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DISSIPATION OF AZOXYSTROBIN 23% SC RESIDUE IN/ON CHILLI FRUITS AND SOIL

Sudeep Mishra, Vikash, Mukesh K Singh, Mahesh K Saini, Shubham Bansal, Vineet, K Pandey, Ajay Singh, Samsul Alam*, Irani Mukherjee, Lalitesh K Thakur and Jitendra Kumar

Institute of Pesticide Formulation Technology, Udyog Vihar, Gurugram – 122016, Haryana

ABSTRACT

The field trials were conducted to evaluate the dissipation pattern of azoxystrobin 23 % SC (Amistar) in/on chilli and field soil. Phule Jyoti variety of chilli was treated at single dose @ 125 g a.i./ha (500 L/ha of water) and double 250 g a.i./ha (500 L/ha of water). The pesticide residues of azoxystrobin were analysed by Liquid chromatography- mass spectrometry (LC-MS/MS). The average initial deposit of 1.766 µg/g for single dose and 3.613µg/g for double dose of spray gradually decreased with time following first order kinetics. The half-life ($T_{1/2}$) values of azoxystrobin were found to be 4.2 and 4.1 days for single and double dose of application, respectively. The daily exposure for each day calculated on basis of ADI- 0.18 mg/kg was in the range 1.73-0.065 mg per person per day. The produce is safe for consumption after5th day both for single and double dose of application.

Keywords: ADI, Azoxystrobin, Chilli, LC-MSMS, Residues

Chilli (*Capsicum annuum* L.) is one of the major spice crops in India. Indian chillies are predominantly popular and have gained global demand due to high colour value and low pungency which is used for culinary purpose (Mathur *et al*., 2000; Pariari *et al*., 2013).

Around 400 different varieties of chillies are cultivated throughout the globe. The spiciest variety being "Carolina Reaper" developed by a grower Ed Currie of West Indies is having the maximum pungency of about 2.2 million SHU (Scoville Heat Units; PuckerButt Pepper Company, 2013). One of the hot chilli varieties of the world "Naga Jalokia," is the native of Tezpur in Assam, India.

The world production of chilli crop is around 7 million tonnes, which is cultivated on 1.5 million hectares of land. According to 2nd advance estimates during 2021-22, chilli was grown in 2.29 lakh hectares with a production of 10.78 lakh tonnes and productivity was 4707 kg/ha. Major chilli producing states include Andhra Pradesh, Maharashtra, Karnataka, and Madhya Pradesh (Post harvest profile of chilli, 2009).

Though, being an important spice crop grown worldwide, many constraints decrease the production, causing significant reduction in yield and seed production. Plant diseases have been a major reason for crop losses worldwide. Chillies are attacked by a number of fungal diseases such as fruit rot (*Colletotrichum truncatum),* Cercospora leaf spot or velvet spot (*Cercospora capsici),* Phytophthora blight (*Phytophthora capsici),* wilting (*Verticillium dahliae),* powdery mildew (*Leveillula taurica*), root rot (*Rhizoctonia solani),* etc. To control these diseases fungicides are used (Saxena *et al.,* 2016).

Azoxystrobin (Fig. 1) [methyl (2*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate—$C_{22}H_{17}N_3O_5$] is a systemic, broad-spectrum fungicide belonging to the class of methoxyacrylates, which are derived from the naturally-occurring strobilurins and oudemansins. The mode of action is through inhibition of mitochondrial respiration by blocking electron transport in fungi (Schirra *et al.,* 2002). It is classified by the World Health Organization (WHO) as slightly hazardous class 3. In view of consumer safety, European Union (EU) (http://ec.europa.eu/sancopesticides/public/index.cfm) has setup Maximum Residue Level (MRL) for azoxystrobin as 3mg/kg in chilli fruits. Trace amounts of pesticide residues remain in food which may pose potential health hazards to consumers. Therefore, field dissipation studies on pesticide persistence in crops and studies of pesticide residues behaviour in agricultural fields are needed (Malhat *et al.,* 2014).

