



Bio-efficacy and post harvest residual toxicity of new fungicides against sheath blight (*Rhizoctonia solani*) of rice (*Oryza sativa*)

PANKAJ KUMAR¹, SUSHIL AHLAWAT², REENA CHAUHAN³, ANIL KUMAR⁴, RAM SINGH⁵ and ASHWANI KUMAR⁶

CCS Haryana Agricultural University, Hisar, Haryana 125 004

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ABSTRACT

Four new fungicidal formulations, viz. tebuconazole 50% + trifloxystrobin 25% WG, difenoconazole 25% SC, propiconazole 13.9% + difenconazole 13.9% EC and kresoxim methyl 44.3% SC were evaluated against *Rhizoctonia solani* Kuhn of rice (*Oryza sativa* L.) *in vitro* and under field conditions during *khariif* 2016 and 2017. *In vitro* growth inhibition tests revealed that the EC₅₀ values of tebuconazole 50% + trifloxystrobin 25% WG, difenoconazole 25% SC, propiconazole 13.9% + difenconazole 13.9% EC and kresoxim methyl 44.3% SC against *R. solani* ranged from 0.398 to 42.35 ppm a.i., whereas the corresponding EC₉₀ values were 8.92 to more than 2000 ppm a.i. Tebuconazole 50% + trifloxystrobin 25% WG was most inhibitory to mycelial growth of the fungus with EC₅₀ and EC₉₀ values of 0.39 and 8.92 ppm a.i. followed by propiconazole 13.9% + difenconazole 13.9% EC. Tebuconazole 50% + trifloxystrobin 25% WG and propiconazole 13.9% + difenconazole 13.9% EC completely inhibited the sclerotia formation at 2.5 ppm and 25 ppm a.i., respectively. In field trials, tebuconazole 50% + trifloxystrobin 25% WG sprayed at 0.4 and 0.8 g/l proved most effective and suppressed sheath blight severity by 50.98 and 79.12%, respectively along with enhancing grain yield of rice followed by propiconazole 13.9% + difenconazole 13.9% EC. However, kresoxim methyl 44.3% SC was the least effective fungicide. Based on bioefficacy and benefit-cost ratio, spray application of tebuconazole 50% + trifloxystrobin 25% WG at 0.4 g/l and propiconazole 13.9% + difenconazole 13.9% EC at 2 ml/l were found highly economical in managing sheath blight of rice with a benefit-cost of 3.52 and 3.02, respectively. Post harvest residues analysis of tebuconazole 50% + trifloxystrobin 25% WG, propiconazole 13.9% + difenconazole 13.9% EC and difenoconazole 25% SC were found below limit of quantification, i.e. 0.01 ppm in rice grains, cropped soil, paddy straw and husk at their single/recommended and double of the recommended doses indicating that tebuconazole 50% + trifloxystrobin 25% WG at 0.4 g/l and propiconazole 13.9% + difenconazole 13.9% EC were safe from the human, animals and environmental point of view.

Key words: Bioefficacy, Fungicides, Post-harvest residue, *Rhizoctonia solani*, Rice, Sheath blight

Rice (*Oryza sativa* L.) is an important cereal crop in India which is grown over an area of 43.39 mha with a production and productivity of 104.32 mt and 2404 kg/ha, respectively (Anonymous 2016). However, in Haryana is occupied an area of 1.422 mha with a production and productivity of 4.880 mt and 3432 kg/ha. Among various biotic stresses known to affect rice production, sheath blight caused by *Rhizoctonia solani* Kuhn (AG 1 IA) is a devastating disease causing substantial quantitative losses

and quality degradation (Singh *et al.* 2013, Laha *et al.* 2017). It has attained the status of major disease on all the commercially cultivated rice varieties and hybrids. The yield losses ranging from 4-58% have been reported depending on the crop stage at the time of infection, severity of the disease and environmental conditions (Singh *et al.* 2016). Due to lack of resistance in rice varieties against sheath blight, the disease management has primarily relied on use of fungicides.

To sustain international trade of rice and the enhance its productivity, it becomes imperative to identify effective and eco-friendly fungicides for the management of devastating diseases like sheath blight.

