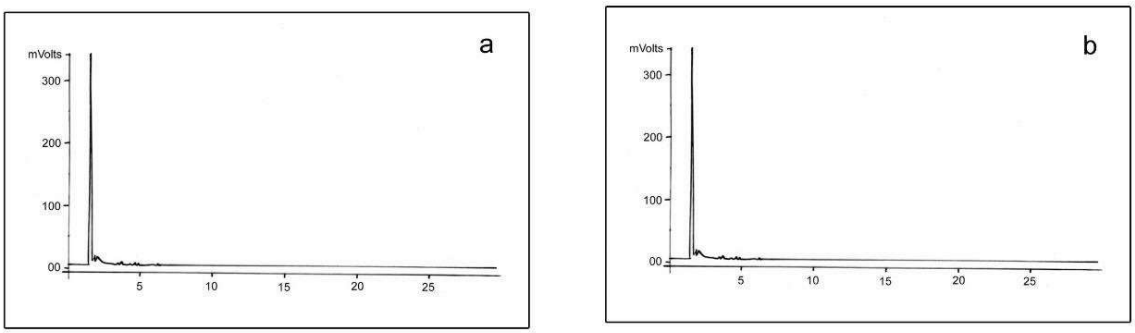
*W/O BF - without bio fertilizer application, **BF- with bio fertilizer application
##BDL - Below Quantifiable Limit, Limit of Quantification = 0.01mgkg^{-1}
#Figures in parenthesis represent percent dissipation of residues from 0 day values.

Estimation of recovery

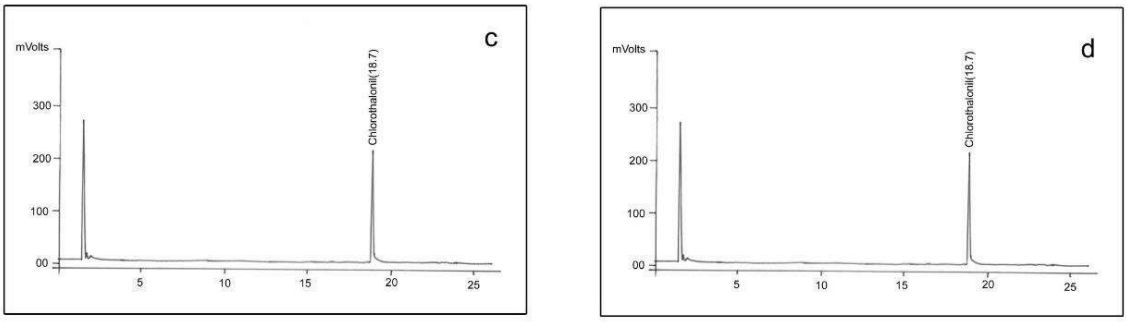
A recovery experiment was carried out to ascertain the efficiency of the analytical method by spiking untreated samples (spinach, green onions and soil) with analytical grade chlorothalonil at the rate of 0.01, 0.05 and 0.1mgkg^{-1}.The spiked samples in five replicates were processed as per the analytical method described above to obtain the percent recovery.