Several studies focused on the analytical methods of azoxystrobin in different matrices. In these studies, dissipation and persistence of azoxystrobin residue have been reported in various crops using different analytical techniques such as in grapes (Hammad *et al.,* 2019) by HPLC-DAD and GC-ECD; by GLC by Gajbhiye *et al.,* 2011; in cumin by GC-MS/MS (Dubey *et al*., 2017) ;in chilli by GC-MS (Ghosh *et al.,* 2014); in banana, by GC-ECD (Huan *et al*., 2013; Wang *et al*., 2013);in green garlic by GC-ECD (Kang *et al.,* 2011) in pomegranate fruits by Q-ToF LC-MS, (Utture *et al*.,

*Corresponding author : alam_samsul@rediffmail.com
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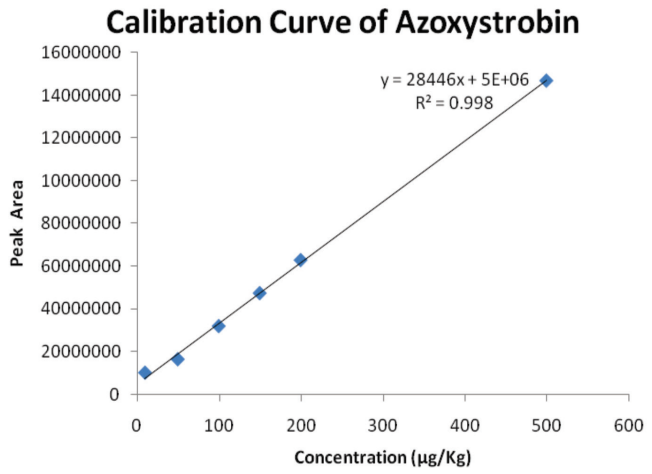

Fig. 1. Linearity curve of Azoxystrobin from LC-MS/MS instrument

2011) in greenhouse legumes by using LC-MS/MS (Wang *et al*. 2010; Itoiz *et al.,* 2012 and Wu *et al.,* 2009).

In the present study, it was aimed to develop and validate an effective method for extraction and quantitative determination of azoxystrobin in chilli fruits and field soil using LC-MS/MS. The analytical method used for the analysis was validated as per method validation guidelines and quality control procedures for pesticide residues analysis in food and feed, SANTE 11312/2021.

MATERIALS AND METHODS

Chemicals and reagents

Certified Reference Material of azoxystrobin (99.3 % purity) was procured from Sigma –Aldrich, USA and the formulation of azoxystrobin 23% SC along with its certificate of analysis, was purchased from local Market. A standard stock solution of 100 mg/ L concentration solution of azoxystrobin was prepared. Necessary dilutions were made from this standard as and when required. Acetonitrile, acetone, n-hexane and methanol of HPLC- grade were procured from J.T. Baker®, USA. Primary secondary amine (PSA) of Agilent Technologies, USA, anhydrous sodium sulphate (Thomas Baker). Anhydrous magnesium sulphate pesticide grade (Merck, India). C-18 (ODS; Agilent®, USA) and water from (Lab-Q® type 1) were procured.

Glassware and apparatus

Micropipette (Eppendorf®, Germany), Analytical precision balance (Sartorius), Mixer grinder (GX7, Bajaj), Vortex (REMI), Rotospin (TARSONS®), Autoshaker (Infros HT), Centrifuge (REMI R-23), TurboVap-Evaporator (Caliper Lifescience), Rotary evaporator (Heidolph®) and Ultrasonic bath (Biogenix) were used. All the chromatography consumables, certified glassware and personal protective equipment (PPE) were used during analysis.

