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Persistence of Combination Fungicide, Fluopyram + Tebuconazole Residues and its Risk Assessment in Kidney Bean

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ABSTRACT

Background: Kidney bean is a protein rich legume crop largely cultivated in hilly tracts of India. Fungal diseases are major biotic constraints causing severe yield losses and fluopyram and tebuconazole are most commonly used for the management of powdery mildew and anthracnose diseases. Hence, studies were taken on dissipation pattern and dietary risk assessment of fungicides to establish consumer safety.

Methods: A simple QuEChERS analytical method was developed and validated for the analysis of fluopyram its metabolite and tebuconazole in kidney bean immature green pods, mature pods, seeds and soil and analyzed in liquid chromatograph mass spectrometer (LC-MS). The combination fungicide was applied as foliar spray at 125+125 and 250+250 g a.i. ha^{-1} on kidney bean thrice at 10 days interval. Residues were determined in immature green pods, dry mature pods and soil. Immature green pods were collected on 0 (within 2 hours after last spray), 1, 3, 5, 7, 10, 15 and 20 days after third spray and in dry mature pods and soil collected at the time of harvest.

Result: The average recovery and RSD were in the range of 81.63-95.48% and 0.55-5.10%, with 0.01 and 0.05 µg mL^{-1} as LOD and LOQ. The initial concentrations were 0.55 and 1.04 mg kg^{-1} for fluopyram and 0.57 and 1.03 mg kg^{-1} for tebuconazole in standard and double dose, respectively. The half-lives were 3.67-4.07 days for fluopyram and 3.86-4.18 days for tebuconazole in standard and double dose. Dietary intake risk of fluopyram and tebuconazole from kidney bean is negligible for adult men, women and children based on RQ values (<1).

Key words: Dissipation, Fluopyram, Kidney bean, QuEChERS, Tebuconazole, Risk assessment.

INTRODUCTION

Kidney bean (*Phaseolus vulgaris* L.) also known as Rajmash or Rajma is an important cool season legume vegetable grown throughout the world. It is consumed as immature green pod as well as mature seed. Seeds are excellent source of protein, complex carbohydrates and other dietary elements such as vitamins and minerals. The crop is affected by a number of biotic and abiotic stresses and among the biotic factors fungal diseases like anthracnose, powdery mildew, yellow mosaic, rust, root rot, wilt and leaf spot are of major concern. For controlling these fungal pathogens, a large number of fungicides are used for the management of several crop diseases of economic importance.

Fluopyram (N-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl}-α,α,α-trifluoro-o-toluamide) is a systemic broad-spectrum molecule acting as succinate dehydrogenase inhibitor (SDHI) and used against a range of ascomycete and deuteromycete diseases in many horticultural crops. The major metabolite is fluopyram benzamide. Tebuconazole 1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol) is a systemic demethylation inhibitor (DMI) used for controlling fungal diseases of fruit and vegetable crops.

The efficacy of combination fungicide, fluopyram + tebuconazole was reported against anthracnose disease in chilli (Saha *et al*., 2014), grapevine (Singh *et al*., 2011), leaf spot and fruit rot of pomegranate (Xavier *et al*., 2020). Fluopyram 17.7% w/w + Tebuconazole 17.7% w/w SC is recommended against powdery mildew and anthracnose in grapes and chilli (CIBRC, 2022).

Hence, with a foreseen widespread and substantial use of these fungicides, it would be indeed crucial to assess the persistence and dissipation behaviour of fluopyram in kidney bean. In the current study, a method for simultaneous determination of fluopyram and tebuconazole residues in kidney bean pods and soil using LC-MS was developed and validated. Persistence of these fungicides through open field trials and their dietary risk assessment were studied.

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MATERIALS AND METHODS

Chemical and reagents

Certified reference materials of fluopyram (99.4%), fluopyram benzamide (99.4%), tebuconazole (99.1%) and the formulation Luna Experience 400 SC were supplied by M/s Bayer Crop Science, Thane, Mumbai, India. The HPLC grade acetonitrile (≥99.99 purity), LCMS grade (LiChrosolv) acetonitrile (≥99.99% purity), Analytical grade sodium chloride (≥99% purity) and anhydrous magnesium sulphate (≥99.5% purity) were obtained from Merck, India. LC-MS grade formic acid was procured from M/s Sigma Aldrich, Bangalore, India. Primary Secondary amine (PSA) and graphitized carbon black (GCB) were supplied by Agilent Technologies, Palo Alto, USA.

