Efficacy of Tebuconazole and Trifloxystrobin against *Colletotrichum Gloeosporiode*s Infestation in Black Pepper (*Piper Nigrum L.*)

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Abstract

Anthracnose caused by *Colletotrichum gloeosporioides* is one of the most damaging disease causing flower set reduction and yield losses in black pepper. Application of fungicide was one of the approaches to control the disease. In the field experiment, Nativo at 0.2 g/L and 0.4 g/L AI significantly suppressed the development of both leaves anthracnose and black berries disease. Pepper vines treated with Nativo produce more quality berries and showed no phytotoxicity. Hence the optimum rate of Nativo was fixed to 0.2 g/L with the pre-harvest interval was on day 15. For the residue analysis, satisfactory recoveries ranging between 77.6%-140.5% were obtained for the fortified pepper berries samples. Results showed low level of tebuconazole and trifloxystrobin residues in dried pepper berries ranging between <0.01 mg/kg to 0.11 mg/kg and <0.01 mg/kg to 1.85 mg/kg respectively. It can be concluded that the pre-harvest interval for application of Nativo fungicide was on day 15 after the last treatment. The determined value of proposed Maximum residue limit (MRL) for tebuconazole and trifloxystrobin were 0.2 and 2.70 mg/kg respectively.

Keywords: black pepper, *C. gloeosporioides,* tebuconazole, trifloxystrobin, MRL, residue

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1. Introduction

Black pepper (*Piper nigrum L.*) is affected by various diseases in Malaysia among which anthracnose caused by *C. gloeosporioides* (Penz) Penz & Sac. is gaining importance in recent years (Yap et al., 2015; Kurian et al., 2000; Wong et al., 1993). The fungus causes damage to pepper vine both in the nursery and field. Infection on spikes results in spike shedding, whereas, infection on immature berries leads to formation of brownish splits on the berries. Spike shedding is more severe at high altitudes. The disease is seen throughout the crop season in plantations and maximum damage is caused during September to December and ranges from 28% to 34% (Nair *et al*., 1987). The damage on the berries due to *C. gloeosporioides* has also been reported to result in 100% yield loss (Santhakumari & Rajagopalan 2000). Successful management of the disease has been achieved through spraying of organic sulphur (Dithio-carbamates) fungicides like maneb and heterocyclic nitrogen compounds. However, these fungicides have shown phytotoxic effect to flowers (McMillan, 1972). Organic sulphur group fungicide, mancozeb was found to be effective in controlling anthracnose, but cannot be used because of ethylene produced as a by-product. Moreover, *C. gloeosporioides* developed resistant to benomyl, a benzimidazoles systemic fungicide for controlling of pre- and post-harvest development of anthracnose (Akthar et al., 1998; Dodd et al., 1991).

Currently, much attention and efforts on anthracnose control has concentrated on the use of fungicides. Long term used of these pesticides will finally lead to the development of pest resistant strain. Therefore, there is a need to seek for alternative pesticide active ingredient in order to sustain the national production of pepper. One of the potential newly developed fungicides that can be used to control anthracnose diseases in pepper industrial is Nativo. This chemical, is a broad spectrum synthetic fungicide which content two active ingredients (trifloxystrobin and tebuconazole) used to control a wide range of fungus disease. Trifloxystrobin is a strobilurin fungicide with excellent biological activity against four major groups of plant pathogenic fungi including Ascomycota, Basidiomycota, Deuteromycetes, and Oomycetes. There are many reports on efficacy of trifloxystrobin against plant diseases such as gray mold (*Botrytis cinerea*) of fruitsand vegetables, leaf spot (*Cercospora beticola*) and powdery mildew (*Erysiphe betae*) of sugar beet, black spot (*Guignardia citricarpa*) of citrus, postharvest rot (*C. gloeosporioides*) of avocado (Ziegler et al., 2004; Hadden and Black, 1898; Lewis Miller, 2003; Slawecki, 2002; Anesiadis et al., 2003). Tebuconazole is another broad spectrum fungicide that is widely used for controlling fungus diseases of fruits and vegetable crop. It gives effective control of black pepper anthracnose disease (Anonymous, 2012). The combination product, trifloxystrobin (25% w/w) and tebuconazole (50% w/w) (Nativo), is a excellent tool for protecting black pepper from anthracnose disease. Even though this product has been registered with Malaysian Pesticide Board for application of various food commodities including fruits, vegetable, but the efficacy and maximum residue level of the said insecticide on black pepper is still unknown. The present study was conducted for the purpose to evaluate the efficacy of the said fungicides and to determine whether residues of the fungicide are present in pepper berries after several treatments with fungicide per season and to establish MRL for trifloxystrobin and tebuconazole, an active ingredient in Nativo fungicide of black pepper.

2. Material and methods

2.1 Isolation of pathogenic fungi

Pieces of pepper leaves and berries obtained from pepper vine that showed symptoms of disease were submerged in 5% sodium hypochloride for five minutes. After this treatment, they were extensively washed with sterile distilled water and placed on Petri dishes containing potato-dextrose-agar (PDA, Difco) and incubated at 30°C for 48 h. The fungi isolates were identified on the basis of cultural and microscopic morphological characters according to the method described by Barnett & Hunter (1972). The purified cultures were then used in *in vitro* experiments.

2.2 Chemicals and reagents

Tebuconazole (purity >98%) and trifloxystrobin (purity 96.4%) were purchased from Sigma-Aldrich, Malaysia. The formulation of tebuconazole (purity >98%) and trifloxystrobin (Nativo) was obtained from Bayer (Co) Sdn Bhd., Malaysia. All the solvent, namely acetonitrile, ethyl acetate and ethanol were HPLC grade. Primary secondary amine (PSA) sorbent was purchased from Agilent Technologies, USA. The other reagents namely acetic acid, ammonium formate, anhydrous sodium sulphate and magnesium sulfate were analytical reagent grade and purchase from the Merch Malaysia Sdn Bhd. All common solvents were redistilled in all-glass apparatus before use.

