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Research Article

DISSIPATION AND HARVEST TIME RESIDUES OF FOLIAR APPLIED FLONICAMID 15% + FIPRONIL 15% WDG ON PADDY

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Abstract: The study on residue and dissipation of flonicamid and fipronil after foliar application of flonicamid 15% + fipronil 15% WDG on rice plant after 2 applications at 15 days interval with the first coinciding with the tillering stage @ 400 g ha^{-1} and 800 g ha^{-1} was conducted at Integrated Farming System Research Station, Kerala Agricultural University, Karamana, Thiruvananthapuram, Kerala. The residues of flonicamid and fipronil were estimated using LCMS/MS. The mean initial deposit of flonicamid at recommended and double the recommended dose were 0.39 and 0.82 mg kg^{-1}, respectively. The residue dissipated with time and reached below limit of quantification of 0.05 mg kg^{-1} within 3 days in the recommended dose and within 5 days in double the recommended dose. The mean initial deposit of fipronil at recommended and double the recommended dose were 2.70 and 3.60 mg kg^{-1}, respectively. The residue dissipated with time and reached below limit of quantification of 0.01 mg kg^{-1} within 10 days both in the recommended and double the recommended dose. However, the harvest time residues of flonicamid and fipronil were below limit of quantification in paddy grain, straw, husk and soil.

Keywords: *Rice, Dissipation, Residues, Flonicamid, Fipronil, Half life*

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Introduction

Improvement in Rice is the important staple food of more than 60 per cent of the world population from which 60-70 % of their energy requirement is met [1]. The increase in the volume of rice production is an immediate requirement in the world due to rapidly growing populations however, achieving this task seems impossible due to various barriers *viz*., pest and disease incidence and weed infestation. It was estimated that an average of 25% of the rice cropis lost due to pests and diseases every year [2]. Farmers resort to various methods to tackle this problem, of which application of pesticides is the popular one. Recommended pesticides should act selectively against certain pests without causing adverse effects to non-target organisms. However, it is not practical to achieve absolute selectively and most pesticides used by farmers are toxic to humans and other non-target organisms. Recently, different pesticide firms have formulated various insecticide mixtures which can take care of both sucking pests as well as leaf feeders/ chewing pests. Pests that are resistant to one or a group of pesticides may be susceptible to a combination of toxicants and synergism may be exhibited by the components. Flonicamid 15% + Fipronil 15% WDG is reported to be the best combination product effective against rice stem borer, leaf folders, brown plant hopper and green leaf hopper incidence [3]. In the present work, the dissipation and harvest time residues of flonicamid and fipronil in rice was studied.

Morita *et al.,* (2007) [4] suggested that the main insecticidal mechanism of flonicamid is starvation based on the inhibition of stylet penetration to plant tissues. Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)-1H-pyrazole-3-carbonitrile) belongs to the phenyl pyrazole or fiprole group of chemicals and is a potent disrupter of the insect central nervous system via interference with the gamma-aminobutyric acid (GABA-) regulated chloride channel [5]. Degradation of Fipronil takes place either by reduction to sulfide, oxidation to sulfone, hydrolysis to amide and photolysis to desulfinyl [6]. Application rates vary between 0.6 and 200 g a.i.ha^{-1}, depending on the target pest and formulation.

Materials and Methods

Chemicals and reagents

Certified reference materials (≥95 % purity) of flonicamid and fipronil and formulation of flonicamid 15% + fipronil 15% WDG were received from M/s UPL Ltd, Mumbai. Standard solution of flonicamid and fipronil prepared with HPLC grade acetonitrile and suitably diluted to obtain the working standards. Acetonitrile, hexane and methanol of LiChrosolv grade, sodium chloride, anhydrous sodium sulphate, and anhydrous magnesium sulphate of GR grade were purchased from Merck Specialities Private Limited, Mumbai and the solid reagents were activated before use. Primary secondary amine (PSA) sorbent was purchased from Agilent Technologies, USA. All the glasswares were thoroughly washed as per the standard operating procedure to avoid the interferences from any contaminants during analysis. The suitability of solvents and other chemicals were ensured by running reagent blanks before actual analysis.

Recovery experiment

Recovery studies were carried out in order to establish the reliability of the analytical methods and to know the efficiency of extraction and clean up method for the present study by fortifying green foliage, grain, straw, husk and soils separately with flonicamid and fipronil. For flonicamid, recovery experiment was done at 0.05mg kg^{-1} (LOQ), 0.25mg kg^{-1} (5 X LOQ) and 0.50 mg kg^{-1} (10 X LOQ) level and for fipronil at 0.01mg kg^{-1} (LOQ), 0.05mg kg^{-1} (5 X LOQ) and 0.10 mg kg^{-1} (10 X LOQ) level.