The previous reports have indicated that the ready mix fungicidal formulations have low risk of resistance development in pathogen due to their broad spectrum and curative action. However, limited information is available on the efficacy of ready mix fungicidal products against sheath blight of rice (Agarwal and Sunder 2013, Singh *et*

¹Ph D Scholar (e mail: bodlapankajbodla@gmail.com),

⁴Professor and Head (e mail: mitharwalani1960@gmail.com),

⁵Principal Scientist cum Regional Director (e mail: singhdraram@gmail.com),

⁶Assistant Professor (e mail: dahiya.ashwani@gmail.com),

Department of Plant Pathology, ²Assistant Professor (e

mail: sushilahlawat08@gmail.com), ³Research Associate (e mail:

reenavansh82@gmail.com), Department of Entomology.

al. 2016, Pal *et al.* 2017) and on their dissipation in rice grains, straw and cropped soil (Kundu *et al.* 2011, Arora *et al.* 2014, Chen *et al.* 2015). Hence, there is a need to study the bio-efficacy and post harvest/terminal residues of new fungicidal formulations to promote international trade and legislation. In the present study, an attempt has been made to evaluate the efficacy of some new fungicidal formulations against *R. solani* AG 1 IA, inciting sheath blight of rice *in vitro* and under field conditions along with the post-harvest residue of the promising fungicides at single/recommended and double of the recommended doses.

MATERIALS AND METHODS

Four fungicides, viz. tebuconazole 50% + trifloxystrobin 25% WG, difenoconazole 25% SC, propiconazole 13.9% + difenconazole 13.9% EC and kresoxim methyl 44.3% SC were evaluated for their toxicity to *R. solani* under *in vitro* conditions at different concentrations using poison food technique (Kumar *et al.* 2016). Observation on mycelial growth and number of sclerotia produced by fungus were recorded in triplicate set of varied concentrations of the test fungicides after 3 and 15 days of incubation at 28 ± 1 °C. The EC₅₀ and EC₉₀ values of the fungicide with respect to mycelial growth inhibition were determined by plotting on log probit scale (Kumar *et al.* 2016).

Field experiment was conducted during *khariif* 2016 and 2017 at CCS Haryana Agricultural University Rice Research Station, Kaul to study the efficacy of fungicides against sheath blight of rice. Thirty days old seedlings of rice cultivar HKR 127 were transplanted in a randomized block design (RBD) with four replications in 3 × 2 m plots at 20 × 15 cm spacing and the crop was raised following recommended package of practices (Fertilizer: 150:60:0:25 kg N:P:K:ZnSO₄/ha in three equal splits as basal dose, 21 and 42 days after transplanting; application of herbicide pretilachlor 50 EC at 2.0 l/ha (1.0 kg a.i./ha) for the control of weeds and need based application of buprofezin 25% EC at 825 ml/ha on formulation basis (206.25 ml a.i./ha) for the control of planthoppers. The inoculum of *R. solani* grown on autoclaved mixture of barley grain and *Typha angustata* (1:1 v/v) was placed in the centre of rice hills 50 days after transplanting to induce sheath blight (Singh *et al.* 2010).

Table 1 *In vitro* toxicity of fungicides to *Rhizoctonia solani* on PDA

Fungicide	Test conc. range (ppm a.i)*	EC50 (ppm a.i)	EC90 (ppm a.i)
Tebuconazole 50% + trifloxystrobin 25% WG	0-10	0.398	8.921
Difenoconazole 25% SC	0-100	4.36	66.07
Propiconazole 13.9% + difenconazole 13.9% EC	0-25	3.71	17.78
Kresoxim methyl 44.3% SC	0-2000	42.35	> 2000

*Initial concentration tested: 0.1-50 ppm on active ingredient (a.i) basis

The fungicides were sprayed twice, one and 11 days after inoculation with the help of knapsack sprayer using 500 l of water/ha. In *khariif* 2017 tebuconazole 50% + trifloxystrobin 25% WG, difenoconazole 25% SC, propiconazole 13.9% + difenconazole 13.9% EC, found effective and economical in managing sheath blight during *khariif* 2016 at single/recommended doses, were also tested on double doses for both efficacy and persistence. The plots sprayed with water served as check. The observations on vertical spread of the disease were recorded 10 days after second spray. Grain yield was recorded on plot basis and expressed as kg/ha. The benefit cost ratio (B:C) of fungicides was worked out by dividing the additional income generated due to yield enhancement in different treatments by the cost of fungicides plus labour required for foliar application.