2.3 Determination of *in vitro* activity of Nativo fungicide against *C. gloeosporioides*

Two independent experiments were conducted to determine the *in vitro* activity of Nativo fungicide against *C. gloeosporioides*. The first experiment was performed by using modified dual cultures techniques. One 10 mm disk of a pure culture of *C. gloeosporioides* was placed at the center of a Petri dish containing PDA amended with Nativo fungicide at 0.1, 0.2 and 0.4 g/L concentration with three replications. Plates were cultured for 72 h at 28± 2°C and growth diameter of the pathogen (fungal growth) was measured and compared to control growth where the fungicide was replaced by sterile distilled water. A positive control was included by adding benomyl into PDA agar. Results obtained are expressed as means % inhibition of the growth of the corresponding pathogen isolate in the presence of the fungicide. Percent inhibition was calculated using the following formula:

% inhibition = [1 - (Fungal growth / Control growth)] × 100

In the second experiment, the effects of Nativo fungicide on conidial germination were determined. *C. gloeosporioides* were grown on potato Dextrose agar for 30 days and further grown in Czapeks medium for 7 days and filtrate was taken. Conidial suspensions of *C. gloeosporioides* were added to the different concentration of Nativo fungicide (0.1, 0.2 and 0.4 g/L) so as to make final count adjusted to 8000-12,000 conidial/ml by using haemacytometer. Conidial germination studies were carried out in cavity slides. Three replicate slides for each pathogen were used. For control, conidial suspension was added with distilled water. Slides were incubated at 28± 2^{o}C for 24 hour. Percentage spore germination was determined by dividing the number of germinating spore by the total number of spore presented per slides as observed under a Nikon Inverted Microcope (IM). Three counts per microscopic field (40 x magnification) were made per slide. A spore was considered to have germinated when the germ tube length was half of the length of the spore (Sariah, 1994). The experiment was repeated three times and plates were arranged in a completely randomized design with five replicates.

2.4 Determination the efficacy of Nativo fungicide under the field condition

Five supervised residue trials were conducted for one pepper production cycle (flowering stage to harvesting stage) at three commercial grower's plots in Kuching, Serian and Padawan, Sarawak from 2015-2016. The plots having 30 mature pepper vines (5 years old vines) each were selected and treated with Nativo using rate as described below. Pepper vine of the variety "Kuching" was planted with spacing of 2.1 m between the row and 2.1 m within the row, with a population of 2,000 plants per ha. The treatment consisted of 3 doses of Nativo (0.1 (11.25g of tebuconazole + 5.65g of trifloxystrobin a.i./ha) , 0.2 g/L (standard rate, (22.50g of tebuconazole + 11.30g of trifloxystrobin a.i./ha) and 0.4 g/L (double rate, 45.0g of tebuconazole + 22.50g of trifloxystrobin a.i./ha) of active ingredient per ha), 1 dose of benomyl (0.5 g/L active ingredient per ha) and untreated control. The pesticide was applied with a motorized sprayer at monthly intervals up to a maximum of 7 applications (complete fruiting cycles) before harvesting. The development of the pepper berries to maturity took 8 months from flowering to full ripeness. Details of the experimental treatment are given in Table 3. The first spray was performed 2 week after flower formation, followed by a spray of monthly interval for 7 month (harvesting stage) between September 2015 to April 2016.

2.4.1 Assessing for the incidence of disease infestation

Observations on the disease incidence was recorded at 4 days interval after last spray by following the score charts as showed in Table 1 below. The percent (%) disease index (PDI) was worked out by using Mckinney's (1923) formula:

$$\text{PDI} = \frac{\text{Sum of all number rating}}{\text{Total number of leave observe}} \times \frac{100}{\text{Maximum grade in the score chart}}$$

trials. The leaves, spike and fruit were regularly examined for injury of leaf tip, leaf surface, wilting necrosis, epinasty and hyponasty. During harvesting season, all remaining pepper vine in the cultivation area of each replicate were harvested for determination of yield per vine. All data was statistically analyzed using analysis of variance (ANOVA) as applicable to a split-plot design (Gomez and Gomez, 1984). The significance of the treatment effect was determined using Duncan's Multiple Range Test (DMRT) and least significant differences (LSD) will be calculated at the 5% probability level.

Table 1: Score chart for pest infestation

Grade	Disease intensity	Description
0	0	No insect
1	1-20	1-5 insects
2	21-40	6-10 insects
3	41-60	11-15 insects
4	61-80	16-25 insects
5	>80	>25 insects

2.4.2 Sampling procedure

The fresh sample of pepper berries collected on 2 hours, 3, 5, 7, 9, 15 and 20 days after the last spray were then undergo series of process to produce black pepper. The pepper berries were separated from pepper spike right after harvesting. The separated green pepper berries were then undergo blanching processing by soaking the pepper berries in boil water for 1 minute and drying for 2 days under sunlight following accepted practices before send for laboratory analysis. All dried black pepper was then powdered using blender from which 3g sample was collected in triplicate for estimation of residues.

2.4.3 Method development and validation

For recovery study, ground pepper sample were fortified with known amount of trifloxystrobin and tebuconazole standards. Appropriate amounts of trifloxystrobin and tebuconazole standards were spiked onto pepper samples to obtain the recoveries at 0.01, 0.1 and 0.5 mg L^{-1} concentration. Three replicates were prepared for each sample. Recovery of trifloxystrobin and tebuconazole was calculated using the following equation:

Recovery of active ingredient = Detected active ingredient (mg/kg) /spiked active ingredient (mg/kg) x 100

2.4.4 Analysis of pesticides

Extraction of trifloxystrobin and tebuconazole residues from pepper berries was carried out using a multi residue method modified from a published method (Anastassiades et al., 2003). Homogenized pepper berries sample (2.5g) was put into a 250 ml bottle, followed by water (7.5ml), acetonitril (10ml), magnesium sulphate (4g) and sodium chloride (1g). The mixture was homogenized using homogenizer (IKA UltraTurrax) for about 1 minute. The entire solution was centrifuged for 5 minutes at 4000 rpm. The supernatant was transferred to a fresh tube, and 1.2g of magnesium sulphate and 0.4 g of Z-Sept was added. Following centrifugation for 5 minutes, 6ml of clean extract was diluted with 600µl of water. The diluted extract was analyzed by liquid chromatography mass spectrometry (LCMS).