Stock solution preparation

Stock solutions (400 µg ml^{-1}) of fluopyram, fluopyram benzamide and tebuconazole were prepared by dissolving the technical material (10±0.5 mg) in acetonitrile (v/v) separately in a calibrated Class A volumetric flask (25 mL) and labeled properly. The calibration and spiking standards were prepared by serial dilution in the range of 0.01-0.50 µg ml^{-1} from the intermediate stock solution and stored at -20°C in a deep freezer.

Field experiment

A field trial was carried out at Kookal Thorai, Kothagiri, The Nilgiris, India (11.48°N, 76.82°E) during March 2021 to April, 2021 to study the persistence and dissipation behaviour of fluopyram and tebuconazole in kidney bean pods. The experiment was laid with randomized block design (RBD) in 25 m^2 plot size /replication and each treatment was replicated thrice. The control plot was maintained with the application of water spray. The combination formulation of fluopyram and tebuconazole (Luna Experience 400 SC) at standard (125+125 g a.i. ha^{-1}) and double dose (250+250 g a.i. ha^{-1}) was applied as foliar spray using knapsack sprayer starting from 52 days after sowing at ten days interval. The maximum and minimum temperature, relative humidity and total rainfall documented during the whole trial were in the range of 22.17 and 10.00°C, 79.46 and 58.99% and 84.90 mm, respectively.

Sample collection and preparation

Samples were drawn randomly from each treatment in polythene bags and brought to the laboratory on the same day for processing. Sample collection was done at 0 (2 h after spraying), 1, 3, 5, 7, 10, 15 and 20 days after last spray for immature pods (500 g) and at harvest for mature pods (2 kg) (25 days after last spray). The green pods were processed by chopping and homogenizing in a high-volume blade homogenizer (Robot Coupe) and seeds from mature dry pods were separated and homogenized. Soil sample collected from 0-15 cm depth was homogenised after shade drying and passed through 2 mm sieve for further analysis. Immature pods, dry pods and soil collected from untreated plots served as control samples. Sample preparation and processing were carried out at Pesticide Toxicology Laboratory, Department of Agricultural Entomology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.

Extraction and cleanup

The extraction of target fungicide residues in kidney bean matrices and soil was carried out by QuEChERS method (Anastassiades *et al*., 2003) with slight modifications.

Extraction and cleanup of kidney bean green pods with immature seeds, dry pods, dry seeds and soil

Representative samples of green immature pods with seeds, mature dried seeds, soil (10 g each), dry pods (5 g sample with 10 mL distilled water) were taken in a 50 mL polypropylene tube and 20 mL of acetonitrile was added as an extraction solvent. Subsequently, 4 g anhydrous $MgSO_4$ and 1 g sodium chloride (NaCl) were added, vortexed for one min. and centrifuged at 6000 rpm for 10 min. After centrifugation, 6 mL of supernatant aliquot was transferred into a 15 mL centrifuge tube containing sorbents namely anhydrous $MgSO_4$ (600 mg), PSA (100 mg) and GCB (10 mg). The mixture was vortexed for one min. to ensure sufficient contact between sorbents and aliquot, then centrifuged for 10 min. at 3000 rpm. After centrifugation, two mL supernatant was concentrated to near dryness under a gentle stream of nitrogen with 15 psi pressure in a turbovap LV at 40°C. The residues were reconstituted in acetonitrile (1 mL) and transferred into 1.5 mL LC-MS autosampler vials for instrument analysis.

LC-MS parameters

Chromatographic analysis was performed using LC-MS (Shimadzu, LCMS 2020) equipped with UHPLC, Agilent shim-pack GIST C_{18} column (250 mm × 4.6 mm, 5 µ particle size and 10 nm pore size) at 40°C column temperature. The eluting solvents consisted of 0.1% formic acid in ultra-pure water and LC-MS grade acetonitrile in an isocratic flow of 40% A and 60% B. The flow rate was 0.6 mL min^{-1} with total run time of 20 min. Mass spectroscopic analysis was done in positive electronic ionization mode and the analytes were scanned in the mass range of 50 to 1000 m/z with time event of 1.2 sec. The m/z ions of fluopyram, fluopyram benzamide and tebuconazole were 308, 190 and 397 in positive ion mode, respectively.