Dissipation of the fungicides found promising against sheath blight, viz. tebuconazole 50% + trifloxystrobin 25% WG, difenoconazole 25% SC, propiconazole 13.9% + difenconazole 13.9% EC was studied in brown rice, husk, straw and soil sample collected from treated plots of single and double dose at the time of harvest, i.e. after 44 days of the last spray application. The harvested paddy plants were allowed to dry for a week. Straw was chopped down to sample pieces using a mechanical slicer while paddy grains were de-husked. Soil collected from a depth of 0-15 cm from 3-4 spots/plot was thoroughly mixed to draw the final sample.

Samples of brown rice were blended in high volume homogenizer (Robot Coupe) and extracted using modified QuEChERS technique (Quick, easy, cheap, rugged and safe). A sample of 10 g of brown rice was drawn in 50 ml centrifuge tube and 30 ml acetonitrile and distilled water mixture (2:1) was added to it. Sample was then homogenized using low volume homogenizer at 14000-15000 rpm till a consistent milky solution is formed. After adding 3 g NaCl, mixture was shaken vigorously and centrifuged at 2500 rpm for 3 minutes. From supernatant organic layer, 12 ml was taken

Table 2 Effect of fungicides on sclerotial formation of *Rhizoctonia solani*

Fungicide	Fungicidal concentration (ppm a.i) in relation to sclerotial formation				
	Abundant*	Many	Few	Rare	Nil
Tebuconazole 50% + trifloxystrobin 25% WG		0.01	0.05-0.5	1	2.5
Difenoconazole 25% SC	0.01-2	3-4	5-10	-	20
Propiconazole 13.9% + difenconazole 13.9% EC	0.1-2.5	5-10	15	20	25
Kresoxim methyl 44.3% SC	0.01-0.05	0.1	0.5-1	2.5	5

*Abundant: >50, many: 20-50, few: 5-20, rare: 1-5 sclerotia per plate, nil: No sclerotia formed, **Concentration of fungicide in ppm a.i.

Table 3 Effect of fungicides on sheath blight severity and grain yield of rice cultivar HKR 127

Treatment	Dose	Vertical disease spread (%)			Disease control (%)	Grain yield (kg/ha)			Increase in grain yield (%)	B:C ratio
		2016	2017	Pooled mean		2016	2017	Pooled mean		
Tebuconazole 50% + trifloxystrobin 25% WG	0.4 g/l	20.61 (26.98)	29.24 (32.68)	24.93 (29.83)	50.98	7850.00	7969.50	7909.75	8.45	3.52
Difenoconazole 25% SC	1 ml/l	26.06 (30.68)	33.48 (35.31)	29.77 (33.00)	41.45	7287.50	8046.50	7667.00	5.13	1.73
Propiconazole 13.9% + difenconazole 13.9% EC	1 ml/l	19.09 (25.85)	33.99 (35.63)	26.54 (30.74)	47.81	7762.50	7603.75	7683.13	5.35	2.80
Kresoxim methyl 44.3% SC	1 ml/l	36.51 (37.16)	46.10 (42.74)	41.31 (39.95)	18.77	7250.00	7815.50	7532.75	3.29	0.96
Tebuconazole 50% + trifloxystrobin 25% WG	0.8 g/l		10.62 (18.95)	18.95 (10.62)	79.12		8162.00	8162.00	11.91	2.66
Propiconazole 13.9% + difenconazole 13.9% EC	2 ml/l		14.43 (22.27)	22.27 (14.43)	71.62		8046.50	8046.50	10.33	3.02
Difenoconazole 25% SC	2 ml/l		16.94 (24.25)	24.25 (16.94)	66.69		7642.25	7642.25	4.79	0.63
Check		42.58 (40.71)	59.13 (50.25)	45.48 (50.85)		7175.00	7411.25	7293.13		
CD (P= 0.05)		(2.56)	(3.81)			333.11	490.61			
CV (%)		4.15	6.58			2.87	4.27			

* Figures in parentheses represent angular transformed values.

out in another centrifuge tube and shaken vigorously for one min after adding 5 g of anhydrous Na₂SO₄ for moisture absorption. Eight ml of aliquot of sample was transferred to 10 ml cleanup tube containing 0.2 g PSA and 0.6 g MgSO₄ and vortexed for 1 min. Tubes were centrifuged for 5 min at 3000 rpm and 4 ml of supernatant was taken, concentrated on rotary vacuum evaporator until dryness and reconstituted in 3 ml n-hexane for residue analysis.