2.5 MRL estimation

A European Union method was employed for calculation of MRL values (Hyder et al., 2003). The estimation was based on the equation shown below:

$MRL = R = KS$

R= Mean of HR** (Highest Residue after Pre-Harvest Interval, PHI)

K= One-sided tolerance factor for normal distribution with 95% confidence interval

S= The standard deviation of HR after PHI

3.0 Results and discussion

3.1 Determination of *in vitro* activity of Nativo fungicide against *C. gloeosporioides*

Results showed that Nativo fungicide significantly reduced the mycelial growths of *C. gloeosporioides* (Table 2). Nativo at 0.1 g/L AI slightly inhibited the mycelial growth with the inhibition percentage over control was 39.36%. This was followed by the application of 0.2 g/L AI with the inhibition percentage of 84.62% whereas 0.4 g/L AI completely inhibited the mycelial growth of *C. gloeosporioides* (Table 2). Similar reading was also recorded on commercialized fungicide (benomyl) with inhibition percentage over control was 100%.

The effects of Nativo fungicide on the conidial germination of *C. gloeosporioides* are presented in Table 2. The results obtained reveal that the maximum inhibition of conidial germination was brought out by 0.4 g/L AI of Nativo with the inhibition percentage over control was 96.30%. This was followed by the application of 0.2 g/L and 0.1 g/L AI with the inhibition parentage over control was 88.48% and 31.55% respectively. No significant different was observed among Nativo at 0.4 g/L AI and benomyl treatment indicated that this concentration was effective to control anthracnose disease in black pepper. Although the efficacy of Nativo at 0.2 g/L concentration recorded lower efficacy value as compared to commercialized fungicide (benomyl), but this concentration was significant superior to control and Nativo 0.1 g/L AI with the efficacy value more than 88.48% indicated that is concentration was on par with commercialized fungicide. This research finding is consistent with the efficacy results of commercialized fungicide (benomyl) with the percentage of conidial germination value was more that 80%.

Table 2: *In vitro* efficacy of trifloxystrobin and tebuconazole on growth of *C. gloeosporioides*

Nativo concentration (g/L)	Mycelial growth (mm)	Percentage of inhibition over control (%)	Conidial germination (%)	Percentage of inhibition over control (%)
0.1	49.12[c]	39.36	68.45[c]	31.55
0.2	12.46[b]	84.62	11.52[b]	88.48
0.4	0.00[a]	100	3.7[a]	96.30
benomyl	0.00[a]	100	4.2[a]	95.80
Control	81.00[d]	/	100[d]	/

3.2 Determination the efficacy of Nativo fungicide under the field condition

Bio efficiency of Nativo against pepper anthracnose was shown in Table 3 and Table 4. Under the field condition, spraying of Nativo significantly reduce the leaves anthracnose and black berries disease of black pepper with the concentration ranging from 0.2 g/L – 0.4 g/L. The incidence of leave anthracnose disease was observed at 4 days interval after last spray till completion of the harvesting stage. Nativo treated vines showed lesser leaf anthracnose than control. The reduction of leave anthracnose varied between the doses of Nativo. Among doses, 0.1 g/L AI slightly reduce the leave anthracnose with the PDI value of 15.09% on days 28 after the last spray. The disease reduction over control was only 49.45%. For Nativo Treatment 2 (0.2 g/L) and Treatment 3 (0.4 g/L), the bioeffiacy was very promising with the disease reduction over control was 100%. This indicated that the bioefficacy of Nativo increased with increase in the concentration (Table 2). Similar reading was also recorded on commercialized fungicide (benomyl) with disease reduction over control was 100% as well.

In term of bioefficacy of Nativo against black berries disease, control vines showed higher disease incidence (49.68 PDI) on day 28 after the last spray with typical symptom like blighted flowers, elongated dark gray or black spots of stalk, aborted flower and mummified fruit. Whereas Nativo treated vines did not show any anthracnose symptom and was completely free from disease. The reduction of black berries disease varied between the doses of Nativo. Among these doses, 0.1 g/L AI slightly reduce the black berries disease occurrence with the final disease reduction over control after 28 days was 67.40%. Whereas other higher doses ranging between 0.2 g/L to 0.4 g/L significantly supressed the black berries infestation with the disease reduction was 100%. This indicated that the optimal dosage for controlling black berries disease in black pepper is ranged between 0.2 g/L – 0.4 g/L.

3.3 Black pepper yield

Due to higher leaves anthracnose and black berries disease, controlled vines produced less spike and more light berries (0.67 kg/vine), whereas Nativo treated trees produced more quality berries which found to be statistically significant (Table 4). Pepper vines treated with 0.2 g/L Nativo produce more quality berries with the production per vine was 1.76 kg, followed by vines treated with 0.4 g/L Nativo (1.65 kg). There is no significant different between this two treatment regimens with commercialized fungicide, benomyl (1.68 kg) indicated that the efficacy of this two treatment regime was in par with commercialized fungicide and can be recommended to be use in pepper industry.

Table 3: Efficacy of Nativo on pepper leaf anthracnose

Amistartop	Conc g/L	Disease Index (PDI) (%)							Disease reduction over control
		4 DAS	8 DAS	12 DAS	16 DAS	20 DAS	24 DAS	28 DAS	
Nativo 1	0.1	13.21[b] (17.56)	12.76[b] (18.68)	12.85[b] (18.93)	13.73[b] (18.02)	14.32[b] (20.15)	12.64[b] (18.52)	15.09[b] (19.82)	49.45%
Nativo 2	0.2	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	100%
Nativo 3	0.4	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	100%
benomyl	0.5	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	100%
Control	/	24.36[c] (29.11)	25.85[c] (30.31)	22.43c (26.10)	23.60[c] (27.83)	25.00[c] (29.99)	27.73[c] (31.77)	29.85[c] (33.01)	

DAS- Days after spray * Mean of three replications

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT, Values in parentheses are arcsine-transformed value.

