Advances in Nutrition and Food Science

Kosmos Publishers

Research Article

Adv in Nutr Fd Sci: ANAFS-113

Dissipation Behaviour of Fluopyram and Tebuconazole in/on Pomegranate

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Citation: CS Patil, Shashi Vemuri, BV Deore, YS Saindane, K Kavitha, V Anitha (2018) Dissipation Behaviour of Fluopyram and Tebuconazole In/On Pomegranate. Adv Nutri and Food Scie: ANAFS-113.

Received Date: 15 September, 2018; **Accepted Date:** 23 October, 2018; **Published Date:** 31 October, 2018

Abstract:

Supervised field trial was conducted in 2016 to determine the residues and dissipation of fluopyram, its metabolite and tebuconazole in/on pomegranate and in soil resulting from spray application of fluopyram + tebuconazole (Luna Experience 400 SC) @ 0.075 % and 0.15 % concentration (75+75 and 150+150 g a.i./ha) by giving two sprays of fluopyram 200 + tebuconazole 200 (Luna Experience 400 SC) at an interval of 10 days, initiating first spray at fruit setting stage. Samples were collected at 0, 1, 3, 5, 7, 10, 15 and 20th day after second spray. Third spray was given 15 days before harvest and mature fruits and soil samples were collected at harvest. Residues of fluopyram dissipated with a half life of 4.90 and 5.29 days, at recommended and double dose, respectively. For tebuconazole, the half life values recorded were 5.02 and 4.86 days respectively. The residues reached below quantification limit (BQL) on 15th and 20th day in both the fungicides at recommended and double the recommended dose, respectively. Results suggest that Pre-Harvest Interval (PHI) of 13.74 and 12.59 at recommended dose whereas 21.92 and 18.97 days at double the recommended dose can be proposed for fluopyram and tebuconazole, respectively when applied at 75 g a.i./ha and 150 g a.i./ha..

1. Keywords: Fluopyram; Dissipation; Residues; Pre-Harvest Interval; Tebuconazole

2. Introduction

Pomegranate (*Punica granatum*) also called as "fruit of paradise" is one of the major fruit crops grown in tropical and subtropical regions of the world. The pomegranate fruit is known for its cool, refreshing juice and valued for its medicinal properties. In India, it is cultivated in an area of 19689 ha with a production of 230644 MT **[1]**. Maharashtra is the leading producer of pomegranate followed by Karnataka, Andhra Pradesh, Gujarat and Tamil Nadu. The crop is under threat due to number of serious diseases such as bacterial blight (*Xanthomonas axonopodis* pv. punicae), wilt (*Ceratocystis fimbriata*) anthracnose (*Colletotrichum gloeosporioides*) and leaf spot and severe fruit rotting due to *Alternaria alternata*, *Cercospora sp*., *Pseudocercospora sp*., *Drechslera sp*. and *Sphaceloma sp*. etc. The application of pesticide during pomegranate cultivation is unavoidable due to attack of these diseases.

Sometimes pesticides are applied during fruiting stage. Indiscriminate use of pesticides has resulted in the accumulation of pesticide residues in the primary agricultural products as well as soil **[2]**. Luna Experience is a combination of fluopyram and tebuconazole and offers two different modes of action. Fluopyram, a pyridyl ethyl amide broad spectrum fungicide belongs to a new chemical class. It is Succinate Dehydrogenase Inhibitor (SDHI) and breaks the respiration chain in the mitochondria of the fungus cell by blocking its energy production. Tebuconazole is a demethylation inhibitor (DMI). It interferes in the process of building structure of fungal cell wall. Finally, it inhibits the reproduction and further growth of fungus. Fluopyram being a new fungicide, no data on its dissipation is available in pomegranate. Hence studies were undertaken to validate the method for analysis of fluopyram and tebuconazole on liquid chromatography and mass spectrometry (LCMS) to determine the dissipation pattern of combination product, fluopyram and tebuconazole in pomegranate in western Maharashtra. The degradation or dissipation of insecticide is influenced by climatic conditions, type of application, plant species, dosage interval between application and time of harvest **[3]**. It is therefore necessary to determine the dissipation pattern of these two fungicides by following Good Agricultural Practices (GAP). Keeping this in view an attempt was made to study the persistence and dissipation pattern of fluopyram, its metabolite and tebuconazole in/on pomegranate. The data thus generated will help in determining safe waiting period for these fungicides in pomegranate.