Instrumental analysis

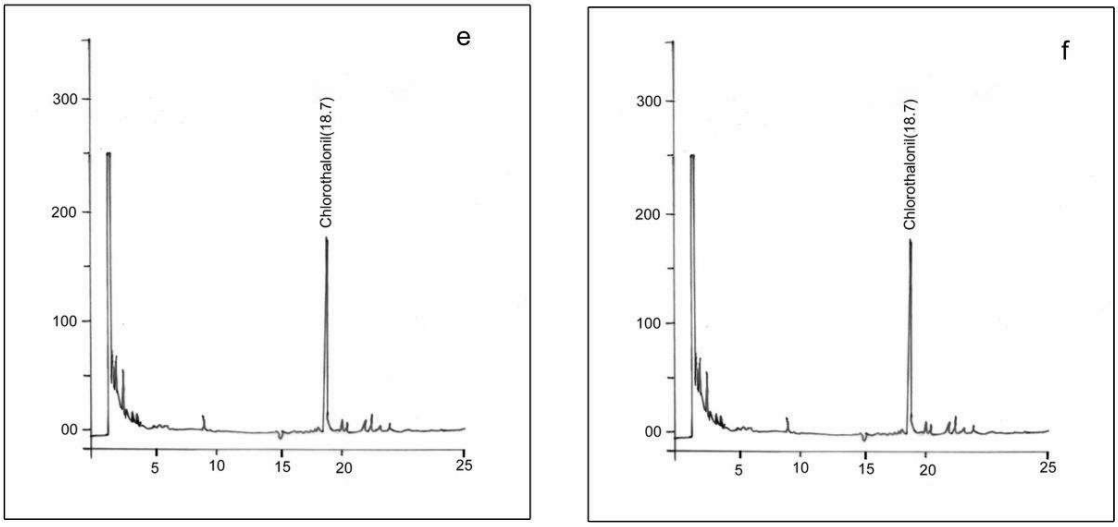
The determination of chlorothalonil residues was carried out using gas chromatograph (GC) Model Varian 3800 equipped with electron capture detector (ECD) and a DB-5 (30 m x 0.25 mm i.d., 0.25 µm film thickness) fused-silica capillary column. The standard split/splitless injector was used for splitless injection at 270 ºC with an injection volume of 1 µL. The ECD detector was maintained at 300 ºC, with the make-up gas nitrogen flow-rates at 30.0 mL min^{-1}. The oven temperature program was 80^{0}C (5 min) ramped to 150 ^{0}C (5 min) @ 15 ^{0}C/min ramp to 250^{0}C (0min) @4^{0}C/min again ramped to 280^{0}C (17min)@10^{0}C/min.The residues of chlorothalonil were confirmed by using a gas chromatograph-mass spectrometer (Shimadzu GCMS -QP2010 SE) operated in single ion monitoring (SIM) mode and full scan mode (40-450 m/z) at the ionization energy of 70eV. The gas chromatograph was operated in split less mode with injector temperature of 250 ^{0}C and ion source temperature of 200 ^{0}C. A capillary column, Restek RXI-5ms (30m x 0.25mm i.d. x 0.25um film thickness) with stationary phase consisting of 5% diphenyl/95% dimethyl polysiloxane was used for analysis. The column temperature was initially maintained at 140 ^{0}C for 5 min, then increased to 250^{0}C @ 4^{0}C min^{-1}and further ramped to 280 ^{0}C @10^{0}C min^{-1} with a hold time of 10 min. Injector and detector temperature was maintained at 250^{0}C and 280^{0}C respectively. The injector was maintained at split



(a) and (b) Spinach and Green onion Blank



(c) and (d) Spinach and Green onion Spiked at 0.1 mg/kg



(e) and (f) 0-day Spinach and Green Onion Biofertilizer treated Sample at recommended dose

Fig 2. Chromatograms for the analysis of Chlorothalonil

mode with a split ratio of 1:10. Auto sampler injection volume was 1µL. Helium was used as carrier gas with a column flow rate of 1mL min^{-1}. Chlorothalonil eluted at 23.9 min under the above GC-MS conditions. Detector voltage was maintained at 0.9 kV in SIM mode. The mass spectrum of chlorothalonil showed ions at m/z 266, 264, 268 and 267. These values were compared with the samples spiked with chlorothalonil and samples collected from treated plots for confirmation of chlorothalonil residues.

RESULTS AND DISCUSSION

Method validation

To ensure that the analytical method used is rugged and effective, the same was validated by evaluating various analytical parameters *viz*. recovery, linearity, specificity, accuracy and precision, limit of detection (LOD), limit of quantification (LOQ), and measurement uncertainty (MU)[18]. The LOD was determinedby injecting known concentrations of certified reference standardof chlorothalonil and reducing the concentration ofstandard solution till a detection peak was obtained at signal to noise (S/N) ratio of 3:1. The LOQ was similarly determined for chlorothalonil peak at S/N ratio of 10:1 in the matrix (spinach/green onion). The limit of quantification (LOQ) of chlorothalonil in spinach, green onions and soil using the above GLC method was 0.01mgkg^{-1}.