Table 4: Efficacy of Nativo on black berries disease

Amistart op	Con ml/L	Disease Index (PDI) (%)							Disease reducti on over control	Yield (kg/ vine)
		4 DAS	8 DAS	12 DAS	16 DAS	20 DAS	24 DAS	28 DAS		
Nativo 1	0.1	12.63[b] (20.64)	13.42[b] (21.34)	14.01[b] (21.92)	14.51[b] (22.25)	15.11[b] (22.75)	15.53[b] (23.09)	17.50[b] (24.56)	67.40%	0.85[ab]
Nativo 2	0.2	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	100%	1.76[b]
Nativo 3	0.4	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	100%	1.65[b]
benomyl	0.5	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	0.00[a] (0.50)	100%	1.68[b]
Control		16.28[c] (23.75)	23.31[c] (28.81)	28.72[c] (31.39)	32.46[c] (35.42)	39.78[c] (40.84)	43.23[c] (41.74)	49.68[c] (45.21)		0.67[a]

DAS- Days after spray * Mean of three replications

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT, Values in parentheses are arcsine-transformed value.

3.4 Fungicide response to rate of application

The fungicide rate of application experiment was useful in determining the optimum rate of Nativo for anthracnose control. The lower concentrations 0.1 g/L had shown higher infection rates, more disease than the higher concentration of 0.2 and 0.4 g/L. The bioefficacy of both higher concentration of Nativo are similar with commercialized fungicide, benomyl. The response of Nativo fungicide to rate applied is illustrated graphically. From the graph (Figure 1), it was clearly indicated that the black berries disease incidence decline drastically from 0.1 g/L to 0.2 g/L and 0.4 g/L. The graph also showed that the optimum rate was arrived at 0.2 g/L by considering the flattening of disease curve between rates (Figure 1). Similar optimum yield was also achieved at 0.2 g/L where yield response continuously to climb with increasing rates (Figure 1). Hence, optimum rate of Nativo was fixed to 0.2 g/L AI for the control of anthracnose disease. Moreover, all the doses of Native had not caused any phytotoxic effect to the pepper vines.

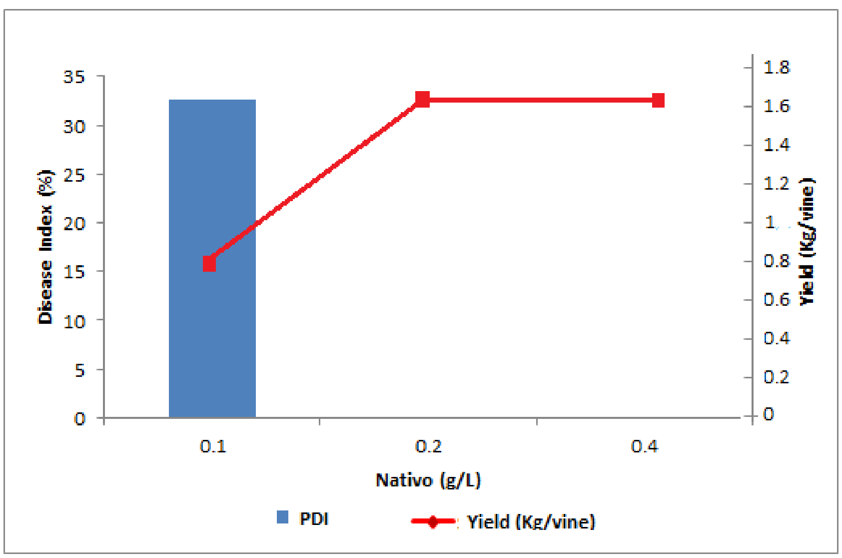

Figure 1: The effect of rate of application of amistartop of disease severity (PDI) and yield (Kg/vine).

3.5 Method Validation

The recovery experiments were carried out at different levels to establish the reliability and validity of the analytical method following the principles as per SANCO document (12495/2011). From the results obtained, the recoveries rate for tebuconazole ranged between 77.6% - 140.5% and trifloxystrobin ranged between 96.4%-102.6% with both relative standard deviation (RSD) of <5.8 % were obtained from overall recovery data of 3 level of spiking suggested that the analytical method used for tebuconazole and trifloxystrobin were effective (Table 5).

The limit of quantification (LOQ) of the analytical method for tebuconazole and trifloxytrobin in pepper berries were 0.01 mg/kg. The LOQ is the lowest level of spiking (0.01) mg/kg that gives acceptable recovery (77.6% - 140%) and precision (relative standard deviation of recoveries <15%). The example of calibration curve (for quantification of detected residue) with good linearity (R^2 = 0.9407 and 0.9999 for tebuconazole and trifloxystrobin respectively) within 0.02 -0.1µl/ml is shown in Figure 2A and 2B. Chromatogram example of tebuconazole and trifloxystrobin peak in standard solution and sample extract are shown in Fig 3A, 3B, 4A, 4B.