3. Material and Methods

3.1. Field Experiment

Residues and dissipation of fluopyram, its metabolite and tebuconazole in/on pomegranate and in soil was studied by conducting supervised field trial during 2016 at Pandharpur in the jurisdiction of Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar. The experiment comprised of three treatments (Recommended dose, Double the recommended dose and untreated control) with three replications. Two sprays of Fluopyram 200 + Tebuconazole 200 : 400 SC were given at an interval of 10 days initiating first spray at fruit setting stage. Water was sprayed in the control plot. Samples were collected at 0, 1, 3, 5, 7, 10, 15 and 20th day after second spray. Third spray was given 15 days before harvest and mature fruits and soil samples were collected at harvest. About 1kg immature fruits, 1kg mature fruits and 1kg of soil were collected from each treated plot. Collected samples were transported to the laboratory, AINP on Pesticide Residues, PJTSAU, Hyderabad in dry ice and analyzed for the estimation of residues of fluopyram, its metabolite and tebuconazole.

3.2. Chemicals and Reagents

Certified Reference Materials (CRM) of fluopyram, its metabolite and tebuconazole with purity of 99.60, 99.40 and 95.60 per cent, respectively were provided by Bayer Crop Science Ltd, Mumbai. The solvents and sorbents used in extraction and analysis were distilled and checked for impurities prior to use.

3.3. Residue Estimation

An accurately weighed 10 mg of an individual standard was dissolved in 10 ml volumetric flask using suitable solvent to prepare the standard stock solution of 1000 mg/ kg. Standard stock solution of each insecticide was serially diluted to obtain intermediate lower concentration of 100 mg/kg. They were stored in a refrigerator at -40^{o}C. From the intermediate standards, working standards were prepared by suitably diluting the stock solution in n-hexane and used as standard check in analysis, linearity and recovery studies.

Prior to analysis of samples, linearity of these fungicides was established on LCMS. Accuracy and precision of the method was determined by per cent mean recovery and per cent relative standard deviation. Linearity was studied by injecting standard solution of fungicides under study at five linear concentrations i.e. 0.05, 0.10, 0.25, 0.50 and 1.00 mg/kg in triplicate. The linearity curve was established **(Figures 1, 2, 3)** with concentration of the standard and corresponding peak area. Recovery study was conducted in different matrices i.e. whole pomegranate fruit, edible arils, juice and cropped soil in order to establish the reliability of the method of analysis [4]. For this purpose, pomegranate samples and soil from control plots were used. The samples were spiked with three different

concentrations viz. 0.05 (LOQ), 0.25 (5×LOQ) and 0.5 (10×LOQ) mg/kg. The extraction and clean up were performed as described hereunder. Per cent recovery was calculated by using following formula.

$$\text{Per cent recovery} = \frac{\text{Quantity of pesticide recovered}}{\text{Quantity of pesticide added}} \times 100$$

3.4. Extraction and Clean Up

3.4.1. Pomegranate Edible Aril and Juice

The pomegranate fruits, edible arils and juice samples were analyzed for fluopyram, its metabolite-fluopyram benzamide and tebuconazole residues following AOAC official method 2007.01 (QuEChERS) after validation of the method at the laboratory. The pomegranate fruits, edible arils and juice samples were homogenized separately with robot coupe blixer and homogenized 15±0.1g sample was taken in 50 ml centrifuge tube. 30±0.1 ml acetonitrile was added to sample tube. The sample was homogenized at 14000-15000 rpm for 2-3 min using Heidolph silent crusher. 3±0.1 g sodium chloride was added to sample, mixed thoroughly by shaking gently followed by centrifugation at 2500-3000 rpm for 3 min to separate the organic layer. The top organic layer of about 16 ml was taken into 50 ml centrifuge tube and added with 9±0.1 g anhydrous sodium sulphate to remove the moisture content. 8 ml of extract was taken in to 15 ml tube containing 0.4±0.01 g PSA sorbent (for dispersive solid phase d-SPE cleanup) and 1.2±0.01 g anhydrous magnesium sulphate. The sample tube was vortexed for 30 sec then followed by centrifugation at 2500-3000 rpm for 5 min. The extract of about 1 ml (0.5 g sample) was taken for analysis on LC-MS/MS under standard operational conditions.