Finely sorted and chopped rice straw and husk were processed following Sondhia *et al.* (2006) with slight modifications. Samples (10 g) were soaked in 60 ml of acetonitrile:water (7:3) mixture for 2 hr followed by continuous shaking on mechanical shaker for 1 hr. Sample were filtered and re-extracted again with 40 ml of acetonitrile:water as described above. The filtrates were pooled together, concentrated and diluted with 5% brine solution (30 ml) and partitioned thrice with 100 ml mixture of hexane:dichloromethane (1:1 v/v), followed by two times extraction with dichloromethane (75 and 50 ml). After each treatment, organic phase was collected and combined by passing through sodium sulphate (Na₂SO₄). Solution obtained was concentrated on rotary vacuum evaporator until dryness and made 3 ml final volume in n-hexane for analysis.

The soil samples from 0-15 cm soil depth were processed as per method of Kumari *et al.* (2008). Ground, sieved and dry representative (15 g) of soil was packed compactly in between two layers of anhydrous sodium sulphate (Na₂SO₄) in glass column (60 cm × 22 mm i.d.) with activated charcoal and activated Florisil (1:5 w/w). The

compact sample was eluted with 125 ml of hexane: acetone (9:1 v/v) mixture at flow rate of 3 ml/min, concentrated the elute on rotary vacuum evaporator and reconstituted in 2 ml in n-hexane for analysis.

Chromatographic separation for three fungicides was performed on a GC System, which is equipped with split/splitless injection port and autosampler coupled with triple-quadrupole (TQ) mass spectrometer. The GC separation was performed in SH-Rxi-5Sil MS (30 m × 0.25 mm × 0.25 µm film thickness) of 5% diphenyl and 95% dimethyl polysiloxane. Argon (99.99% Kumar *et al.* 2016) was used as a carrier gas at initial flow of 1.46 ml/min. Oven temperature was programmed as 80°C for 2 min, 20°C/min to 180°C for 0 min, 5°C/min to 300°C for 3 min, injector port temperature 250°C, ion source temperature 200°C, interface temperature 250°C and loop time 0.3 second. The flow rate of gas was 1.46 ml/min through the column with split ratio 1:10. All three fungicides were eluted within 30.0 min.

Table 4 Molecular mass (g/mol), retention time (min.), quantification and diagnostic ion used in GC-MS/MS analysis

Pesticide	Molecular mass (g/mol)	RT (min)	Products (m/z)
Tebuconazole	307.82	19.00	→ 125 153 70
Trifloxystrobin	408.37	18.31	→ 190 130 162
Propiconazole	342.22	18.52	→ 191 173 69
Difenoconazole	406.26	27.70	→ 265 202 209

Blank samples of rice were analyzed to verify the absence of interfering species at about the retention time of the analytes. The linearity of the method was studied by analyzing different matrix matched standard solutions in triplicate at seven concentrations ranging from 0.01 to 1 mg/l. The parameters of linear regression equations, the standard deviation and the correlation coefficient (R^2) were calculated (Table 5). The recovery experiments were conducted to check efficiency of extraction and cleanup and reliability of analytical conditions by spiking at three (0.01, 0.05 and 0.5 ppm) different levels with five replicates and a non-spiked blank sample as the control. The recoveries were found to be >80% (Table 6). Therefore, the method was adopted as such without any recovery factor for terminal residue studies. The recovery experiment of trifloxystrobin, tebuconazole, difenconazole and propiconazole were conducted.

The matrix-dependent LOQ and LOD were calculated for the analytical methodology by using the blank and calibration standards of rice. The LODs of fungicides were the concentrations that produced a three times higher signal than noise of the base line at the retention time of the peak of interest. The LOQs were determined by multiplying LOD to a factor of 3 to accommodate matrix effect and to be more precise.

RESULTS AND DISCUSSION

In vitro evaluation of fungicides against *R. solani*

The fungicides, viz. tebuconazole 50% + trifloxystrobin 25% WG, difenconazole 25% SC, propiconazole 13.9% + difenconazole 13.9% EC and kresoxim methyl 44.3% SC varied considerably in their toxicity to *R. solani* in vitro. The EC_{50} values of fungicides ranged from 0.398 to 42.35 ppm a.i., whereas the corresponding EC_{90} values were 8.921 to >2000 ppm a.i. Tebuconazole 50% + trifloxystrobin 25% WG was the most inhibitory fungicide with EC_{50} and EC_{90} values of 0.398 and 8.921 ppm a.i. followed by propiconazole 13.9% + difenconazole 13.9% EC in which EC_{50} and EC_{90} values were 3.71 and 17.78 ppm a.i. (Table 1). The present study is in conformity to the findings of Pal *et al.* (2017) who reported that trifloxystrobin + tebuconazole at 200 ppm completely inhibited the mycelial growth of *R. solani* with ED_{50} value of 3.19 ppm. Mishra and Sinha (1999) reported a high toxicity of propiconazole, carbendazim and cyproconazole with ED_{50} <10 ppm. Higher concentration of carbendazim at 5 µg/ml and propiconazole at 10 µl/ml resulted in complete inhibition of mycelial growth. It was