Field experiment

Paddy (var. Uma) was raised at Integrated Farming System Research Station, Kerala Agricultural University, Karamana, Thiruvananthapuram, Kerala adopting the Package of Practices Recommendations of Kerala Agricultural University to conduct the studies on dissipation of flonicamid and fipronil. The trial was laid out in randomized block design (RBD) replicated thrice with a plot size of 25 m^2 with

Table-1 *Multiple-reaction monitoring (MRM) and LC parameters for flonicamid and fipronil*

Compound	RT (min)	MRM transitions		Declustering Potential	Entrance Potential	Collision Cell Entrance Potential	Collision Energy	Collision Cell Exit Potential
		Quantitative ion	Qualitative ion pair					
Flonicamid	0.33	230.1	203.1	43	10	21	24	2
			174	43	10	21	27	1
Fipronil	2.98	434.9	330	-36	-6	-23	-23	-6
			250	-36	-6	-23	-36	-6

three treatments, *i.e.* recommended (X), double the recommended dose (2X) and control. The first application of flonicamid 15% + fipronil 15% was given in paddy plants at tillering stage in two doses 400 g ha^{-1} (X), and 800 g ha^{-1} (2X). The second application of pesticide was given at fifteen days interval. The dissipation of residues in paddy was carried out from the day of the second application. About 500 g samples of paddy was collected at 0 (within 2 hrs), 1, 3, 5, 7, 10, 15 days after the last application. Three samples were collected from each replication corresponding to each treatment. The harvest time residues of paddy grain, straw, husk and soil were also estimated.

Extraction and cleanup

Green foliage

500g of green foliage of paddy was blended and from which 25g was taken, added 50 ml acetonitrile and homogenized at 14,000 rpm for 2 min. The samples were shaken for 4 minutes after adding 10 g sodium chloride. The samples were then centrifuged for 5 min. at 2500 rpm. A 16 mL supernatant was transferred in to 50 mL centrifuge tube containing 6 g anhyd. Na_2SO_4 and mixed well using high speed vortex shaker for 2 min. 12 ml extract was transferred to a 15 mL centrifuge tube containing 0.2 ± 0.01 g PSA sorbent and 1.2 ± 0.01 g anhy. $MgSO_4$. The sample was shaken and centrifuged for about 3 min at 2500 rpm. 5 ml of the extract was evaporated in a turbovap and made up to 2 ml using methanol for LC-MS/MS analysis

Paddy Grain

At the time of harvest, 500 g each of paddy grain was collected from three treatments. It was coarsely ground and from which 25g was taken to a 200ml centrifuge tube, added 50 ml acetonitrile and 25ml distilled water. The samples were shaken for 30 minutes. Added 12 g sodium chloride and shook well. The samples were then centrifuged for 5 min. at 2500 rpm. A 16 mL supernatant was transferred in to 50 mL centrifuge tube containing 2 g anhyd. Na_2SO_4, 2 g anhyd. $MgSO_4$ and mixed well using high speed vortex shaker for 2 min, then centrifuged for 5 min. at 2500 rpm. 12ml supernatant was transferred to a 15 mL centrifuge tube containing 0.10 ± 0.01 g PSA sorbent and 0.75 ± 0.01 g anhy. $MgSO_4$. The sample was mixed well using high speed vortex shaker for 2 min and centrifuged for about 5 min at 2500 rpm. 5 ml of the extract was evaporated in a turbovap at 450c and made up to 2 ml using methanol for LC-MS/MS analysis.

Straw/husk

100g of straw/husk taken from three treatments were powdered and from which 5g was taken, added 40ml distilled water containing 10g sodium chloride and kept for 1 hour, mixed well for uniform wetting and then 50ml acetonitrile was added. The samples were shaken for 10 minutes and were centrifuged for 5 min. at 2500 rpm. A 25 mL supernatant was transferred in to 50 mL centrifuge tube containing 5 g anhyd. Na_2SO_4 and mixed well using high speed vortex shaker for 2 min, then centrifuged for 3 min. at 2500 rpm. 10 ml supernatant was transferred to a 15 mL centrifuge tube containing 0.125 ± 0.01 g PSA sorbent and 2.00 ± 0.01 g anhy. $MgSO_4$. The sample was mixed well using high speed vortex shaker for 2 min and centrifuged for about 3 min at 2500 rpm. 5 ml of the extract was evaporated in a turbovap at 45°C and made up to 2 ml using methanol for LC-MS/MS analysis. Injected at LC-MS/MS with Atlantic dc-18 column, at 40°C using methanol-water mobile phase.