Table 5: Percentage recoveries of tebuconazole and trifloxystrobin from spiked pepper berries

Active ingredient	Spike concentration	Percentage recovered (%)				SD	% RSD
		R1	R2	R3	Average		
Tebuconazole	0.01	140.5	129.3	132.1	134.0	5.8	5.8
	0.1	82.1	90.4	92.5	88.3	5.5	5.2
	0.5	77.6	80.5	85.5	81.2	4.0	3.9
Trifloxystrobin	0.01	96.4	101.9	102.6	100.3	3.4	3.5
	0.1	104.1	95.6	99.1	99.60	4.3	4.2
	0.5	99.6	92.6	100.0	100.0	4.2	4.3

SD= Standard deviation RSD= Relative standard deviation

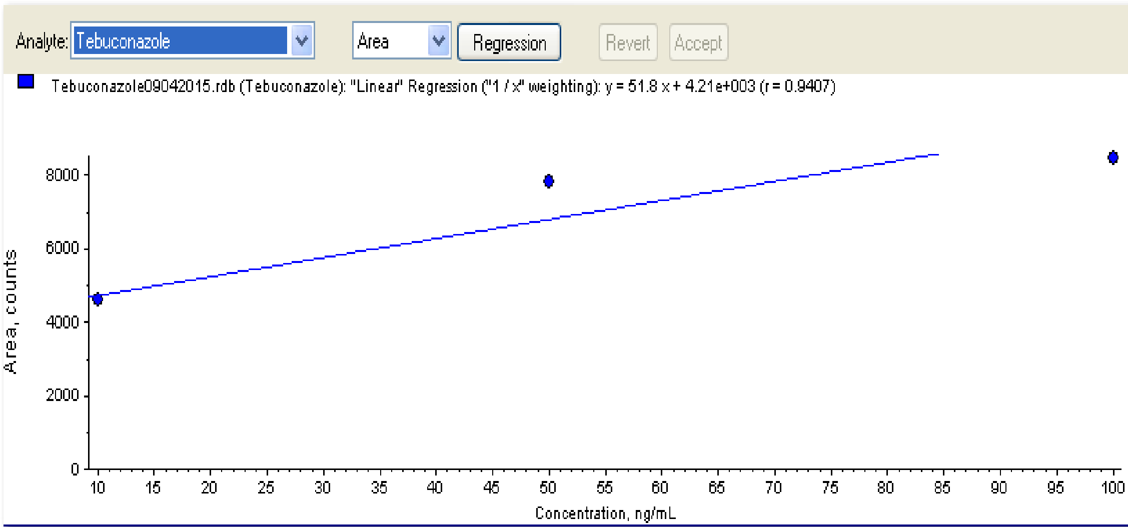

Figure 2A: Calibration curve of tebuconazole.

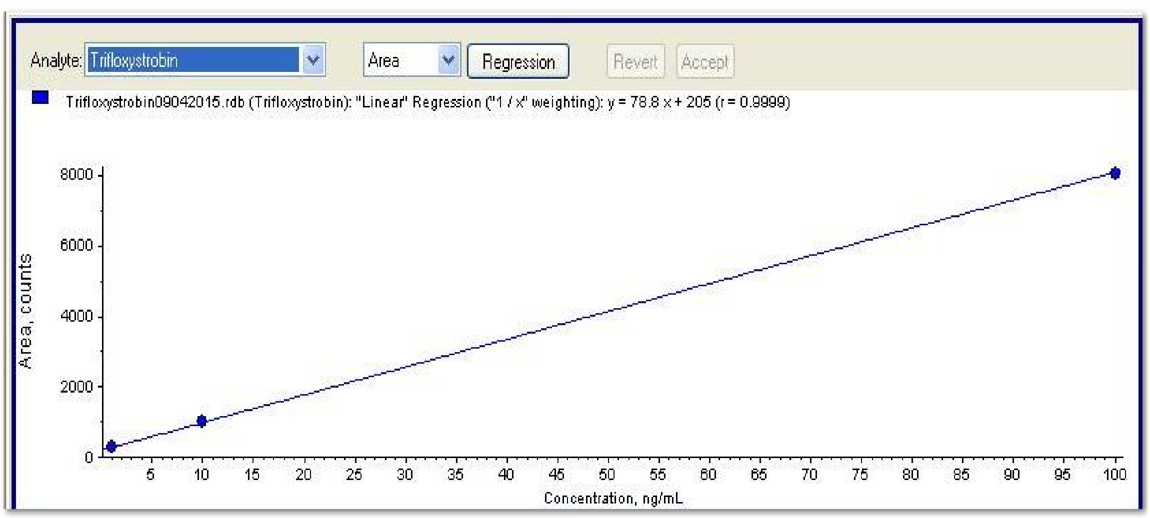

Figure 2B: Calibration curve of trifloxystrobin.

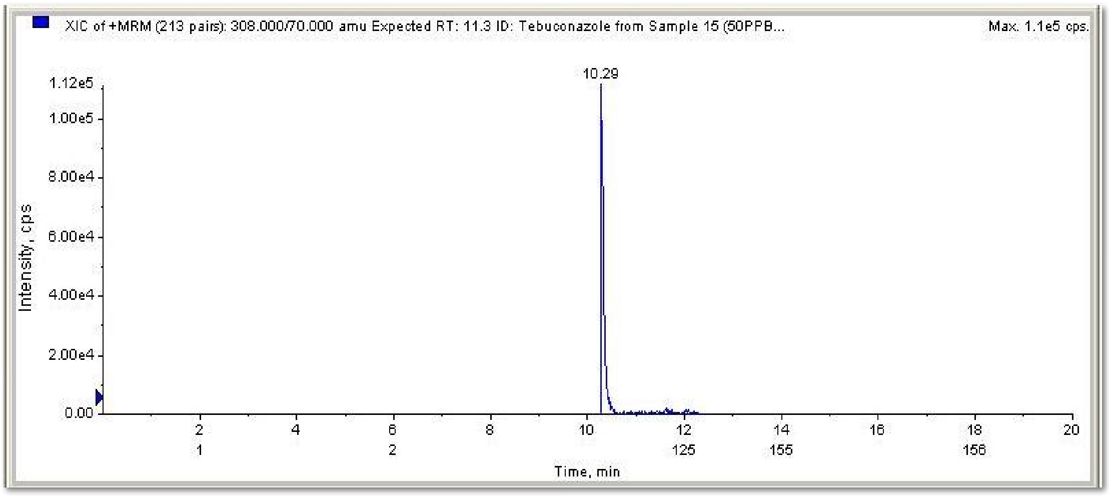

Figure 3A. LCMS chromatogram of tebuconazole standard at retention time of 11.30 min.