Table 5 Regression equation, correlation coefficient (R^2), limit of quantification (mg/kg), and limit of detection (mg/kg) of various fungicides

Compound	Matrix	Regression equation	R^2	LOD	LOQ
Trifloxystrobin	Rice	Y= 40162x-12727	0.999	0.005	0.01
Tebuconazole	Rice	Y= 40901x-17520	0.999	0.005	0.01
Propiconazole	Rice	Y= 62802x-19848	0.999	0.005	0.01
Difenconazole	Rice	Y= 24550x-11059	0.991	0.005	0.01

interesting to note that kresoxim methyl 44.3% SC, which inhibited 50% of the mycelial growth at 42.35 ppm a.i., failed to inhibit 90% of mycelial growth even at a concentration as high as 2000 ppm a.i.

The test fungicides also varied considerably in their ability to inhibit sclerotia formation of the fungus. *R. solani* did not form any sclerotia even at 2.5 ppm of tebuconazole 50% + trifloxystrobin 25% WG followed by kresoxim methyl 44.3% SC which completely inhibited sclerotial formation at a concentration of 5 ppm a.i. However, difenconazole 25% SC and propiconazole 13.9% + difenconazole 13.9% EC imparted complete sclerotial inhibition at 20 and 25 ppm a.i., respectively (Table 2). Carbendazim, hexaconazole, epoxiconazole, carbendazim + epoxiconazole and propiconazole have been observed to completely inhibit the growth of *R. solani* at 25-50 and 1-2 ppm, respectively (Tiwari *et al.* 2002). Previous reports have indicated complete inhibition of growth and sclerotia formation of the pathogen by thiafluzamide and hexaconazole at 0.5 ppm a.i. (Sunder *et al.* 2003)

Field trials conducted during 2016 and 2017 to study the efficacy of fungicides against sheath blight revealed that all the test fungicides reduced disease severity significantly during both the years. Tebuconazole 50% + trifloxystrobin 25% WG was found most effective and provided 50.98% and 79.12% disease control at single and double dose,

Table 6 Recoveries of azoxystrobin, difenconazole and propiconazole in four matrices at three fortified levels

Compound	Level of fortification (µg/ml)	Recovery from rice grains (%)	Recovery from cropped soil (%)	Recovery from paddy straw and husk (%)
Tebuconazole	0.01	82.35 (±0.98)	82.38 (±1.07)	83.45 (±0.23)
	0.05	86.48 (±0.34)	86.54 (±0.88)	89.28 (±0.48)
	0.1	90.15 (±0.92)	92.69 (±.29)	93.46 (±1.12)
Trifloxystrobin	0.01	81.75 (±0.92)	80.89 (±0.98)	82.29 (±0.92)
	0.05	88.00 (±0.69)	83.78 (±0.68)	87.58 (±0.64)
	0.1	93.58 (±0.47)	95.43 (±0.24)	94.58 (±0.23)
Pencycuron	0.01	80.28 (±0.14)	82.45 (±1.09)	80.00 (±0.99)
	0.5	83.80 (±0.63)	86.38 (±0.69)	86.82 (±0.49)
	0.1	89.75 (±0.32)	91.67 (±0.45)	91.25 (±0.31)
Difenconazole	0.01	79.50 (±1.92)	86.29 (±1.72)	81.38 (±0.91)
	0.05	81.25 (±0.89)	88.46 (±0.81)	87.48 (±0.46)
	0.1	86.70 (±0.58)	99.09 (±0.22)	93.29 (±0.32)

Table 7 Terminal residue (mg/kg) of ready mix fungicides in different substrate of paddy