Soil

Soil samples (500 g) taken from three treatments were air dried and sieved through 2 mm sieve. Ten-gram soil sample was transferred to a 50 mL polypropylene tube to which 20 mL acetonitrile, 4 g $MgSO_4$ (activated) and 1g NaCl were added and shaken vigorously for one minute. The contents were centrifuged at 3300 rpm for 4 minutes and 10 mL of the supernatant was transferred to another 15 mL polypropylene centrifuge tube containing 1.5 g of magnesium sulphate and 0.25 g of primary secondary amine (PSA). The contents were shaken for 30 seconds and then centrifuged for 10 minutes at 4400 rpm from which 4 mL aliquot of the supernatant was taken and evaporated to dryness using Turbovap at 40°C. The dry residue was reconstituted to 1 ml in methanol for LC-MS/MS analysis.

Instrumentation

Estimation of flonicamid and fipronil in LC MS/MS

Analysis of flonicamid and fipronil was carried out in LC-MS/MS (Applied Biosystems API-3200) triple quadrupole MS/MS with electro spray ionization (ESI) in the positive mode coupled to a Waters LC (Acquity UPLC TM), which includes a binary pump, column oven and auto sampler.

Mass spectrometry parameters

The chromatographic separation was achieved using Waters Acquity UPLC system equipped with a reversed phase Atlantis d C-18 (2.1 × 100 mm, 5-micron particle size) column. A gradient system involving the following two eluent components: A: 10 % methanol in water + 0.1 % formic acid + 50 mM ammonium acetate; B: 10 % water in methanol + 0.1 % formic acid + 50 mM ammonium acetate were used as mobile phase for the separation of residues. The gradient elution was as follows: 0 min isocratic 20 % B, 0.0 - 0.4 min linear from 20 % to 90 % B, 4.0 - 5 min linear from 90 % to 95 % B, and 5 - 9 min linear from 95 % to 100 % B, 9-10 min linear from 100 % to 20 % B, with 10-12 min maintained the same polarity of 20 % B. The flow rate remains constant at 0.8 mL min^{-1} and injection volume was 10 µL. The column temperature was maintained at 40°C. The effluent from the LC system was introduced into Triple quadrapole API 3200 MS/MS system equipped with an electrospray ionization interface (ESI), operating in the positive ion mode. The source parameters were temperature 600°C, ion gas (GSI) 50 psi, ion gas (GS2) 60 psi, ion spray voltage 5,500 V and curtain gas 13 psi. Under these operating conditions the retention time of flonicamid and fipronil were found to be 0.33, and 2.98 minutes respectively. For each analyte, two MRM transitions were monitored and the parameters are listed in [Table-1].

LC- Separation

All LC separations were carried out using a reversed phase column, Atlantis d C18 (2.1X100 mm) with 5µm spherical porous particles. The elution was performed using gradient between methanol and water. Mobile phase A contained 5 milli molar ammonium acetate in water and B contained 5 milli molar ammonium acetate in methanol. Flow rate 0.80 mL min^{-1}, column temperature 40oC, sample temperature 5 °C, and the injection volume 10 µL were used in all the estimation.

MS/MS - The MS/MS conditions were optimised using direct infusion in to ESI source in positive mode to provide the highest signal/noise ratio for the quantification ion of each analyte. Two MS/MS transitions were made in case of chemical interferences observed in the quantitation ion chromatogram and for qualitative purpose. The ion source temperature was 550°C with ion spray voltage of 5500 V. Chromatographic elution zones were divided into appropriate number of time segments. In each segment corresponding MS/MS transitions were monitored using multiple reactions-monitoring (MRM) mode.