Method validation

The analytical method used for determination of fluopyram, fluopyram benzamide and tebuconazole in kidney bean and soil was validated by assessing the parameters *viz*., linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision as per SANTE/12628/2019 guideline. The fungicide mix of fluopyram, fluopyram benzamide and tebuconazole was utilized for validating the parameters. Linearity was established using calibration curve created using seven concentrations in the range of 0.01-0.50 µg mL^{-1}. LOD and LOQ were calculated using a signal to noise ratio of 3 and 10, respectively. Precision, the measure of closeness between the analytical results is expressed as per cent relative standard deviation (RSD).

Method accuracy was ascertained by fortifying pod, seed and soil matrices at five levels (0.05, 0.125, 0.25, 0.375 and 0.50 µg mL^{-1}) in six replicates and samples were extracted by above mentioned procedure. The matrix effect was established by comparing peak area of the neat standard and matrix standard and threshold level is < 20%.

The dietary intake risk of fluopyram and tebuconazole in green immature kidney bean was carried out by estimating risk quotient (RQ) by dividing estimated daily intake (EDI) and average daily intake (ADI). The ADI for fluopyram is 0.01 mg kg^{-1} body weight and 0.03 mg kg^{-1} body weight for tebuconazole.

$$EDI = DC \times MIR/bw$$

Where

DC= Daily consumption of kidney bean (40 g/ person/day);

MIR= Maximum initial residue concentration of target fungicides (mg kg^{-1});

bw= Weight of an average Indian adult men (60 kg), adult women (55 kg) and children 6-7 years (25.3 kg) (Patel *et al*. (2016), Lozowicka *et al*. (2014).

RESULTS AND DISCUSSION

In the present study, QuEChERS analytical method was validated for simultaneous extraction and determination of fluopyram, its metabolite fluopyram benzamide and tebuconazole from kidney bean immature pod, dry pod, seed and soil matrices. A good linear relationship was obtained (R^2 >0.9997) for matrix matched standards (0.01-0.50 mg kg^{-1}) of the three analytes studied. The LOD and LOQ for fluopyram, its metabolite fluopyram benzamide and tebuconazole were 0.01 and 0.05 mg kg^{-1}, respectively. The recovery chromatograms of fluopyram, its metabolite fluopyram benzamide and tebuconazole at LOQ (0.05 mg kg^{-1}) level are presented in Fig 1. The method developed was considered satisfactory for determination of fluopyram its metabolite flupoyram benzamide and tebuconazole residues in kidney bean and soil with acceptable limit of 70-120% recovery and <20% RSD (Table 1). In all the four matrices, the matrix effect was within the acceptance criteria of ±20% (SANTE, 2019).

Dissipation of fluopyram, fluopyram benzamide and tebuconazole residues

The dissipation of fluopyram, fluopyam benzamide and tebuconazole residues in kidney bean green immature pods was analyzed using first-order kinetics equation.

$$Ct = Coe^{-kt}$$

Where,

Ct= Pesticide concentration (mg kg^{-1}) at the time (days).