A calibration curve was prepared by plotting concentration of chlorothalonil injected versus peak areas which showed linearity in the range of 0.01 - 10 mg kg^{-1}. Specificity was validated by analyzing respective blank samples (n = 3) and ensuring that no interfering peaks appeared at the same retention time. The precision was expressed as the percentage relative standard deviation (%RSD) of repeatability at the concentrations of 0.01, 0.05, and 0.1 mg kg^{-1} for chlorothalonil by analyzing five replicates in 1day over a period of 5 days (inter-day). Recoveries of chlorothalonil residues from fortified samples of spinach, green onions and soil using the GLC analytical protocol described is presented in Table1. Recovery of chlorothalonil residues from spinach, green onions at fortification levels of 0.01 mgkg^{-1}, 0.05 mgkg^{-1} and 0.1 mgkg^{-1}was in the range of 85.5 -90.7 percent, 90.2 to 92.9 per cent and 90.3 -97.2 percent respectively indicating high efficiency of analytical method used.

Residues of chlorothalonil were analysed in spinach and green onions over a period of 20 days.Initial residue deposit of chlorothalonil after the foliar spray at recommended dose in bio fertilizer untreated spinach was 112.0mgkg^{-1},whereas in bio fertilizer treated spinach it was 116.8mgkg^{-1} (Table 2). These dissipated to below LOQ level of 0.01 mgkg^{-1}on 20th day of chlorothalonil application in bio fertilizer untreated spinach where as in bio fertilizer treated crop, the residues reached below determination level on 15thday.The half -life period ($t_{1/2}$) of chlorothalonil residues in untreated spinach was 1.3 days, whereas in bio fertilizer treated spinach it was 1.2days. In case of foliar application of chlorothalonil at double the recommended dose of 4g/L in spinach, the initial residues of chlorothalonil was 192.9mgkg^{-1}in bio fertilizer untreated crop while the same was 191.2mgkg^{-1}in bio fertilizer treated crop. The residues dissipated with a half-life of 1.3days in former and 1.1 days in latter. No residues of chlorothalonil was detected in untreated spinach and soil. Green onions have much lesser surface area than spinach and therefore, the initial residues of chlorothalonil in green onions were comparatively less viz.9.6mgkg^{-1}following application of chlorothalonil at 2gL^{-1},while the same was16.7 to 16.9 mgkg^{-1} following application of chlorothalonil at 4gL^{-1}(Table 3).The residues of chlorothalonil were not detectable in green onions grown with bio fertilizer application on 10thday after chlorothalonil application at recommended as well as double the recommended doses. The residues of chlorothalonil persisted longer and was not detectable on 20th day after application in bio fertilizer untreated green onions. The half- life of chlorothalonil residues was 1.4 to 1.2 days in bio fertilizer untreated green onions while the same was 0.9 to 1.1days in the bio fertilizer treated crop. The average biomass increase resulting from bio fertilizer application in spinach was 1.2 kg to 1.5 kg per m^2 while that resulting from bio fertilizer application in green onions was 35g to 55g per m^2. In terms of per cent increase in dry weight, the dry weight of spinach leaves increased by 14.2 to 15.0 to per cent while that of green onions increased by 11.3 to12.1 percent. The hypothesis that increase in biomass would result in residue dilution seemed to hold true as the persistence of chlorothalonil was higher in bio fertilizer untreated crop. On the basis of the dissipation pattern and MRL (EU) of chlorothalonil in spinach (0.01mgkg^{-1}) which is fixed at the level of quantification, the waiting period recommended for safe harvest of spinach crop treated with chlorothalonil was 19.5 to 20.2 days for bio fertilizer untreated crop while the same for bio fertilizer treated crop was in the range of 17.5 to 17.6 days respectively. Similarly, the waiting period recommended for green onions based on MRL(EU) of 10mgkg-^{1}(Anonymous,2016)following application of chlorothalonil was 1.1 days in case of bio fertilizer untreated crop and 1.0 day in case of bio fertilizer treated crop. Chlorothalonil residues in spring cabbage have been shown to depend on application schedule, dosage and weather (Zhang et al. 2007) and the average weather parameters during this study were as follows, temperature(maximum) –29.7 ^{0}C , temperature

(minimum)- 17.6 ^{0}C, relative humidity (maximum)- 73.9 %, relative humidity (minimum) – 45.6%, rainfall- 10.1mm.