Certified reference materials of pesticides and stock solutions were prepared using pesticide grade solvents. Single laboratory method validation was performed to establish the recovery of pesticides. Spiking solutions for measuring per cent recovery were prepared from stock solutions of concentration 1000 mg L^{-1}. Calibration was performed with six levels of serially diluted standard mixture,

Table 2- *Percent recovery of flonicamid in paddy grain, husk, straw and soil*

SN	Fortification (mg kg^{-1})	Recovery (%)					RSD (%)				
		Green foliage	Grain	Straw	Husk	Soil	Green foliage	Grain	Straw	Husk	Soil
1	0.05	83	82	82	81	81	2.80	1.80	3.40	3.40	7.50
2	0.25	81	85	83	84	86	2.00	3.00	2.90	2.30	2.80
3	0.50	85	86	85	82	84	5.40	2.60	1.60	4.50	3.00

Table 3- *Percent Recovery of fipronil in paddy grain, husk, straw and soil*

SN	Fortification (mg kg^{-1})	Recovery (%)					RSD (%)				
		Green foliage	Straw	Grain	Husk	Soil	Green foliage	Straw	Grain	Husk	Soil
1	0.01	85	85	85	88	85	2.03	2.10	2.40	5.60	2.60
2	0.05	82	83	82	85	82	1.80	2.90	1.60	3.30	1.90
3	0.10	80	81	84	83	81	1.42	3.20	1.90	2.50	5.30

Table-4 *Persistence of flonicamid and fipronil in green foliage of paddy plant at different intervals*

Days after treatment	Mean Residues of flonicamid and fipronil (mg kg^{-1})					
	Flonicamid			Fipronil		
	T1= 400 g ha^{-1}	T2=800 g ha^{-1}	T3- Untreated control	T1= 400 g ha^{-1}	T2=800 g ha^{-1}	T3- Untreated control
0	0.39	0.82	<LOQ	2.7	3.6	<LOQ
1	0.06	0.09	<LOQ	2.3	2.4	<LOQ
3	<LOQ	0.05	<LOQ	2	2.3	<LOQ
5	<LOQ	<LOQ	<LOQ	0.96	1.9	<LOQ
7	<LOQ	<LOQ	<LOQ	0.14	1.2	<LOQ
10	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Half-life (days)	0.33	0.81	-	3.91	5.19	-

prepared from stock solutions. Calibration curves of working standards were used to evaluate the linearity of the gas chromatograph response in each day of analysis and pesticide residues were quantified based on these standards. The concentration of pesticide residue was calculated as follows,

Concentration of pesticide residues (mg kg^{-1}), y = a/b

a = Area of sample peak X Concentration of standards X Dilution Factor

b = Area of the standard peak

Dilution factor, D.F. = c /d

c = Sample Wt. X Volume of Aliquot

d = Volume of acetonitrile

Studies on linearity check

Flonicamid

Studies on linearity check was carried out with the help of analytical standard of flonicamid. In this study calibration curve was prepared by taking the areas corresponding to different concentrations of calibration standard, against which final quantification was done. Analytical grade (0.0100 g; 99.50 %) flonicamid was weighed and was transfer to a 25 mL volumetric flask using the methanol. The volume was made up to the mark with methanol to give 400 mg kg^{-1} stock solution of flonicamid. From this stock solution, 10 mg kg^{-1} intermediate standard was prepared and sequential concentrations of 1, 0.5, 0.25, 0.10, 0.05 mg kg^{-1} were prepared.

Calibration of the equipment was performed using pure analytical standard of the test material at concentration ranging from 0.05 to 1 mg kg^{-1} and the response/ area obtained was plotted against concentration. The response was found linear in the concentration tried (0.05-1 mg kg^{-1}). The correlation coefficient (r^2) value obtained was 0.9982 indicating perfect linearity.

Fipronil

Analytical grade (0.0101 g; 98.7 %) fipronil was weighed and transferred to a 25 mL volumetric flask using methanol. The volume was made up to the mark with methanol to give 400 mg kg^{-1} stock solution of fipronil. From this stock solution 10 mg kg^{-1} intermediate standard was prepared. From this 10 mg kg^{-1}, sequential concentrations of 1, 0.5, 0.25, 0.10, 0.05, 0.025 and 0.01 mg kg^{-1} were prepared.

Calibration of the equipment was performed using pure analytical standard of the test material at concentration ranging from 0.01 to 1 mg kg^{-1} and the response/ area obtained was plotted against concentration. The response was found linear in the concentration tried (0.01-1.00 mg kg^{-1}). The correlation coefficient (r^2) value obtained was 0.9963 indicating perfect linearity.