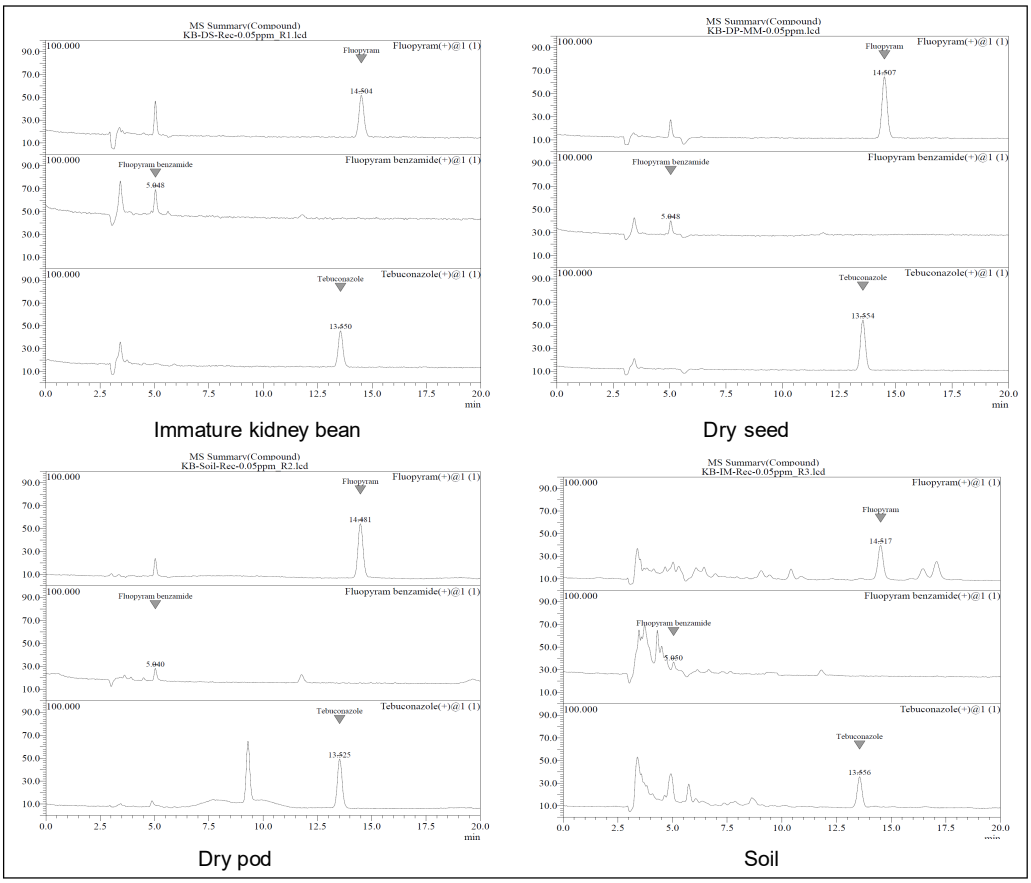

Fig 1: Recovery chromatograms of kidney bean and soil matrices spiked at 0.05 kg/mg level.

Co= Apparent initial concentration (mg kg^{-1}).
k= Dissipation rate constant.

The initial concentration of fluopyram in immature green kidney bean pod was 0.55 mg kg^{-1} and 1.04 mg kg^{-1} at 125 g a.i. ha^{-1} and 250 g a.i. ha^{-1}, respectively. The residue of fluopyram dissipated slowly with time and reached below LOQ (0.05 mg kg^{-1}) within 15 and 20 days after third spraying in the standard and double doses. The residue of the metabolite fluopyram benzamide was < LOQ (0.05 mg kg^{-1}) in all the samples collected in both treatments (Table 2). The initial concentration of tebuconazole was 0.57 mg kg^{-1} and 1.03 mg kg^{-1} from treatments at 125 g a.i. ha^{-1} and 250 g a.i. ha^{-1},

Table 1: Recovery results of Fluopyram, its metabolite and tebuconazole in kidney bean matrices and soil.

Matrix	Spiking level (µg ml^{-1})	Fluopyram		Fluopyram benzamide		Tebuconazole	
		Mean recovery (%) ± SD	RSD (%)	Mean recovery (%) ± SD	RSD (%)	Mean recovery (%) ± SD	RSD (%)
Immature green pods with seeds	0.05	94.12±3.01	3.20	105.05±5.21	4.96	90.52±7.18	7.93
Dried seeds		97.84±5.05	5.16	106.73±6.82	6.39	103.03±6.45	6.26
Dried pods		86.31±3.00	3.48	85.05±5.65	6.65	87.75±6.18	7.04
Soil		90.75±1.55	1.71	84.30±2.22	2.64	92.04±0.69	0.75
Immature green pods with seeds	0.125	99.78±1.74	1.74	105.46±2.25	2.14	97.45±2.21	2.27
Dried seeds		91.60±0.66	0.72	109.95±2.96	2.70	103.30±1.35	1.31
Dried pods		80.88±0.95	1.17	89.20±2.47	2.77	90.06±0.86	0.96
Soil		96.24±1.63	1.70	99.40±1.81	1.82	96.78±1.51	1.56
Immature green pods with seeds	0.250	92.78±2.81	3.02	92.21±4.81	5.22	95.52±4.09	4.28
Dried seeds		96.86±0.69	0.72	113.90±3.00	2.64	107.06±1.27	1.18
Dried pods		89.52±0.45	0.50	94.73±1.47	1.56	94.13±1.03	1.09
Soil		89.59±2.35	2.62	94.67±1.12	1.19	91.61±1.34	1.46
Immature green pods with seeds	0.375	93.89±3.33	3.54	90.23±1.01	1.12	94.40±3.17	3.36
Dried seeds		89.16±0.58	0.65	102.69±3.02	2.94	99.40±0.65	0.65
Dried pods		82.98±0.53	0.64	85.85±2.93	3.41	87.56±0.38	0.43
Soil		90.63±2.07	2.28	91.71±4.32	4.71	93.02±1.59	1.71
Immature green pods with seeds	0.50	88.26±0.47	0.54	84.01±2.57	3.06	89.34±0.58	0.65
Dried seeds		93.31±0.58	0.62	104.98±1.26	1.20	105.37±0.46	0.43
Dried pods		86.79±0.47	0.54	86.90±1.61	1.85	93.00±0.28	0.31
Soil		91.77±1.46	1.59	93.31±3.01	3.23	95.24±1.67	1.75