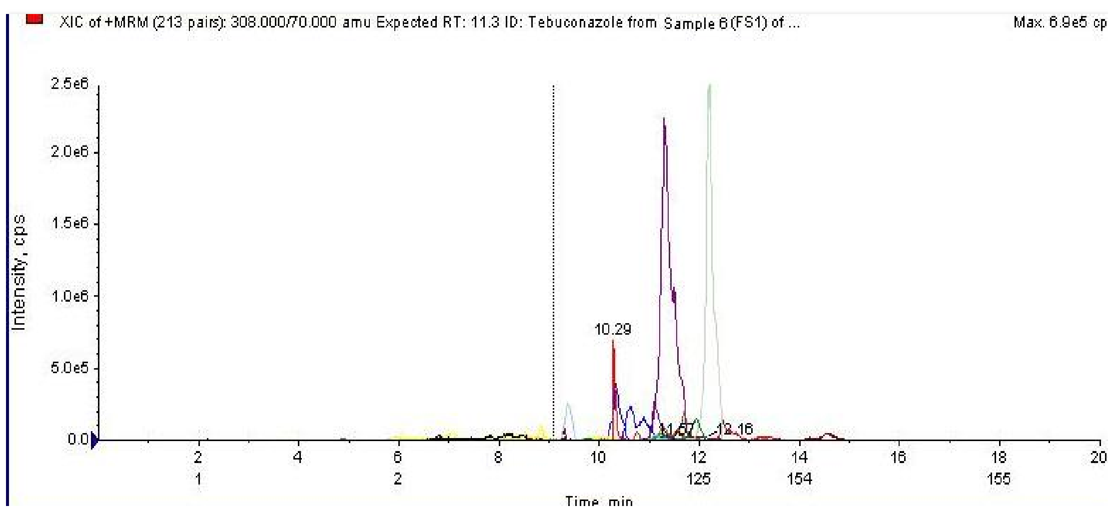

Figure 3B. LCMS chromatogram of pepper berries extract sample at retention time of 11.30 min.

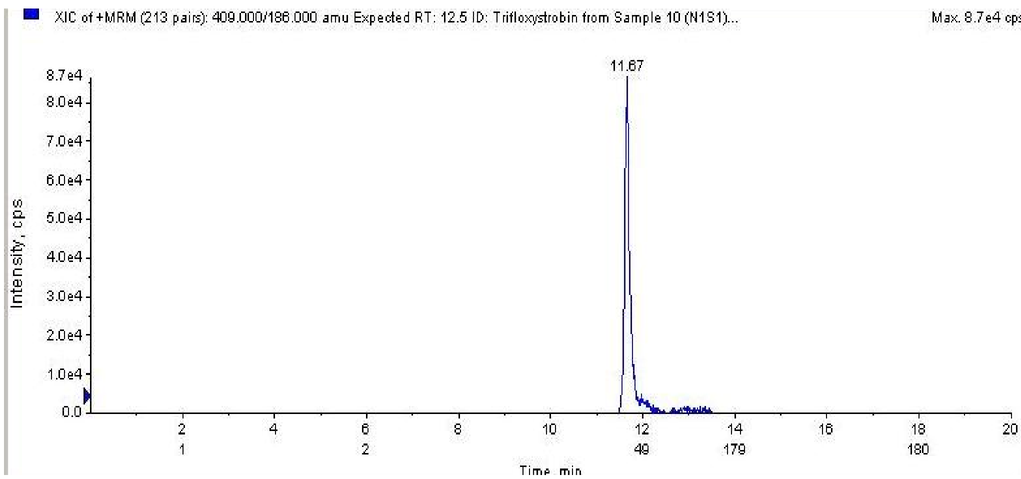

Figure 4A. LCMS chromatogram of trifloxystrobin standard at retention time of 12.50 min.

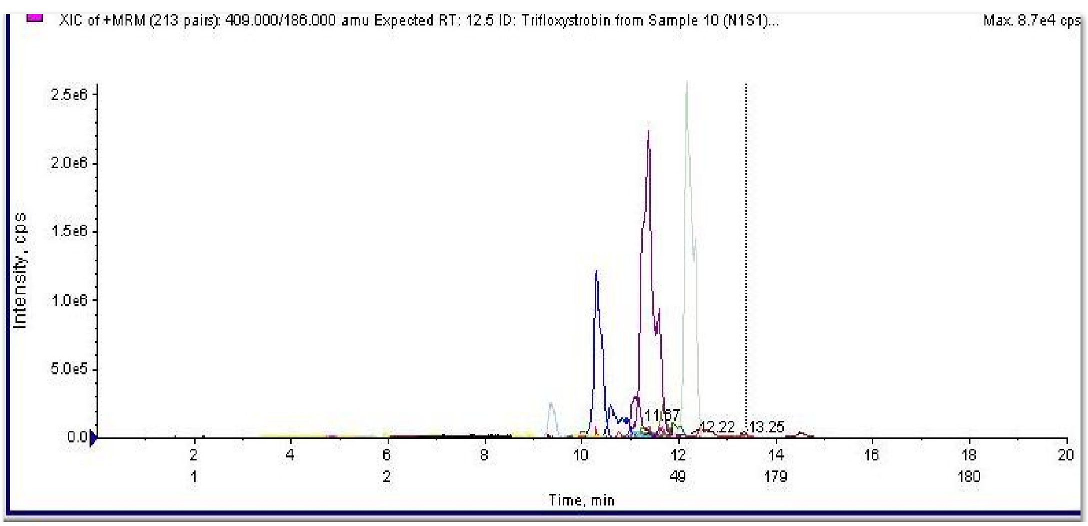

Figure 4B. LCMS chromatogram of pepper berries extract sample at retention time of 12.50 min.