3.4.2. Soil

The soil samples were analyzed following the QuEChERS method after validation of the method at the laboratory. The soil samples were dried at room temperature under shade, ground, passed through 2 mm sieve and a representative 10 g sample was taken in to 50 ml centrifuge tube. 20 ml acetonitrile was added to sample tube and shaken vigorously for 2 min. The samples were then added with 4±0.1 g magnesium sulphate and 1±0.1 g sodium chloride and centrifuged at 2500-3000 rpm for 5 min to separate the organic layer. The top organic layer of about 10 ml was taken into 15 ml centrifuge tube and added with 250±0.1 mg PSA sorbent and 1.5±0.01 g magnesium sulphate and sonicated for 1 min then centrifuged at 2500-3000 rpm for 10 min. The extract of about 1 ml (0.5 g sample) was taken for analysis on LCMS/MS under standard operational conditions.

LC-MS/MS Parameters for Fluopyram and Tebuconazole

LC-MS/MS	**SHIMADZU LCMS/MS - 8040.**
Detector	Mass Spectrophotometer
Column	Kinetex, 2.6µ, C18 Column, 100 x 3.0.
Column oven temperature	40^{o}C
Retention time	Tebuconazole - 5.2 min Fluopyram - 5.4 min
Nebulizing gas	Nitrogen
Nebulizing gas flow	2.0 litres/min
Pump mode/ flow	Gradient / 0.4 ml/ min
Solvents	A: Ammonium Formate in Water(10Mm) B: Ammonium Formate in Acetonitrile (10Mm)

LC programme	Time solvent Conc. 0.01 B Conc. 50% 1.00 B Conc. 80% 4.00 B Conc. 50% 8.00 B Conc. 50%
Total Time Programme	8 min

LC-MS/MS PARAMETERS FOR FLUOPYRAM METABOLITE - FLUOPYRAM BENZAMIDE

LC-MS/MS	**SHIMADZU LCMS/MS - 8040.**
Detector	Mass Spectrophotometer
Column	Kinetex, 2.6µ, C18 Column, 100 x 3.0.
Column oven temperature	40°C
Retention time	Fluopyram Benzamide - 1.5 min
Nebulizing gas	Nitrogen
Nebulizing gas flow	2.0 litres/min
Pump mode/ flow	Gradient / 0.4 ml/ min
LC programme	A: Ammonium Formate in Water (10Mm) - 40% B: Ammonium Formate in Methanol (10Mm) - 60%
LC programme	Time solvent Conc. 0.01 B Conc. 50% 2.00 B Conc. 80% 3.00 B Conc. 50% 4.00 B Conc. 50%
Total Time Programme	4 min

4. Results and Discussion

The detector response to the neat standards of the fungicides was studied by injecting five linear concentrations of these fungicides. The calibration curve was plotted with detector response against respective concentrations and linearity line was drawn. The response of the instrument was linear over the range tested and R^2 value was 0.99 for all the fungicides under study **(Figures 1, 2, 3**). These results indicated that the LC-MS analysis is a valid method for residue determination of the tested fungicides in pomegranate fruits.

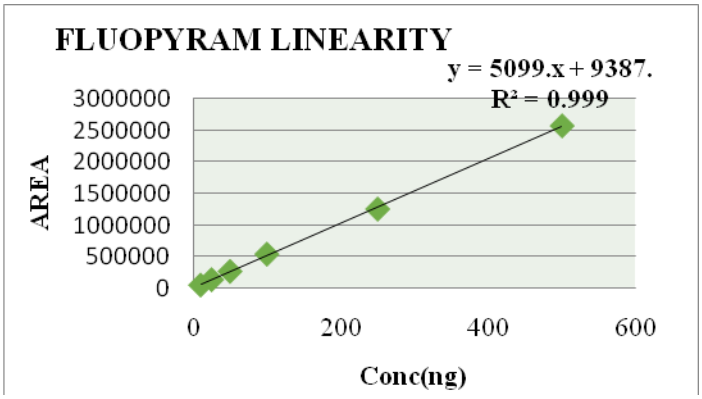

Figure 1: Calibration curve of Fluopyram.