Small microbial consortiums have been used as bio-control agents to control plant pathogens and also to improve plant growth and health (Sarma et al. 2015). Microbial consortiums have also been used in the past for bioremediation of sites or fields highly contaminated with pesticides (Pino Penuela 2011; Ravi et al. 2015). The small microbial consortium (AMC) used in this study resulted in increase in biomass upto 15 per cent, thus reducing persistence of the insecticide in leafy vegetables by three to five days and there by reducing the effective waiting period for obtaining safe produce from 20 or 21 days to 18 days. Thus, in addition to the beneficial effect of AMC on environment, crop yield and crop health, it's use may result in lesser pesticide residues in horticultural produce at harvest. Abiotic dissipation of chlorothalonil has been reported to be accelerated in a soil amended with a high rate of FYM by Katayama *et al.* (1995) but use of small microbial consortia such as AMC for improved plant growth, also resulting in faster chlorothalonil residue dissipation is reported for the first time in this study.

ACKNOWLEDGEMENTS

The authors thank the Director, Indian Institute of Horticultural Research, Bengaluru for providing the facilities for carrying out this study.

REFERENCES

Ashok Kumar, C.K.; Divya Sree, M.S.; Joshna, A; Mohana Lakshmi, S; Satheesh Kumar, D. 2013. A review on South Indian edible leafy vegetables. *Journal of Global Trends in Pharmaceutical Sciences*, *4*(4), 1248-1256

Anonymous, 2016. https://secure.pesticides.gov.uk/MRLs/main.asp accessed on 4th Aug.,

Correll, J.C.; Morelock, T.E.; Black, M.C.; Koike, S.T.; Brandenberger, L.P.; Dainello, F.J. 1994 Economically important diseases of spinach. *Plant Disease*, *78*, 653-660

Fuentes-Davila, G. 1988 Studies on a leaf spot disease of spinach caused by Cladosporium variable (Cooke) de Vries. Ph.D. thesis, Washington State University, Pullman, WA, 49 pp

KurzMarcia, H.S.; Goncalves, F.F.;, Adaime,M.B.; da Costa, Ivan, F.D.; Prime, E.G.;

Zanella, R. 2008. A gas chromatography method for the determination of fungicide chlorothalonil in tomatoes and cucumbers and its application to dissipation studies in experimental greenhouses. *Journal of Brazilian Chemical Society*. , 19(6), 590-598

Katayama, A.; Mori, T. Kuwatsuka, S. 1995. Abiotic dissipation of chlorothalonil in soil accelerated by amendment with high applications of farmyard manure. *Soil Biology and Biochemistery*, *27*(2): 147-151

Miller, M.E.; Bruton, D.; Amador, J.M. 1986. Effects of number and timing of chlorothalonil applications on onion yield. *Plant Disease*, *70*(9), 875-876

Panneerselvam, P.; Selvakumar, G; Ganeshamurthy, A.N. 2012. Microbial Consortium- A speciality bioinoculant for sustainable vegetable production. *Bio fertilizer News Letter*, 20(2), 12- 14

Pino, N.; Penuela, G. 2011. Simultaneous degradation of the pesticides methyl parathion and chlorpyrifos by an isolated bacterial consortium from a contaminated site. *International Biodeterioration and Biodegradation*, *65*(6) :827-831

Ravi, R.K., Pathak ,B. Fulekar, M.H .(2015) Bioremediation of persistent pesticides in rice field soil environment using surface soil treatment reactor. *International journal of Current Microbiology and applied sciences 4*(2): 359-369

Sarma, B.K.; Yadav, S.K.; Singh,,S.; Singh, H.B. 2015 Microbial consortium-mediated plant defense against phytopathogens: Readdressing for enhancing efficacy. *Soil Biology and Biochemistery* , *87*: 25-33

Zhang, Z-Y.; Liu, X-J.; Yu, X-Y.; Zhang, C-Z.; Hong, X-Y. 2007. Pesticide residues in the spring Cabbage(Brassica oleracea L. var. capitata) grown in open field. *Food Control* 18: 723– 730

MS Received 17 March 2019
MS Accepted 29 April 2019