Data of three replicates (n= 3); SD- Standard deviation; RSD = Relative standard deviation (intra-day repeatability check)

Table 2: Residues and persistence of fluopyram and tebuconazole in immature beans, dried seeds, dry pods and soil.

Matrix	Days after application	Fluopyram		Tebuconazole	
		(125 g a.i. ha^{-1})	(250 g a.i. ha^{-1})	(125 g a.i. ha^{-1})	(250 g a.i. ha^{-1})
		Mean residue (mg kg^{-1})	Mean residue (mg kg^{-1})	Mean residue (mg kg^{-1})	Mean residue (mg kg^{-1})
Immature beans	0	0.55	1.04	0.57	1.03
with seeds	1	0.47	0.94	0.43	0.95
	3	0.28	0.78	0.25	0.81
	5	0.21	0.60	0.19	0.59
	7	0.15	0.43	0.14	0.48
	10	0.08	0.14	0.09	0.16
	15	<LOQ	0.10	<LOQ	0.10
	20	<LOQ	<LOQ	<LOQ	<LOQ
Dry pods	25	<LOQ	<LOQ	<LOQ	<LOQ
Dry seeds	25	<LOQ	<LOQ	<LOQ	<LOQ
Soil	25	<LOQ	<LOQ	<LOQ	<LOQ
Regression equation		Y=2.735-0.082x	Y=3.070-0.074x	Y=2.701-0.078x	Y=3.077-0.072x
Half-life ($t_{1/2}$) days		3.67	4.07	3.86	4.18

respectively. The residues of fluopyram, fluopyram benzamide and tebuconazole were less than LOQ (<0.05 mg kg^{-1}) in dry pods, dry mature seeds and soil collected at the time of harvest in both the doses.

The half-life ($t_{1/2}$) was determined by DT50=log2/k (Hoskins, 1961), where k=slope of regression equation of the log residues determined in mg kg^{-1}. The half-lives of fluopyram residues were 3.67 and 4.07 days and that of tebuconazole residues were 3.86 and 4.18 days, at standard and double doses, respectively (Table 2).

Several previous studies have reported the half-lives of fluopyram and tebuconazole in various crops and were 3.4 and 3.8-3.9 days in French bean (Katna *et al*., 2018), 8.85-9.12 and 6.7-7.7 days in onion (Patel *et al*., 2016), 5.6-5.7 and 3.5-4.5 days in chilli (Shukla *et al*., 2017), 6.48-6.60 days and 5.87-6.93 days in watermelon (Dong and Hu, 2014). In a multilocation trials conducted in three different places, the half lives of 4.40-6.40 days and 3.60-7.40 days in mango and pomegranate, respectively, for fluopyram and 2.90-5.20 days and 3.50-6.10 days in mango and pomegranate, respectively, for tebuconazole (Tripathy *et al*., 2022) were reported. The crop stage and varieties, pesticide chemistry, dosage and method of application, environmental factors such as relative humidity, temperature, precipitation and sun light are the major deciding factors of dissipation pattern of pesticides and thereby influence the half life values.

Dietary risk assessment of combination of fluopyram and tebuconazole through risk quotient (RQ) by comparing the values of EDI with ADI was studied. Overall, the RQ values of the two target analytes in the kidney bean matrix were < 1, indicating negligible level of dietary intake risk for all three analytes by consuming kidney bean.

CONCLUSION

A simple and rapid QuEChERS method was validated for simultaneous determination of fluopyram, its metabolite fluopyram benzamide and tebuconazole in kidney bean using LC-MS. The dissipation pattern of fluopyram and tebuconazole residues followed first-order kinetics with half-lives of 3.67 to 4.07 days and 3.86 and 4.18 days for fluopyram and tebuconazole, respectively. The terminal residues of all three analytes in dry pods, dry seeds, soil were below LOQ. The dietary risk assessment (RQ <1) of combination product of fluopyram and tebuconazole showed that even at the highest initial residue concentration the risk was at acceptable level.

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Conflict of interest: None.

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