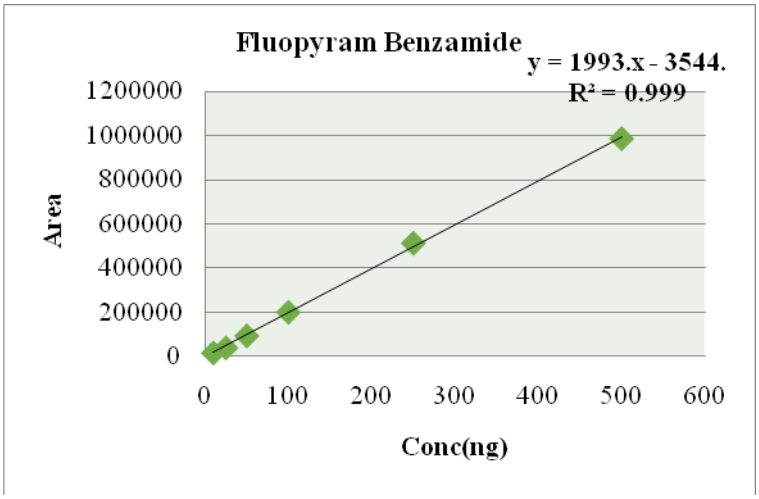

Figure 2: Calibration curve of Fluopyram Benzamide.

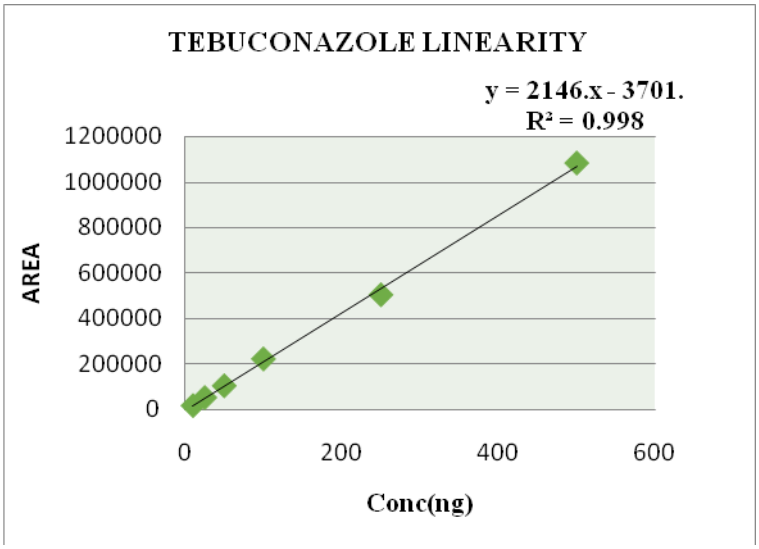

Figure 3: Calibration curve of Tebuconazole.

Accuracy of the analytical method was determined by recovery studies. The per cent recovery was within acceptable range of 70-120 per cent prescribed by SANCO (2011) **[5]** and mentioned in (**Table 1).**

Matrix	Fortification level	Recovery (%)		
		Fluopyram	Fluopyram Benzamide	Tebuconazole
Edible arils	0.05 mg/kg	106.66 (3.10)	88.0 (3.78)	114.0 (0.95)
	0.25 mg/kg	83.46 (2.64)	87.73 (0.65)	95.46 (2.46)
	0.50 mg/kg	102.53 (3.10)	102.0 (4.51)	117.2 (1.63)
Juice	0.05 mg/kg	102.0 (2.16)	88.0 (2.10)	112.60 (4.42)
	0.25 mg/kg	85.6 (2.66)	96.26 (2.33)	94.80 (1.98)
	0.50 mg/kg	99.53 (3.80)	107.13 (0.88)	115.00 (0.82)
Soil	0.05 mg/kg	103.33 (1.63)	96.0 (5.14)	114.0 (2.25)
	0.25 mg/kg	84.53 (1.72)	96.4 (2.13)	102.8 (13.13)
	0.50 mg/kg	106.13(3.25)	105.86 (0.37)	117.13 (1.59)

Table 1: Recoveries of fluopyram, fluopyram benzamide and tebuconazole at various fortification levels in different matrices.

4.1. Dissipation of Insecticides

The initial residues of 0.323 mg/kg of fluopyram was detected in pomegranate samples collected from plots sprayed with Fluopyram 200+ Tebuconazole 200:400 SC @ 0.75 ml/L. of water (Recommended dose), which dissipated to 0.068 mg/kg by 10th day and Below Quantitation Level by 15th day **(Table 2, Figure 4,5)**.