Field experimentation and sampling

The supervised field trial was conducted at IPFT, to evaluate the dissipation pattern of Azoxystrobin 23 % SC (Amistar) in/on chilli and field soil. The meteorological conditions recorded during the duration of the experiment (January 2021) were average temperature °C/(F): Max 20.1/(68.2), Min 7.6/(45.6), Precipitation (23mm/0.9inch) and Humidity 66 %. The study was conducted during winter season in the month of January and no precipitation occurred during the study period. The experimental field layout was Completely Randomized Block Design (CRBD).The chilli variety PhuleJyoti was grown in experimental plot size of 20 m^2 each plot; 4×5 meter for three treatment dosages i.e., recommended, T_1= 125 g a.i./ha (500 mL/ ha of water) and double the recommended,T_2 = 250 g a.i./ha (500 mL/ha of water) along with untreated control plots (T_3 = untreated). The pesticide was applied with a Knapsack sprayer fitted with hollow cone nozzle and no other crop protection measures were used during field application. Azoxystrobin 23 % SC was applied only once during 50% fruit formation stage. After the application, three replicates of treated and untreated chilli fruit (approx. 250g) were randomly taken at initial time between 12.00-14.00 hrs . The samples were drawn at 0 (2hr after spraying), 1. 3, 5, 7, 10, and 15 days after the spray. Soil samples (approx. 1kg) were withdrawn from both treated and untreated plots on 15th day of spray using soil auger from the plough layer (15 cm) from different places. The collected samples were organized, labelled and sealed in plastic bag then frozen immediately at -18°C till sample processing and pesticide residue analysis. The samples were processed and analysed within 48 hrs of sampling.

Sample extraction and cleanup procedure

Collected samples were extracted by modified QuEChERS (quick, easy, cheap, effective, rugged and safe) method of pesticide residue analysis (Lehotay, 2007).

Extraction of chilli fruit samples

The samples were homogenized in a grinder. About 10 g (±1 g) of homogenized samples were taken using an analytical balance in a 50 mL Teflon centrifuge tube with three replicates of each treatment (T_1, T_2 and T_3). Ten mL of acetonitrile was added to each centrifuge tube and vortex for 2 minutes and kept aside for half an hour. 10 g of anhydrous magnesium sulphate ($MgSO_4$) was added to each tube and mixed by manual shaking

followed by roto-spin. After half an hour, the samples were centrifuged at 3000 rpm for 3 minutes. For clean-up, 5 mL of supernatant solution was transferred into another 15 mL centrifuge tube containing dispersive solid phase extraction (d-SPE) sorbents : 125 mg of primary secondary amine (PSA), 125 mg of C-18 and 750 mg of anhydrous magnesium sulphate ($MgSO_4$) and subjected to vortex for 2 minutes and then centrifuged at 3000 rpm for 3 minutes. After centrifugation, 1 mL upper solvent layer was filtered using 0.22 µm nylon syringe filter in LC-MS/MS vials.

Extraction of soil samples

The soil samples were homogenised and weighed approx 20 g in 250 mL stoppered conical flask (Borosil) followed by addition of 50 mL of methanol to each conical flask and subjected to auto-shaker for 1 hr at 160 rpm. After 1 hr of shaking, samples were left undisturbed on flat surface for half an hour for settling of soil particles. After that, 20 mL of clear supernatant was pipetted out and filtered through anhydrous sodium sulphate (Na_2SO_4) bed into 50 mL flat bottom round flask. Anhydrous sodium sulphate bed was finally washed by 10 mL of methanol into same flat bottom round flask. Then the organic solvent was evaporated near to dryness on rotary evaporator. Later reconstituted the residues with 1 mL of methanol, then syringe filtered in LC-MS/MS vials.