3.6 Residue and dissipation of tebuconazole and trifloxystrobin in black pepper

Data on tebuconazole and trifloxystrobin residue in dried pepper berries from 5 supervised residue trials are presented in Table 6. Results from the residue trials indicated that tebuconazole applied at standard dose, 0.2 g/L AI resulted in low residue level in pepper berries ranging from <0.01 mg/kg to 0.11 mg/kg. The highest recorded for tebuzonazole residue was detected in trial farm 1, treated with 0.4 g/L AI with the residue value of 0.24 mg/kg, sample collected 2 hour after the spray. The initial tebuconazole residue concentration were range between 0.08 – 0.11 mg/kg in dried pepper berries treated with 0.2 g/L AI whereas for the berries that are treated with double dose concentration, the initial tebuconazole residue were slightly higher with the residue value ranged between 0.19 - 0.24 mg/Kg. After 5 days, nearly half of the residue in both treatment plots degraded, but it remained at a low level for up to 9 days at standard dose treatment and 15 days at double dose treatment.

In term of trifloxystrobin residue, the residue value is much higher than tebuconazole residue, which may be due to the higher concentration of the former in the combination formulation. The trifloxystrobin residue can be detected in almost all the sample collected with the residue value ranging between <0.01 mg/kg to 3.12 mg/kg. The highest trifloxystrobin residue was detected in farm 3 that are treated with 0.4 g/L AI with the residue value of 3.12. The dissipation pattern of trifloxystrobin showed a constant decrease of residue from first day to 20th days. The pre-harvest interval (PHI) for application of 0.2 g/L AI of Nativo fungicide were on the day 15 after the last treatment whereas for berries that are treated with 0.4 g./L AI, the pre-harvest interval was on days 20.

The observed low residue levels of Nativo residue could be due to low dosage used and the compound's rapid degradation. In addition, for the last 4 application, between January-April 2015, the field condition was considered dry and might attribute to rapid degradation of this residue. Rapid degradation of Nativo fungicide in crop has also been reported by other researchers (Braun *et al*., 1982). Besides, another reason contributes to the low level of Nativo residue might probably due to the blanching method used in the drying process. Based on the overall results obtained, it can be concluded that the pre-harvest interval for application of Nativo fungicide was on day 15 after the last treatment.

Table 6: Residue data summary from supervised trials of tebuconazole and trifloxystrobin in pepper berries

Trial	Residue	No. of sample taken for residue analysis	Control	0.2 g/L AI		0.4 g/L AI	
				Means Residue (mg/kg)	Sampling Interval (DAT)	Means Residue (mg/kg)	Sampling Interval (DAT)
1	Tebuconazole	4	ND	0.10	0	0.24	0
				0.05	3	0.15	3
				0.01	5	0.07	5
				<0.01	9**	0.021	9
				<0.01	15	<0.01	15**
				<0.01	20	<0.01	20
	Trifloxystrobin	4	ND	1.83	0	2.32	0
				0.62	3	1.35	3
				0.21	5	0.75	5
				0.06	9	0.13	9

				<0.01	15**	0.04	15
				<0.01	20	<0.01	20**
2	Tebuconazole	4	ND	0.11	0	0.23	0
				0.06	3	0.12	3
				0.01	5	0.07	5
				<0.01	9**	0.03	9
				<0.01	15	<0.01	15**
				<0.01	20	<0.01	20
	Trifloxystrobin	4	ND	1.47	0	3.09	0
				0.73	3	2.51	3
				0.31	5	1.26	5
				0.08	9	0.82	9
				<0.01	15**	0.05	15
				<0.01	20	<0.01	20**
3	Tebuconazole	4	ND	0.09	0	0.21	0
				0.05	3	0.12	3
				0.02	5	0.05	5
				<0.01	9**	0.2	9
				<0.01	15	<0.01	15**
				<0.01	15	<0.01	20
	Trifloxystrobin	4	ND	1.28	0	3.12	0
				0.79	3	1.73	3

				0.29	5	0.93	5
				0.11	9	0.42	9
				<0.01	15**	0.14	15
				<0.01	15	<0.01	20**
4	Tebuconazole	4	ND	0.08	0	0.19	0
				0.04	3	0.10	3
				0.01	5	0.06	5
				<0.01	9**	0.02	9
				<0.01	15	0.01	15
				<0.01	20	<0.01	20**
	Trifloxystrobin	4	ND	1.42	0	2.34	0
				0.86	3	1.25	3
				0.35	5	0.82	5
				0.11	9	0.31	9
				<0.01	15**	0.05	15
				<0.01	20	<0.01	20**
5	Tebuconazole	4	ND	0.10	0	0.19	0
				0.03	3	0.13	3
				0.01	5	0.06	5
				<0.01	9**	0.02	9
				<0.01	15	<0.01	15**
				<0.01	20	<0.01	20

	Trifloxystrobin	4	ND	1.35	0	2.02	0
				0.69	3	1.05	3
				0.31	5	0.51	5
				0.12	9	0.21	9
				<0.01	15**	0.05	15
				<0.01	20	<0.01	20**

0* 2 hours after last spray

** Recommended pre-harvest interval

3.7 Maximum residue limit (MRL) estimation

MRL for tebuconazole and trifloxystrobin were calculated using a European Union method (Hyder et al., 2003). This method required residue data collected from standards dose with a minimum of 5 residue trials for a minor crop which is defined as crop which has a mean dietary intake of less than 7.5g/person/day. Black pepper is considered as a minor crop with the food consumption data by United Stated Department of Agriculture (USDA) recorded its mean dietery intake is 6.9g/person/day (USDA reference number: 02030). For MRL calculation, the highest residue from each trial will be chosen as one data point; these data points are group and computed for relevant statistical values.