Days after 2nd Application	Residues of Fluopyram (mg/kg)					
	Fluopyram		Fluopyram benzamide		Tebuconazole	
	Recommended Dose	Double Recommended Dose	Recommended Dose	Recommended Dose	Recommended Dose	Recommended Dose
Pomegranate fruits	Mean SD(±)	Mean SD(±)	Mean SD(±)	Mean SD(±)	Mean SD(±)	Mean SD(±)
0 Day	0.323 (0.052)	0.716 (0.043)	BQL	BQL	0.267 (0.048)	0.634 (0.046)
1 Day	0.290 (0.033)	0.685 (0.107)	BQL	BQL	0.256 (0.032)	0.579 (0.096)
3 Days	0.226 (0.080)	0.530 (0.154)	BQL	BQL	0.174 (0.055)	0.415 (0.123)
5 Days	0.188 (0.030)	0.512 (0.067)	BQL	BQL	0.151 (0.023)	0.401 (0.030)
7 Days	0.168	0.502	BQL	BQL	0.131	0.384

	(0.028)	(0.047)			(0.024)	(0.031)
10 Days	0.068 (0.012)	0.380 (0.095)	BQL	BQL	0.062 (0.029)	0.289 (0.063)
15 Days	BQL	0.076 (0.037)	BQL	BQL	BQL	0.055 (0.018)
Edible aril at harvest	BQL	BQL	BQL	BQL	BQL	BQL
Juice at harvest	BQL	BQL	BQL	BQL	BQL	BQL
Soil at harvest	BQL	BQL	BQL	BQL	BQL	BQL
Regression Equation	y = -0.0614x + 2.543	y = -0.0569x + 2.9463			y = -0.0587x + 2.4646	y = -0.0619x + 2.8732
Regression coefficient	R^2 = 0.9154	R^2 = 0.8145			R^2 = 0.950	R^2 = 0.846
Half life (Days)	4.90	5.29			5.02	4.86
PHI (Days)	13.74	21.92			12.59	18.97

Table 2: Residue data of Fluopyram in pomegranate at regular intervals in edible aril, juice and soil at harvest.

LOQ: Fluopyram-0.05 mg/kg.
Fluopyram benzamide-0.05 mg/kg.
Tebuconazole-0.05 mg/kg.
Figures in parenthesis are ± SD values.

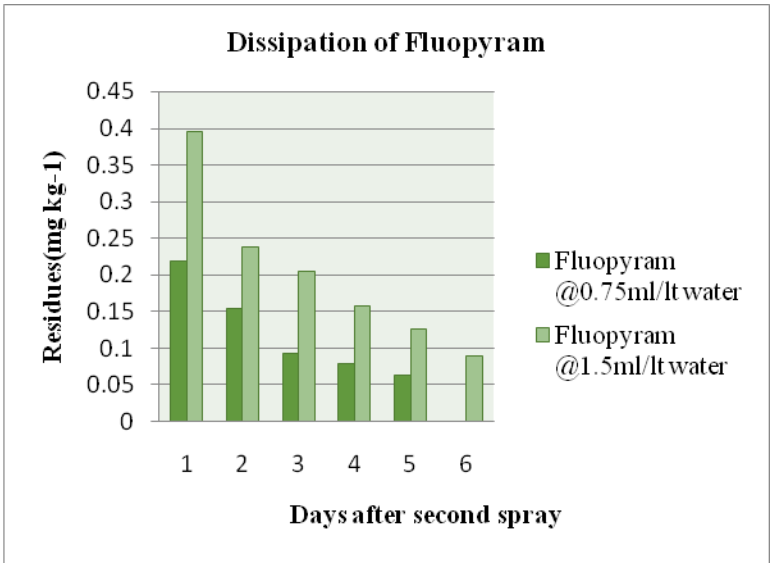

Figure 4: Dissipation Pattern of Fluopyram in/on Pomegranate.