Instruments and apparatus

Instrumental analysis

The pesticide residues of azoxystrobin were analyzed by Liquid chromatography mass spectrometry (LC-MS/MS) using Thermo Scientific Hypersil GOLD C-18 (length 50mm x 2.1 ID mm, 1.9µm film thickness), with mobile phase (HPLC-grade) acetonitrile (A) and water (C) (buffered by 0.1% formic acid) in gradient flow: A-0%,C-100% for 2.2 min, A-100%, C-0% for 3.0 min ; A-100%, C-0%, for 5 min ; A-0%, C-100%, for 6 min ; A-0%, C-100%, for 7min). Flow of mobile phase was 400 µL/min with injection volume of 10 µL with positive Electrospray Ionization (ESI); Spray Voltage: 4500 V; Sheath Gas pressure: 50; Ion Sweep Gas pressure: 0; Auxiliary Gas pressure: 18; Capillary temperature: 350°C; Tube lens offset: 46; Skimmer offset: 0. SRM transition: 404.10 m/z → (372.07 m/z at Collision energy = 13 and 344.12 m/z at Collision energy = 23). Total run time of acquisition was 7 minutes, scan time 0.2, scan width (m/z) 0.1 and retention time. The overall performance of the analytical method satisfied the quality control procedures for pesticide residue analysis (DG-SANTE 2017) indicates that the analytical method was efficient enough for analysis of azoxystrobin.

Method validation

According to document SANTE 11312/2021, a within laboratory method validation was performed to provide evidence that the method is fit for the extraction and quantitative determination of azoxystrobin in green chilli fruits. The analytical method was validated by evaluating quality parameters such as linearity, precision (repeatability), selectivity, limits of detection and quantification and recovery values. The linearity (r^2) and sensitivity of the method were checked by plotting calibration curve (10, 50, 100, 150, 200, 500 µg/kg). Limits of detection (LOD) and quantification (LOQ) were calculated as three and ten times the signal-to-noise ratio, respectively (DG-SANTE 2017). Trueness and precision of the method has also been tested through recovery experiments which was carried out by spiking the respective control matrix at concentration level of 0.03, 0.15, 0.3µg/L. Precision was evaluated by calculating relative standard deviation. A stock standard solution about (100 mg/L) of fungicide was prepared in acetonitrile by weighing approximately 1mg of certified reference material. All standard solutions were stored in the dark at 4˚C. Repeatability (expressed as relative standard deviation, RSD %) was determined by analyzing samples spiked with azoxystrobin at all the concentrations used to determine the linear range on the same day. Triplicate chilli fruit and soil samples were spiked with 0.03µg/kg, 0.15 and 0.3 µg/kg of standard solutions for azoxystrobin.

Linearity of instrument

Linearity of the instrument was checked for azoxystrobin and it was found linear in the range of 10 µg/L -500 µg/L concentration with r^2 value > 0.998. Each calibration level was injected thrice to check the repeatability of injections. The repeatability at each level complies with the guidelines. Retention time accuracy and sensitivity were recorded.

LOD, LOQ and recovery

The limit of detection (LOD) and limit of quantification (LOQ) of the method were optimised at 0.01 and 0.03 µg/g, respectively which complies European Union, EU protocols (DG-SANTE, 2017) for method validation i.e. LOQ≤ maximum residue level (MRL). The current available MRLs of azoxystrobin in chilli are 1.0 µg/g (FSSAI 2018) and 3 µg/g (EU maximum residue limit). Recovery studies were carried out in order to establish the authenticity of the analytical method employed. According to EU the recovery study should be in the range of 70-120% with RSD below 20% (DG-SANTE 2017). Chilli fruit and soil samples were fortified at the level of 0.03 (LOQ), 0.15(5LOQ) and 0.3µg/kg (10LOQ) with the standard solutions of azoxystrobin and were