For values that are below the limit of quantification (LOQ), they are assumed to be at the LOQ. The estimation was based in the equation shown below:

MRL for tebuconazole = R + KS

= 0.096 + (4.310 x 0.02)

= 0.1822 mg/kg

MRL for trifloxystrobin = R + KS

= 1.47 + (4.215 x 0.28)

= 2.6502 mg/kg

R is the mean of the highest residue (HR) of very trial after PHI (3 trials with 3 highest residues in ascending order are 0.08, 0.09, 0.10, 0.10 and 0.11mg/kg for tebuconazole and 1.28, 1.35, 1.42, 1.47, 1.83, and, mg/kg for trifloxystrobin). S is the standard deviation and K is the one sided tolerance factor for normal distributions with 95% confidence level. It should be noted that HR of each trial will be assumed to be at the LOQ when all residue results after the PHI are < LOQ. The estimated MRL value is 0.1822 for tebuconazole and 2.6502 mg/kg for trifloxystrobin. This value is rounded up to 0.20 and 2.70 following the rules set by Codex in having the common classes of MRL value such as 0.01, 0.02, 0.05, 0.1, 0.5, 1.0 etc. Therefore, the proposed MRL of tebuconazole and trifloxystrobin in black pepper based on residue data are 0.2 mg/kg and 2.70 mg/kg.

4. Discussion

Nativo is considered as new generation of fungicide that contain of 2 active ingredients namely, tebuconazole and trifloxytrobin. In pepper cultivation, qquality is the only driver for a successful crop with the main focus on visual quality. Nativo delivers this through the protection and strobilurin boost of trifloxystrobin, safely combined with the curative and protectant triazole properties of tebuconazole. Both of these fungicides are one among the strobilurin and triazole class of systemic fungicide, with bioefficacy against all fungal species since it inhibit mitochondrial respiration. Although, the field test bio efficacy results of these fungicides are promising in various agro-climatic zones of the country, none of this fungicide are currently recommended for black pepper cultivation in Malaysia because of the lack information regarding their residue dynamics.

Spraying of Nativo has limited the anthracnose development. The disease incidence reduced was greater when Nativo sprayed at 0.2 and 0.4 g/L. Treating vines with these concentration provided 100% reduction of leaves anthracnose and black berries diseases compared to untreated vines for which disease incidence were 29.85 and 49.68 PDI, respectively. The controlling effect was mainly due to translaminar and systemic movement of tebuconazole and trifloxytrobin inside the tissue. Nativo at said concentration not only suppressed development of anthracnose incidence but also increase the production of pepper berries per vine basis. Several other studies demonstrated the efficacy of tebuconazole and trifloxytrobin in reducing disease severity. For examples, Li et al., (2014) reported that trifloxytrobin effectively controlled downy mildew (*Plasmopora viticola*) and powdery mildew (*Uncinula necator*) of grapevines as against control.

The fungicide rate of application experiment was useful for determining the optimum rate of fungicide application. Prior to commencement of this study no work has been undertaken on optimum rates of application of fungicides for anthracnose disease in black pepper. The results of the experiment in this study indicated response to rate was fixed to 0.2 g/L AI. Moreover, all the doses of Nativo had not caused any phytotoxic effect to the pepper vines.

From the results obtained, it was found out that the trifloxytrobin residue remained on pepper berries for 15 (standard dose) and 20 days (Double dose) after the last spray. This finding is consistent with the finding reported by Mohapatra et al., 2010, where they reported that the persistence of trifloxytrobin in cash crop like banana dan grapes was more than 20 days. This phenomena might probably due to the long maturity of period of pepper berries that may slow down the dilution of pesticides (per unit weight of fruit), thereby increasing it persistence.

This research finding also revealed that the dissipation rate of tebuconazole is faster than trifloxytrobin. More than 80% of the tebuconazole was degraded 9 days after the application of fungicide. In addition, lower solubility in trifloxytrobin (610µg/L at 25^{o}C) as compared to tebuconazole was another factor contributes to the long persistence of trifloxytrobin in berries (Mohapatra, 2015).

The PHI for treatment with the combination formulation of trifloxytrobin and tebuconazole on pepper was 15 dan 20 days for standards and doubles dose treatments respectively. The research find was on par with research finding reported by Mohapatra, 2015. Based on the residue trial data conducted on five supervised areas, it is estimated that MRL value for tebuconazole is 0.19 and 2.71 mg/kg for trifloxystrobin. This value is rounded up to 0.20 and 2.70 following the rules set by Codex in having the common classes of MRL value such as 0.01, 0.02, 0.05, 0.1, 0.5, 1.0 etc. Therefore, the proposed MRL of tebuconazole and trifloxystrobin in dried black pepper based on residue data are 0.2 mg/kg and 2.70 mg/kg. The MRL result for black pepper obtained in this experimental trial was slightly higher than MRL for other fresh crop stated in the Food Act 1983 (Malaysia) (Food Act, 2014). This results is expected cause both trifloxytrobin and tebuconazole residue were found to get concentrated during sun drying which could be due to high adsorption of the pesticide to the skin and the stability of the molecule to high temperature and weight reduction consequent to dehydration. The result obtained in this study corroborated the finding of George et al., 2014 wherein the sun light increase the pesticide residue in dried foodstuffs as compared to fresh dried foodstuffs.

5. Conclusion

The result obtained indicated that spraying Nativo during pepper production cycle in field showed to be very efficient to control antharacnose diseases. Therefore, it can be concluded that the optimal dosage of Nativo to control anthracnose disease in black pepper was fixed to 0.2 g/L with the PHI with the preharvest interval was on day 15.

Lack of MRLs for agriculture commodities may give rise to non-tariff trade barrier for the country bounded by WHO trade agreement. This study was part of an effort by Malaysian Pepper Board to establish MRLs of fungicides for export commodities. Based on the overall results obtained, it can be concluded that the pre-harvest interval for application of Nativo fungicide was on day 15 after the last treatment. A proposed MRL of 0.2 mg/kg for tebuconazole and 2.70 mg/kg for trifloxystrobin were determined for pepper berries (*Piper nigrum L*.) based on residue trials data.

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