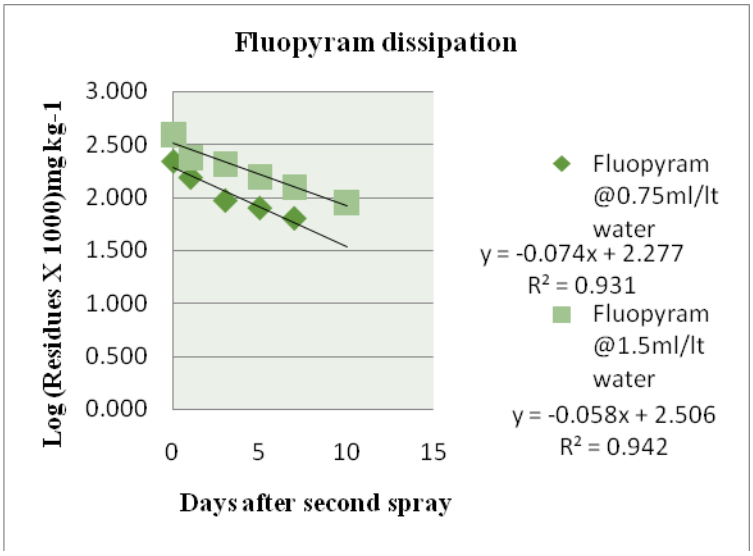

Figure 5: Semi logarithmic graph showing dissipation kinetics of Fluopyram in pomegranate.

The initial residues of 0.716 mg/kg of Fluopyram was detected in pomegranate samples collected from plots sprayed with Fluopyram 200+Tebuconazole 200:400 SC @1.5 ml/lit. of water (Double the recommended dose), which dissipated to 0.076 mg/kg by 15th day and Below Quantitation Level by 20th day **(Table 2)**. As regards, fluopyram benzamide, a metabolite of fluopyram, no sample irrespective of dose, didn't record any residues. The initial residues of 0.267 mg/kg of Tebuconazole was detected in pomegranate samples collected from plots sprayed with Fluopyram 200+ Tebuconazole 200:400 SC @0.75 ml/L. of water (X dose), which dissipated to 0.256 mg/kg by 1st day, 0.174 mg/kg by 3rd day, 0.151 mg/kg by 5th day, 0.131 mg/kg by 7th day and 0.062 mg/kg by 10th day Below Determination Level by 15th day **(Table 2, Figure 6,7).**

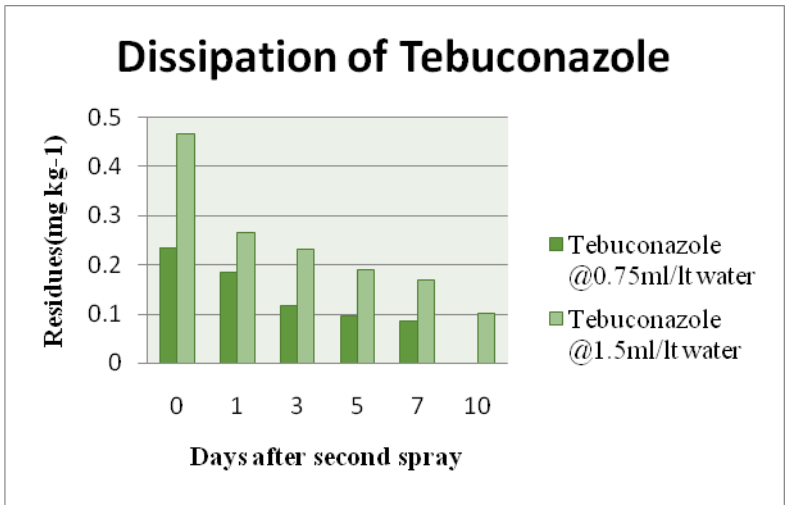

Figure 6: Dissipation Pattern of Tebuconazole in/on Pomegranate.