Table 1. Residues of azoxystrobin in chilli fruit

Treatment	Days	Residues in (µg/g)				
		R1	R2	R3	Mean ± SD	Percent dissipation
T_1	0	1.806	1.756	1.738	1.766 ± 0.035	-
	1	1.556	1.545	1.463	1.521 ± 0.050	13.87
	3	1.246	1.177	1.191	1.204 ± 0.036	31.82
	5	0.967	0.99	0.965	0.974 ± 0.0139	44.85
	7	0.822	0.77	0.773	0.788 ± 0.029	55.38
	10	0.497	0.554	0.557	0.536 ± 0.034	69.65
	14	0.147	0.151	0.134	0.144 ± 0.008	91.85
	0	3.735	3.567	3.539	3.613 ± 0.106	-
	1	2.998	3.148	2.881	3.009 ± 0.134	16.72
	3	2.666	2.553	2.610	2.609 ± 0.056	27.79
T_2	5	2.132	2.027	1.996	2.052 ± 0.072	43.21
	7	1.556	1.503	1.607	1.555 ± 0.052	56.96
	10	0.976	0.930	0.933	0.947 ± 0.026	73.79
	14	0.302	0.293	0.286	0.293 ± 0.008	91.89

analyzed following the aforementioned procedure. Results of recovery study are shown in table 2. According to the document SANTE 11312/2021, the acceptable drift between two bracketing injections of the same concentration should not exceed 30%. The three level of 0.03, 0.15 and 0.3 µg/L were injected in triplicates, the repeatable area does not exceed above 10%. According to document SANTE 11312/2021, acceptable mean recoveries lie within the range of 70-120%. The recovered amount of the spiked samples at all the LOQ values lies between 88-91 % with relative standard deviation lies below 5%.

Dissipation study

Day-wise residue data were subjected to first order kinetics by using the formula, $C_t=C_0e^{-kt}$. Where, C_t denotes the concentration (µg/g) after time t (day); C_0 =initial conc., k=rate constant (day-1). The regression coefficient (R^2) represents the relationship between residual data with time and residual half life was calculated using formula $t_{1/2}=\ln 2/k$.

The test results (Result =Instrument Reported Concentration X Dilution Factor or divided by concentration factor) regarding the residue of azoxystrobin in chilli are summarized by the regression equation and half-life values for the different treatments. Analysis of the samples was done by injecting calibrated standards of different concentration levels with samples in the same sequence. The sequence was processed and concentrations of samples were calculated.

RESULTS AND DISCUSSION

The results regarding the residue of azoxystrobinin chilli and soil samples have been summarized in Table 1. The per cent dissipation of azoxystrobin for the treatment T_1 was 13.8% after 1 day which increased

Table 2. Dietary exposure of azoxystrobin in chilli

	Days wise concentration (µg/g)						
(g ai/ha) Dose	0	1	3	5	7	10	14
125	(1.76)	(1.46)	(1.19)	(0.96)	(0.77)	(0.55)	(0.13)
250	(3.53)	(2.88)	(2.61)	(1.99)	(1.60)	(0.93)	(0.29)
Dietary exposure (mg per person per day) day-wise = ADI x Average Body Weight (56 kg) x residue level of the day/ Average per capita consumption/day							
125	0.88704	0.73584	0.59976	0.48384	0.38808	0.2772	0.06552
250	1.735727	1.416117	1.283356	0.978498	0.57038	0.457288	0.142595

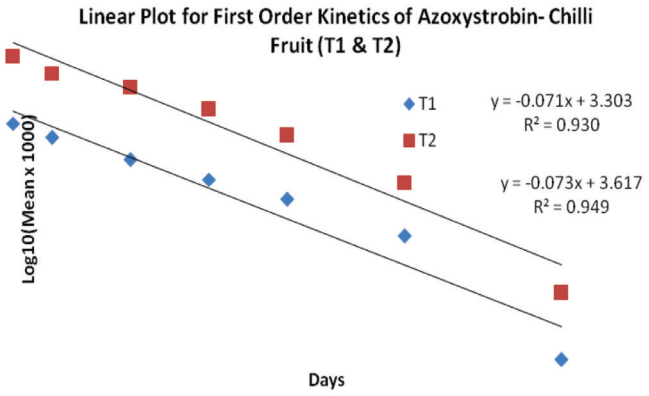

Fig. 2. Linear plot for 1st order kinetics of Azoxystrobin (in Chilli fruit).