Result and discussion

The mean recovery percentage of flonicamid ranged between 81 to 85 in green foliage, 82-86 in grain, 82-85 in straw, 81-84 and 81-86 in soil with relative standard deviation of repeatability (RSDr) between 2.00 - 5.40 %, 1.80 – 3.00 %, 1.60-3.40 %, 2.30-4.50 % and 2.80 to 7.50 % respectively [Table-2]. The mean recovery percentage of fipronil ranged between 80 to 85 in green foliage, 82-85 in grain, 81-85 % in straw, 83-88 and 81-85 in soil with relative standard deviation of repeatability (RSDr) between 1.42 - 2.03 %, 1.60 – 2.40 %, 2.10-3.20 %, 2.50-5.60 and 1.90-5.30 % respectively [Table-3]. The satisfactory recovery values indicated the accuracy and repeatability of this method and are within the accepted range for residue estimation

The mean initial deposit of flonicamid at recommended and double the recommended dose were 0.39 and 0.82 mg kg^{-1}, respectively. The residue dissipated with time and reached below limit of quantification of 0.05 mg kg^{-1} within 3 days in the recommended dose and within 5 days in double the recommended dose. The mean initial deposit of fipronil at recommended and double the recommended dose were 2.70 and 3.60 mg kg^{-1}, respectively. The residue dissipated with time and reached below limit of quantification of 0.01 mg kg^{-1} within 10 days both in the recommended and double the recommended dose [Table-4].

In the present study, the harvest time residues of flonicamid and fipronil in paddy grain, husk, straw and soil were below limit of quantification. Kumar and Singh (2013) [7] studied the dissipation of fipronil in rice plant and they reported that the residues of fipronil and its metabolites were reached below the limit of quantification (0.01 mg kg^{-1}) after 45 and 90 days at recommended and 4 times of recommended doses respectively and the result is not in agreement with the present study. However, the harvest time residues in paddy straw, rice grain, bran, husk did not reveal the presence of fipronil and its metabolites which is in accordance with present findings.

The studies on dissipation of flonicamid in crop plants are so meagre. Xu *et al*., (2011) [8] conducted a safety evaluation of flonicamid and its metabolites in vegetables using QuEChERS. They reported the sublethal concentration of flonicamid was 0.44 mg L^{-1} as LC10 and 1.25 mg L^{-1} as LC30 against green peach aphid, *Myzus persicae* Sulzer which revealed the safety of the chemical.

Another study, conducted at Kerala on persistence of fipronil in chilli (*Capsicum anuum* L) by George *et al*. (2014) [9] revealed that initial deposits of total fipronil on fresh chilli fruits at single and double doses were 0.69 and 1.43 µg g^{-1} respectively and were dissipated to below limit of quantification at 27 days after application. In 2018, Anju, (2018) [10] studied the dissipation pattern of fipronil in cabbage in plains and hills.

She reported that the residues of fipronil reached below limit of quantification on three and seven days after spraying in plains and hills respectively. Half-life of fipronil was 0.22 and 1.96 days in plains and hills. This finding is in agreement with present study.

Mandal and Singh (2013) [11] studied the dissipation and persistence of fipronil in soil and reported that the total residues of fipronil and its metabolites in soil after 7 days of its application @ 75 and 300 g a.i. ha $^{-1}$ were found to be 0.025 and 0.098 mg kg^{-1}, respectively. These residues could not be detected after 210 and 240 days following the application of fipronil at lower and higher dosages, respectively. The present study also revealed that residues of fipronil was below the limit of quantification in soil sampled at the time of harvest of crop

Conclusion

Dissipation studies of flonicamid 15% + fipronil 15% WDG in rice green foliage revealed the presence of residues up to 5-10 days in paddy foliage. However, the harvest time residues of paddy grain, straw and husk revealed the safety of the combination product, flonicamid 15% + fipronil 15% WDG to the end users. As compared to vegetables, no frequent harvest in rice and hence low risk in paddy than vegetables. Moreover, the residue data combined with all other parameters will help in registration of the combination product under CIB & RC, India against pest omplex *viz*., stem borer, leaf folders, brown plant hopper and green leaf hopper etc.

Application of research: To study the dissipation of insecticide mixture in rice to ensure its safety to the consumers

Research Category: Pesticide Residue Analysis

Abbreviations: LC-MS/MS- liquid chromatography mass spectrometer
WDG- Water dispersible granules
DAS- Days after spraying
LOQ- Limit of quantification
RSD- Relative standard deviation
CIB & RC-Central Insecticide Board and Registration committee
QuEChERS-Quick easy cheap efficient rugged and safe

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Study area/ Sample collection: Integrated Farming System Research Station, Kerala Agricultural University, Karamana, Thiruvananthapuram, Kerala.

Cultivar/Variety name: Paddy-Variety -Uma

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
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