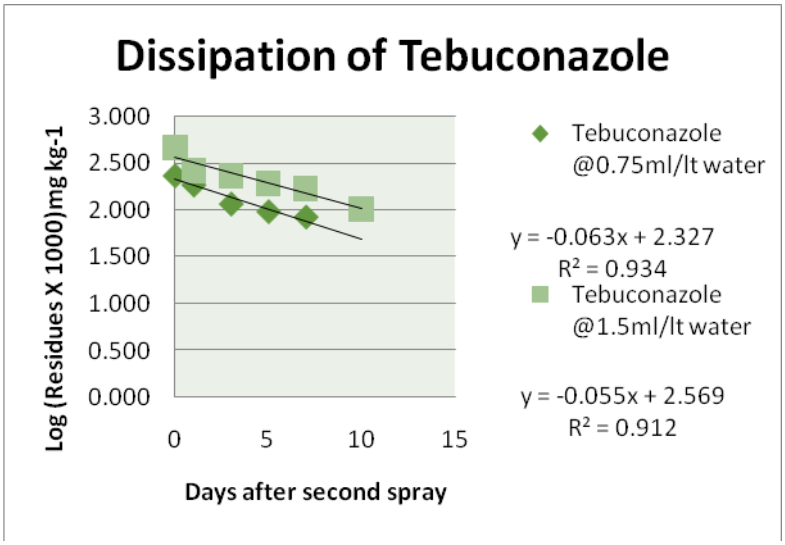

Figure 7: Semi logarithm graph showing dissipation kinetics of Tebuconazole in/on pomegranate.

The initial residues of 0.634 mg/kg of Tebuconazole was detected in pomegranate samples collected from plots sprayed with Fluopyram 200+ Tebuconazole 200:400 SC @1.5 ml/L. of water (2X dose), which dissipated to 0.579 mg/kg by 1st day and 0.055 mg/kg by 15th day and Below Determination Level by 20th day **(Table 2).** Dong and Hu (2014) **[6]** reported the half-lives of fluopyram were 6.48–6.60 days in watermelon and 15.8–24.8 days in soil. The half-lives of tebuconazole were 5.87–6.93 days in watermelon and 11.2–14.4 days in soil. The terminal residues of fluopyram and tebuconazole were all below the maximum residue limits (MRLs) value set by USA (fluopyram, 1 mg/kg; tebuconazole, 0.09 mg/kg). Patel *et al* (2016) **[7]** reported the initial residues of 1.14 and 2.86 mg/kg of fluopyram on spring onion at standard and double dose, respectively. The levels of fluopyram residues gradually declined and recorded 0.25 and 0.58 mg/kg on 20th day of application with half-lives of 8.8 and 9.1 days at standard and double dose, respectively. For tebuconazole, the corresponding residues observed after 1 h (0 day) of application were 0.92 and 2.29 mg/kg. The levels declined gradually to 0.12 and 0.33 mg/kg on 20th days with half-life of 6.7 to 7.7 days at standard and double dose, respectively. Further they proposed a pre-harvest interval of 7 day for fluopyram and tebuconazole in spring onion when applied at 75 + 75 g a.i./ha (400 SC).

The dissipation of residues of fluopyram and tebuconazole (Luna Experience 400SC) was studied in chilli **[8]**, onion **[7]** and watermelon **[6]** and tebuconazole alone applied in/on onion **[9,10]**, mango **[11]**, chilli **[12]**, tomato **[13]** and apple **[14]**. According to Patel *et al* (2016) **[7]**, half life of fluopyram was 8.85 and 9.12 days, respectively in recommended and double dose. Dong and Hu (2014) **[6]** showed half life of 6.48 days for fluopyram in watermelon. In chilli, Saha (2016) **[8]** found a half life of 1.161 and 1.241 days for single (100 g a.i./ha) and double (200 g a.i./ha) application rate. For tebuconazole, half life of 6.69 and 7.72 days was reported in onion **[7]** and 0.866 and 1.083 days in case of chilli **[8]** at the single and double dose, respectively. In other studies, the reported half life was 1.7 days (Mohopatra *et al* 2018) and 6 days in onion **[10]** and mango **[11]** 5.87 and 6.93 days in watermelon **[6]** 4.49 days in ginseng **[16]**, 1 day in chilli **[12]** and 0.9 days in tomato **[13]**. However, half life of tebuconazole ranged between 19.38 and 25.99 days and 19.84 and 28.86 days at the application rate of 200 and 400 g ai/ha in apple **[14]**. A PHI of 21 days was recorded for tebuconazole on onion by CIB & RC of India Mohopatra *et al* (2014) **[10]** suggested a PHI of 16 days and 35 days for tebuconazole at 187.5 and 375 g a.i./ha in immature onion bulb with leaves. From the present study, the pre harvest interval (PHI) of 7.76 and 9.91 days for fluopyram and tebuconazole can be considered safe for harvesting residue free pomegranate at application rate of 75 and 150 g a.i./ha.

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