to 91.8% by14th day, similarly for the double dose (T_2) the initial dissipation was 16.7 %, which increased to 91.8 % after 14thday. The regression equation and half-life values for the different treatments are given in Table 1. The results showed that the average initial deposit was 1.766 µg/g for T_1, and 3.613µg/g for T_2 . The residue gradually decreased with time following first order kinetics for both the treatments (Fig. 2). The residues of azoxystrobin were quantifiable even in the samples after 14 days for $T_1$and for T_2. However, in soil samples the residues were below quantifiable limit (< 0.03 µg/g). The half-life ($T_{1/2}$) values of azoxystrobin in chilli fruit were found to be 4.2 and 4.1 days for T_1 and T_2, respectively. Untreated controls (T_3) were free from any residues of azoxystrobin.

Many studies have reported the degradation behaviour of azoxystrobin in different vegetables and fruits. It is reported that the azoxystrobin applied on grapes @ 150 g a.i/ha and 300 g a.i/ha had a residual half-life which varied from 5.4-11.2 days (Gajbhiye *et al.,* 2011). Similar studies reported that azoxystrobin residues on green garlic and tomato leaves (green house condition) dissipated with a half-life varying from 1.2-1.4 days (Kang *et al*., 2011) and 13 days (Szpyrka *et al*., 2009), respectively. The rate of dissipation ($T_{1/2}$) of azoxystrobin was 5.4-8.9 days at recommended dose (150 g a.i/ha) and 7.0-10.4 days at double the recommended dose (300 g ai/ha). Dissipation of azoxystrobin in grapes at various locations in India followed the order kinetics, with half-life values ranging from 5.4 days, (Maharashtra) > 6.1 days (Tamil Nadu) and 7.0 days in, Karnataka at recommended dose of application (Gajbhiye *et al.,* 2011). Bursi *et al.* (2007) cited that the residues of azoxystrobin in cucumber samples collected 7 days after treatment were below the MRL. Azoxystrobinis being non-volatile compound does not leach. Photolysis accounts for the majority of the initial loss of the compounds, the remaining being degraded microbially.

The dietary exposure of azoxystrobin residues were calculated and presented in Table-2.The ADI is 0.18 mg/kg body weight for azoxystrobin (FAO, 2008). The per capita consumption of green chilli is 20.5 g/person/day (Household Consumption of Various Goods and Services in India 2011-12, NNS, 68THround July 2011-June 2012; National Sample Survey Office Ministry of Statistics and Programme Implementation June 2014). Maximum permissible intake (MPI) was calculated by multiplying ADI x average body weight (56 kg),which is 9 mg azoxystrobin per person per day. Dietary exposure was calculated by dividing average residue concentration on a particular day in a sample with average per capita consumption per day of chilli. The daily exposure values of each day were in the range 1.73-0.065 mg per person per day (Table-2). Dietary exposure (ADI=0.18 mg/kg) after 5th day of application could be considered safe for human consumption both for single and double dose, as it is much lower than the MRL of 3mg/kg in chilli [CODEX Alimentarius Commission, 2009].

To conclude, azoxystrobin residue was found below quantifiable limit i.e. 0.03 µg/g in all the harvest time samples of chilli and field soil as well as in all the control samples. Azoxystrobin may not create any residual toxicity problem in chilli. Initial concentration of azoxystrobin at 5th days in chilli fruit of both recommended single and double dose was below MRL value according to Codex Alimentarious Commission (2009) which is 3mg/kg of Indian recommendation irrespective of any dose. So, it may be stated that chilli may safely be consumed without any hazard.

Authors' contribution

Conceptualization and designing of the research work (JK, LKT, SA, IM); Execution of field/lab experiments and data collection (V, SB, VKP, AS); Analysis of data and interpretation (SM,MKS,MKS); Preparation of manuscript (SM,MKS,MKS)

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