Substrate	Single dose				Double dose			
	Tebuconazole	Trifloxy-strobin	Propi-conazole	Difeno-conazole	Tebuconazole	Trifloxy-strobin	Propi-conazole	Difeno-conazole
Cropped soil	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Straw	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Husk	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Grain	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

respectively along with maximum increase in grain yield of paddy followed by propiconazole 13.9% + difenoconazole 13.9% EC, whereas kresoxim methyl 44.3% SC was the least effective fungicide. The bio-efficacy and B:C ratio of different fungicides indicated that tebuconazole 50% + trifloxystrobin 25% WG and propiconazole 13.9% + difenoconazole 13.9% EC sprayed at 0.4 g/l and 2 ml/l were effective and economical treatments in managing sheath blight of rice with a benefit cost ratio of 3.52 and 3.06, respectively (Table 3). Tebuconazole 50% + trifloxystrobin 25% WG and propiconazole 250 EC have also been reported to exhibit high level of efficacy against *R. solani* and *Drechslera oryzae* *in vitro* and against sheath blight and brown spot of rice in terms of disease reduction and yield enhancement under field conditions (Hunjan *et al.* 2011). Recently, Pal *et al.* (2017) also reported that trifloxystrobin + tebuconazole provided effective disease control under field conditions.

Lore *et al.* (2012) observed that application of kresoxim methyl 40% + hexaconazole 8% at 0.1% was highly effective against sheath blight of rice. However, in present studies, kresoxim methyl 44.3% SC proved to be the least effective treatment. The difference in results obtained in previous and present studies could be attributed to variation in fungicidal formulation, variation in time of fungicide application in rice field and variation in weather conditions such as temperature and rainfall after application as reported by earlier researchers (Lin *et al.* 2001).

Residue persistivity in harvest samples

The most considerable challenges in residue analyses are matrix interferences even after QuEChERS extraction

and cleanup. Triple quadrupole GC-MS/MS has become a progressive approach for the analysis of trace level residues in multiple reactions monitoring (MRM) mode. On the basis of m/z ratio scanned in MRM, details of retention time, collision energy, precursor ions and productions are given in Table 4.

Both trifloxystrobin 25% WG and tebuconazole 50% WG were found below the detectable limit of the instrument (< 0.01 ppm) irrespective of doses in brown rice, cropped soil paddy straw and husk when sprayed at 50 and 61 days after transplanting in paddy variety HKR 127 with a pre-harvest period of 44 days indicating that both the rice grains and paddy straw were safe for human and animal consumption (Table 7). Wang *et al.* (2012) reported that application of difenoconazole at 135 g a.i./ha possessed no residual activity at harvest time in rice hull and husked rice at pre-harvest intervals of 30, 40 and 50 days after fungicides application. However, the fungicidal concentration in straw at these intervals ranged from 0.037 mg/kg and 2.53 mg/kg. Use of another triazole fungicide, i.e. tebuconazole @ 750 and 1500 ml/ha has also been found safe in paddy, wheat and grapes as it did not possess any residual toxicity problem (Kundu *et al.* 2011).

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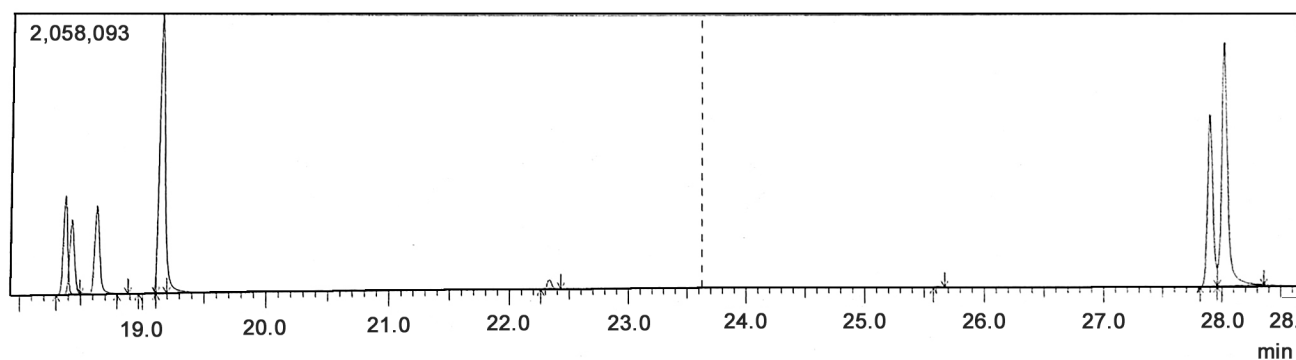


Fig 1 Tebuconazole, trifloxystrobin, propiconazole and difenoconazole eluted at different retention times

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